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Prebiotics and Probiotics

Edited by
Flavia Indrio

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Special Issue Editor

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About the Special Issue Editor

Flavia Indrio is an Associate Professor of Pediatrics, Senior Consultant in Pediatric Gastroenterology in the Department of Pediatrics, University of Bari; Responsible for the Pediatric Gastroenterology and Motility Unit; Teacher of Pediatrics, Gastroenterology and Nutrition for the Resident in Pediatrics, University of Bari School of Medicine; Tutor of medical students at the University of Bari, School of Medicine; Member of the Committee of Nutrition ESPGHAN and eLearning Program for ESPGHAN.

Preface to ”Prebiotics and Probiotics”

The huge impact that the billons of microscopic cells living in our gut have on our gastrointestinal and systemic health, even our thinking processes and our mood, is now became more and more clear. The alteration of the normal composition of the microbiota disturbs the mutualistic relationship between the host and the microbiota that becomes ”dysbiotic” and can cause or contribute to the development of pathologies, increasing the risk of infections, the growth of potentially pathogenic microorganisms and the development of inflammatory diseases. Microflora-specific aberrations detected in specific diseases are defined as 'microbial signature', indicating that a certain microbial structure may be a hallmark of a specific disease

The intestinal microbiota must therefore be considered a fully functional organ that forms a super-organism with its host and whose structure is the result of millions of years of evolution and early life events, such as nutrition, diseases and therapies. antibiotic. A healthy microbiota, or in eubiosis, protects from disease, while an abnormal microflora structure, or in dysbiosis is associated with an increased risk of disease. Understanding the close connections that exist between the microbiota, the host and the pathogens, allows to obtain a new interpretative key to the pathogenetic mechanisms of various diseases as well as to open new frontiers for the research of innovative strategies for the prevention and treatment of gastrointestinal diseases and systemic.

The purpose of this volume is to provide an overview of what is and how one can study the microbiota and under what circumstances the manipulation of the microbiological pattern can have a therapeutic role.

Flavia Indrio
Special Issue Editor

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Article

Physiological Translocation of Lactic Acid Bacteria during Pregnancy Contributes to the Composition of the Milk Microbiota in Mice

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Abstract: The human milk microbiota is a complex and diverse ecosystem that seems to play a relevant role in the mother-to-infant transmission of microorganisms during early life. Bacteria present in human milk may arise from different sources, and recent studies suggest that at least some of them may be originally present in the maternal digestive tract and may reach the mammary gland through an endogenous route during pregnancy and lactation. The objective of this work was to elucidate whether some lactic acid bacteria are able to translocate and colonize the mammary gland and milk. For this purpose, two lactic acid bacteria strains (*Lactococcus lactis* MG1614 and *Lactobacillus salivarius* PS2) were transformed with a plasmid containing the *lux* genes; subsequently, the transformed strains were orally administered to pregnant mice. The murine model allowed the visualization, isolation, and Polymerase Chain Reaction (PCR)-detection of the transformed bacteria in different body locations, including mammary tissue and milk, reinforcing the hypothesis that physiological translocation of maternal bacteria during pregnancy and lactation may contribute to the composition of the mammary and milk microbiota.

Keywords: human milk; translocation; *Lactobacillus salivarius*; *lux*; bioluminescence; pregnancy; lactation

1. Introduction

Human milk contains microorganisms that, together with other milk components, play a pivotal role in the early colonization of the infant gut. The use of diverse culture-dependent or-independent techniques has allowed either the isolation or detection of a wide spectrum of commensal bacteria in milk samples provided by healthy women worldwide [1]. Most research efforts have been focused on lactobacilli and bifidobacterial isolates because of their potential to be used as probiotics [2].

Traditionally, the presence of bacteria in human milk was related to contamination from the mother's skin or infant's oral cavity [2]. In addition, bacterial translocation from the digestive tract of healthy women has been proposed as a source of bacteria for the mammary gland during late pregnancy and lactation [3]. In fact, physiological translocation of commensal bacteria from the gut to different mucosal surfaces has already been shown in vitro and in vivo through a mechanism involving complex interactions between bacteria, epithelial cells, and immune cells (including dendritic cells and macrophages) [3,4]. Previous studies have reported that oral administration of some lactobacilli

strains to lactating women leads to their presence in milk [5,6]. However, more mechanistic studies focused on the potential bacterial transfer from the gut to the mammary glands of healthy hosts are required to further confirm such findings.

Bioluminescent whole-body imaging allows rapid and real-time monitoring of bacteria in vivo. This system captures photons of light emitted by naturally luminescent bacteria or by those that have been genetically manipulated to produce bioluminescence. The bioluminescence reaction involves a luciferase-catalyzed intracellular oxidation of a long-chain fatty aldehyde (R-CHO) together with a concomitant reduction of flavin mononucleotide ($FMNH_2$). Such reactions lead to the generation of blue-green light, as follows: $FMNH_2 + O_2 + R\text{-}CHO \rightarrow FMN + H_2O + R\text{-}COOH + Light$ (~495 nm). The *lux* operon (*luxABCDE*) contains the genetic determinants for bioluminescence and includes the structural genes (*luxA* and *luxB*) coding for the two subunits (α and β, respectively) of the luciferase enzyme as well as three additional genes (*luxC*, *luxD*, and *luxE*) encoding the fatty acid reductase complex responsible for fatty aldehyde synthesis [7].

Bioluminescence imaging has successfully demonstrated in vivo translocation of pathogenic strains of *Escherichia coli* and *Citrobacter rodentium* in mice [8–10], and persistence of *Lactococcus lactis* and *Lactobacillus plantarum* strains in the murine gastrointestinal tract [11]. In addition, translocation of bioluminescent bifidobacteria from the gastrointestinal tract of mice, and their subsequent selective recruitment by tumoral cells, have been shown using such an approach [12]. In this context, the objective of this work was to obtain bioluminescent lactic acid bacteria (LAB) strains to provide in vivo evidence of their physiological translocation in mice during late pregnancy and lactation.

2. Material and Methods

2.1. Bacterial Strains and Media

The LAB strains used in this work as recipients for the *lux* genes were *Lactococcuslactis*MG1614, a laboratorial strain widely used in studies dealing with molecular biology of lactococci [13], and *Lactobacillus salivarius* PS2, a probiotic strain originally isolated from human milk [14–16]. *L. lactis*MG1614 was routinely grown in M17 (Oxoid, Basingstoke, UK) supplemented with 0.5% (*w*/*v*) glucose (GM17 medium) and incubated at 30 °C, while *L. salivarius*PS2 was grown in Man, Rogosa, and Sharpe (MRS) (Oxoid) medium and incubated at 37 °C. Competent *Escherichia coli* cells were purchased from Bioline (BIO-85027; Bioline Reagents Ltd., London, UK). *E. coli* was grown in Luria Bertani (LB) medium and incubated at 37 °C. When required, erythromycin (Em) (Sigma-Aldrich, St. Louis, MI, USA) was added to the cultures at the following concentrations: 150 μgmL^{-1} for *E. coli*, 2.5 μg mL^{-1} for *L. lactis*MG1614, and 5 μg mL^{-1} for *L. salivarius* PS2. Previously, the Em resistance of *L. salivarius* PS2 had been tested in MRS broth containing Em concentrations ranging from 0.25 to 5 μg mL^{-1} at 37 °C for 24 h and bacterial growth was detected by measuring the OD_{600} of the cultures.

2.2. Construction of pMG36e::luxAB and pMG36e::luxABCDE

Plasmid pXen-5 (Xenogen Bioware, Alameda, CA, USA) was used as template to amplify the *luxABCDE* operon using primers XAF1/XBR1 (which generate a DNA fragment containing only the structural genes *luxAB*) and XAF1/XER2 (which generate a DNA fragment containing the complete *luxABCDE* operon) [17]. However, primers XAF1/XBR1 were modified to add restriction sites *Sac*I/*Sma*I and *Sac*I/*Sal*I, respectively, at the ends of the amplicons (Table 1). PCR was performed using Phusion Hot Start II DNA High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) with the following conditions: 98 °C for 30 s; 35 cycles at 98 °C for 10 s, 72 °C for 1 min, 72 °C for 5 s; and finally, 72 °C for 5 min. PCR products were purified using Sure Clean Plus (Bioline, London, UK) following the manufacturer's instructions.

After digestion of the vector (plasmid pMG36e) with the corresponding enzymes (New England Biolabs. *Sma*I, at 25 °C; *Sac*I and *Sal*I at 37 °C; all incubations occurred overnight), ligations were performed overnight at 16 °C with T4 DNA ligase (Roche, Mannheim, Germany) to generate plasmids

pMG36e::*luxAB* and pMG36e::*luxABCDE*, respectively. Plasmid pMG36e is a 3.6 kb expression vector carrying an Em resistance gene and the strong P32 promoter [18]. Both plasmids were separately introduced into competent cells of *E. coli* (BIO-85027; Bioline), following the manufacturer's instructions. Cells were plated on LB plates supplemented with Em (150 μg mL^{-1}) in order to select for transformants.

Table 1. Primers used in this study for the Polymerase Chain Reaction (PCR) detection of *lux* genes.

Name	Sequence (5′-3′)	Reference or Source
XAF1	CCC CGA GCT CAT GAA GCA AGA GGA GGA CTC TCT ATG	Modified from [17]
XBR1	GGC CCC GGG TTA GGT ATA TTC CAT GTG GTA C	Modified from [17]
XAF1	CCC CGA GCT CAT GAA GCA AGA GGA GGA CTC TCT ATG	Modified from [17]
XER2	GGC GGC GTC GAC TTA ACT ATC AAA CGC TTC GGT TA	Modified from [17]
lux1280	ACG CCG CAG GAA TGT ATT GA	This study
lux1732	TAT GGC GAC AGG ATG ATG AG	This study
lux4807	GTC AAT GAA CGC CGA ATG AG	This study
lux5068	GTC ACT ACT GTC AGG CAC AC	This study

2.3. Transformation of L. lactis MG1614 and L. salivarius PS2

Both plasmids were extracted from transformed *E. coli* cells using the Qiagen plasmid mini kit (Qiagen, Hilden, Germany) and electroporated into *L. lactis*MG1614 cells as described previously [19]. After electroporation, *L. lactis* cells were plated on GM17 supplemented with Em (2.5 μg mL^{-1}) and incubated at 30 °C for 48 h. Subsequently, both plasmids (containing P32-*luxAB* and P32-*luxABCDE*, respectively) were isolated from the previously transformed *L. lactis* MG1614 cells and electroporated into competent *L. salivarius*PS2 cells, which had been obtained as previously described [20]. Then, bacterial cells were plated on MRS plates supplemented with Em (5 μg mL^{-1}) and incubated at 37 °C for 48 h. The stability of the recombinant plasmids was assayed by daily sub-culturing of the recombinant strains in non-selective media during seven consecutive days. Aliquots of each subculture were plated on selective (Em-supplemented MRS) and non-selective (MRS) agar plates [21]. Plasmid maintenance was determined by comparing the numbers of colonies that grew on both types of media.

Presence of the plasmids in the transformant cells was confirmed by PCR. Primers lux1280F/lux1732R allow the amplification of a 452 bp fragment located between *luxA* and *luxB* genes, while primers lux4807F/lux5068R were designed to amplify a 261 bp fragment between *luxD* and *luxE* genes (Table 1). PCR was performed using My Taq™ Red DNA Polymerase (Bioline) and the following conditions: 94 °C for 4 min; 25 cycles at 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The amplified fragments were visualized by electrophoresis in a 1.2% agarose gel after 90 min at 90 V.

2.4. Bioluminescence Assays

Bioluminescence assays were performed in collaboration with the Biolum Lab of the "Red de Laboratorios de la Comunidad de Madrid". Production of bioluminescence by the bacterial cultures was measured directly in broth medium (1 mL) with a luminometer (Biocounter® M1500 Lumacbv, Landgraaf, The Netherlands), and the bioluminescence was expressed as Relative Light Units (RLUs). The presence of bioluminescent signals was also analyzed on the surface of agar plates using the IVIS 100 imaging system (In Vivo Imaging System, Xenogen, Perkin Elmer, Hopkinton, MA, USA), which consists of a cooled charge-coupled-device (CCD) camera mounted on a light-tight specimen chamber. The signal intensity was quantified as photon counts per second (p/s).

2.5. In Vivo Translocation Model

Ten-week-old pregnant Balb/c mice (Day 5 of gestation) were used to assess the potential in vivo translocation of the transformed *L. lactis* and *L. salivarius* strains. Animals were kept in the Animal

Facility of Centro de Biología Molecular Severo Ochoa (CBM-CSIC, Madrid, Spain), and housed individually (one pregnant mouse per cage) in 1264C Eurostandard Type II cages (26.7 × 20.7 × 14.0 cm-floor area 370 cm^2, Tecniplast, Buguggiate, Italy) with bedding, food (Diet 2018, Harlan, Correzzana, Italy) and water available ad libitum, under a temperature (22 °C) and light-controlled (12 h) cycle. All experimental procedures complied to the principles of good laboratory animal care, were carried out in compliance with national legislation following the EU-Directive 2010/63/EU for the protection of animals used for scientific purposes, and were approved by the ethics committee for animal experimentation and the Animal Welfare Body of CBM/Complutense University of Madrid, Spain (Code 15/017E; approval date: January 2015). All adequate measures were taken to minimize animal pain or discomfort.

Bacterial strains were grown overnight in GM17 supplemented with Em (2.5 μg mL^{-1}) at 32 °C (*L. lactis* cells) or MRS supplemented with Em (5 μg mL^{-1}) at 37 °C (*L. salivarius* cells). Cells were harvested by centrifugation (18,800× *g* for 10 min at 4 °C) and the pellet was resuspended in a mixture of 10% skimmed milk with 10% of 2.5 M sucrose. The mix was dispensed as single-doses (200 μL, ~10^9 CFU/dose), lyophilized, and kept at −20 °C until their administration to mice. The stability of the doses was assessed weekly by plate count on GM17 (Em: 2.5 μg mL^{-1}) or MRS (Em: 5 μg mL^{-1}) agar plates.

Test mice (n = 6) received a daily dose (at the same time each day) of transformed *L. lactis* (n = 2) or *L. salivarius* (n = 4; two pregnant and two non-pregnant females) intragastrically via oral gavage until delivery (~20 days). Control mice (n = 5) received a daily dose (200 μL) of the probiotic matrix (10% skimmed milk with 10% of 2.5 M sucrose) also by intragastric administration.

At Day 15 of pregnancy and within the first 6 h after delivery, in vivo bioluminescence imaging of the mice was performed using the multimodal IVIS 100 imaging system described above. For this purpose, mice were anesthetized with 2% isoflurane and placed into the camera chamber, where a controlled flow of 1.5% isoflurane-supplemented air was administered through a gas anesthesia system. Bioluminescence was quantified as p/s using the Living Image® software (Caliper Life Sciences, Waltham, MA, USA).

Within the first 12 h after delivery, the female mice and their offspring (before weaning) were euthanized using a mixture of CO_2/O_2 according to the EU guidelines and necropsies performed under sterile conditions. Samples of feces, milk, urine, blood (EDTA tubes), and biopsies of intestine (large and small), stomach, liver, spleen, kidneys, mammary glands, uterus, Peyer's patches, and mesenteric nodes were collected and stored at 4 °C for microbiological and microscopy-based analysis.

To evaluate potential translocation of the transformed strains from the gastrointestinal tract to different tissues, the biological samples (biopsies, urine, milk, and feces) were homogenized in peptone water (with the exception of urine and milk) and decimal serial dilutions were spread on selective GM17 (Em: 2.5 μg mL^{-1}) or MRS (Em: 5 μg mL^{-1}) agar plates.

Tissue biopsies were fixed, processed, and analyzed by transmission electron microscopy (TEM) as described previously [22]. For this purpose, blood was centrifuged (620× g for 20 min 4 °C), to collect the cells and the pellet was embedded in an agarose matrix before fixing in paraformaldehyde and glutaraldehyde.

2.6. Statistics Analysis: Sample Size Calculation

The recommended number of animals included in this trial was determined using the G*Power 3.1.9.2 program [23], and previous results obtained after oral administration of a genetically labeled *Enterococcus faecium* strain to pregnant mice [24]. The minimum value for the frequency of detection of the *lux*-labeled strain in the experimental group was estimated to be 80%, while it was not expected to be found in the control group. The minimum sample size was estimated to be 12 animals, using a 1:1 allocation ratio, and considering a one-tailed test, a 5% alpha level, and a statistical power of 90% to demonstrate a significant difference. Since one mouse in the control group was lost (a mouse that did not become pregnant) and the real proportion of animals containing *lux*-labeled *Lactococcus*

in the experimental group was higher than 80%, a post hoc analysis was performed. This analysis confirmed that the achieved power was higher (>99%) than the value that had been selected initially for the calculation.

3. Results

3.1. Transformation of E. coli and LAB Strains with pMG36e::luxAB and pMG36e::luxABCDE

Transformation of competent *E. coli* cells with plasmid pMG36e::*luxAB* or pMG36e::*luxABCDE* led to bacterial grow in selective conditions. Some colonies showed bioluminescence both in broth (RLU > 1500) and agar plates and, therefore, were selected for further studies (Figure 1). The presence of the *lux* operon in the transformed cells was also confirmed by PCR amplification of *lux* operon-specific fragments.

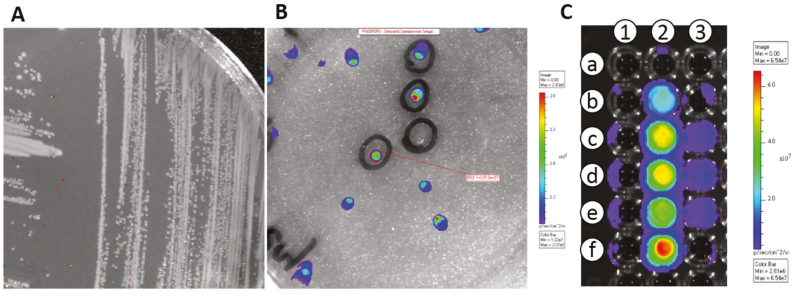

Figure 1. Transformation of *E. coli* with the *lux* operon. (**A**) LB agar plate showing non-transformed *E. coli* cells (negative control). (**B**) LB agar plate showing *E. coli* cells transformed with pMG36e::*luxABCDE*. (**C**) Microtiter plate showing *E. coli* cells transformed with pMG36e::*luxABCDE*; Column 1: non-inoculated LB medium (negative control); Column 2: files b to f: serial dilutions of *E. coli* cells transformed with pMG36e::*luxABCDE*.

Similarly, the successful transformation of *L. lactis* MG1614 with either pMG36e::*luxAB* or pMG36e::*luxABCDE* and that of *L. salivarius* PS2 with pMG36e::*luxAB* was confirmed by bioluminescence and PCR (Figure 2). However, the transformation of *L. salivarius* PS2 with pMG36e::*luxABCDE* was not possible after several electroporation attempts. The addition of D-luciferin Firefly (Xenogen) to the growth medium (150 µg/mL) was required in order to generate and detect bioluminescence by *L. salivarius* PS2 containing only the *luxAB* genes.

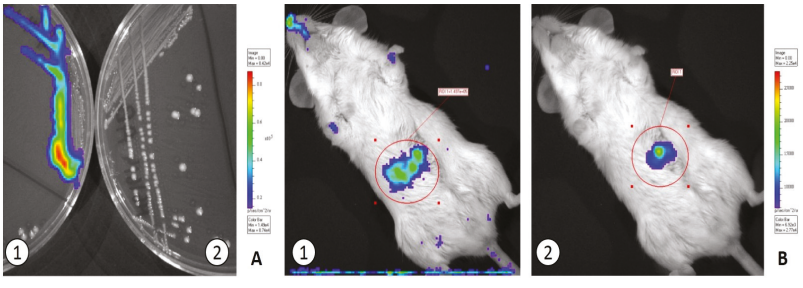

Figure 2. In vitro and in vivo detection of *L. lactis* MG1614 transformed with pMG36::*luxABCDE*. (**A**) GM17 agar plate with transformed (left) and non-transformed (right) *L. lactis* MG1614 cells. (**B**) Mouse immediately (left) and 20 min (right) after being fed with *L. lactis* pMG36e::*luxABCDE*.

The stability of *L. salivarius* PS2 transformed with pMG36e::*luxAB* was measured during~100 generations under selective (Em-supplemented) and non-selective conditions. The growth of the transformed strain in selective medium was ~1 $\log_{10}$ cycle higher in comparison to that observed in the non-selective one. Such difference remained constant along time, indicating that recombinant plasmid was stable in *L. salivarius* PS2.

3.2. In Vivo Translocation Model

A strong bioluminiscence signal was detected in the stomach of mice during the first 20 min after oral administration of the transformed strains (Figure 2). The bacteria gradually entered (and became diluted) in the gut compartment and, as a consequence, bioluminescence was lost ~1 h after their administration.

In relation to recombinant *L. lactis* (pMG36::e*luxABCDE*), mice were sacrificed at the end of the assay and biological samples (milk, urine, and feces) and biopsies of different organs and tissues were collected, homogenized (when required), and seeded on plates of agar GM17 supplemented with Em (2.5 μg mL^{-1}). After incubation, bioluminescent *L. lactis* colonies could be isolated from the samples of milk, urine, and feces and from the biopsies of mammary gland, liver, kidney, intestine, stomach, Peyer patches, spleen, and uterus (Figure 3).

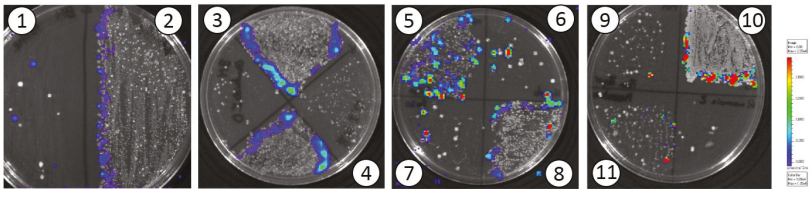

Figure 3. Isolation of transformed *L. lactis*MG1614 cells on GM17 agar plates from different maternal biological samples and organ biopsies: 1: milk; 2: feces; 3: small intestine; 4: large intestine; 5: kidney; 6: liver; 7: spleen; 8: stomach; 9: Peyer's patch; 10: urine; and 11: mammary gland.

With respect to recombinant *L. salivarius* (pMG36::e*luxAB*), the four mice that received the strain and one from the control group were sacrificed within one day after delivery. Feces and biopsies from small intestine, cecum, mammary gland, spleen and mesenteric lymph nodes were collected, homogenized (when required), and seeded on MRS and on MRS supplemented with Em(5 μg mL^{-1}) agar plates. PCR analyses were also carried out to detect the presence of the *luxAB* genes in the biological samples. The counts obtained from the different samples analyzed in this study are shown in Table 2. Bacterial growth was observed in the fecal samples from all the animals. As expected, fecal bacterial counts (expressed as cfu/g) were 3–4 $\log_{10}$ cycles higher in MRS (non-selective medium) than in Em-supplemented MRS (selective medium) plates. The *luxAB* fragment could be detected by PCR from feces of the four mice that received the recombinant strain but not from those collected from the control animal. In addition, the strain could be isolated and PCR-detected from mammary biopsies of the pregnant mice but not from those collected from the non-pregnant mice. Bacterial counts in the mammary samples were notably lower than fecal counts obtained from the same media and from the same animal (Table 2).

The *luxAB* fragment could not be detected either in spleen or in mesenteric lymph node samples. However, bacteria were observed in mesenteric lymph nodes (Figure 4A) and spleen (Figure 4B) by TEM image analysis. Using such a technique, bacterial cells appeared as a double membrane surrounding a dense content (chromatin) (Figure 4). Similar structures were detected in mammary gland biopsies.

Table 2. *L. salivarius* PS2 counts (log_{10} cfu/g) in the biological samples collected from mice (1 and 2: non-pregnant mice; 3 and 4: pregnant mice) that were fed with *L. salivarius* PS2 cells transformed with plasmid pMG36e::*luxAB*.

Sample	Mice	Growth Medium	
		MRS	MRS-Em [a]
Feces	1	na	5.92
	2	7.96	4.72
	3	7.54	4.26
	4	7.96	3.48
Small intestine	1	4.80	3.00
	2	4.64	nd
	3	4.96	nd
	4	na	na
Large intestine	1	6.65	5.54
	2	na	na
	3	6.48	2.70
	4	6.23	4.56
Spleen	1	nd	nd
	2	nd	nd
	3	nd	nd
	4	3.65	3.90
Mammary gland tissue	1	nd	nd
	2	nd	nd
	3	4.98	3.30
	4	4.98	3.00

[a.] MRS supplemented with erythromycin; na: sample not available; nd: not detected.

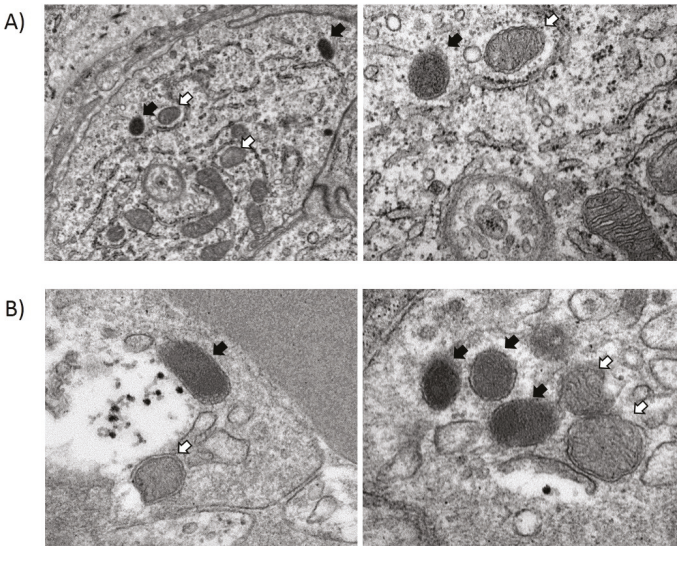

Figure 4. Transmission electron microscopy (TEM) images. Bacteria (black arrows) and mitochondria (white arrows) present in samples from a mesenteric lymph node (**A**) and spleen (**B**).

4. Discussion

In this work, two LAB strains (*L. lactis* MG1614 and *L. salivarius* PS2) were genetically modified to harbor genes belonging to the *lux* operon. This strain-labeling method constitutes an excellent tool for in vivo whole-body detection of bacteria in animal models [25]. Its advantages include (a) a very sensitive and rapid detection of luciferase activity, (b) a luminescent response that is linearly dependent on the amount of luciferase, and (c) the possibility of using real-time non-invasive techniques when combined with low-light imaging CCD cameras [25,26].

The recombinant plasmid pMG36e::*luxABCDE* was successfully transformed into *L. lactis* MG1614, but it could not be successfully electroporated into *L. salivarius* PS2. The large size of the plasmid (>10 kb) and the fact that *L. salivarius* PS2 is a wild strain may explain our inability to obtain transformants. Fortunately, *L. salivarius* PS2 cells were transformed with the smaller pMG36e::*luxAB* plasmid. In the absence of the *luxCDE* genes, strain-specific bioluminescence can still be detected provided that D-luciferin is added to the growth medium.

Both strains could be isolated (and the *lux* genes detected by PCR) from either milk or mammary gland biopsies after their oral administration to pregnant mice. While some may argue that their presence in milk might be the result of superficial fecal contamination in mice, such a route can hardly explain their isolation and detection from mammary biopsies. In a previous work, oral administration of *L. salivarius* PS2 to pregnant women led to the presence of the strain in the milk of some of the women after delivery [16]. It must be noted that, in contrast to mice, fecal contamination of human milk is highly improbable and, in fact, enterobacteria are usually absent in milk samples collected by manual expression from healthy women [27]. Our results reinforce the hypothesis that, at least, some members of the milk microbiota may arise from the digestive tract of the mother.

In the last years, different studies have shown that milk from healthy women contain bacteria that are subsequently transferred to the infant gut [1,28–36]. The detection of cells and/or DNA belonging to anaerobic species that are related to the adult gut environment (*Blautia*, *Bifidobacterium*, *Bacteroides*, *Parabacteroides*, *Clostridium*, *Collinsella*, *Coprococcus*, *Faecalibacterium*, *Roseburia*, *Subdoligranulum*, or *Veillonella*) has ledtothe hypothesisthat some of the human milk bacteria may originate in the maternal digestive tract (mouth, gastrointestinal tract) and reach the mammary gland through an endogenous route [3,4,31,33,37].

So far, culture-independent studies have not provided information on microbial viability and have not allowed strain level discrimination. A second caveat of molecular methods is the low amount of DNA typically extracted from milk samples, making the relative proportion of contaminant DNA from sample manipulation and from DNA extraction reagents more important than when analyzing other biological samples, such as feces [33,38]. However, the role of breastfeeding in the vertical mother-to-infant transfer of specific bacterial strains (including bifidobacteria and lactic acid bacteria strains) has already been demonstrated [6,16,31,33–35,38–45]. Some studies have revealed the ability of certain gut bacteria to spread to extra-digestive locations in healthy hosts [46–50], while others (including in vitro, animal, and human studies) have shown that physiological bacterial translocation during late pregnancy has a scientifically plausible basis and may involve complex interactions between microbes, immune cells, and gut epithelial cells [4–6,16,47,51–56].

Gut bacteria translocation has usually been associated with pathogenic conditions [57–60], but a low rate of bacterial translocation (involving *Bacteroides*, lactobacilli, bifidobacteria, or enterococci) occurs in healthy hosts [12,60–66] and may be associated to physiological immunomodulation [4,67,68]. Some bacterial strains seems to specifically mediate their own translocation without collateral translocation of other bacteria from the host digestive tract [12,69]. Many transient anatomical and physiological changes that occur during pregnancy and lactation may favor an increased bacterial translocation during such periods [1,62,70–72] and have been reviewed in [37].

At present, it is widely accepted that microbes are present in diverse organs and tissues previously thought to be sterile environments, including tumors [12]. Oral administration of a probiotic *Bifidobacterium breve* strain harboring a *lux*-expressing plasmid to mice bearing subcutaneous tumors

led to its presence in tumors at levels similar to intravenous administration [12]. Similarly, another probiotic strain (*E. coli* Nissle 1917) robustly colonized tumor tissue in rodent models of liver metastasis after oral delivery but did not colonize healthy organs or fibrotic liver tissue [69]. The authors suggested that oral delivery could lead to the preferential colonization of liver tumors by allowing probiotics to follow physiological blood flow patterns, wherein the venous outflow from the gut is directed to the liver via the hepatic portal vein. This phenomenon has been attributed to at least three different factors: (a) suppressed immune surveillance within the tumor, (b) tumor vascularization, and (c) increased availability of nutrients in the necrotic tumor core [73–76]. It has been proposed that the entry, survival, and replication of bacteria in tumors depends on the vascularization and immune-privileged nature of solid tumors, which may provide a suitable microenvironment for a small spectrum of bacterial species [74]. The nutrient-rich environment may also play an important role since tumors can support the growth of auxotrophic *S. typhimurium* strains [76–78]. Interestingly, the same factors are also present in the mammalian glands during late pregnancy and may explain the selective tropism or homing effect that the mammary gland seems to exert on some maternal bacterial species during this life stage: (a) there is a physiological immunodepression state in order to tolerate the fetus; (b) as stated above, there is a formidable angiogenesis process; and (c) pre-colostrum starts to fill the mammary duct during the last third of pregnancy providing a rich nutrient environment for bacteria.

Further studies are required to elucidate the mechanisms by which some bacterial strains may translocate physiologically in certain hosts or life stages. The existence of such bacterial oral- and entero-mammary pathways would provide new opportunities for manipulating an altered maternal-fetal microbiota, reducing the risk of preterm birth or infant diseases.

5. Conclusions

Human milk is one of the main sources for the vertical mother-to-infant transmission of bacteria in early life. Although the origin of the bacteria naturally present in this biological fluid can be diverse, the results of this study confirm that there may be a physiological translocation of certain bacterial strains from the maternal digestive tract to the mammary gland and milk.

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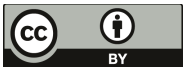

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Live *Faecalibacterium prausnitzii* Does Not Enhance Epithelial Barrier Integrity in an Apical Anaerobic Co-Culture Model of the Large Intestine

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Abstract: Appropriate intestinal barrier maturation during infancy largely depends on colonization with commensal bacteria. *Faecalibacterium prausnitzii* is an abundant obligate anaerobe that colonizes during weaning and is thought to maintain colonic health throughout life. We previously showed that *F. prausnitzii* induced Toll-like receptor 2 (TLR2) activation, which is linked to enhanced tight junction formation. Therefore, we hypothesized that *F. prausnitzii* enhances barrier integrity, an important factor in appropriate intestinal barrier maturation. In order to test metabolically active bacteria, we used a novel apical anaerobic co-culture system that allows the survival of both obligate anaerobic bacteria and oxygen-requiring intestinal epithelial cells (Caco-2). The first aim was to optimize the culture medium to enable growth and active metabolism of *F. prausnitzii* while maintaining the viability and barrier integrity, as measured by trans-epithelial electrical resistance (TEER), of the Caco-2 cells. This was achieved by supplementing the apical cell culture medium with bacterial culture medium. The second aim was to test the effect of *F. prausnitzii* on TEER across Caco-2 cell layers. Live *F. prausnitzii* did not improve TEER, which indicates that its benefits are not via altering tight junction integrity. The optimization of the novel dual-environment co-culturing system performed in this research will enable the investigation of new probiotics originating from indigenous beneficial bacteria.

Keywords: intestinal barrier maturation; intestinal microbiota; obligate anaerobic bacteria; tight junctions

1. Introduction

Faecalibacterium prausnitzii is one of the most abundant bacterial species in the colon of healthy human adults [1,2]. This bacterium is thought to be critical for maintaining colonic health because its abundance is reduced in people with gastrointestinal diseases [3–10]. Therefore, increasing the abundance of *F. prausnitzii* in the colonic microbiota has become the target of much research, either by directly delivering the bacterium as a probiotic [11] or by using food ingredients that preferentially stimulate the growth of endogenous *F. prausnitzii* [12]. Despite this, little is known about the role of *F. prausnitzii* in appropriate development of the intestinal barrier during infancy and whether it has the potential to be a probiotic during early life.

Intestinal maturation, including the development of the intestinal barrier integrity and immune function as well as the establishment and stabilization of the microbiota, occurs throughout the first two years of life. Much of this process is regulated by diet (e.g., breast milk versus infant formula),

which in turn influences the colonization patterns of the early microbiota and their interactions with the host [13]. *F. prausnitzii* colonizes the large intestine between six and 12 months of life [14–16], so it is likely to have an impact on intestinal maturation during weaning.

One key area of intestinal maturation is the education of the immune system by the resident bacteria. *F. prausnitzii* has been associated with anti-inflammatory effects in adult gnotobiotic rodents colonized with *Escherichia coli* [17] or *Bacteroides thetaiotaomicron* [18]. However, *F. prausnitzii* is unable to mono-colonize gnotobiotic rodents [17], which means that targeted in vivo studies are not possible. An alternative is to use in vitro techniques to investigate the specific immune-modulatory effects of *F. prausnitzii* on host cells. Such studies have been limited due to the difficulty of co-culturing obligate anaerobes and human oxygen-requiring cells using conventional culturing systems. Using a novel dual-environment co-culturing system we previously showed that live *F. prausnitzii* induced TLR2 activation in transfected human embryonic kidney cells (HEK293) [19], which has been implicated in maintaining homeostasis between immunity and tolerance in the intestinal epithelium [20].

Another key to appropriate intestinal maturation is development of the barrier integrity, which is crucial not only for nutrient absorption but also to prevent the entry of bacteria and food antigens from the lumen into underlying tissues [21]. *F. prausnitzii* improved barrier integrity in mice with DSS-induced colitis [22]. However, our previous study using Caco-2 cell monolayers as a model of the large intestinal epithelium showed that *F. prausnitzii* did not alter ion permeability, as measured by the trans-epithelial electrical resistance (TEER) assay, and increased small molecule permeability, as measured by the ^{3}H-mannitol flux assay, which could be considered detrimental [23]. In the study described above using the dual-environment co-culturing system the viability of *F. prausnitzii* in apical anaerobic conditions was improved compared to when cultured in the presence of oxygen, but the bacterium was not actively growing. The discrepancy between the in vivo and in vitro results may be due to this lack of growth, especially since mammalian cells have been shown to respond differently to the same bacterium depending on its growth phases [24].

Therefore, the specific hypothesis of this research was that actively growing *F. prausnitzii* improves intestinal barrier integrity, as measured by the TEER across Caco-2 cells, indicating that it has potential to be a probiotic to improve intestinal barrier maturation during early life. In order to test the hypothesis the first aim of this study was to optimize the apical medium to suit the requirements of both the bacterium and the intestinal epithelial cells, and in particular to encourage growth and active metabolism of *F. prausnitzii*. The second aim was then to test the effects of three *F. prausnitzii* strains, A2-165, American Type Culture Collection (ATCC) 27768, and HTF-F, on TEER across Caco-2 cells to ensure that our results were not limited to one strain.

2. Materials and Methods

2.1. F. prausnitzii Culturing Conditions

The three *F. prausnitzii* strains A2-165 (DSM 17677), ATCC 27768, and HTF-F (DSM 26943) were kindly provided by Hermie J. M. Harmsen (Department of Medical Microbiology, University of Groningen, Groningen, The Netherlands). Bacteria were cultured anaerobically in Brain Heart Infusion (BHI) broth containing 3.7% (*w*/*v*) BHI powder (Becton Dickinson, Auckland, New Zealand) supplemented with 0.5% (*w*/*v*) yeast extract, 0.0005% (*w*/*v*) hemin, 0.0005% vitamin K and 0.2% L-cysteine (Sigma-Aldrich, Auckland, New Zealand) in an atmosphere of 10% CO_2, 10% H_2 in N_2 at 37 °C (Concept Plus Anaerobic Workstation, Ruskinn Technology Ltd., Bridgend, UK) as previously described [19].

2.2. Caco-2 Cell Culturing Conditions

Caco-2 cells (HTB37) were obtained from the ATCC and used in experiments between passage 25 and 35. Caco-2 cells were maintained in cell culture flasks (Corning, New York, NY, USA) in Medium 199 (M199) cell culture medium supplemented with 10% fetal bovine serum (FBS),

1% non-essential amino acids (NEAA; 100× solution), and 1% penicillin-streptomycin (10,000 units/mL penicillin, and 10 mg/mL streptomycin (Life Technologies, Auckland, New Zealand), referred to as M199 Standard medium (M199 Std) and incubated at 37 °C in 5% CO_2. Medium was replaced twice a week. For co-culture experiments Caco-2 cells were seeded on semi-permeable polyester membranes (Transwell inserts, 6.5 mm diameter, 0.4 μm pore size, Corning, New York, NY, USA) at a density of 8×10^4 cells per insert and cultured for 16–18 days to attain differentiated cell monolayers (TEER over 400 $\Omega \cdot cm^2$).

2.3. Apical Anaerobic Co-Culture Model

The apical anaerobic co-culture model utilized an in-house designed and built dual-environment co-culture chamber inside an anaerobic workstation (Figure 1). The Caco-2 cells received oxygen through the semi-permeable membrane from the oxygenated medium in the basal compartment and the bacterial cells were maintained in the apical anaerobic medium. The chamber was sterilized and consumables and solutions were made anaerobic before each experiment as previously described [19].

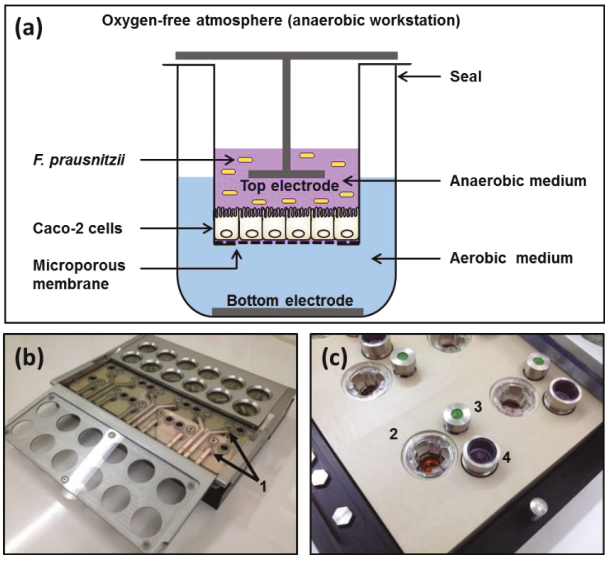

Figure 1. Apical anaerobic co-culture model. (**a**) Schematic diagram of a single well of the apical anaerobic co-culture model used for the co-culture of Caco-2 cell monolayers with anaerobic *F. prausnitzii*. The top and bottom electrodes enable the determination of the effect of *F. prausnitzii* on TEER across the Caco-2 cell monolayers; (**b**,**c**) Photographs of the co-culture chamber including details of the components. 1: Top electrodes; 2: Transwell insert containing Caco-2 cell monolayer; 3: Septum for basal media sampling; 4: One-way pressure relief valve.

2.4. TEER Experiment Protocol

Twenty-four hours before the experiments, the apical and basal M199 Std was removed from the Caco-2 cell monolayers grown on Transwell inserts and replaced with M199 supplemented only with 10% FBS and 1% NEAAs, referred to as M199 TEER, in order to remove the antibiotics before co-culturing the cells with bacteria. On the day of the experiment, the basal wells of the co-culture chamber were filled with 3 mL of M199 TEER. The Transwell inserts containing the cell monolayers were carefully inserted into the co-culture chamber using a twisting motion. The co-culture chamber was transferred into the interlock chamber of the anaerobic workstation, purged with nitrogen and

after finishing the interlock cycle moved into the anaerobic workstation. The co-culture chamber was connected to the commercially available CellZscope automated TEER monitoring system (CellZscope controller and CellZscope software version 2.2.3; nanoAnalytics, Münster, Germany). The TEER across each Caco-2 cell monolayer was measured twice and the second reading (after 1 h adaptation to the environment) was used as a baseline reading. The apical aerobic medium was removed and replaced with 260 µL of anaerobic medium or treatments. The TEER measurements were resumed and recorded hourly over 12 h. Since the initial TEER for each insert was different, the effect on TEER over time was expressed as the change in TEER compared to the initial TEER for each insert using the following Equation (1):

$$\text{Change in TEER } (\%) = \frac{(\text{TEER}_{\text{current}} - \text{TEER}_{\text{initial}})}{\text{TEER}_{\text{initial}}} \times 100. \quad (1)$$

2.5. Survival of F. prausnitzii in Anaerobic Cell Culture Media

The three *F. prausnitzii* strains were incubated in M199 TEER medium to monitor their survival. Secondary bacterial cultures in stationary phase were pelleted by centrifugation at 2500× *g* for 6 min (11180/13190 rotor, Sigma 3-18K centrifuge, Osterode am Harz, Germany) and resuspended in anaerobic media inside the anaerobic workstation. Bacterial number was estimated using a Petroff-Hauser counting chamber and solutions diluted to yield a concentration of 2.4×10^7 bacterial cells/mL. This bacterial density was chosen so that when used in the co-culture experiments described below it resulted in a multiplicity of infection (MOI) of 100 bacterial cells per intestinal epithelial cell. Triplicates of the bacterial solutions were incubated at 37 °C and the optical density at a wavelength of 600 nm (OD_{600nm}) was recorded (Implen OD600 DiluPhotometer with DC10 DiluCell cuvettes; Total Lab Solutions, Auckland, New Zealand) at 2-h intervals over 24 h.

2.6. TEER and Viability of Caco-2 Cells Using a Combination of Cell and Bacterial Culture Media

The effect of combining M199 TEER medium and BHI medium on the TEER and viability of Caco-2 cells was examined using the apical anaerobic co-culture model. Following the measurement of the initial resistance after two hours the apical medium was removed and replaced with 260 µL of anaerobic M199 TEER medium supplemented with increasing concentrations of anaerobic BHI (0%, 25%, 50%, 75%, or 100% BHI). After incubation for 12 h with automated hourly TEER measurements, the inserts were removed and the viability of the Caco-2 cells was determined using the neutral red uptake assay. Neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride; Sigma-Aldrich) was dissolved in phosphate-buffered saline (PBS) at 5 mg/mL, filter sterilized (0.22 µm filter), and diluted with M199 Std to a concentration of 50 µg/mL (referred to as neutral red medium). The medium was removed from all the Caco-2 cell monolayers and replenished with 200 µL neutral red medium. After incubation for 2 h at 37 °C in a CO_2 incubator, the neutral red medium was removed and cell monolayers washed twice with PBS. The dye was extracted from viable cells by adding 200 µL solubilization solution (1% acetic acid–50% ethanol) and incubating at room temperature on a plate shaker at 200 rpm for 7 min. 150 µL of extract was transferred to a 96-well plate and the absorbance of neutral red was determined on a microplate reader at 540 nm (FlexStation 3, Molecular Devices, Sunnyvale, CA, USA). The background absorbance of the 96-well plate was measured at 690 nm and subtracted from the 540 nm measurement. The experiment was completed in five blocks with four replicates per treatment group in each block.

2.7. Viability of the F. prausnitzii Strains in the Apical Anaerobic Co-Culture Model Using a Combination of Cell and Bacterial Culture Media

Combinations of anaerobic M199 TEER medium and BHI medium were used for co-culturing Caco-2 cells and the three *F. prausnitzii* strains in the apical anaerobic co-culture model and it was determined if the bacteria were growing in these adapted conditions. The media compositions chosen were anaerobic M199 TEER supplemented with 25% or 50% of anaerobic BHI (referred to as 25% and

50% BHI). This adapted medium was only used for the apical compartment of the co-culture chamber; the basal compartments had aerobic M199 TEER medium. The bacterial suspensions were diluted with 25% and 50% BHI to a concentration of 2.4×10^7 bacterial cells/mL as described previously. The viability of the *F. prausnitzii* strains in the co-culture model was assessed by determining the viable colony forming units (CFU) of the bacteria at 0 h and 12 h after co-culture with the Caco-2 cells in both the cell supernatant and cell lysate. Duplicate ten-fold serial dilutions of the bacterial solutions were made in 96-well plates for each sample. Three 20-µL spots of each dilution were pipetted onto anaerobic BHI agar, allowed to dry, and then incubated anaerobically for 48 h at 37 °C. Spots with between 10 and 100 colonies were counted and the CFU were calculated. This experiment was done in five blocks, with three replicates per treatment group per block.

2.8. TEER Assay Using F. prausnitzii in Different Apical Media

To assess the effects of *F. prausnitzii* on TEER, each of the strains (A2-165, ATCC 27768, or HTF-F) was co-cultured with differentiated Caco-2 monolayers in the apical anaerobic co-culture model using 25% or 50% BHI as apical medium. There were eight treatment groups: the two control media (25% and 50% BHI) and the three *F. prausnitzii* strains diluted with the two different media. The TEER was recorded hourly for 12 h. The experiment was done in five blocks, with three replicates per treatment in each block.

2.9. TEER Assay Using Live and UV-Killed F. prausnitzii

The effect of both live and UV-killed *F. prausnitzii* on TEER across Caco-2 monolayers in the apical anaerobic co-culture model was determined using 50% BHI as the apical medium. The bacterial suspensions of the three *F. prausnitzii* strains were prepared for the co-culture experiments as follows. A 2-mL aliquot was removed from each bacterial suspension, and transferred to wells of a six-well plate. With the lid removed, the plate was placed on ice and the bacterial suspension treated with a UV lamp (UVP 3UV-38, Bio-Strategy Ltd., Auckland, New Zealand). The bacteria were exposed for 15 min to UVC light. The UV-treated bacteria were plated on anaerobic BHI agar plates and incubated anaerobically for 48 h at 37 °C to confirm that the bacteria were dead. For the TEER experiment there were seven treatment groups: the control medium (50% BHI), the three live *F. prausnitzii* strains and the three UV-killed *F. prausnitzii* strains. The experiment was done in three blocks, with three replicates per treatment per block.

2.10. Statistical Analysis

The statistical analyses were performed using SAS (SAS/STAT version 9.3; SAS Institute Inc., Cary, NC, USA). An analysis of repeated measures was conducted to test the effect of the treatment and time and their interaction on the response variables (change in TEER or change in OD_{600nm}). The most appropriate covariance structure of the mixed model for each response variable was selected after fitting the models by restricted maximum likelihood method and comparing them using the log-likelihood ratio test. When an interaction was not significant it was removed from the model. An analysis of variance (ANOVA) was also conducted to test the effect of the BHI concentration on the viability of Caco-2 cells in apical anaerobic conditions. When the F-value of the analyses were significant ($p < 0.05$), the means were compared using Tukey tests. A two independent samples *t*-test procedure was performed to compare the viability of the three *F. prausnitzii* strains before and after the co-culture with Caco-2 cells in the apical anaerobic co-culture model. Additionally, a paired *t*-test was conducted to compare the viability of the three *F. prausnitzii* strains before and after the incubation in 50% BHI. For all the analyses the model assumptions (e.g., normal distribution and the homogeneity of variance) was evaluated using the Output Delivery System (ODS) graphics in SAS. When the response variable did not fulfil these assumptions a $\log_{10}$ transformation was performed to reach these assumptions.

3. Results and Discussion

3.1. F. prausnitzii Did Not Grow in an Anaerobic Cell Culture Medium

For bacterial–mammalian cell co-culture experiments, it is common to harvest bacterial cells by centrifugation and then resuspend the bacteria in mammalian cell culture media [23,25,26]. Studies have shown that there was no difference in the viability for strains of *Escherichia coli*, *Salmonella typhimurium*, and *Lactobacillus fructosus* in mammalian and bacterial culture medium [25]. In contrast to this, our results showed that the three *F. prausnitzii* strains did not grow in the anaerobic cell culture medium over the period of 24 h of incubation at 37 °C, as shown in the change in the OD_{600nm} graph (Figure 2). There were no significant differences in the change of the OD_{600nm} until 8 h of incubation for each of the three strains. However, after 24 h of incubation, all three strains showed a decrease in the OD_{600nm} compared to the previous time points (0 to 8 h; $p < 0.05$). Therefore, it concluded it was necessary to supplement the cell culture medium to stimulate the growth and metabolic activity of *F. prausnitzii.*

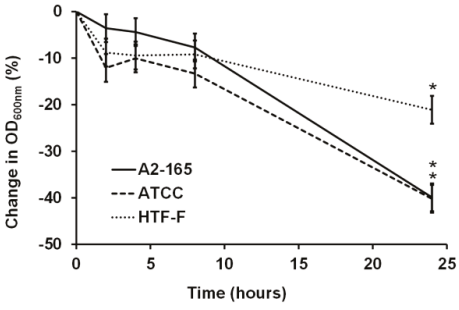

Figure 2. Normalized change in OD_{600nm} (%) of the three *F. prausnitzii* strains in an anaerobic cell culture medium. The three *F. prausnitzii* strains (A2-165, ATCC 27768, and HTF-F) in stationary phase were resuspended in anaerobic M199 TEER, incubated at 37 °C, and the OD_{600nm} was measured over 24 h. The graph shows the mean values (±SEM; $n = 3$) after normalizing by the OD_{600nm} at time 0 h for each of the three *F. prausnitzii* strains over 24 h. * Change in OD_{600nm} different compared to previous time point for the same strain ($p < 0.05$).

When considering which supplements should be added to the medium first the nutrient composition of M199 TEER was investigated. Iron and vitamin K_1 were identified as nutrients that may be lacking in this medium. Bacterial culture media often contain hemin as the iron source [1,23,27], whereas ferric nitrate is the only iron source in the M199 cell culture medium. This may not be optimum for *F. prausnitzii* since bacteria have developed heme acquisition systems to obtain iron from host heme-sequestering proteins [28]. Similarly, bacterial culture media often contain vitamin K_1 [27,29], which bacteria convert into the vitamin K_2 required for electron transport processes during anaerobic respiration [30]; whereas M199 only contains vitamin K_3 (menadione), a synthetic type of vitamin K, which is a provitamin that requires conversion to menaquinone-4 in order to be active [23,31]. The bioavailability of vitamin K_3 may therefore be lower for *F. prausnitzii* compared to other vitamin K sources.

Further investigation in the literature indicated that *F. prausnitzii* likely requires a complex medium to grow. A recent study used a combined approach of computational modeling, in vitro experiments, metabolomic analysis and genomic analysis to identify the metabolic capabilities of *F. prausnitzii* A2-165 and to develop a chemically defined medium, CDM1, for this bacterium [32]. However, CDM1 did not enable growth of *F. prausnitzii* A2-165, and even when enriched with additional vitamins, amino acids,

and bases, *F. prausnitzii* A2-165 growth was still less than on the bacterial medium YCFAG [32]. Therefore, it was decided to supplement cell culture medium with a complex bacterial culture medium.

3.2. Bacterial Medium Did Not Affect Caco-2 Viability, but Reduced TEER at High Concentrations

As our previous experience indicated that bacterial medium can be detrimental to mammalian cells, initially the effect of the bacterial medium on the Caco-2 cell monolayers was investigated. The viability of the Caco-2 cells treated with anaerobic M199 TEER mixed with anaerobic BHI in different ratios (0%, 25%, 50%, 75%, or 100% BHI) in apical anaerobic conditions (co-culture chamber inside the anaerobic workstation) over 12 h was determined using the neutral red viability assay (Figure 3a). There was no significant difference ($p = 0.07$) between the viability of Caco-2 cell monolayers treated with 0%, 25%, 50%, 75%, or 100% BHI in the apical anaerobic co-culture model.

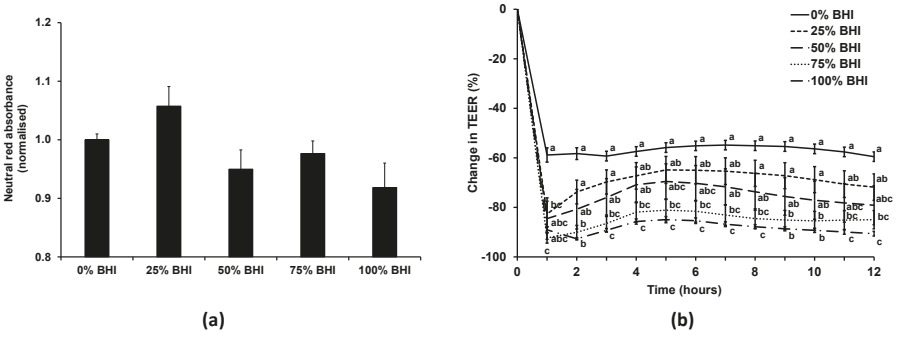

Figure 3. Effect of combining cell and bacterial culture medium on the viability and TEER of Caco-2 cells in the apical anaerobic co-culture model. The apical compartment of Caco-2 cell monolayers were exposed to non-supplemented anaerobic M199 TEER medium (0% BHI), or M199 TEER medium supplemented with increasing concentrations of anaerobic BHI medium (25%, 50%, 75%, or 100% BHI). (**a**) Viability (mean ± SEM; $n = 20$) of Caco-2 cells after 12 h incubation. Neutral red absorbance was normalized by adjusting the 0% BHI exposed cells to 1. Viability of the Caco-2 cells was unchanged as a consequence of differing culture medium composition ($p = 0.07$). (**b**) Mean (±SEM; $n = 20$) change in TEER as a percentage of initial TEER across Caco-2 cell monolayers over 12 h for each medium. There was a significant interaction between the time and the BHI concentration on the change in TEER ($p < 0.01$). Treatments that do not share the same letters are significantly different ($p < 0.05$).

The effect of the different M199 TEER-BHI media combinations on TEER across Caco-2 monolayers in apical anaerobic conditions was tested over 12 h (Figure 3b). There was a significant interaction between the time and the BHI concentration on the change in TEER ($p < 0.01$). When treated with 25% BHI, the normalized TEER across Caco-2 cell monolayers was significantly lower at one hour after adding the treatments compared to 0% BHI, however after that time point onwards there was no difference between 0% and 25% BHI. Caco-2 cell monolayers treated with 50% BHI showed no difference in TEER to Caco-2 cells treated with 0% BHI across all time points. In contrast, when 75% or 100% BHI were added to the apical side of Caco-2 cell monolayers, the normalized TEER values were significantly lower compared to cells treated with 0% BHI across all time points ($p < 0.05$).

The TEER of the Caco-2 cells treated with 50% BHI reached a plateau of approximately 300 $\Omega \cdot cm^2$ following the initial drop in TEER, whereas those with cell culture medium had TEER values that plateaued at approximately 600 $\Omega \cdot cm^2$. The Caco-2 cell monolayers treated with this combination of bacterial and cell culture medium had TEER values that were more comparable to those reported for human colon tissues (100 to 300 $\Omega \cdot cm^2$) [33,34].

3.3. Two F. prausnitzii Strains Grew in 50% Bacterial Medium When Co-Cultured with Caco-2 Cells

Based on the results above, the viability of the *F. prausnitzii* strains was tested using the anaerobic M199 TEER: BHI ratio (1:1), which was referred to as 50% BHI. The CFUs of the three *F. prausnitzii* strains were determined before and after their incubation in 50% BHI over 12 h (Figure 4a). The number of CFU of *F. prausnitzii* A2-165 increased by 1.7 log ($p < 0.001$), while the other two strains showed no significant differences between the CFU before and after 12 h of incubation in 50% BHI.

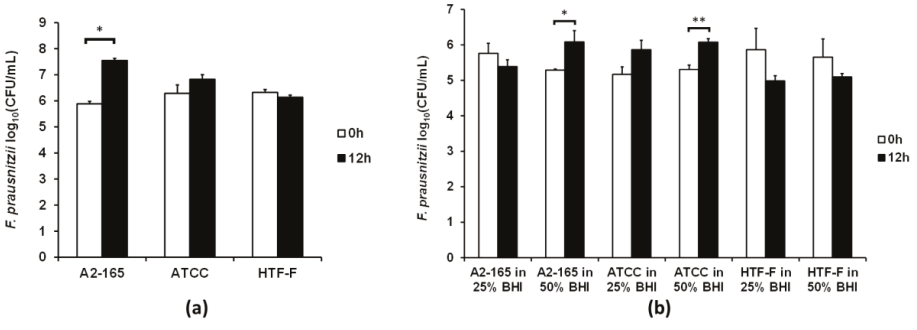

Figure 4. Viability of the three *F. prausnitzii* strains in a cell culture medium supplemented with bacterial culture medium. (**a**) In Hungate culture tubes. The three *F. prausnitzii* strains in stationary phase were resuspended in anaerobic M199 TEER: BHI (1:1) (referred to as 50% BHI) and incubated anaerobically at 37 °C. The graph shows the mean (±SEM; $n = 3$) $\log_{10}$(CFU/mL) of the three *F. prausnitzii* strains before and after 12 h of incubation in 50% BHI. * Mean $\log_{10}$(CFU/mL) differ between 0 and 12 h at $p < 0.05$. (**b**) In the apical anaerobic co-culture model with Caco-2 cells. The three *F. prausnitzii* strains were co-cultured with Caco-2 cells using two different media on the apical side of the Caco-2 cell monolayer (25% and 50% BHI). The graph shows the mean (±SEM; $n = 4$ and 8 for time 0 and 12 h, respectively) $\log_{10}$(CFU/mL) of the bacteria at 0 and 12 h of incubation with Caco-2 cells in the co-culture model. Mean $\log_{10}$(CFU/mL) differ between 0 and 12 h at * $p < 0.05$ and ** $p < 0.01$.

It was proposed that the *F. prausnitzii* strains may grow better in the presence of intestinal epithelial cells. Therefore the three strains were co-cultured with differentiated Caco-2 cell monolayers in the apical anaerobic co-culture model using M199 TEER supplemented with 25% or 50% BHI medium on the apical side of the cell monolayers. These medium compositions were chosen as they did not compromise the TEER and viability of the Caco-2 cell monolayers. The viability of the bacterial strains was determined by comparing the CFU before and after 12 h of co-culture with Caco-2 cells. No bacteria were able to be cultured from the Caco-2 cell lysate, therefore indicating that none of the three strains adhered to the Caco-2 cells. When 25% BHI was used at the apical side of the Caco-2 cell monolayer none of the three *F. prausnitzii* strains had an increase in CFU (Figure 4b). The number of CFU of both *F. prausnitzii* A2-165 and *F. prausnitzii* ATCC 27768 increased by 0.8 log after the 12 h of incubation ($p < 0.05$) when 50% BHI was used as the apical culture medium. However, there was no significant difference in CFU of *F. prausnitzii* HTF-F at 0 and 12 h of co-culture with Caco-2 cells when 50% BHI was used as the apical culture medium.

In agreement with published results using a simple dual-environment co-culture model [35], the presence of the Caco-2 cells improved the growth of the *F. prausnitzii* strains. This may be due to the presence of mucins. Though Caco-2 cells do not express mucin-2, the predominant mucin in the gastrointestinal tract, they express mucins 3 and 5A/C [36,37]. Furthermore, *F. prausnitzii* may benefit from the oxygen gradient close to the Caco-2 cell monolayer. *F. prausnitzii* uses an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen [38]. Small amounts of oxygen may diffuse from the aerobic basal compartment of the apical anaerobic co-culture model through the Caco-2 cell monolayer to the apical side. The *F. prausnitzii* strains may be able to use riboflavin

(vitamin B_2), one component of M199, for its extracellular electron transfer, which may benefit growth at this oxic–anoxic interphase [39].

3.4. Live F. prausnitzii Did Not Alter TEER across Caco-2 Cells

To determine whether live *F. prausnitzii* was able to improve TEER, differentiated Caco-2 monolayers were co-cultured with the three *F. prausnitzii* strains (A2-165, ATCC 27768, or HTF-F) in the apical anaerobic co-culture model using 25% or 50% BHI as apical medium. These apical media were chosen as one of them enabled growth of *F. prausnitzii* A2-165 and ATCC 27768 in co-culture with Caco-2 cells (50% BHI), whereas the other medium did not enable growth of any of the three strains (25% BHI) in the previous experiment. It could therefore be determined if change in TEER over time across Caco-2 monolayers differed when co-cultured with growing or non-growing bacteria. The interaction between the bacterial treatment and time was significant for both media (Figure 5; $p < 0.001$). However, there were no differences between the TEER of Caco-2 cells treated with either the 25% or 50% BHI medium or the three *F. prausnitzii* strains in the respective medium for each time point ($p > 0.05$).

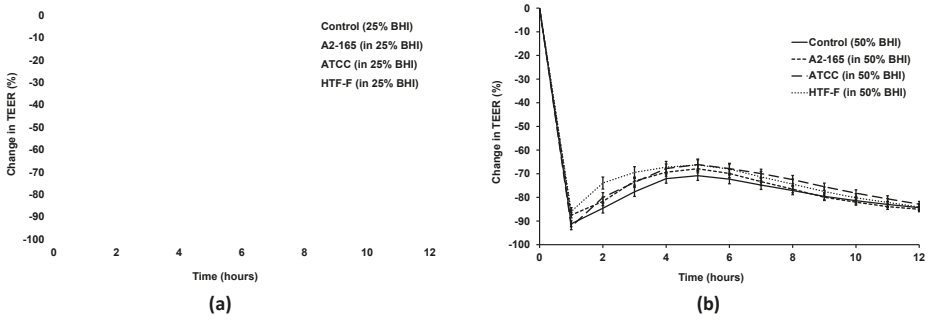

Figure 5. Change in TEER across Caco-2 monolayers co-cultured with live *F. prausnitzii* using 25% and 50% BHI as apical medium. Differentiated Caco-2 monolayers were co-cultured with the three *F. prausnitzii* strains for 12 h in the apical anaerobic co-culture model using 25% or 50% BHI as apical medium. The interaction between the treatment and time was significant for the change in TEER ($p < 0.001$). The graphs show the mean (±SEM; $n = 12$) change in TEER across Caco-2 monolayers when using (**a**) 25% BHI and (**b**) 50% BHI as apical medium. No differences were determined between the two control media and the three *F. prausnitzii* strains in the respective medium at any time point ($p > 0.05$).

Based on these results, it is likely that the maintenance of colonic health by *F. prausnitzii* is not mediated through enhancement of epithelial barrier integrity. Instead, the beneficial effects of *F. prausnitzii* may be due to it supporting immune homeostasis, as previously shown [19]. However, it is possible that metabolites secreted by live *F. prausnitzii* may require an increased treatment time than that undertaken here to exert their effects on intestinal barrier integrity. Specific probiotics and commensal bacteria influence intestinal barrier integrity through secreted metabolites [40–42], for example butyrate, a short chain fatty acid produced during bacterial fermentation, enhanced the barrier integrity through the regulation of tight junction assembly [43]. However, the barrier enhancing properties of butyrate occurred only after 24 h of incubation and TEER values reached maximum levels between 48 to 72 h [43]. In order to determine the effects of *F. prausnitzii* on TEER over a prolonged incubation time in this model, further validation studies would be necessary to ensure survival of the Caco-2 cells since the initial validation studies were performed for 12 h [23]. In addition, although *F. prausnitzii* is known as one of the major butyrate producers in the colon [1], it is unknown whether it

produced butyrate when using 50% BHI as apical medium; therefore, further studies could analyze the composition of the apical medium after the co-culture of *F. prausnitzii* with Caco-2 cells.

3.5. UV-Killed F. prausnitzii Decreased TEER

Caco-2 cells in the apical anaerobic co-culture model were co-cultured with live or UV-killed *F. prausnitzii* (strains A2-165, ATCC 27768, or HTF-F) in 50% BHI for 12 h (Figure 6). The interaction between the treatment and time was not significant, so it was removed from the statistical analysis. There was a significant treatment effect ($p = 0.002$), so between-treatment comparisons were warranted. No differences were observed between the TEER across Caco-2 monolayers exposed to the bacterial treatments and the untreated controls ($p > 0.05$). However, the TEER across Caco-2 monolayers co-cultured with live or UV-killed *F. prausnitzii* HTF-F was significantly different ($p < 0.05$) with higher TEER values recorded for cells treated with live bacteria. It is likely that this detrimental effect of the UV-killed bacteria is due to bacteria surface proteins interacting with the host cells. It is also possible that this negative effect is mitigated by metabolites produced by the live bacterium. Live *F. prausnitzii* may also maintain barrier integrity of Caco-2 monolayers through the activation of innate signaling. For example, commensal induced TLR2 signaling was shown to enhance intestinal barrier function and thereby limit mucosal inflammation [44,45]. We have previously shown that live *F. prausnitzii* induced higher TLR2 activation compared to dead *F. prausnitzii* [19], which may cause the barrier-protecting properties.

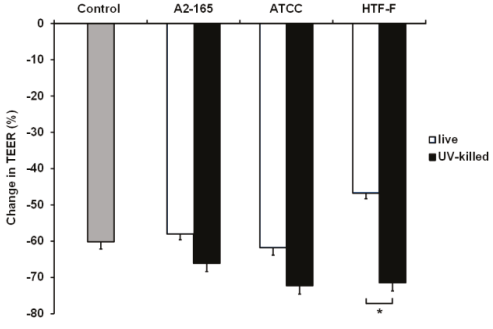

Figure 6. Change in TEER across Caco-2 monolayers co-cultured with live or UV-killed *F. prausnitzii* using 50% BHI as apical medium. Caco-2 monolayers were co-cultured with live or UV-killed *F. prausnitzii* (strains A2-165, ATCC 27768, or HTF-F). The TEER across the Caco-2 monolayers was recorded hourly over 12 h. The interaction between the treatment and time was not significant and so was removed from the statistical model. There was a significant treatment effect on the change in TEER ($p = 0.002$). The graph shows the mean (±SEM; $n = 9$) change in TEER across Caco-2 monolayers co-cultured with live or UV-killed *F. prausnitzii*. * indicates significant difference in TEER ($p < 0.05$).

4. Conclusions

In conclusion, this research resulted in further optimization of the novel dual-environment co-culturing system, which will enable the investigation of new probiotics originating from indigenous beneficial bacteria. Contrary to our hypothesis, actively growing *F. prausnitzii* (strains A2-165, ATCC 27768, or HTF-F) did not improve intestinal barrier integrity, as measured by the TEER of Caco-2 cells. This result indicates that the benefits of *F. prausnitzii* are likely not due to it altering intracellular tight junction integrity.

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Author Contributions: Eva Maier, Rachel C. Anderson and Nicole C. Roy conceived and designed the experiments; Eva Maier performed the experiments and analyzed the data; Eva Maier and Rachel C. Anderson wrote the paper. All authors commented, edited, approved and are responsible for the final version of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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A Metagenomic and in Silico Functional Prediction of Gut Microbiota Profiles May Concur in Discovering New Cystic Fibrosis Patient-Targeted Probiotics

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Abstract: Cystic fibrosis (CF) is a life-limiting hereditary disorder that results in aberrant mucosa in the lungs and digestive tract, chronic respiratory infections, chronic inflammation, and the need for repeated antibiotic treatments. Probiotics have been demonstrated to improve the quality of life of CF patients. We investigated the distribution of gut microbiota (GM) bacteria to identify new potential probiotics for CF patients on the basis of GM patterns. Fecal samples of 28 CF patients and 31 healthy controls (HC) were collected and analyzed by 16S rRNA-based pyrosequencing analysis of GM, to produce CF-HC paired maps of the distribution of operational taxonomic units (OTUs), and by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) for Kyoto Encyclopedia of Genes and Genomes (KEGG) biomarker prediction. The maps were scanned to highlight the distribution of bacteria commonly claimed as probiotics, such as bifidobacteria and lactobacilli, and of butyrate-producing colon bacteria, such as *Eubacterium* spp. and *Faecalibacterium prausnitzii.* The analyses highlighted 24 OTUs eligible as putative probiotics. Eleven and nine species were prevalently associated with the GM of CF and HC subjects, respectively. Their KEGG prediction provided differential CF and HC pathways, indeed associated with health-promoting biochemical activities in the latter case. GM profiling and KEGG biomarkers concurred in the evaluation of nine bacterial species as novel putative probiotics that could be investigated for the nutritional management of CF patients.

Keywords: cystic fibrosis; gut microbiota profiling; KEGG prediction-tailored probiotic design

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive condition occurring among people with European origins, which is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR mutation leads to the failure or the absence of functional CFTR proteins at the apical membrane of epithelial cells in several body systems [1]. The CFTR protein, in addition to functioning as a chloride channel, can also affect bicarbonate transport. Protein mutations cause the formation of viscous and dehydrated mucus followed by the establishment of aberrant mucosa in

the lungs and digestive tract. This condition increases the risk of recurrent and chronic pulmonary infection and inflammation, pancreatic insufficiency (PI), CF-related liver disease, and diabetes [2].

The recurrent destructive airway infections, determined by the progressive inflammatory lung diseases, represent the principal cause of mortality, morbidity, and altered quality of life in CF patients, resulting in respiratory failure in 90% of patients with CF [3].

To reduce pulmonary exacerbation, patients are subjected to an antibiotic therapy which leads to the modification of the gut microbiota (GM) [4]. The CFTR mutations also lead to the alteration of intestinal permeability, determining an impaired composition and function of the intestinal barrier. The production of immune mediators is altered alongside with mucosal inflammation, triggering an increase in the concentrations of fecal calprotectin and rectal nitric oxide [5]. The mutations can also affect the body's endocrine, neural, and immune systems [6]. This clinical status also leads to a compromised nutritional status associated with the severity of CF disease, which unfortunately affects the quality of life and life expectancy [7].

The maintenance of an optimal nutritional status may ameliorate the quality of life of CF patients, especially during rehabilitation programs and therapies targeting the respiratory infections [8]. GM modulation induced by nutritional intervention may have implications in the management of CF-related malnutrition and comorbidities, since diet is perhaps the most modifiable factor that shapes microbiota profiles [9].

The diet-driven functional evolution of the GM has been thoroughly discussed in mammalian species, starting at neonatal age [10,11]. The maintenance of microbiota eubiosis seems to contribute to the prevention and clarification of complex disease phenotypes [12]. In particular, the administration of probiotics also contributes to GM eubiosis maintenance and restoration in CF patients [13].

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [14]. They colonize the intestine and affect either microbiota composition or function, acting on the host epithelial and immunological responses [15], reducing intestinal inflammation, and hence improving the intestinal functions at clinical and biochemical levels [16–18] even when they were altered by an antibiotic therapy [17]. Probiotics have been used with positive outcomes in childhood gastroenteritis, atopic diseases, and *Helicobacter pylori* infection [19]. Specifically, the administration of *Lactobacillus* GG can decrease the incidence of exacerbations and reduce the intestinal inflammation in CF patients, as reported by Bruzzese [20]. Potential mechanisms of action for probiotics in CF include their influence on gut motility and intestinal barrier function and the inhibition of pathogenic bacteria colonization [2].

There is evidence that probiotic administration in these patients reduces pulmonary exacerbation rate and hospital admission [21]. Clinical trials on probiotic administration in CF patients are on the rise [17,22], but there is no evidence of an optimal patient-tailored probiotics regimen to be administered for this chronic disease.

The aim of this study was to evaluate the distribution of *Bifidobacterium* spp., *Lactobacillus* spp., *Eubacterium* spp., and *Faecalibacterium prausntizii* in the GM of CF and healthy subjects. We focused on species commonly claimed as targets for the design of novel probiotics [23]. According to a targeted metagenomics analysis and functional prediction of related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, we propose potential probiotics species for CF management.

2. Material and Methods

2.1. Patients

This study was conducted on 28 consecutive CF patients aged 1 to 6 years (average age 3.5 years, SD $\pm$ 1.69; 11 males and 17 females), recruited at the Cystic Fibrosis Unit of the Bambino Gesù Children's Hospital (OPBG, Rome, Italy) over one year (2012). The diagnosis of CF was made on the basis of the results of a pathological sweat test (chloride > 60 mmol/L, reference value), as described by Gibson and Cooke [24], or by the presence of two CF-causing mutations in the CFTR gene [25].

The study protocol was approved by the OPBG Ethics Research Committee (protocol No. 534/RA), and was conducted in accordance with the Declaration of Helsinki (as revised in Seoul, Korea, October 2008). A signed informed consent was obtained from the parents of the enrolled subjects. The patients were age-matched with 31 healthy controls (HC) screened by means of a survey of the OPBG Human Microbiome Unit on pediatric gut microbiota programming.

Inclusion criteria for HC were: absence of any inflammatory, infectious, and chronic diseases at the time of the microbiota analysis and no antibiotic and pre-probiotic intake in the previous two months.

For CF patients, the inclusion criteria consisted of being recruited under clinical stability (i.e., absence of infectious exacerbation of pulmonary symptoms) and no pre-probiotic intake in the previous two weeks.

2.2. Anamnestic and Laboratory Features

Age, gender, and body mass index (BMI) (for patients over 2 years of age) or Z-score (Weight/Length (W/L) of patients under 2 years of age) were collected for both CF patients and HC, whereas sweat chloride test values, pancreatic status (PS), and antibiotic data for chronic regimen were collected only for CF patients (Table 1).

Table 1. Cystic fibrosis (CF) Patients and healthy controls (HC) features.

Subjects	Males	Mean Age	Mean W/L or BMI Z-Score *	Pancreatic Insufficiency: Yes/Not	Mean Value of Sweat Test	Chronic Use of Antibiotic: Yes/No	Disease Severity: Mild/Severe
CF	11/28 (39%)	3.5	±0.9	22/6 79/21 (%)	93	12/16 43/57 (%)	4/24 14/86 (%)
HC	20/31 (64.5%)	3.06	±0.51	nda **	nda	nda	nda

* BMI/Z-Score: body mass index (BMI) (for patients over 2 years of age) or Z-score (Weight/Length (W/L) (for patients under 2 years of age); ** nda: no data associated.

2.3. DNA Extraction and Next Generation Sequencing (NGS) Analysis

Fecal samples (59) were collected from each subject during clinical examination and stored until metagenomics analysis. The genomic DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The V1–V3 region (520 bp) of the 16S ribosomal RNA locus was amplified for pyrosequencing analysis using a 454-Junior Genome Sequencer (Roche 454 Life Sciences, Branford, CT, USA) according to Del Chierico et al. [26]. The nucleotide barcodes, added in forward primers, were composed of 8 unique nucleotides (Roche 454 Life Sciences). The polymerase chain reactions were performed using Hi-Fi Polymerase Chain Reaction (PCR) Taq polymerase (FastStart™ High Fidelity PCR System, dNTPack, Roche Diagnostics, Mannheim, Germany), guaranteeing high specificity, sensitivity, and accuracy during PCR amplification.

2.4. Statistical Analysis

Reads were analyzed with Qiime 1.8 (Quantitative Insights Into Microbial Ecology, http://qiime.org/1.4.0/) using the default pipeline [27]. After demultiplexing, reads with an average quality score lower than 25, shorter than 300 bp, and with an ambiguous base calling were excluded from the analysis to guarantee a higher level of accuracy in terms of detection of the operational taxonomic units (OTUs). Sequences that passed the quality filter were denoised [28], and singletons were excluded. The denoised sequences *were chimera-checked by identify_chimeric_seqs.py*.

To characterize the taxonomic structure of the samples, the sequences were organized into OTUs by clustering at a threshold of 97% pairwise identity and by classifying the representative sequences using the Greengenes 13_8 database [29]. The representative sequences were submitted to PyNAST for sequence alignment [30] and to UCLUST for sequence clustering [31].

The OTU Kruskal–Wallis tests were performed by QIIME software (http://qiime.org/1.4.0/) using "group_significance.py" script [32]. The Kruskal-Wallis test was performed on OTU distribution with False Discovery Rate (FDR) correction (p-value ≤ 0.1). To gain more insight into the metagenomics-based function of the microbiome of the CF patients and HC, the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) v1.1.0 tool was used [33], and the resulting function prediction was analyzed using the HUMAnN2 v0.99 program to get KEGG pathways (http://huttenhower.sph.harvard.edu/humann2) [34]. To find possible OTUs and KEGG biomarkers associated with CF and HC, a linear discriminant effect size (LEfSe) analysis was performed [35] with the α value of the statistical test equal to 0.05 and the logarithmic Linear Discriminant Analysis (LDA) score threshold equal to 2.0.

3. Results

3.1. Putative Probiotic Distribution in the GM Profiles

By targeted metagenomics, a total of 316,000 reads was obtained with an average of 5356 reads/sample and an average length of 487 bp. Genus-level comparisons were performed on 24 OTUs belonging to *Bifidobacterium* spp., *Lactobacillus* spp., *Eubacterium* spp., and *F. prausnitzii* chosen from a total dataset of 165 OTUs, considering their putative probiotic role.

The profiling of targeted metagenomic sequencing pointed out a distribution of 11 bacterial species prevalently associated with the GM of the CF patients (Figure 1, Panel A), and 9 species prevalently associated with the GM of the HC (Figure 1, Panel B) (Table 2). The Kruskal–Wallis test identified a statistically significant difference for *F. prausnitzii* distribution between CF patients and HC, highlighting a higher relative abundance in HC.

On the contrary, the remaining 4 OTUs, namely, *Lactobacillus brevis*, *L. delbrueckii*, *L. helveticus*, and *Eubacterium cylindroides* were comparably distributed in the GM profiles of the CF patients and HC (Figure 1, Panel C).

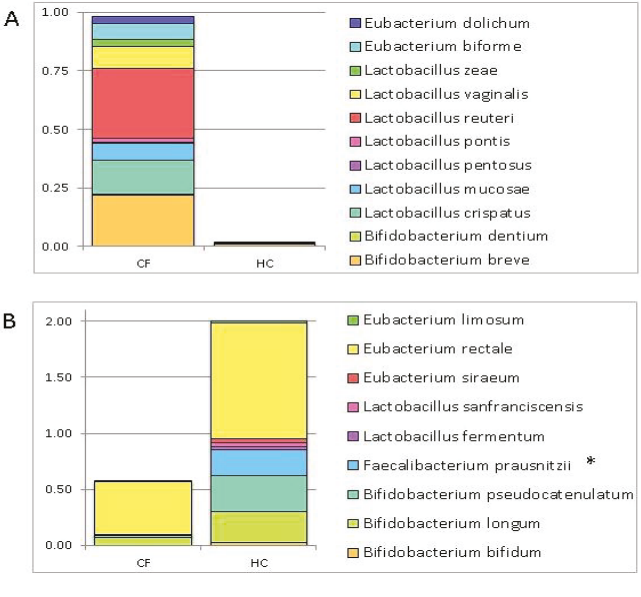

Figure 1. *Cont.*

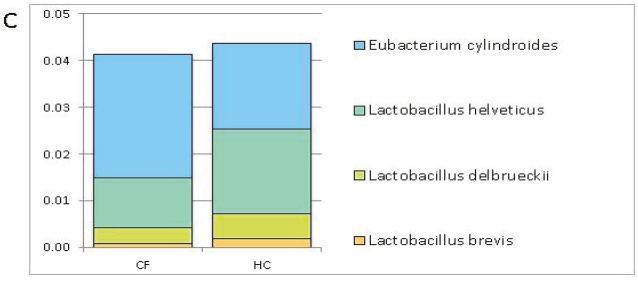

Figure 1. Histograms of the relative abundance of 24 selected operational taxonomic units (OTUs) in the gut microbiota (GM) patterns of cystic fibrosis (CF) patients and healthy controls (HC). These OTUs were chosen for their putative probiotic role. The histograms show the relative abundance of the searched putative probiotic bacteria scanned through the GM patterns of the CF patients and HC. (**Panel A**): 9 OTUs prevalently distributed in the GM profile of the CF subjects (relative abundance > 0.001); (**Panel B**): 11 OTUs prevalently distributed in the GM profile of the HC (relative abundance > 0.02). *Fecalibacterium prausnitzii* shows a statistically significant value False Discovery Rate (FDR) adjusted p value ≤ 0.1); (**Panel C**): 4 OTUs comparably distributed in the GM profiles of the CF patients and HC.

Table 2. List of 20 bacteria prevalently associated with the GM profile of HC and CF patients. These OTUs were chosen for their putative probiotic role.

Bacteria	Group of Subjects
Bifidobacterium bifidum	HC
Bifidobacterium longum	
Bifidobacterium pseudocatenulatum	
Faecalibacterium prausnitzii	
Lactobacillus fermentum	
Lactobacillus sanfranciscensis	
Eubacterium siraeum	
Eubacterium rectale	
Eubacterium limosum	
Bifidobacterium breve	CF
Bifidobacterium dentium	
Lactobacillus crispatus	
Lactobacillus mucosae	
Lactobacillus pentosus	
Lactobacillus pontis	
Lactobacillus reuteri	
Lactobacillus vaginalis	
Lactobacillus zeae	
Eubacterium biforme	
Eubacterium dolichum	

3.2. Metabolic Pathways of Probiotics

To better define the metabolic role of the detected putative probiotic species, a supervised comparison of CF patients' and HC's KEGGs was inferred by LEfSe on the 24 OTU matrix.

The predicted microbial function highlighted differences in metabolic pathways associated with the 24 selected OTUs (Figure 2). In particular, 24 pathways resulted associated with CF and 39 with HC (Table 3).

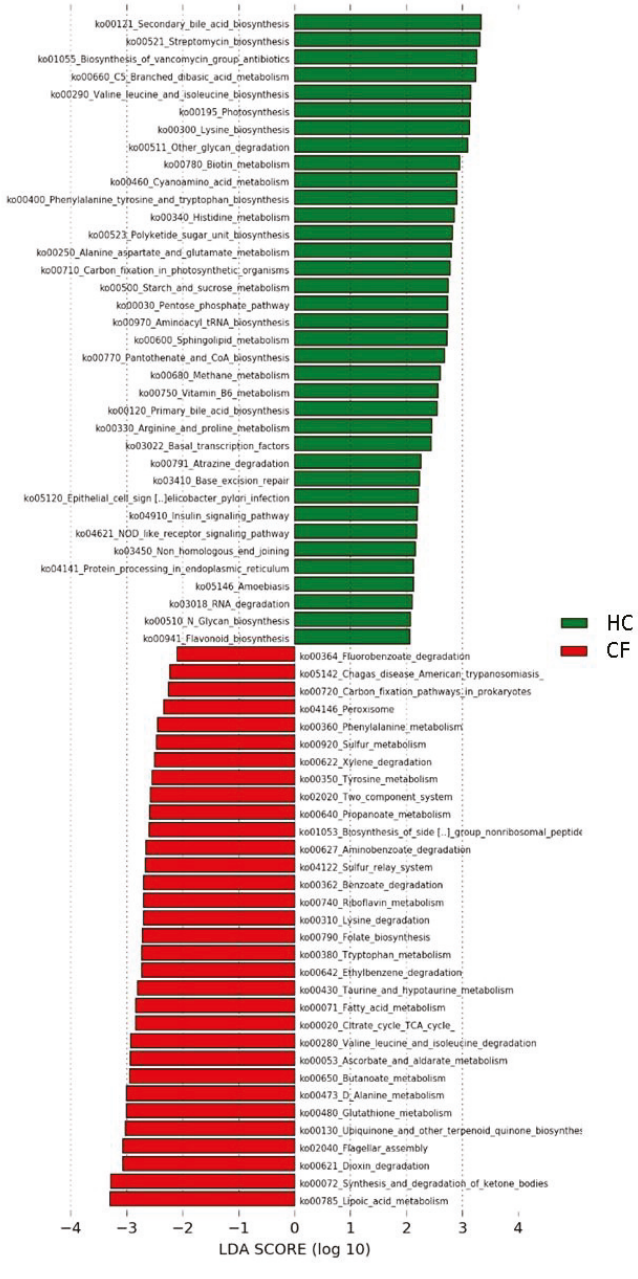

Figure 2. Kyoto Encyclopedia of Genes and Genomes (KEGG) biomarkers inferred from the whole set of 24 OTUs of putative probiotic bacteria scanned through the GM patterns of CF patients and HC subjects. A linear discriminant effect size (LeFse) analysis was performed ($\alpha = 0.05$, logarithmic Linear Discriminant Analysis (LDA) score threshold = 2.0).

Table 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways associated with HC and CF subjects.

KEGG Pathways	Class *	Subclass	Group	KEGG Pathways	Class	Subclass	Group
Carbon fixation in photosynthetic organisms	1	Energy metabolism	HC	Lysine degradation	1	Amino acid metabolism	CF
Alanine aspartate and glutamate metabolism		Amino acid metabolism		Phenylalanine metabolism			
Arginine and proline metabolism				Tryptophan metabolism			
Histidine metabolism				Tyrosine metabolism			
Lysine biosynthesis				Valine leucine and isoleucine degradation			
Phenylalanine tyrosine and tryptophan biosynthesis				Ascorbate and aldarate metabolism		Carbohydrate metabolism	
Valine leucine and isoleucine biosynthesis				Butanoate metabolism			
Flavonoid biosynthesis		Biosynthesis of other secondary metabolites		Citrate cycle TCA cycle			
Streptomycin biosynthesis				Carbon fixation pathways in prokaryotes		Energy metabolism	
C5 Branched dibasic acid metabolism		Carbohydrate metabolism		Sulfur metabolism			
Pentose phosphate pathway				Fatty acid metabolism		Lipid metabolism	
Propanoate metabolism							
Starch and sucrose metabolism							
Methane metabolism		Energy metabolism		Synthesis and degradation of ketone bodies			
Photosynthesis							
N Glycan biosynthesis		Glycan biosynthesis and metabolism		Folate biosynthesis		Metabolism of cofactors and vitamins	
Other glycan degradation				Lipoic acid metabolism			
Primary bile acid biosynthesis		Lipid metabolism		Ubiquinone and other terpenoid quinone biosynthesis			
Secondary bile acid biosynthesis							
Sphingolipid metabolism							
Biotin metabolism		Metabolism of cofactors and vitamins		Glutathione metabolism		Metabolism of other amino acids	
Pantothenate and CoA biosynthesis				Taurine and hypotaurine metabolism			
Riboflavin metabolism							
Vitamin B6 metabolism							
Cyanoamino acid metabolism		Metabolism of other amino acids		Biosynthesis of siderophore group nonribosomal peptides		Metabolism of terpenoids and polyketides	
D Alanine metabolism							
Biosynthesis of vancomycin group antibiotics		Metabolism of terpenoids and polyketides		Aminobenzoate degradation		Xenobiotics biodegradation and metabolism	
Polyketide sugar unit biosynthesis				Benzoate degradation			
Atrazine degradation		Xenobiotics biodegradation and metabolism		Dioxin degradation			
Protein processing in endoplasmic reticulum	2	Folding, sorting and degradation		Ethylbenzene degradation			
RNA degradation				Fluorobenzoate degradation			
Base excision repair		Replication and repair		Xylene degradation			
Non homologous end joining				Sulfur relay system	2	Folding, sorting and degradation	
Basal transcription factors		Transcription		Two component system	3	Signal transduction	
Aminoacyl tRNA biosynthesis		Translation					

Table 3. Cont.

KEGG Pathways	Class *	Subclass	Group	KEGG Pathways	Class	Subclass	Group
Insulin signaling pathway	5	Endocrine system		Flagellar assembly	4	Cell motility	
Nucleotide oligomerization domain (NOD) like receptor signaling pathway		Immune system		Peroxisome		Transport and catabolism	
Amoebiasis	6	Infectious diseases		Chagas disease American trypanosomiasis	6	Infectious diseases	
Epithelial cell signaling in *Helicobacter pylori* infection							

* Class: 1. Metabolism; 2. Genetic Information Processing; 3. Environmental Information Processing; 4. Cellular Processes; 5. Organismal Systems; 6. Human Diseases.

4. Discussion

4.1. Putative Probiotic Distribution in the GM Profiles

Bifidobacteria and lactobacilli are recognized as beneficial bacteria for their intrinsic probiotic features [23]. *Eubacterium* spp. may aid in the digestion, the absorption, or both of food ingredients and minerals, especially under malnutrition conditions usually occurring in CF because of nutrient absorption defects [36]. More generally, *F. prausntizii* has been recently proposed to provide high butyrate production in the gut [37].

Among the bacteria associated with the CF gut profiles (Table 2), some are actually linked to different pathologic conditions. Indeed, *Bifidobacterium dentium* was detected in the oral cavity in association with dental caries [38], while *Eubacterium dolichum* was associated with frailty in the elderly, a condition that represents the biggest problem associated with population aging [39]. *Lactobacillus mucosae* was detected in the microbiota of short bowel syndrome patients [40]. Other bacterial strains identified for CF patients were associated with metabolic disorders, such as high total cholesterol and low-density lipoprotein levels (*Eubacterium biforme*) [41], obesity (*Lactobacillus reuteri*) [42], and nonalcoholic fatty liver disease (NAFLD) (*L. zeae* and *L. vaginalis*) [24].

Unlike the aforementioned negative roles of the previous reported bacteria in human health, *B. breve* is considered a commensal or even a health-promoting microorganism [43] because it improves symptoms in necrotizing enterocolitis [44] and atopic dermatitis [45], as well as those associated with HIV-induced damages [46]. Moreover, *B. breve* shows antimicrobial activity [47], induces innate immune responses, and has anti-inflammatory effects [48]. Also, *L. pentosus* was reported to ameliorate colitis in the aged rodent by inhibiting the activation of nuclear factor (NF)-κB, activator protein 1 (AP1), and mitogen-activated protein kinases (MAPKs) [49].

No role in the human GM or putative effects as probiotics have been reported for *L. crispatus* and *L. pontis*.

The GM in the HC group seemed to be enriched in species involved in gut integrity and mobility, digestion of specific dietary compounds, and immune system modulation

Indeed, in the research of Kanauchi and co-workers [50], *E. limosum* was presented as an important probiotic candidate for its short-chain fatty acid (SCFA) production, role in maintaining and enhancing mucosal integrity, and anti-inflammatory properties in the intestinal mucosa [50]. Moreover, Bruzzese et al. found that both *E. rectale* and *F. prausnitzii* were reduced in the GM of CF children compared to HC, confirming our results [51].

Eubacterium siraeum is able to degrade wheat bran, contributing to the beneficial effects of cereal fiber in human health through their impact on the GM [52].

L. sanfranciscensis is generally considered the most important lactic acid bacterium in the fermentation of rye and wheat sourdoughs [53]. The strain *L. sanfranciscensis* LBH1068, tested in an induced chronic colitis mouse model, improved mouse health by reducing weight loss, decreasing gut permeability, and modulating cytokine production [54].

In addition, *L. fermentum* demonstrated intestinal anti-inflammatory effects in the model of sodium dextran sulfate-induced colitis in mice. Among the mechanisms proposed, *L. fermentum* restored GM composition and modulated the altered immune response by preserving intestinal barrier integrity, decreasing pro-inflammatory cytokine production, and modulating the expression of Th1-, Th17- and T_{reg}-related cytokines [55].

The study of Moya-Pérez and colleagues demonstrated that *B. pseudocatenulatum* modulated immune cell infiltration and inflammation in the gut in obesity [56].

A reduction in bifidobacteria in CF, especially *B. longum*, was already reported by Duytschaever et al. [57]. High richness of bifidobacteria species was positively correlated with the maturation of the mucosal immune system [58]. *B. longum* was found to be an inhibitor of rotavirus, the predominant cause of sporadic diarrhea in infants [59]. A recent study has demonstrated that

acetate produced by *B. longum* acts as an essential cosubstrate for butyrate production and for *E. rectale* growth [60].

B. bifidum and *B. longum* possess numerous pathways involved in the catabolism of human milk oligosaccharides (HMO) and may also consume carbohydrates released by other bacteria [61]. *B. bifidum* and *B. longum* were described as being more abundant in healthy subjects compared to NAFLD patients, suggesting a protective and beneficial role also in obesity and NAFLD [62].

Finally, each of these microbial species, especially those lacking in patients' GM (Table 2, HC-related species), could be considered suitable for the design of CF patient-tailored probiotics.

4.2. Metabolic Pathways of Probiotics

To evaluate the microbial metabolic and functional KEGG pathways of the chosen putative probiotic species, a supervised comparison of CF patients' and HC's KEGGs was performed by LEfSe (Table 3). Pathways associated with fatty acid biosynthesis, metabolism, and synthesis and degradation of ketone bodies were significantly associated with CF patients, as already described by Fouhy et al. [63]. This increase in fat metabolism probably occurs as a result of a combination of factors, including a reduced intestinal absorption and an altered GM in CF patients. Reduced fat absorption is one reason why most CF patients are traditionally prescribed a high-fat diet to ensure adequate weight maintenance. Moreover, it is possible that the altered GM might contribute to the increase in fat metabolism [63].

On the contrary, primary and secondary bile acid biosynthesis pathways were associated with the GM of HC. It is known that CF patients have a variety of intestinal abnormalities in bile acid metabolism at the intestinal level, including increased fecal bile acid losses, reduced bile acid pool size, and duodenal bile acid concentration [64]. These abnormalities appear to be associated with exocrine pancreatic insufficiency and steatorrhoea. Indeed, improvement of bile abnormalities with amelioration in fat malabsorption was reported after pancreatic enzyme therapy [64].

Pathways involved in xenobiotic metabolism have been significantly observed in the GM pattern of CF patients, including benzoate, fluorobenzoate, dioxin, xylene, aminobenzoate, and ethylbenzene degradation pathways. Bacterial pathways involved in xenobiotic metabolism were also observed by Fouhy et al. in CF patients [63]. The increase in bacteria capable of degrading xenobiotic compounds is probably due to the higher exposure to antibiotics and pharmacological treatments, recurrent in CF patients [65]. An enhanced ability of CF patients' GM to metabolize proteins was highlighted by the increase in amino acid catabolism (e.g., valine, leucine, isoleucine, and lysine degradation) prediction. Indeed, the increase in protein catabolism in CF individuals has been well documented, probably due to the breakdown of both cellular and connective tissue proteins, which is related to the degree of impaired lung function and to the systemic inflammatory response [66]. Moreover, also valine, leucine, isoleucine, lysine, phenylalanine, tyrosine, and tryptophan biosynthesis pathways were linked to putative probiotics in HC. Our results nicely agree with the findings of a study carried out by Palmer et al., in which 11 genes involved in branched-chain and aromatic amino acid catabolism were highly upregulated in CF patients' sputum, while genes involved in the biosynthesis of these amino acids were repressed [67].

Moreover, the flagellar assembly pathway was associated with putative probiotics in CF. Bacterial flagellin is classified as a potent mediator of virulence of Gram-negative bacteria. Recurrent infections caused by Gram-negative strains could be linked to this inferred pathway [68].

Finally, the prediction of lipoic acid metabolism and folate biosynthesis pathways were associated with CF patterns. Consistently, Quinn and colleagues reported the abundance of lipoic acid metabolism in the lung of CF patients [65]. Lipoic acid is an antioxidant and a potent quencher of reactive oxygen species (ROS) [69], and it is used as a metabolic cofactor by Proteobacteria, Gram-positive bacteria, and *Pseudomonas aeruginosa* [69,70]. Quinn reported also high abundance of folate synthesis in CF patients [65]. Sulfonamides, such as sulfamethoxazole and trimethoprim, are antibiotics commonly used to treat CF infections that target microbial enzymes required for folate biosynthesis [71].

Prolonged exposure to sulfonamides may select microbes with multiple copies of these genes to overcome the drug's effect on folate synthesis [72].

5. Conclusions

In conclusion, patients with CF usually have an abnormal intestinal microbiota and dysregulated immune mediators resulting from a massive exposure to antibiotics. Probiotics as immunomodulatory and anti-inflammatory substances are considered to improve both the clinical and the biochemical intestinal and pulmonary function in CF patients. The results reported in this study may point out new putative probiotic species on the basis of the GM differential profiles and predicted metabolic pathways of CF patients compared to HC.

On the basis of our data, we speculate that some putative probiotic species, such as *B. longum*, *E. rectale*, *E. limosum*, *E. siraeum*, *L. sanfranciscensis*, *L. fermentum*, *B. pseudocatenulatum*, *B. bifidum*, and *F. prausnitzii* and their produced metabolites may have a protective role against CF disorders. Nevertheless, further in vitro studies and clinical trials should focus on these probiotics to assess whether the administration of selected strains, alone or in combination, may improve the quality of life and the clinical management of CF patients.

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Meta-Analysis of Fecal Microbiota and Metabolites in Experimental Colitic Mice during the Inflammatory and Healing Phases

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Abstract: The imbalance of gut microbiota is known to be associated with inflammatory bowel disease, but it remains unknown whether dysbiosis is a cause or consequence of chronic gut inflammation. In order to investigate the effects of gut inflammation on microbiota and metabolome, the sequential changes in gut microbiota and metabolites from the onset of colitis to the recovery in dextran sulfate sodium-induced colitic mice were characterized by using meta 16S rRNA sequencing and proton nuclear magnetic resonance (^{1}H-NMR) analysis. Mice in the colitis progression phase showed the transient expansions of two bacterial families including Bacteroidaceae and Enterobacteriaceae and the depletion of major gut commensal bacteria belonging to the uncultured Bacteroidales family S24-7, Rikenellaceae, Lachnospiraceae, and Ruminococcaceae. After the initiation of the recovery, commensal *Lactobacillus* members promptly predominated in gut while other normally abundant bacteria excluding the Erysipelotrichaceae remained diminished. Furthermore, ^{1}H-NMR analysis revealed characteristic fluctuations in fecal levels of organic acids (lactate and succinate) associated with the disease states. In conclusion, acute intestinal inflammation is a perturbation factor of gut microbiota but alters the intestinal environments suitable for *Lactobacillus* members.

Keywords: gut microbiota; dysbiosis; inflammatory bowel disease; metabolome; meta 16S rRNA analysis; ^{1}H-NMR analysis; experimental colitic mice; *Lactobacillus*

1. Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is characterized by chronic and relapsing inflammation of the gut. Although the definite pathogenesis of IBD remains unclear, numerous studies have suggested the genetic and environment factors associated with chronic intestinal inflammation. Meta-analysis of genome-wide association studies have discovered many IBD susceptibility genes including cytokine regulation, innate immunity, and lymphocyte activation [1]. In many immune-deficient mice that spontaneously develop colitis, the presence of gut commensal bacteria is essential for the development of intestinal inflammation [2–4].

A recent molecular phylogenetic analysis revealed the imbalanced bacterial composition (dysbiosis) in human IBD patients [5–7] and IBD model animals [8,9]. However, it is not clear whether dysbiosis is actually a cause or consequence of dysregulated mucosal immune response in IBD.

Gut environments including microbiota and its metabolites influence the maintenance of gut homeostasis. Short chain fatty acids (SCFAs, e.g., acetate, butyrate, and propionate), which are produced by gut commensal bacteria from dietary fiber, have profound effects on energy metabolism, hormone production, intestinal epithelial homeostasis, mucosal immunity, and virulence regulation of pathogens [10,11]. Mice lacking SCFA receptors such as GPR43 and GPR109a are highly susceptible to chemical-induced colitis [12,13]. Among SCFAs, butyrate produced by gut commensal bacteria plays an essential role in the induction of regulatory T cell (Treg), which is involved in immunosuppressive mechanisms in the colon [14,15]. Furthermore, microbiome studies in IBD patients have reported the reduction in these Treg-inducing bacterial population including *Clostridia* clusters IV and XIVa [5,6]. These findings indicate the possibility that dysbiosis-induced changes in gut metabolites are responsible for the pathogenesis of IBD.

The manipulation of gut microbiota with antibiotics administration [16,17], probiotics administration [18–20], or fecal microbiota transplantation (FMT) [21–23] provides prospective therapeutic options for IBD. As with any medication, these therapies come with potential risks and side effects [24–26]. Several studies of FMT application have reported the risk of relapse or worsening of gut inflammation [25,26] and the development of obesity [27]. Antibiotic treatment has also some concerns such as the onset of *Clostridium difficile* infection [28] and the overgrowth of mucosal-associated bacteria after its cessation [29]. Thus, accumulating knowledge of how dysbiosis is developed in IBD patients is required for disease control of IBD over the long-term period.

This study aimed to characterize the dynamics of gut microbiota and metabolites from the onset of colitis to the recovery period by using a multi-omics approach consisting of meta 16S rRNA sequencing and proton nuclear magnetic resonance (^{1}H-NMR) analysis. We used a dextran sulfate sodium (DSS)-induced colitis model. Mice given DSS exhibit reproducibly acute colitis, while mice gradually convalesce after its removal. This feature is useful for characterizing the relationships between gut environments and intestinal inflammation. We demonstrate here characteristic dynamics of commensal bacteria and organic acids associated with disease states.

2. Materials and Methods

2.1. Animal Experiment

Six-week-old female C57BL/6J mice (n = 6) were obtained from CREA Japan, Inc. (Tokyo, Japan) and co-housed for 2 weeks before the start of DSS administration. For chemical-induced colitis, mice were fed with 5% DSS (molecular weight: 5000 Da; Wako, Osaka, Japan) ad libitum for 5 days. Then, mice were given regular water in the remaining periods. Body weight and bleeding score (no bleeding, 0; bleeding, 1; gross bleeding, 2) were recorded. A total of 46 fecal samples were collected on days 0, 5, 6, 8, 9, 11, 13, 15, and 20 for the gut microbiome and metabolome analysis. Animal experiments in this study were approved by the ethical committee of Waseda University Academic Research Ethics Committee (2013—A073a, 2014—A035a, 2015—A066).

2.2. 16S rRNA Gene-Based Microbiome Profiling

We extracted the DNA from fecal samples using ISOFECAL for Beads Beating (Nippon Gene, Inc., Toyama, Japan). The V1-2 region of 16S rRNA gene was amplified using the 27F/357R primer set [30] with the Ion Xpress Barcode Adaptor sequences. The concentration and fragment size of PCR amplicons purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The diluted pooled amplicons were clonally amplified by emulsion PCR and enriched template-positive particles using an Ion OneTouch 2 instrument (Life Technologies, Carlsbad, CA, USA) and an Ion

PGM Template OT2 400 Kit (Life Technologies) according to their instructions. Sequencing was carried out using an Ion Sequencing 400 kit and Ion 314 chip (Life Technologies) on the Ion PGM system. Sequence data were processed using QIIME 1.8.0 [31] to determine the operational taxonomic unit (OTU) as the same phylotype at 97% identity threshold.

2.3. Fecal Metabolites Analysis Using ^{1}H-NMR Analysis

The freeze-dried and grounded feces were extracted by mixing with 100 mM potassium phosphate buffer in D_2O (pH 7.4) containing 1 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard. After centrifugation, the supernatants of water-soluble components were collected for NMR measurements with AVANCE II 700 MHz Bruker Biospin (Bruker, Rheinstetten, Germany) as described previously [32–35]. The NMR spectra were processed using TopSpin 3.1 software (Bruker) and each of the peak signal intensities were calculated. For statistical analysis using R software [36], the spectra data were reduced by subdividing spectra into sequential 0.02 ppm. As mentioned in previous reports [37–41], the NMR peaks of interest were assigned using two web databases (SpinAssign, HMDB) with assistance of 2-dimension NMR spectra (^{1}H-^{13}C heteronuclear single quantum coherence) data of fecal samples in DSS-treated mice, which was collected in another research project.

2.4. Measurements of Lactate and Succinate in Fecal Samples

Fecal samples collected from five mice were suspended in distilled water. After homogenization with a handy homogenizer NS-310EIII (Microtech Corp., Chiba, Japan), fecal suspensions were heated for 15 min at 50 °C in a water bath, and filtered through a 0.2-μm cellulose acetate syringe filter (Advantech, Tokyo, Japan). Fecal extracts were mixed with an equal volume of chloroform, and centrifuged at 15,000 rpm for 10 min. The aqueous phase was mixed with two volumes of acetonitrile, and centrifuged at 15,000 rpm for 10 min. For succinate analysis, the aqueous phase was further purified with nine volumes of acetonitrile. After centrifugation, lactate and succinate in the deproteinized supernatants were measured with an ion chromatography ICS2100 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and a liquid chromatography electrospray quadrupole time-of-flight mass spectrometry Xevo G2-XS QTof (Waters Corp., Milford, MA, USA).

2.5. Statistical Analysis

Multidimensional scaling (MDS) of fecal microbiome and principal component analysis (PCA) of fecal metabolome were performed using the R software package, ver 2.15.3 [36]. Using the statistical software JMP® 12 (SAS Institute Inc., Cary, NC, USA), the correlations between microbiome and metabolites were screened, and analyses of variance with a post hoc Turkey-Kramer honestly were performed to identify significant differences in relative abundance of bacterial taxa or peak signal intensities of metabolites among the pathological phase.

3. Results

3.1. Clinical Manifestation of DSS-Treated Mice

Oral administration of 5% DSS for 5 days was carried out to induce colitis in wild type C57BL/6 mice (n = 6). The body weight began to decrease 2 to 6 days after DSS administration (Figure 1). Two mice (No. 3 and No. 5) that exhibited high sensitivity to DSS showed a drastic decrease in body weight. One of these mice (No. 3) died on day 5 due to massive bleeding. For the remaining five mice, the bleeding disappeared and the body weight began to increase 1 to 4 days after the removal of DSS from the drinking water. The periods required for the mice to recover to their initial body weights ranged between 3 to 14 days after the removal of DSS. The body weight loss was well-recognized as the primary indicator of DSS-induced colonic damage. Thus, we categorized the disease phases according to body weight changes during the experimental period: before DSS administration (phase 1), progressive phase (phase 2), a peak of weight loss (phase 3), recovery phase (phase 4).

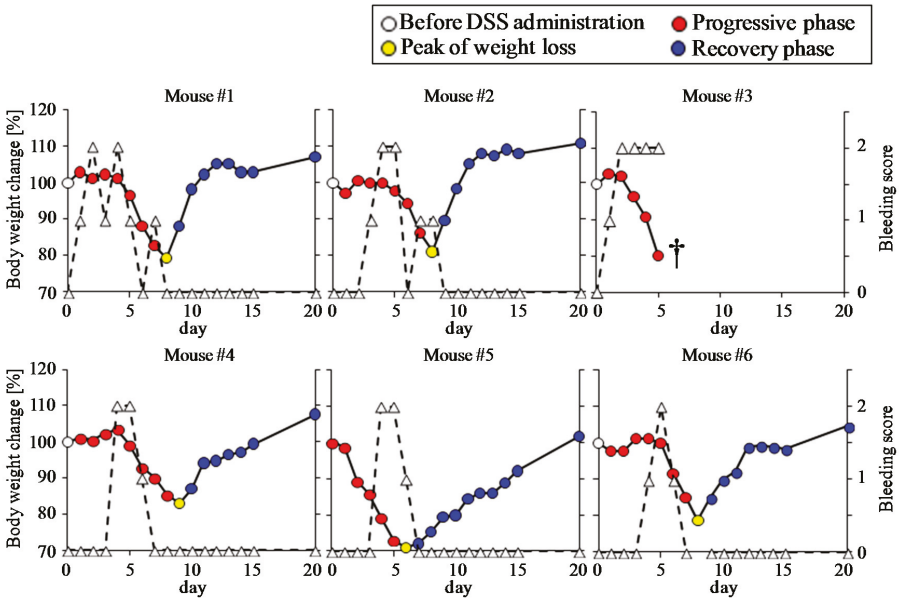

Figure 1. Monitoring of body weight changes and bleeding score of dextran sulfate sodium (DSS)-treated mice. Body weight changes (solid lines) are expressed as the percent of initial body weight. Bleeding (dashed lines) in mice was scored according to the following criteria: no bleeding, 0; bleeding, 1; gross bleeding, 2. The disease phases were categorized according to body weight changes during the experimental period: before DSS administration (phase 1, white), progressive phase (phase 2, red), a peak of weight loss (phase 3, yellow), recovery phase (phase 4, blue). One of the mice (No. 3) died on day 5 (†).

3.2. Time-Course Transitions in Colonic Bacterial Compositions in DSS-Treated Mice

The colonic bacterial community compositions in each mouse during the experimental period were monitored by 16S rRNA gene (V1–V2 regions) amplicons sequencing. A total of 147,794 sequences (an average of 3213 sequences per sample) obtained from 46 fecal samples were clustered into 2625 OTUs. Time-course changes in relative abundance of taxa classified at the family level within individual DSS-treated mouse are shown in Figure 2A. Prominent bacterial members in DSS-untreated mice, corresponding to the data at day 0 in Figure 2A, included members of eight families: Lactobacillaceae (34.1 ± 6.3%), uncultured Bacteroidales family S24-7 (18.3 ± 2.2%), Bacteroidaceae (13.8 ± 5.4%), Lachnospiraceae (10.0 ± 2.0%), Ruminococcaceae (7.4 ± 1.3%), Erysipelotrichaceae (6.8 ± 0.8%), Rikenellaceae (3.5 ± 0.5%), and Bifidobacteriaceae (1.8 ± 0.4%). Notably, the abundance of the Bacteroidaceae in the two mice with high sensitivity to DSS (NO.3, 37.9%; NO.5, 20.6%) was much higher than the other four mice (3.2% to 8.1%).

To characterize the time-course transitions in microbiota from the onset of colitis to the recovery phase, we performed a MDS analysis. The MDS plot of bacterial community composition showed a gut microbiota disturbance by the treatment with DSS (Figure 2B). Next, we characterized the relative abundance in eleven bacterial families in each phase after DSS administration. The abundances of the Bacteroidaceae (38.7 ± 16.5%), Enterobacteriaceae (25.0 ± 15.4%), Clostridiaceae (2.8 ± 1.2%), and Porphyromonadaceae (1.6 ± 0.7%) increased in the phase of colitis progression but rapidly decreased in the recovery phase (Figure 3A). On the other hand, the onset of DSS-induced colitis resulted in a significant decrease in the abundances of Bifidobacteriaceae (0.7 ± 0.4%), uncultured Bacteroidales family S24-7 (0.5 ± 0.5%), Rikenellaceae (0.1 ± 0.1%), Lachnospiraceae (0.3 ± 0.1%),

and Ruminococcaceae (0.03 ± 0.03%). Furthermore, these four bacterial families did not increase in the recovery phase. The abundances of two bacterial families (i.e., Lactobacillaceae, Erysipelotrichaceae) did not change during the colitis progression, but the Lactobacillaceae increased beyond the baseline level immediately after weight gain in the recovery phase. Taken together, our results show the characteristic population changes of bacterial families associated with the development and termination of intestinal inflammation.

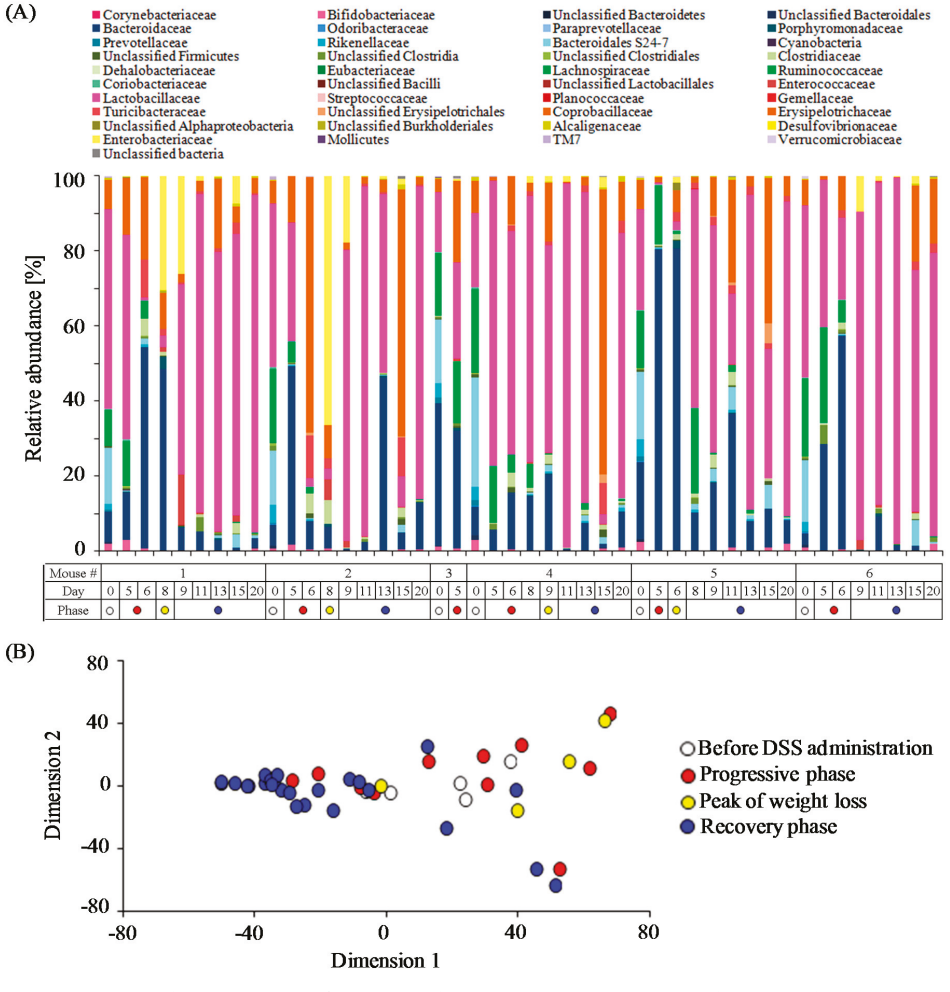

Figure 2. Fecal microbiome profiles during the experimental periods. (**A**) Changes in the relative abundance at the family level in each DSS-treated mouse; (**B**) Multidimensional scaling (MDS) plot of fecal microbiome (at the family level) at each time point.

Furthermore, we identified abundant bacterial phylotypes in each phase. When viewed at the OTU level, eleven OTUs were found in the top five most abundant OTUs in four phases (Table 1). Changes in the relative abundances of these OTUs among the disease phases are shown in Figure 3B. The OTU414 (*Lactobacillus johnsonii*-related 16S rRNA sequence) was most abundant in all of OTUs detected in this study. The relative abundance of the OTU414 showed an increasing tendency after DSS treatment, but this was not statistically significant. On the other hand, the relative abundance of other two OTUs

(OTU826 and OTU2301) affiliated with the genus *Lactobacillus* during the experiment period showed different trends from the OTU414. Treatment with DSS resulted in a rapid decrease in the abundance of the OTU2301 (*L. intestinalis*), and then the abundances of OTU826 (*L. murinus*) and OTU2301 were dramatically elevated in the recovery phase. DSS-induced colitis led to a transient overgrowth during the weight-loss period in the following OTUs: OTU527 (*Bacteroides uniformis*), OTU629 (*Escherichia coli*), OTU1387 (*E. coli*), and OTU2095 (*B. acidifaciens*). Unlike the above *Bacteroides*-related OTUs, the OTU2045 (*Bacteroides* sp.) was slightly reduced after DSS treatment. Three phylotypes (OTU682, OTU759, and OTU1332) tended to increase after the onset of colitis, and then remained stable during the experiment period. Thus, we screened the abundant bacterial phylotypes that responded to gut environmental changes induced by acute colitis.

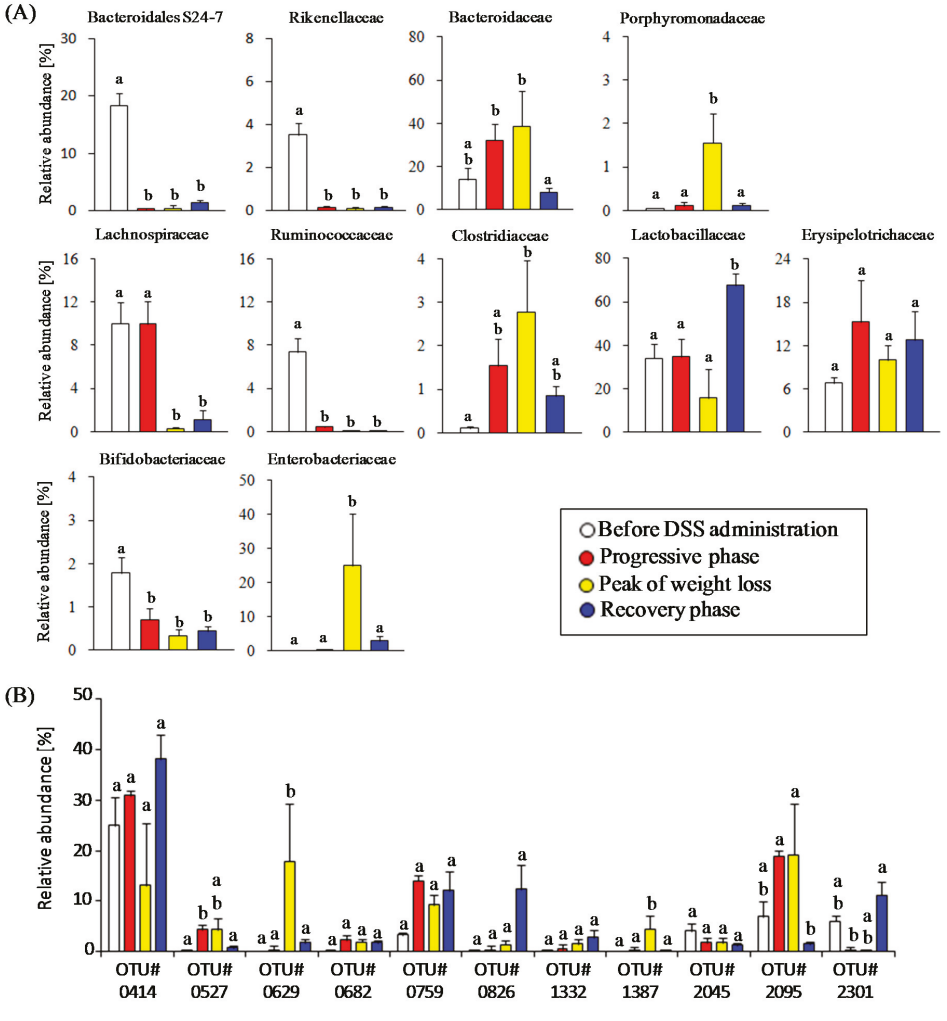

Figure 3. Characteristics of changes in relative abundances of major commensal bacterial groups. (**A**) Changes in the abundances of eleven bacterial families in four pathological phases; (**B**) Changes of the top five most abundant operational taxonomic units (OTUs) in each phase. Different letters indicate significant differences between bars.

Table 1. List of the top five most abundant operational taxonomic units (OTUs) in each phase. Taxonomic assignment of eleven OTUs was carried out with the basic local alignment search tool (BLAST) program in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database.

OTU #	Closest Reference Strain (Acsession No.)	Identities	Ranking			
			Phase 1	Phase 2	Phase 3	Phase 4
414	*Lactobacillus johnsonii* strain UMNLJ22 (CP021704)	99.2%	1	1	3	1
527	*Bacteroides uniformis* strain CECT 7771 (AB021157)	97.7%		4		
629	*Escherichia coli* strain 4060 (FJ405333)	99.4%			2	
682	*Turicibacter* sp. LA62 (AB727349)	100%		5		
759	*Clostridium* sp. ARIAKE1333 (AB809059)	98.5%	5	3	4	3
826	*Lactobacillus murinus* (LC159538)	99.5%				2
1332	*Bacteroides caecimuris* strain I48 (KR364741)	99.4%				5
1387	*Escherichia coli* strain D1 (CP010134)	99.7%			5	
2045	*Bacteroides* sp. SLC1-38 (AB599946)	99.7%	4			
2095	*Bacteroides acidifaciens* (AB510696)	99.4%	2	2	1	
2301	*Lactobacillus intestinalis* strain ls74 (EU381126)	99.2%	3			4

3.3. Characterization of Fecal Metabolites in DSS-Treated Mice

The changes in bacterial metabolite profiles can be inferred from the perturbation of gut microbiota caused by acute intestinal inflammation. In the present study, ^{1}H-NMR analysis was employed to evaluate time-course profiles of fecal metabolites in DSS-treated mice. The PCA score plot of ^{1}H-NMR data showed a fluctuation of gut metabolites of DSS-treated mice during the experimental period (Figure 4A). As a result, it was found that DSS treatment altered the signal intensity of several metabolites including essential amino acids (isoleucine, leucine, lysine, phenylalanine, tryptophan, and valine), non-essential amino acids (alanine, glutamate, glutamine, glycine, and tyrosine), succinate, and lactate (Figure 4B,C). In particular, the levels of succinate and lactate were elevated after the progressive phase. SCFAs (acetate, butyrate, and propionate) did not change in DSS-treated mice. In another animal experiment, time-course profiles of succinate and lactate in DSS-treated mice (n = 5) were re-evaluated with ion-chromatography and liquid chromatography with mass spectrometry, respectively (Figure 5). Fecal levels of succinate transiently increased around the peak of weight loss and decreased to the baseline levels in the recovery phase. On the other hand, fecal levels of lactate gradually decreased after the onset of colitis but increased to the baseline level in the recovery phase. Notably, PCA plots demonstrated that ^{1}H-NMR-based metabolite profiles of mice recovered from DSS-induced colitis (at day 20) were similar with those of DSS-untreated mice (Figure 4A). Taken together, these results suggest the association of gut bacteria-derived metabolites, especially lactate and succinate, with the disease states of DSS-colitis mice.

3.4. Estimation of Gut Microbiota Associated with Succinate Production

We performed correlation analyses of gut microbiome (abundances of all OTUs) and ^{1}H-NMR data (signal intensity of succinate) for screening the bacteria responsible for succinate production. A change in the fecal succinate level was positively correlated with seventeen OTUs ($r > 0.5$, $p < 0.0004$). Nine OTUs (r = 0.51–0.63) had 96.0–99.4% similarities with 16S rRNA sequence of *Lactobacillus intestinalis* (EU381126), four OTUs (r = 0.52–0.57) had 95.8–99.7% similarities with *Bacteroides caecimuris* (KR364741), two OTUs (r = 0.51–0.63) had 97.4% and 98.0% similarities with *B. thetaiotaomicron* (LC033799 and CP012937), one OTU (r = 0.51) had 97.6% similarity with *B. acidifaciens* (AB021159), and one OTU (r = 0.53) had 92.0% similarity with *Parasutterella excrementihominis* (LT558827).

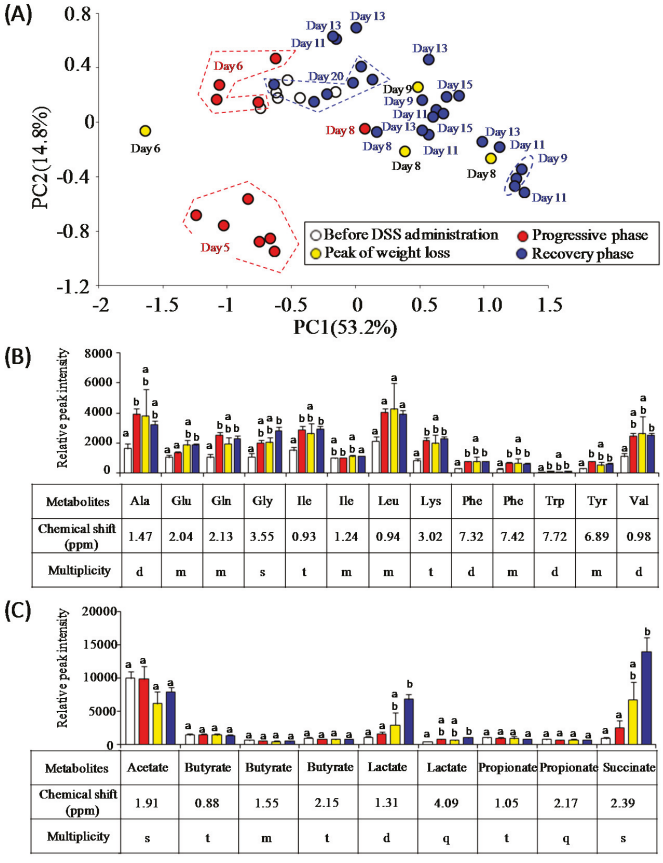

Figure 4. Characteristics of changes in fecal metabolites of DSS-treated mice. (**A**) Principal component analysis (PCA) score plots of fecal metabolites at each time point; (**B**) Changes in relative peak intensities of amino acids; (**C**) Changes in relative peak intensities of short chain fatty acids and organic acids. Different letters indicate significant differences between bars.

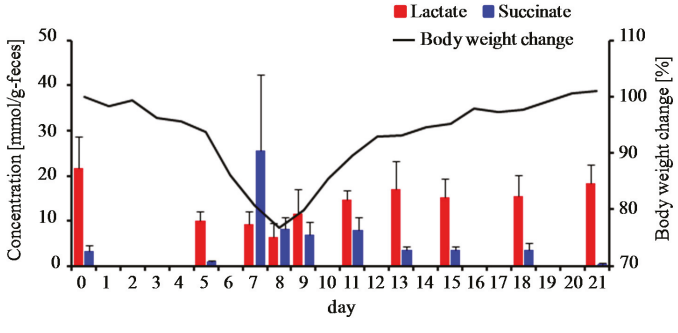

Figure 5. Monitoring of fecal lactate and succinate levels in DSS-treated mice. The solid lines show the average body weight change of five mice during the experimental period. Data of fecal lactate and succinate levels are mean ± standard error of the mean (SEM) (n = 5).

4. Discussion

Our results on the microbiome during the colitis progressive phase demonstrate transient expansions of two specific bacterial families including the Bacteroidaceae and the Enterobacteriaceae. Previous studies have also reported the high abundance in the Bacteroidaceae in a DSS-induced colitis mouse model [8,42] and the colitogenic property of commensal *Bacteroides* species [43]. Deletion of commensal *Bacteroides* species by the metronidazole has been known to be effective for the prevention of the colitis development in DSS-treated mice [17,44]. These previous findings are consistent with our results that mice with high abundance of the Bacteroidaceae are highly sensitive to DSS. The expansion of Enterobacteriaceae is the common feature in experimental colitis models [8,45,46] and IBD patients [5,6], and may contribute to the perpetuation of intestinal inflammation [47]. Reactive oxygen species play an essential role in the pathogenesis of IBD [48]. Inflammation-mediated increases in intestinal oxygen levels may support the ecological niche of facultative anaerobic bacteria such as *Escherichia coli* [45] and the reduction of anaerobic bacteria including Bacteroidetes and Firmicutes in IBD animals [8,9] and patients [5,6]. Our results also demonstrate that some major anaerobic commensal bacteria (e.g., Lachnospiraceae, Ruminococcaceae, Bacteroidales S24-7 family) were greatly depleted after the onset of colitis. The members in the Lachnospiraceae and Ruminococcaceae play an essential role in the maintenance of gut immune homeostasis as inducers of colonic regulatory T cells [14,15]. It remains unclear whether the Bacteroidales S24-7 family contribute to the gut homeostasis because they have not yet been cultured. A recent metagenomic analysis described the ability of oxidative stress protection of the Bacteroidales S24-7 family, suggesting that they as well as *Bacteroides* species behave as anaerobes capable of growing under marginally oxic conditions [49,50]. Thus, it might be premature to ascribe the depletion of the Bacteroidales S24-7 family to changes in oxygen levels. Interestingly, Choo et al. (2017) also reported the depletion of the Lachnospiraceae and Ruminococcaceae and the increases in the Enterobacteriaceae and Lactobacillaceae in mice treated with vancomycin and imipenem [51], suggesting that they may be competitors with each other under non-inflammatory conditions. Further studies of how these commensal bacteria establish their ecological niches in the gut will help to illustrate the mechanisms for the improvement of the IBD-associated dysbiosis.

Time-course monitoring of fecal metabolites using ^{1}H-NMR analysis revealed the association of organic acids (i.e., lactate and succinate) with the disease state of DSS-treated mice. Okada et al. (2013) reported that lactate produced by commensal lactobacilli is an essential trigger to induce the proliferation of intestinal epithelial cells (IECs) arrested by starvation [52]. Thus, we speculated that the elevated lactate levels associated with the remission affect the maintenance of IECs damaged by the exposure to DSS, although we have not yet obtained the direct evidence. Future studies focusing on the suppression of bacterial lactate production in the recovery phase using drugs (e.g., antibiotics, or lactate dehydrogenase inhibitors) are expected to provide new insights into the role of lactate in gut maintenance. Further, we observed a positive correlation ($r > 0.5$) between the succinate production and the relative abundances of some bacteria belonging to the genera *Bacteroides*, *Lactobacillus*, and *Parasutterella*. Some gut commensal bacteria, such as *Bacteroides* and *Prevotella*, have been reported as the producers of succinate [53]. However, the functional exploration of several genes associated with succinate biosynthesis, such as with a metagenomic approach, is required to precisely identify the succinate-producing bacteria in the recovery phase of DSS-induce colitis.

Succinate has its pros and cons in relation to gut health. A recent report indicated *Bacteroides*-derived succinate plays an essential role in promoting the colonization of strict anaerobes such as Clostridia, which prevents the colonization and the growth of exogenous pathogens [54]. This might be due to the reduction in intestinal oxygen levels by the growth of aerobic and facultative anaerobic bacteria utilizing succinate. There may be the possibility that succinate supports the reconstitution of the gut bacterial ecosystem after colitis. As per its disadvantages, previous studies reported the contribution of *Bacteroides*-derived succinate to the development of colonic ulceration in DSS-treated mice [55], the exacerbation of enteric infection [56], and the expansion of *C. difficile*

in the gut perturbed by antibiotics treatment or diarrhea [57]. Thus, further characterizations for judging whether the deletion of the succinate-producing bacteria is effective in protecting the colitis progression and secondary enteric infections are needed in future studies.

Another finding of ours is the characteristic changes in members of the genus *Lactobacillus* in DSS-treated mice. The onset of DSS-induced colitis leads a reduction in the abundance of *L. intestinalis* but no significant change in *L. johnsonii*, indicating different capacities for the adaptation to inflammation-mediated environmental changes among *Lactobacillus* species. On the other hand, the increased abundances of commensal *Lactobacillus* members seem to be associated with the recovery from colitis. Furthermore, the predominance of *Lactobacillus* members in the remission phase is accompanied by the regaining of the fecal metabolite profile, suggesting that the *Lactobacillus* promotes the improvement in the gut ecosystem. Their expansion may be attributed to the inflammation-mediated environment suitable for its growth. If this hypothesis is correct, the supplementation of *Lactobacillus*-containing probiotics in IBD animals and patients might confer beneficial effects such as dysbiosis correction and remission induction. Previous studies reported that a probiotic cocktail VSL#3 consisting of eight strains of lactic acid bacteria including four *Lactobacillus* species is effective in inducing the remission and manipulation of gut microbiota in experimental colitis animal [20] and IBD patients [18,19]. Establishing a further understanding of the ecophysiology of the commensal and probiotic *Lactobacillus* in the healthy or dysbiotic disease states will provide useful information for the successful treatment and prevention of chronic intestinal inflammation.

5. Conclusions

Chemical-induced colitis causes a transient expansion of colitogenic commensal bacteria, the depletion of beneficial commensal bacteria, and the fluctuation of intestinal lactate and succinate levels. Intestinal environments changed by host-mediated inflammation are favorable for the growth of commensal *Lactobacillus* members. These fundamental understandings of how intestinal inflammation impacts the gut ecosystem will provide the mechanistic explanation for and the correction of IBD-associated dysbiosis.

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Exploring the Impact of Food on the Gut Ecosystem Based on the Combination of Machine Learning and Network Visualization

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Abstract: Prebiotics and probiotics strongly impact the gut ecosystem by changing the composition and/or metabolism of the microbiota to improve the health of the host. However, the composition of the microbiota constantly changes due to the intake of daily diet. This shift in the microbiota composition has a considerable impact; however, non-pre/probiotic foods that have a low impact are ignored because of the lack of a highly sensitive evaluation method. We performed comprehensive acquisition of data using existing measurements (nuclear magnetic resonance, next-generation DNA sequencing, and inductively coupled plasma-optical emission spectroscopy) and analyses based on a combination of machine learning and network visualization, which extracted important factors by the Random Forest approach, and applied these factors to a network module. We used two pteridophytes, *Pteridium aquilinum* and *Matteuccia struthiopteris*, for the representative daily diet. This novel analytical method could detect the impact of a small but significant shift associated with *Matteuccia struthiopteris* but not *Pteridium aquilinum* intake, using the functional network module. In this study, we proposed a novel method that is useful to explore a new valuable food to improve the health of the host as pre/probiotics.

Keywords: gut ecosystem; food intake; metabolic response; machine learning; network analysis

1. Introduction

The gastrointestinal tract has a wide variety of functions, mainly in food digestion and nutrient absorption as well as in the development of systemic immunity, regulation of behavior, and targeting of drug delivery [1–3]. Gastrointestinal functions are directly and/or indirectly augmented by the gut microbiota [4–7]. However, dysbiosis can occur, which involves the disruption of proper microbial function; a typical case of this results from consuming an unbalanced diet, resulting in undesirable conditions in the host, such as inflammatory bowel disease and obesity [8–10]. Therefore, it is important to obtain a detailed understanding of the interaction between the gastrointestinal tract and intestinal microbiota.

The intestinal microbiota consists of many bacterial species, whose composition and metabolic function responds to the daily diet; probiotics and prebiotics positively impact the health of an individual [11,12]. Additionally, specific bacteria play a role in digesting specific molecules and providing metabolites to the host in cooperation with other bacteria, which are regulated by diet [13–15]. Any component of the daily diet and additional food have the potential of developing a pre/probiotic effect, thus emphasizing the need of a precise evaluation method to determine the impact of food on the intestinal microbiota; this information can be utilized to regulate the intestinal microbiota by controlling the daily diet (pre/probiotics), meal frequency, and time of food intake [16–18].

Many researchers have attempted to clarify effect of microbiota to host responses using mice, because the results obtained from such studies could be applied to humans [19,20]. Although many researchers have attempted to determine the factors that impact the intestinal microbiota, including antibiotics, pre/probiotics, and food intake, the data obtained from the microbiota analysis are affected by daily diet, stress, environment, and individual characteristics, including gene-based factors [11,15,21–23]. To overcome these problems, intestinal microbiota and host responses were measured by multiple methods, including next-generation DNA sequencing (NGS), nuclear magnetic resonance (NMR), mass spectrometry (MS), and biochemical analyses [10,24–28]. However, the data subjected to a multivariate analysis, including principal component analysis (PCA), correlation analysis, factor analysis, clustering analysis, and network analysis [26,27,29,30], remain potential options for further improvement of data mining.

In this research, we selected *Pteridium aquilinum* and *Matteuccia struthiopteris* as the representative components of the daily diet. *P. aquilinum* is consumed in Japan, Korea, and China, while *M. struthiopteris* is widely consumed in the Northern hemisphere; both plants are pteridophytes, which may have varied nutrient compositions from those of land plants and seaweed. This suggests that pteridophytes have a comparatively simple nutrient profile compared with higher plants, thus enabling easy evaluation [31]. In addition, *P. aquilinum* and *M. struthiopteris* may be beneficial prebiotics in daily diet because of their easy availability (Figure S1) [31]. We chose Random Forest, which is a machine learning tool, superior to classification by decision tree, to identify important factors associated with the impact of food, such as age and geography, on intestinal microbiota composition and applied these important factors to the network community of heterogeneous measurement data (Figure 1) [32–34]. This combination of machine learning and network visualization methods works well in identifying significant factors that are difficult to detect by other analytical approaches. A flow chart of the analysis protocol is presented in supplementary Figure S2, which indicates the novel part of this study.

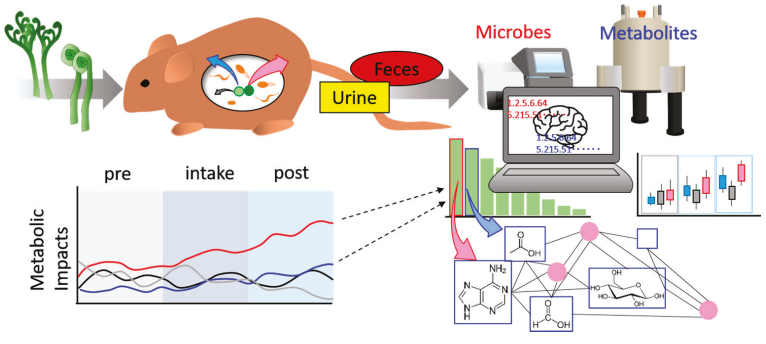

Figure 1. Schematic illustration of the novel method for exploring the impact of food, as proposed in this study. Mice were fed additional food and samples were collected (urine, feces). The samples were measured by multiple methods (e.g., NGS for the microbiome, NMR for the metabolome). These multiple data were analyzed by machine learning and important factors of response to food intake were determined. Furthermore, we calculated the network of normalized multiple data. Finally, combined functional network and important factors revealed masked impacts of food intake.

2. Materials and Methods

2.1. Animals

Male C57BL/6NCrl mice (3 weeks old) were obtained from Oriental Yeast (Tokyo, Japan) and habituated to the conditions in the animal facility before the start of the experiment (at 8 weeks old). All animal experiments were carried out in accordance with the Guidelines for the Laboratory Animal Facility of Showa Pharmaceutical University (ethic code number: P-2015-10).

2.2. Food Preparation

P. aquilinum and *M. struthiopteris*, used in this study, were developed in Yamagata Prefecture. *P. aquilinum* was boiled in 3% $NaHCO_3$ water and then soaked for at least 3 h in the same solution. Soaked *P. aquilinum* was rinsed thrice in sterile water and soaked in new sterile water for over 3 h. *M. struthiopteris* was boiled in 2% NaCl solution for 1 min and then soaked in cool water. These materials were then freeze-dried and crushed.

2.3. Animal Experiment and Sample Collection

We fixed the food-intake period (2 weeks) and the pre/post-food-intake periods. Cellulose (Wako), dried *P. aquilinum*, and *M. struthiopteris* were re-suspended (50 mg/mL) in 0.9% NaCl solution. Next, 400 μL of these solutions were orally administered to mice three times every other day in a week. Biological samples (feces, urine) were collected on the same day of food intake. A total of 216 fecal and 183 urinary samples were collected from mice (n = 4 for each group) throughout the experimental period for metabolic and microbial analyses. These samples were stored at −80 °C until sample preparation for analysis.

2.4. NMR Measurements

For the characterization of the components of *P. aquilinum* and *M. struthiopteris*, freeze-dried powder was suspended in 1 mL of KPi/D_2O solvent with 1 mmol/L sodium 2, 2-dimethyl-2-silapentane-5-sulfonate (DSS) and heated at 65 °C for 15 min with shaking (1400 rpm). After centrifugation, the supernatants of water- and methanol-soluble components were collected for NMR measurements. For the extraction of macromolecular components, 100 mg of the powder was rinsed with hexafluoroacetone (HFA) and ultrapure water, with a subsequent milling step, in accordance with a slightly modified version of the protocol in a previous study [35]. The milled samples were dissolved in 600 μL of DMSO-d_6/pyridine-d_5 (4:1) and the supernatant was used for NMR measurements. The collected urine and feces were prepared for NMR measurements, in accordance with the protocol used in a previous study [36]. All samples were measured by AVANCE II 700 MHz Bruker BioSpin (Bruker, Rheinstetten, Germany), metabolite annotations were performed using ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC), HSQC-total correlation spectroscopy (TOCSY), double-quantum single-quantum (DQ-SQ), and two-dimensional (2D) ^{1}H-^{1}H *J*-resolved NMR spectroscopy. In the HSQC NMR measurements, the Bruker standard pulse program "hsqcetgpsisp2.2" was used with the following parameters: number of data points, 1024 (F2) and 256 (F1); number of scans, 128; spectral widths, 9803.922 (F2) and 24,649.248 (F1) Hz; and D1, 1 s. In the HSQC-TOCSY NMR measurements, the Bruker standard pulse program "hsqcdietgpsisp.2" was used with the following parameters: number of data points, 2048 (F2) and 256 (F1); number of scans, 128; and spectral widths, 9803.922 (F2) and 28,170.570 (F1) Hz. In the DQ-SQ NMR measurements, the Bruker standard pulse program "dqseagp90" was used with the following parameters: number of data points, 16,384 (F2) and 512 (F1); number of scans, 64; spectral widths, 9803.0922 (F2) and 14,003.065 (F1) Hz; and D1, 1 s. In the 2D *J*-resolved NMR measurements, the Bruker standard pulse program "jresgpprqf" was used with the following parameters: number of data points, 16,384 (F2) and 32 (F1); number of scans, 8; spectral widths, 12,500 (F2) and 50 (F1) Hz; and D1, 2 s. In addition, metabolic profiling for feces and urine was also performed using 2D *J*-resolved NMR with skyline projection [37]. The detected peaks in NMR spectra were annotated using SpinAssign (http://dmar.riken.jp/spinassign/)

and SpinCouple (http://dmar.riken.jp/spincpl/) programs, as well as the Human Metabolome Database (http://www.hmdb.ca/) [38–40].

2.5. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) Measurement

A sufficient amount of each of 138 urinary samples was diluted approximately 100 times and measured by ICP-OES (SPECTRO BLUE FMX26 EOP; SPECTRO, Tokyo, Japan). As the operating parameters, main argon pressure, plasma power, plasma gas flow, auxiliary gas flow, nebulizer gas flow, and pump speed were set to 6.5 bar, 1400 W, 12 L/min, 1 L/min, 1 L/min, and 30 rpm, respectively.

2.6. Fecal Microbiome Analysis

Fecal microbes were disrupted using 5 mm stainless beads and a medicine spoonful of 0.3 mm zirconia beads in 200 µL of SDS-TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 2% SDS) using a vortex mixer for a total of 60 s. Disrupted feces were subjected to three freeze–thaw cycles and then centrifuged (10,000× *g*, 4 °C, 5 min), after which 100 µL of the supernatant was transferred to a new tube. DNA was extracted by phenol:chloroform:isoamyl alcohol (25:24:1) from the supernatant. Extracted DNA was dissolved in TE buffer and stored at −80 °C until microbiome analysis. The region encoding 16S rRNA was amplified using PCR with a TaKaRa Ex Taq HS kit (TaKaRa Bio, Shiga, Japan) and a primer set (Table S1) [41]. DNA was amplified by the following program: preheating at 94 °C for 4 min; 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min; and a final terminal extension at 72 °C for 10 min. The 16S rRNA gene amplicon was purified using AMPure XP (Beckman Coulter, Brea, CA, USA) with the manufacturer's protocol. The purified double-stranded DNA concentration was determined by Picogreen (Thermo Fisher Scientific, Waltham, MA, USA) and sequencing of prepared library was performed using a MiSeq sequencer (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. Obtained DNA sequence data were analyzed by QIIME (http://qiime.org/) and detected bacteria are listed in Table S2 [42]. These bacterial data were handled as percentages in the following analysis. The sequences that passed the QIIME of 16S rRNA OTU had a mean of 5619.26, and SD of 1653.89.

2.7. Data Analysis

Peaks of one-dimensional skyline projection (^{1}H-NMR) data were picked up by rNMR on the "R" platform [43,44]. The peak-picking data and other microbial and elemental data were normalized by unit variance. PCA, Random Forest, and network analyses were performed using the packages "muma," "randomForest," and "igraph" on the R platform, respectively [45–47]. These packages were used with their default settings. Calculated network data were depicted by Cytoscape (http://www.cytoscape.org/) [48]. The top 30 important factors were extracted from the mean decrease in accuracy of the Random Forest analysis. These factors were applied to calculate the community. The important factors of the included community were statistically analyzed. Statistical significance was determined using the Holm method, in which the false discovery rate was intermediate between Benjamini–Hochberg method and Bonferroni method [49,50].

2.8. Data Deposition

DNA sequences of the study have been deposited in DDBJ (http://www.ddbj.nig.ac.jp/). The accession number was DRA006349.

3. Results and Discussion

3.1. Sample Measurement and Multivariate Analysis

NMR measurements of the components of *P. aquilinum* and *M. struthiopteris* revealed that the plants contained some sugar groups and organic acids, chlorogenic acid, shikimate, and quinate (Figure S3a,b), the metabolites of which are derived from the shikimate pathway [51]. In particular, chlorogenic acid was

reported to reduce blood pressure, indicating that the plants have the possibility of improving health [52]. Additionally, the plants have almost the same high-molecular-weight components, except for olefin and starch (Figure S4a,b). Next, the contents of urine and feces from throughout the experimental period were annotated and used to create a PCA score plot, which is widely used to obtain an overview of multivariate data for easy visualization (Table S3, Figures S5 and S6, Figure 2a–c). Although the PCA score of feces from cellulose-treated mice moved slightly in the PC2 negative direction from the pre to post period (Figure 2a), the PCA scores of *P. aquilinum* and *M. struthiopteris* transitioned from the PC1 negative to the positive side (Figure 2b,c). This suggested that *P. aquilinum* and *M. struthiopteris* intake had a greater impact than cellulose intake, which may have arisen from the plants' sugar groups and organic acids. However, we were unable to determine the exact causal factor from the PCA score loading, as there was substantial noise. In addition, we measured intestinal microbiota by MiSeq and analyzed the data using PCA. The results of the microbiome data from all groups were not clearly separated among the groups (Figure 2d–f). Furthermore, we estimated the Firmicutes/Bacteroidetes ratio, which are major phyla in the gut and indicators of host life-style [12], to be almost the same (mean = 1.13, SD = 0.42, for the whole experimental period). These results suggest that additional food intake of six times every other day for two weeks could not make a major difference to gut microbiota composition. The urinary profiles based on NMR and elements by ICP-OES indicated a complex dispersion on the PCA score plot (Figure S7a–f). These results appear to demonstrate that PCA was unsuitable for exploring the impact of food in this case.

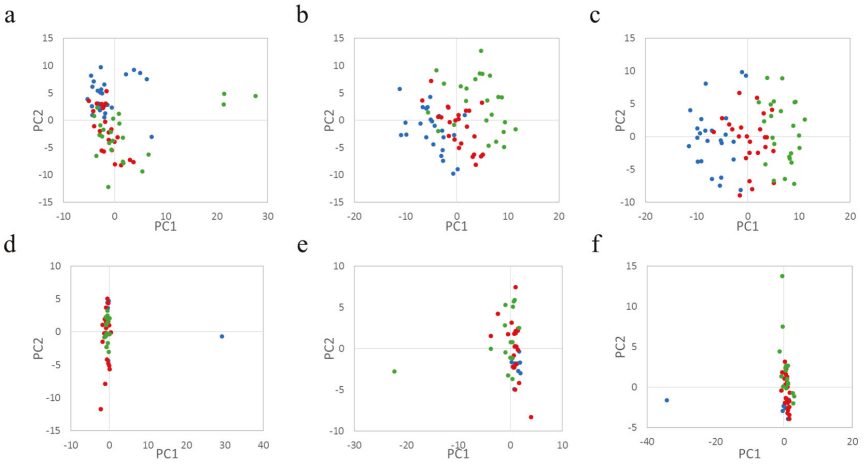

Figure 2. Principal component analysis of fecal metabolites and the microbiome. Red, blue, and green spheres represent single data points from pretreatment, during treatment, and posttreatment, respectively. Upper panels indicate the results of fecal NMR measurement analyzed by PCA. The data for the experimentally treated mouse groups (**a**–**c**) were from the administration of cellulose, *Pteridium aquilinum*, and *Matteuccia struthiopteris*, respectively; (**d**–**f**) show PCA score plots of the MiSeq data for each group; (**d**–**f**) show the results for the microbiota of mice treated with cellulose, *P. aquilinum*, and *M. struthiopteris*, respectively. The data analysis was performed using the package "muma" in R [45].

3.2. Selection of Important Variables by Determining the Food Impact

Although the impact of additional food intake was detected using fecal metabolite PCA, others indicated complex dispersion. It was suggested that the same composition microbiota shifted to another state of metabolism or that treated mice responses of food intake were masked by other factors, such as mouse-specific characteristics and stress [22]. We managed to mine the data on the impact of food by machine learning using Random Forest, which was applied to all datasets and was used to create a multi-dimensional plot, indicating that each group had a specific factor that separated

it from the others (Figure 3a, Figures S8a and S9a); we then selected the factors important for this separation, with careful cross-validation, to avoid over-training, using the Random Forest package (Figure 3b, Figures S8b and S9b). Random Forest revealed differences that were undetectable on PCA. The representative factors were clustered in the functional network module of the following network analysis (Figure 3c, Figures S8c and S9c). In addition, urinary ICP-OES data could not be separated using Random Forest analysis. These results indicate that urinary elements were not affected by food intake in this case.

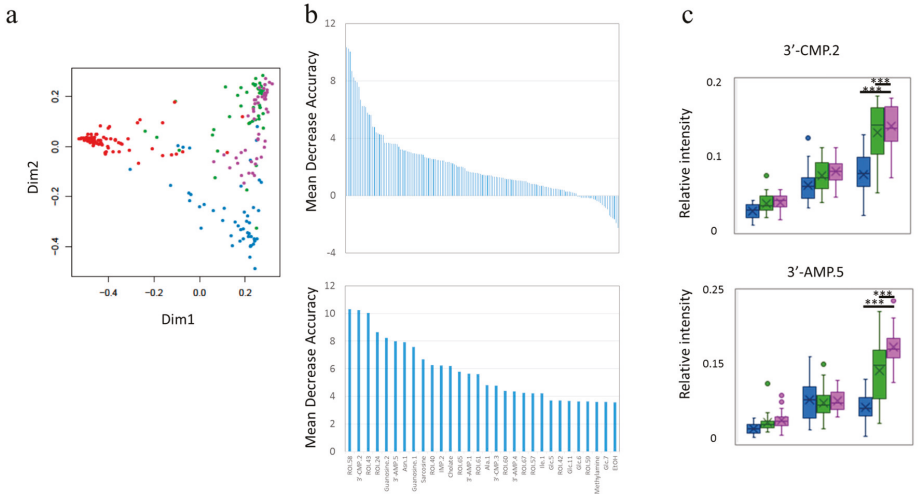

Figure 3. The results of Random Forest for fecal metabolites. Multi-dimensional score (MDS) plots (**a**) and important factors separating the groups (**b**) upper panel indicates all important factors and lower panel indicates the top 30 most important factors with annotation. In (**a**), red, blue, green, and purple circles represent single data points from the pre-treatment period for all groups, and the cellulose-, *Pteridium aquilinum*-, and *Matteuccia struthiopteris*-treated groups, respectively. (**c**) Shows a boxplot of representative important factors that can be applied on the network module. The upper panel and lower panel depict the results for 3′-CMP.2 and 3′-AMP.5, respectively. Blue, green, and purple boxes represent cellulose-, *P. aquilinum*-, and *M. struthiopteris*-treated groups, respectively. *** $p < 0.001$ calculated by the Holm method.

3.3. Application of Important Factors to Network Modules for Separation

We were able to detect important variables from the multivariate data by Random Forest and then attempted to find important variables in the network modules concerning the impact of additional food intake. We calculated the Spearman rank correlation coefficient with the combined dataset of the group treated with both *Pteridium aquilinum* and *Matteuccia struthiopteris* (metabolites of urine and feces and microbiome), performed analyses based on the correlation coefficients, using the R [45] package "igraph", and then described the network community, using Cytoscape (Figure 4, Figures S10 and S11). The purine/pyrimidine network of *M. struthiopteris* indicated that the impact of *M. struthiopteris* intake affected the microbiota, metabolites, and host metabolism. Dietary purine/pyrimidine was important to avoid an allergic state and to develop gastrointestinal systems [53,54]. The bacterium that was an important factor in the *M. struthiopteris*-treated group, from the genus-associated purine/pyrimidine metabolism community, was *Prevotella*, which is a gram-negative bacterium, reported to be associated with fiber [15]. In addition, the genus *Akkermansia*, which is known to affect metabolism and reduce obesity and inflammation [55,56], responded to *M. struthiopteris* intake. Another community, including glucose signal-associated bacterium, was described (Figures S10b and S11c); however, important bacterium and

important glucose signals were indirectly connected. Furthermore, the community did not associate with urinary metabolites. The results suggested that the impact of *M. struthiopteris* intake affects the purine/pyrimidine network more than glucose network. The important community and its associated bacterium *Akkermansia* were reported to be useful for health, so the impact of *M. struthiopteris* intake may involve a prebiotic-like effect of shifting nucleotide availability. Our method, involving a combination of machine learning and network visualization, detected a less significant food impact, which may help in the identification of prebiotics and probiotics that could be useful in the daily diet.

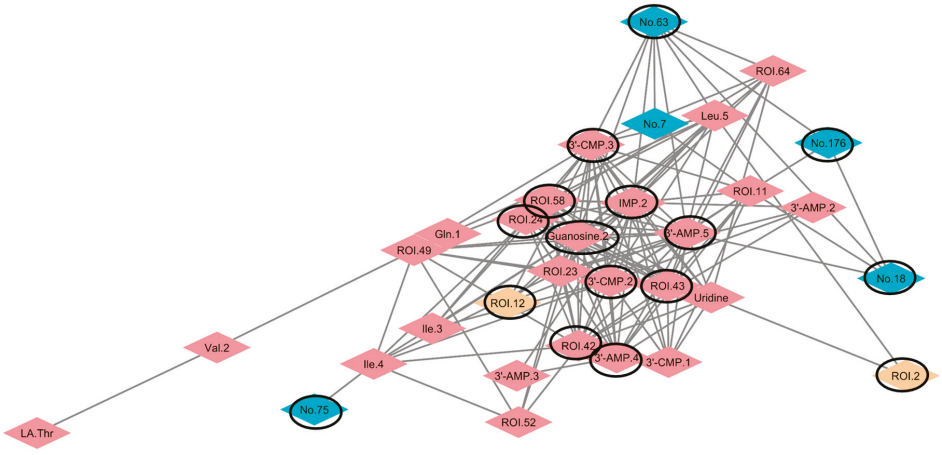

Figure 4. Purine/pyrimidine network with important bacteria and urinary metabolites of *Matteuccia struthiopteris*-treated mice based on the combined data set. The depicted network is based on calculations using the igraph package with Cytoscape. Blue, red, and light-yellow diamonds represent bacteria, fecal metabolites, and urinary metabolites, respectively (Tables S2 and S3). Black-line-circled diamonds represent factors selected by Random Forest as factors important for separating the groups.

4. Conclusions

In this paper, we selected two pteridophytes, which may have led to varied nutrient compositions between land plants and seaweed, as targets to study the impact of food on the gut ecosystem in mice. These pteridophytes may be beneficial prebiotics in the daily diet because of their easy availability. We proposed a novel methodology for exploring the impact of food on the gut ecosystem based on a combination of machine learning and network visualization. We obtained total of 490 variables by multiple measurements from non-invasive, time-course sampled urine and feces, and finally detected two important network modules, based on 30 selected variables of importance, based on Random Forest calculations. This novel analytical method could detect the impact of a small, but significant, shift associated with *Matteuccia struthiopteris* intake, using the functional purine/pyrimidine network module.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/12/1307/s1. Figure S1: Tree diagram of plant components based on data set obtained by NMR measurement, Figure S2: Flow chart of the analysis procedure in this study, Figure S3: ^{1}H-^{13}C HSQC NMR spectra of *Pteridium aquilinum* and *Matteuccia struthiopteris* extracted by KPi/D_2O solvent, Figure S4: ^{1}H-^{13}C HSQC NMR spectra of *Pteridium aquilinum* and *Matteuccia struthiopteris* components extracted by DMSO-d_6/pyridine-d_5 (4:1) solvent, Figure S5: Annotation of metabolites in urine NMR spectra, Figure S6: Annotation of metabolites in fecal NMR spectra, Figure S7: Principal component analysis of urinary metabolites and ions, Figure S8: The results of Random Forest for the fecal microbiome, Figure S9: The results of Random Forest for urinary metabolites, Figure S10: Network community correlation coefficients for *Matteuccia struthiopteris*-treated mice based on the combined data set, Figure S11: Network community correlation coefficients for *Pteridium aquilinum*-treated mice based on the combined data set, Table S1: Information on primers used for performing microbiome analysis, Table S2: List of sequential numbers for bacterial detection by MiSeq analysis, Table S3: List of annotated metabolites and unknown signals from NMR measurements.

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Hypocholesterolemic Effects of Probiotic Mixture on Diet-Induced Hypercholesterolemic Rats

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Abstract: Growing evidence has indicated that supplementation with probiotics improves lipid metabolism. We aimed to investigate the beneficial effects of a probiotics mixture (PM) of three strains belonging to the species *Bifidobacterium* (*B. longum*, *B. lactis*, and *B. breve*) and two strains belonging to the species *Lactobacillus* (*L. reuteri* and *L. plantarum*) on cholesterol-lowering efficacy in hypercholesterolemic rats. A hypercholesterolemic rat model was established by feeding a high-cholesterol diet for eight weeks. To test the effects of PM on hypercholesterolemia, hypercholesterolemic rats were assigned to four groups, which were treated daily with low (1.65×10^9 cfu/kg), medium (5.5×10^9 cfu/kg), or high (1.65×10^{10} cfu/kg) doses of probiotic mixture or simvastatin for eight weeks. Significant reductions of serum total cholesterol (TC), triacylglycerol (TG), and low-density lipoprotein (LDL)-cholesterol levels, but increases of high-density lipoprotein (HDL)-cholesterol were observed after supplementation of PM in hypercholesterolemic rats. In PM-supplemented hypercholesterolemic rats, hepatic tissue contents of TC and TG also significantly decreased. Notably, the histological evaluation of liver tissues demonstrated that PM dramatically decreased lipid accumulation. For their underlying mechanisms, we demonstrated that PM reduced expressions of cholesterol synthesis-related proteins such as sterol regulatory element-binding protein 1 (SREBP1), fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC) in the liver. Taken together, these findings suggest that PM has beneficial effects against hypercholesterolemia. Accordingly, our PM might be utilized as a novel therapeutic agent for the management of hypercholesterolemia.

Keywords: hypercholesterolemia; high-cholesterol diet; probiotics mixture; *Bifidobacterium longum*; *Bifidobacterium lactis*; *Bifidobacterium breve*; *Lactobacillus reuteri*; *Lactobacillus plantarum*; liver steatosis

1. Introduction

Hypercholesterolemia is a risk factor of cardiovascular disease (CVD), type 2 diabetes mellitus, and metabolic syndrome [1]. The WHO has predicted that up to 40% of all deaths will be related to CVD by 2030, affecting approximately 23.6 million people around the world [2]. Indeed, the risk of heart attack is three times higher in patients with hypercholesterolemia than those who have normal blood lipid contents. Furthermore, a 1% increase in serum cholesterol concentration results in 2%–3% increase in the occurrence of CVD [3]. In addition, it is a major cause of atherosclerosis

and atherosclerosis-associated diseases such as coronary disease and peripheral vascular disease [4]. It is characterized by high levels of total cholesterol (TC), low-density lipoprotein (LDL)-cholesterol, and triacylglycerol (TG), but low levels of high-density lipoprotein (HDL)-cholesterol in the blood vessels [5]. Hypercholesterolemia is also believed to be a crucial factor in the development of non-alcoholic fatty liver disease (NAFLD) [6]. The excess of TG by hypercholesterolemia can be stored as lipid droplets in the liver. Furthermore, excessive accumulation of TG within hepatocytes causes NAFLD [7]. NAFLD is considered as the most common causes of liver injury, and often occurs along with diabetes and obesity [8]. It can further progress to severe liver diseases such as liver fibrosis, cirrhosis, and end stage liver failure, and rarely hepatocellular carcinoma [9–11].

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" by the Expert Panel commissioned in 2001 by the Food and Agriculture Organization of the United Nations, and supported by the World Health Organization [12]. Probiotics could improve gut health by inhibiting the growth and attachment of harmful bacteria [13]. In addition to improving gut health, probiotics have also been reported to exert to other health-promoting effects against many kinds of diseases, including hypertension [14], cancer [15], allergic symptoms [16], arthritis [17], hyperlipidemia [18], and so on. In particular, probiotics have also been studied for their cholesterol-lowering effects in animal and human studies. Among them, Lactobacilli and Bifidobacteria are well studied in terms of their cholesterol-lowering effects. *Lactobacillus acidophilus* reduces blood cholesterol by breakdown of cholesterol and de-conjugation of bile salts [19]. Another study has demonstrated that *Bifidobacterium longum* BL1 decreases serum TC, LDL-cholesterol, and TG, and increases HDL-cholesterol in humans [20]. However, certain strains of probiotics have demonstrated cholesterol-lowering properties while some strains have not. Furthermore, evidence and proposed mechanisms targeting cholesterol-lowering effects remain controversial.

In the present study, we investigated cholesterol-lowering effects of five potential probiotic strains (*Bifidobacterium longum* CBG-C11, *Bifidobacterium lactis* CBG-C10, *Bifidobacterium breve* CBG-C2, *Lactobacillus reuteri* CBG-C15, and *Lactobacillus plantarum* CBG-C21) on high cholesterol diet (HCD)-induced hypercholesterolemic rats. In addition, we also evaluated potential mechanisms underlying their cholesterol-lowering effect.

2. Materials and Methods

2.1. Bacteria and Culture

Each probiotic strain (*Bifidobacterium longum* CBG-C11, *Bifidobacterium lactis* CBG-C10, *Bifidobacterium breve* CBG-C2, *Lactobacillus reuteri* CBG-C15, and *Lactobacillus plantarum* CBG-C21) were obtained from Chebigen Co. Ltd. (Jeonju, Korea) and grown in Man Rogosa Sharpe (MRS) medium containing 0.5 mg/mL linoleic acid at 37 °C for 15 h. Cells were collected by centrifugation at 4000× *g* for 10 min at 4 °C. The bacteria were mixed with freezing conservative containing 10% creaming powder, 10% dextran, and 5% sorbitol and lyophilized. The lyophilized formulation containing 5.5×10^{10} cfu/g of five probiotic strains was used in animal study. The viabilities of the administered strains were confirmed.

2.2. Animals and Study Design

All animal experiments in this study were approved by the Animal Care Committee of Wonkwang University (Approval number: WKU16-24) and were performed according to the guidelines from the Wonkwang University IACUC the NIH principles for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats aged 7 weeks (Samtako Biokorea, Daejeon, Korea) were used for all experiments. Rats were individually housed in cages maintained at 23 ± 2 °C with 50% ± 5% humidity and subjected to a 12 h light/dark cycle, and fed with either standard food diet (Std) or high cholesterol diet (HCD). HCD was prepared according to Paigen atherogenic diet (1.25% cholesterol, 0.5% cholate, and 15%

fat) [21] for 8 weeks. After 1 week of acclimation, rats were divided into six groups: (1) control group treated with saline; (2) HCD-fed group; (3) HCD-fed + low dose 1.65×10^9 cfu/kg/day probiotics mixture (PM); (4) HCD-fed + medium dose (5.5×10^9 cfu/kg/day) PM; (5) HCD-fed + high dose (1.65×10^{10} cfu/kg/day) PM; (6) HCD-fed + 4 mg/kg/day simvastatin (as a positive drug which is commonly used for the treatment of hypercholesterolemia) treatment ($n = 10$ in each group). In the control group, rats were administered saline daily by oral gavage. For PM and simvastatin supplements, rats were administered indicative doses of PM or simvastatin daily by oral gavage. The animals were weighed and the remaining foods were weighed weekly to calculate food intake.

2.3. Measurement of Serum Lipids and Alanine Transaminase (ALT) and Aspartate Transaminase (AST)

At the end of 8 weeks, all rats were sacrificed after 12 h fasting and blood was then collected from the abdominal vein by heparinized syringe. Serum was collected by centrifugation at $1500\times$ *g* for 15 min. A Hitachi 7020 system (Hitachi, Tokyo, Japan) was used for analysis of serum TC, TG, LDL-, and HDL-cholesterol levels.

2.4. Liver Histological Analysis

Liver tissues were carefully removed, rinsed, and fixed with 10% formalin solution and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin and eosin (H & E) and examined by light microscopy. The evaluation of liver steatosis score was conducted according to the previous study [22].

2.5. Measurement of Hepatic TC and TG

The hepatic lipids were extracted from the liver tissue using a chloroform/methanol mixed solution (2:1, *v*/*v*). Samples were then centrifuged at $12{,}000\times$ *g* for 10 min. After obtaining supernatants, hepatic TC and TG levels were quantified by using commercial enzymatic kits (Asan Pharmaceutical Co., Asan, Korea).

2.6. Western Blot Analysis

Liver tissues were homogenized in RIPA lysis buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein homogenates were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking for 1 h with 5% non-fat dry milk, the membranes were incubated overnight at 4 °C with antibodies against sterol regulatory element-binding protein 1 (SREBP1), fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and β-actin (Santa Cruz Biotechnology, Dallas, TX, USA). Next, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Tech, Beverly, MA, USA) for 1 h, and the bands were detected using enhanced chemiluminescence. The blots were scanned by a Bio-Rad ChemiDoc XRS and the intensity of each protein was quantified by Quantity One 4.5.0 software (Bio-Rad, Hercules, CA, USA).

2.7. Statistical Analysis

All data are reported as the mean ± SEM. Statistical significance was analyzed by using repeated measures or one-way ANOVA with Bonferroni post-hoc test (Prism 5.0.3, GraphPad Software Inc., San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of Probiotics Mixture on Hypercholesterolemic Rats

Figure 1 shows the growth curves on body weight changes during the experimental period. Body weight was steadily increased in each group. At 8 weeks, the body weight was significantly increased in HCD-fed group compared with control group (15.5% increase vs. control group). However,

no difference in food intakes was observed between HCD-fed and control groups. No differences in food and water intakes were observed in probiotics mixture (PM)-supplemented groups (low, medium, and high doses, respectively) and simvastatin-treated group compared with control group (Table 1).

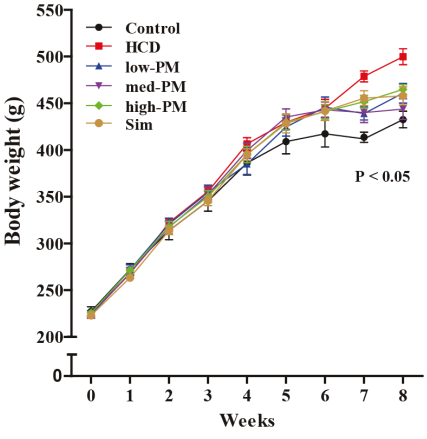

Figure 1. Body weight (BW) changes during experimental procedure. Rats were divided into control, high cholesterol diet (HCD)-fed, and PM-treated groups (n = 10 per group). BW was measured once a week in each group. p-value for ANOVA for repeated measures is given. HCD, high cholesterol diet-fed group; low-, med-, and high-PM: low (1.65×10^9 cfu/kg/day), medium (5.5×10^9 cfu/kg/day), and high doses (1.65×10^{10} cfu/kg/day) of probiotic mixture-treated group, respectively.

Table 1. Body weight (BW) and daily food and water intake during experimental procedure.

Parameter	Control	HCD	Low-PM	Med-PM	High-PM	Simvastatin
Initial BW (g)	227.2 ± 5.1	224.8 ± 2.6	224.3 ± 3.6	223.8 ± 3.6	226.0 ± 2.3	223.1 ± 2.2
Final BW (g)	432.7 ± 8.8	499.8 ± 8.5 #	460.8 ± 10.5 *	443.8 ± 11.0 *	464.8 ± 5.1 *	458.0 ± 9.6 *
Food intake (g/day)	19.0 ± 0.5	18.37 ± 0.3	19.2 ± 0.6	19.1 ± 0.3	19.0 ± 0.5	18.9 ± 0.5
Water intake (g/day)	33.4 ± 1.5	28.0 ± 0.8	30.0 ± 1.7	31.2 ± 1.0	30.5 ± 0.7	27.5 ± 0.4

Data are expressed as mean ± SEM. Significance was measured by performing a one-way ANOVA followed by Bonferroni's post-hoc test. # $p < 0.05$ vs. control. * $p < 0.05$ vs. HCD-fed group. HCD, high cholesterol diet-fed group; low-, med-, and high-PM: low (1.65×10^9 cfu/kg/day), medium (5.5×10^9 cfu/kg/day), and high doses (1.65×10^{10} cfu/kg/day) of probiotic mixture-treated group, respectively.

3.2. Effects of Probiotics Mixture on Serum Lipid Levels in Hypercholesterolemic Rats

At 8 weeks after PM treatment, serum TC, TG, LDL-, and HDL-cholesterol in the HCD-fed group were significantly elevated compared with control group (6.4-fold, 11.4-fold, 16.0-fold, and 6.0-fold increases for TC, TG, LDL-, and HDL-cholesterol levels, respectively, vs. control group). Otherwise, PM treatment dramatically attenuated elevated levels of these lipid parameters compared with the HCD-treated group. TC level was significantly lower by 1.2-fold, 1.5-fold, and 1.3-fold in low, medium, and high doses of PM-treated groups, respectively, compared to the HCD-treated group. TG level was dramatically inhibited, by 1.32-fold, 1.4-fold, and 1.4 fold in low, medium, and high doses of PM-treated groups, respectively, compared to the HCD-treated group. LDL-cholesterol level was inhibited by 1.3-fold, 1.4-fold, and 1.5 fold in low, medium, and high doses of PM-treated groups, respectively, compared to the HCD-treated group. On the contrary, HDL-cholesterol level was increased by 1.4-fold, 0.9-fold, and 1.1 fold in in low, medium, and high doses of PM-treated groups, respectively compared to the HCD-treated group. Simvastatin (as a hypercholesterolemia drug) treatment has similar patterns with PM-treatment (Figure 2). Thus, PM supplementation could inhibit increased serum lipids under HCD-induced hypercholesterolemia.

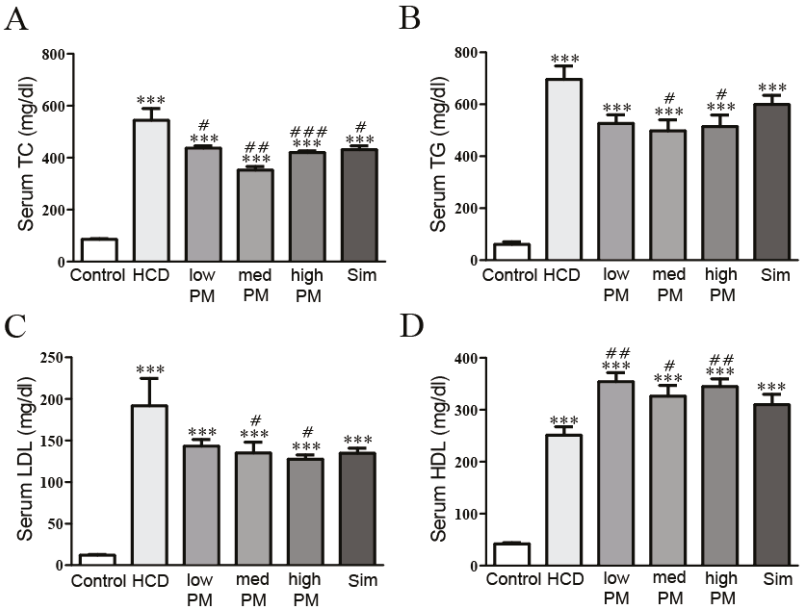

Figure 2. Effects of PM treatment on serum levels of (**A**) TC, (**B**) TG, (**C**) LDL-cholesterol, and (**D**) HDL-cholesterol in hypercholesterolemic rats. Data are expressed as mean ± SEM. Significance was measured by performing a one-way ANOVA followed by Bonferroni's post-hoc test. *** $p < 0.005$ vs. control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.005$ vs. HCD-fed group. HCD, high cholesterol diet-fed group; low-, med-, and high-PM: low (1.65×10^9 cfu/kg/day), medium ($5.5E \times 10^9$ cfu/kg/day), and high doses (1.65×10^{10} cfu/kg/day) of probiotic mixture-treated group, respectively; Sim, simvastatin-treated group; TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol.

3.3. Effects of Probiotics Mixture on Hepatic Steatosis in Hypercholesterolemic Rats

Hypercholesterolemia is an important risk factor for NAFLD, which is characterized by steatosis, lobular inflammation, and hepatocellular ballooning [23]. To determine the effects of PM on NAFLD development in hypercholesterolemic rats, we examined the hepatic morphology in rats. As shown in Figure 3, while there was no obvious steatosis with clear hepatic cord and sinusoid in the control group, liver cells in the HCD-fed group exhibited massive fatty changes and severe steatosis with cytoplasmic vacuoles, many fat vacuoles, and microvesicles filled with small lipid droplets compared with control group. Otherwise, in PM-treated groups, the degrees of hepatic steatosis were remarkably improved compared with the HCD-fed group. Similarly, the simvastatin treatment also decreased the severity of hepatic steatosis by HCD (Figure 3). In addition, we also determined the score of hepatic steatosis according to the percentage of hepatocytes containing lipid droplets [19]. The score in the HCD-fed group was dramatically increased relative to the control group (3.75 score in HCD-fed group). Notably, treatment with probiotics mixture reduced the scores compared with HCD-fed group, with the most marked effect in the group treated with high dose of PM (3.35, 3.29, and 2.5 scores in low, medium, and high doses of PM-treated groups, respectively). A similar result was found for simvastatin treatment, with the score reduced to 2.5 (Figure 3).

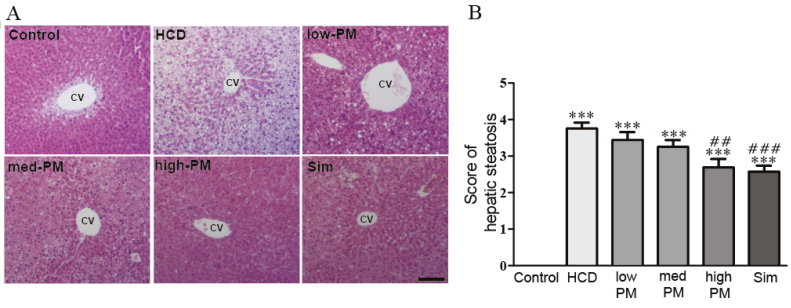

Figure 3. Effects of PM on hepatic steatosis in hypercholesterolemic rat. (**A**) Histological analysis of liver tissue in hypercholesterolemic rats. Liver sections were stained with hematoxylin and eosin (H & E) and examined under a light microscope (n = 8 per group); (**B**) Scores of hepatic steatosis of hypercholesterolemic rat livers. Scores were determined according to hepatocytes containing lipid droplets (n = 8 per group). Data are expressed as mean ± SEM. Significance was measured by performing a one-way ANOVA followed by Bonferroni's post-hoc test. *** $p < 0.005$ vs. control. ## $p < 0.01$, ### $p < 0.005$ vs. HCD-fed group. HCD, high cholesterol diet-fed group; low-, med-, and high-PM: low (1.65×10^9 cfu/kg/day), medium (5.5×10^9 cfu/kg/day), and high doses (1.65×10^{10} cfu/kg/day) of probiotic mixture-treated group, respectively; Sim, simvastatin; CV, central vein. Scale bar, 50 μm.

3.4. Effects of Probiotics Mixture on Hepatic ALT, AST, TC, and TG in Hypercholesterolemic Rats

To determine whether PM treatment could mitigate the HCD-induced liver injury, the levels of hepatic TC and TG were examined. The levels of ALT and AST in the HCD-fed group were significantly higher than that of the control group (1.6-fold and 1.3-fold increases for ALT and AST, respectively, vs. control group). In contrast, hypercholesterolemic rats treated with PM or simvastatin had significantly attenuated levels of hepatic ALT and AST (64.4%, 59.2%, 79.2%, and 70.0% decreases for ALT; 71.3%, 60.2%, 78.9%, and 69.1% decreases for AST in low, medium, and high doses of PM, and simvastatin-treated groups, respectively, vs. HCD-fed group) (Table 2).

In addition, hepatic TG and TC levels in the HCD-fed group were also significantly higher than those of the control group (3.2-fold and 3.7-fold increases for TG and TC, respectively, vs. control group). In PM and simvastatin-treated groups, these levels were decreased compared to the HCD-fed group (91.6%, 83.3%, 63.8%, and 66.0% for TG; 94.0%, 89.6%, 88.5%, and 83.3% for TC in low, medium, and high doses of PM, and simvastatin-treated groups, respectively, vs. HCD-fed group) (Table 2). These data indicated that PM treatment improved the liver injury and hepatic lipid profiles in the hypercholesterolemic rats.

Table 2. Levels of hepatic ALT, AST, and lipids in hypercholesterolemic rats.

Parameter	Control	HCD	Low-PM	Med-PM	High-PM	Sim
ALT (IU/L)	72.3 ± 4.8	117.7 ± 18.5 ##	75.8 ± 17.9 *	69.7 ± 14.7 **	93.2 ± 10.7 *	82.3 ± 14.4 *
AST (IU/L)	136.0 ± 12.9	171.3 ± 18.1 #	122.2 ± 13.9 **	103.2 ± 9.4 **	135.2 ± 11.9 *	118.4 ± 12.7 *
TG (mg/dL)	43.8 ± 4.3	140.3 ± 54.9 ##	128.6 ± 32.4 *	116.9 ± 34.5 *	89.54 ± 14.5 **	92.58 ± 16.6 *
TC (mg/dL)	41.6 ± 11.4	152.4 ± 27.1 ##	143.3 ± 32.2 *	136.5 ± 61.4 *	134.9 ± 46.8 *	127.0 ± 52.1 *

Data are expressed as mean ± SEM. Significance was measured by performing a one-way ANOVA followed by Bonferroni's post-hoc test. # $p < 0.05$, ## $p < 0.01$ vs. control. * $p < 0.05$, ** $p < 0.01$ vs. HCD-fed group. HCD, high cholesterol diet-fed group; low-, med-, and high-PM: low (1.65×10^9 cfu/kg/day), medium (5.5×10^9 cfu/kg/day), and high doses (1.65×10^{10} cfu/kg/day) of probiotic mixture-treated group, respectively; ALT, alanine transaminase; AST, aspartate transaminase; Sim, simvastatin-treated group; TG, triglycerides; TC, total cholesterol.

3.5. Effects of Probiotics Mixture on Expressions of Hepatic Cholesterol Homeostasis-Related Proteins in Hypercholesterolemic Rats

To explore the mechanisms underlying the effects of PM in hypercholesterolemia, expression levels of cholesterol homeostasis-related proteins such as SREBP1, FAS, and ACC were determined by Western blot analysis. The results showed that SREBP1 and its target proteins, FAS, and ACC in the HCD-fed group were significantly elevated compared with the control group (2.9-fold, 2.1-fold, and 2.4-fold increases for SREBP1, FAS, and ACC, respectively, vs. control group). In contrast, PM and simvastatin treatments significantly diminished the expression levels of these proteins in hypercholesterolemic rats (74.4%, 56.0%, 59.8%, and 61.3% decreases for SREBP1; 80.0%, 54.6%, 55.2%, and 70.6% decreases for FAS; 47.3%, 32.4%, 34.4%, and 47.7% decreases for ACC in low, medium, and high doses of PM, and simvastatin-treated groups, respectively, vs. HCD-fed group) (Figure 4).

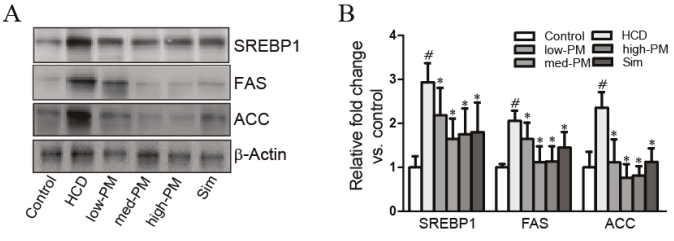

Figure 4. Effects of PM treatment on hepatic cholesterol homeostasis-related proteins in hypercholesterolemic rats. (**A**) Liver extracts from HCD diet, PM, and simvastatin treated groups were used for Western blot analysis; (**B**) Expression levels of SREBP1, FAS, and ACC were quantified by measuring band densities with National Institutes of Health (NIH) Image J software. β-actin was used as a loading control. Data are expressed as mean ± SEM. Significance was measured by performing a one-way ANOVA followed by Bonferroni's post-hoc test. $^{\#}$ $p < 0.05$ vs. control group. * $p < 0.05$ vs. HCD-fed group. HCD, high cholesterol diet-fed group; low-, med-, and high-PM: low (1.65×10^9 cfu/kg/day), medium (5.5×10^9 cfu/kg/day), and high doses (1.65×10^{10} cfu/kg/day) of probiotic mixture-treated group, respectively; Sim, simvastatin-treated group.

4. Discussion

Hypercholesterolemia is a common cause for many diseases [24,25]. Therefore, intensive efforts have been made to develop lipid-lowering drugs, such as statins (e.g., pitavastatin, atorvastatin, simvastatin, etc.). Statins are most commonly used to reduce cholesterol levels, and are a well-established class of drugs in the treatment of hypercholesterolemia. Furthermore, statin drugs have been proven to improve liver functions by inhibiting enzymes involved in cholesterol synthesis in patients with hypercholesterolemia [26]. However, there are also reported side effects of statins, such as myopathy (the symptoms of which are muscle weakness and muscular pain) and rhabdomyolysis affecting 0.1%–0.2% of patients taking statins [27].

Therefore, the natural and safe properties of probiotics can be a useful strategy for the treatment of hypercholesterolemia. Among them, *Lactobacillus* and *Bifidobacterium* species have been well-studied on their hypolipidemic effects in animal and human studies. *L. plantarum* used as single or mixed with *L. paracasei* exhibited blood cholesterol lowering effect in high fat and cholesterol diet-fed rats [28]. *L. rhamnosus* hsryfm has beneficial effects on lipid metabolism in hyperlipidemic rats by regulating the gut microbiota [29]. Supplement of milk-yogurts fermented with *B. longum* could reduce about 50% TC, LDL-cholesterol, and TG concentrations in albino hypercholesterolemic rats. Another study also observed decreases of these lipid parameters after 4 weeks supplement of yogurt containing *B. longum* in humans [20].

Recently, some studies have attempted to elucidate their effects using multi-strain probiotics against diseases. A meta-analysis conducted by literature review showed that multiple-strain probiotics are more effective than a single strain in reducing NEC (necrotizing enterocolitis) and mortality in infants [30]. Probiotics mixture, which contains five strains, was effective in the treatment of NAFLD by ameliorating increased lipid profiles, liver function, and inflammatory markers [31].

We have screened the probiotic strains that have cholesterol-lowering effects by bile salt hydrolase (BSH) activity assay and cholesterol-removal assay, and found five bacteria having cholesterol-lowering effects, which were used for this study. In the present study, significant reductions in serum TC, TG, and LDL-cholesterol were observed after eight weeks of supplementation with probiotics mixture containing two lactobacilli (*L. reuteri* and *L. plantarum*) and three bifidobacteria (*B. longum*, *B. lactis*, and *B. breve*) strains in hypercholesterolemic rats (Figure 2). Indeed, since LDL-cholesterol is a major component of serum cholesterol, LDL-cholesterol level may be an important factor for reducing total cholesterol. Our data indicated that probiotic mixture is a good substance for treating hypercholesterolemia.

Generally, excess cholesterol in the body is deposited in hepatic cells and eventually causes NAFLD. As expected, we demonstrated that high-cholesterol diet induced increases of hepatic TC, TG, LDL-cholesterol, and liver injury markers such as ALT and AST in rats (Table 2). Moreover, hypercholesterolemia induced the liver steatosis—characterized by cytoplasmic vacuoles, many fat vacuoles, and microvesicles filled with small lipid droplets. Similar results were also shown in our liver histology (Figure 3). In contrast, hypercholesterolemic rats supplemented with the probiotics mixture displayed significant reductions in these hepatic lipid profiles and liver injury markers. In addition, our results from liver histology proved that supplementation with probiotics mixture had a potential effect in alleviating hepatic steatosis in hypercholesterolemic rats.

Several mechanisms by which probiotics affect hypercholesterolemia have been proposed: reduction of plasma cholesterol through reduction of the enterohepatic circulation of bile salts by bile salt hydrolase activity; reducing the bioavailability of cholesterol from the diet; and a decrease in systemic levels of blood lipids by inhibiting hepatic cholesterol synthesis [32]. In this study, we found that probiotics mixture ameliorated increased levels of SREBP1 and their target proteins, such as FAS and ACC in hypercholesterolemic rats (Figure 4). In previous report on hepatic steatosis, these proteins were found to be elevated in hepatic steatosis, implicating increased cholesterol and fatty acid biosynthesis as potential causes of lipid deposition. SREBP1 protein is an important liver transcription factor controlling many genes involved in the metabolism of cholesterol and other lipids [33]. Thus, our data indicated that probiotics mixture could lower the lipid levels via the inhibition of SREBP1-related lipid biosynthesis mechanism in the liver.

5. Conclusions

In conclusion, our results suggested that probiotic mixture of two lactobacilli and three bifidobacteria has the potential to reduce serum total cholesterol, triglycerides, and LDL-cholesterol levels in hypercholesterolemic rats. We also demonstrated that our probiotic mixture inhibited the hepatic steatosis and cholesterol synthesis signaling pathway in the liver. Therefore, our study provides that using this probiotic mixture is a potential strategy for the prevention of hypercholesterolemia.

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Author Contributions: S.-J.K. and D.K.Y. designed the experiments and prepared the manuscript. H.-S.S., S.-H.J., S.-W.L., S.H.P., S.Y.K., B.K., K.-Y.Y. and S.-Y.K. performed experiments and analyzed data. H.-S.S., S.-H.J. and S.-W.L. contributed materials.

Conflicts of Interest: The authors declare no conflict of interest.

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Impact of High-Dose Multi-Strain Probiotic Supplementation on Neurocognitive Performance and Central Nervous System Immune Activation of HIV-1 Infected Individuals

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Abstract: Background: Gut microbiota has metabolic activity which influences mucosal homeostasis, local and systemic immune responses, and other anatomical systems (i.e., brain). The effects of dysbiosis are still poorly studied in Human Immunodeficiency Virus-1 (HIV-1) positive subjects and insufficient data are available on the impairment of the gut-brain axis, despite neurocognitive disorders being commonly diagnosed in these patients. This study evaluated the impact of a probiotic supplementation strategy on intrathecal immune activation and cognitive performance in combined antiretroviral therapy (cART) treated HIV-1 infected subjects. **Methods:** Thirty-five HIV-1 infected individuals were included in this study. At baseline (T0) a battery of tests was administered, to evaluate neurocognitive function and a lumbar puncture was performed to determine neopterin concentration in cerebrospinal fluid (CSF), as a marker of Central Nervous System (CNS) immune activation. Subsequently, a subgroup of participants underwent a 6-month course of multi-strain probiotics supplementation; this intervention group was evaluated, after probiotic treatment, with a second lumbar puncture and with repeated neurocognitive tests. **Results:** At T0, all participants showed impaired results in at least one neurocognitive test and elevated neopterin concentrations in CSF. After supplementation with probiotics (T6), the interventional group presented a significant decrease in neopterin concentration and a significant improvement in several neurocognitive tests. In contrast, no significant modifications were observed in the neurocognitive performance of controls between T0 and T6. The CNS Penetration Effectiveness Score of antiretroviral therapy did not show an influence from any of the investigated variables. **Conclusions:** Multi-strain probiotic supplementation seems to exert a positive effect on neuroinflammation and neurocognitive impairment in HIV-1 infected subjects, but large trials are needed to support the concept that modulation of the gut microbiota can provide specific neurological benefits in these patients.

Keywords: HIV; asymptomatic neurocognitive impairment; immune activation; central nervous system; neopterin; probiotic; supplementation; multi-strain; dysbiosis; gut-brain axis

1. Introduction

Following the widespread use of combined antiretroviral therapy (cART) the prevalence of severe neurocognitive impairments, among HIV-1 infected individuals, considerably decreased, while the prevalence of milder extents of HIV-1 associated neurocognitive disorders, such as Asymptomatic Neurocognitive Impairment, persisted at a stable rate among this population [1]. Wider evidence shows that changes in the qualitative and quantitative composition of the gut's microbiome can affect the modulation of the gut-brain axis, thus resulting in the onset of behavioral and neurocognitive alterations [2]. For these reasons, the use of probiotics represents a novel potential approach to manage conditions, such as stress-related behaviors, and to ameliorate cognitive function in several pathological settings [3,4].

HIV-1 infected patients usually suffer from two conditions that negatively affect neurocognitive function: the alteration of the normal gut flora composition (dysbiosis), with the overgrowth of detrimental bacterial strains [5] and elevated cerebrospinal fluid (CSF) concentrations of neopterin, a biomarker of Central Nervous System (CNS) immune activation [6]. Neopterin—a biochemical product of the guanosine triphosphate pathway—is a recognized marker of monocyte activation, and an association between its expression and the development of HIV-associated neurocognitive disorders (HAND), Acquired Immuno-Deficiency Sindrome (AIDS) dementia complex, and HIV encephalitis have been proposed [7–10]. The expression of neopterin has been found to be higher in HIV-1-positive patients, compared to healthy people, and in naïve HIV-1-positive patients, compared to those on effective cART [10,11]. In this study, we evaluated the impact of a 6 month course of high dose multi-strain probiotic supplementation on CSF immune activation and neurocognitive impairment of HIV-1 positive patients.

2. Materials and Methods

2.1. Study Design, Recruitment, Study Eligibility Criteria and Ethics Statement

The present study included 35 HIV infected individuals, enrolled at the HIV Outpatient Clinic of the Department of Public Health and Infectious Diseases of University of Rome "Sapienza". The study protocol was approved by the internal committee of the Department of Public Health and Infectious Diseases of "Sapienza" University of Rome and by the Ethics Committee of Policlinico Umberto I Hospital, Rome (ethical approval code Rif# 2970). All participants agreed to the enrolment by signing a written informed consent form.

The design of the study is shown in Figure 1. Inclusion criteria for the enrolment in the study were: age >18 years old, a stable and effective (plasma HIV RNA <37 copies/mL) cART regimen for at least 6 months prior to enrolment. Exclusion criteria were: education below primary school, Mini Mental State Examination <26, any impairment in daily living activities, as defined in the Instrumental Activities of Daily Living scale, previous history or actual diagnosis of any neurologic or psychiatric condition, positive Polymerase Chain Reaction (PCR) on CSF for any of the following pathogens: Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Herpes simplex virus-1 (HSV-1), Herpes simplex virus-2 (HSV-2), Varicella-zoster virus (VZV), Human herpesvirus-8 (HHV-8), BK virus (BKV), JC virus (JCV). At baseline (T0) all participants underwent (I) a lumbar puncture, to assess HIV-RNA, PCR for CMV, EBV, HSV-1, HSV-2, VZV, HHV-8, BKV, JCV, and neopterin concentration in CSF, (II) an array of neuropsychological tests.

Subsequently, we divided the enrolled patients into two groups, on the basis of their neuroinflammation levels (interventional group with higher level of CSF neopterin and control

group with lower levels of CSF neopterin). Nine participants with higher levels of CSF neopterin (interventional group) underwent a six month course of supplementation with oral probiotics (2 sachets, each containing 450 $\times$ 10^9 billion bacteria, twice a day); the selected probiotic was a commercially available product with the following composition: *Lactobacillus plantarum* DSM 24730, *Streptococcus thermophilus* DSM 24731, *Bifidobacterium breve* DSM 24732, *Lactobacillus paracasei* DSM 24733, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734, *Lactobacillus acidophilus* DSM 24735, *Bifidobacterium longum* DSM 24736, and *Bifidobacterium infantis* DSM 24737 (Vivomixx®, Dupont, Madison, WI, USA).

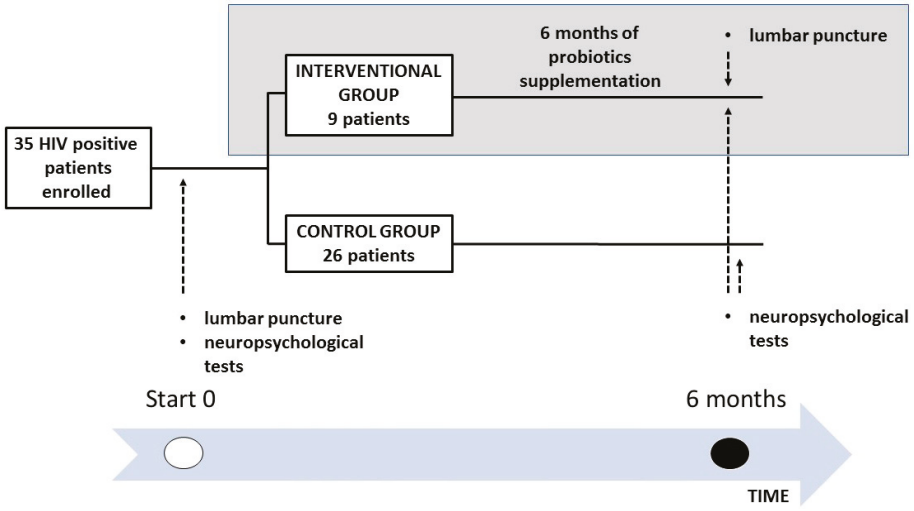

Figure 1. The design of the study. (Abbreviations. HIV: Human Immunodeficiency Virus).

At the end of the supplementation period (T6) participants from the intervention group underwent a second lumbar puncture and a second neurocognitive assessment battery, while controls (the remaining 26 subjects) were assessed with a second assay of neurocognitive tests (a parallel version of the Rey Auditory Verbal Learning Test (RAVLT) was administered at T6; parallel versions with Italian validation for the other tests are not available).

2.2. Neuropsychological Test Battery

Neuropsychological tests, administered by a trained neuropsychologist, explored verbal areas, language, attention, working memory, abstraction, executive, learning memory, processing speed of information, sensory-perceptual and motor skills. The tests included the Rey–Osterrieth Complex Figure Test (ROCF), to evaluate participants' recognition and recall skills for non-verbal contents, the Rey Auditory Verbal Learning Test (RAVLT) to evaluate short term auditory-verbal memory, rate of learning and retention of information, the Test of Weights and Measures Estimation (STEP), to evaluate abstraction skills, the Visual Search Test (Attention Matrices Test) to evaluate attention skills, the Verbal Fluency test (FAB), to evaluate executive functions and the ability to switch between different tasks, the Test of Phonological and Semantic Verbal Fluency (respectively PVF and SVF) to evaluate phonological and semantic supplies and the ability to access them, Raven's Standard Progressive Matrices (SPM), to evaluate abstract reasoning and problem solving capabilities, the Digit Span test, to evaluate short term memory and executive functions, the Corsi Block Tapping Test (CBTT) to evaluate short term spatial memory and executive functions, the Aachener Aphasia Test (AAT), to evaluate the presence of aphasia among study participants, the Trail Making Test A and B (TMT A and TMT B), to evaluate

visual-spatial attention and motor skills. On average, the neuropsychological evaluation required 45 min for the operator.

2.3. Evaluation of Neopterin Levels by ELISA Assay

CSF was collected by lumbar puncture, and cell-free centrifuged supernatant samples were stored at −80 °C. CSF neopterin levels were determined by a commercially available solid phase enzyme-linked immunosorbent assay (ELISA), based on the basic principle of a competitive ELISA (IBL International GmbH, Hamburg, Germany). The upper normal reference value was previously determined to be 5 nmol/L (upper limit of the 99% confidence interval) [12].

2.4. Bacterial DNA Isolation from Fecal Samples

Faecal samples from patients enrolled in the intervention group, were collected at T0 and T6, in order to evaluate adherence to the treatment and the efficacy of the multi-strain probiotic supplementation in changing the microbiota composition. For this reason, the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instructions: 200 mg of frozen samples were suspended in 1.4 mL of ASL lysis buffer from the stool kit, added with glass beads (150–212 μm, Sigma–Aldrich, St. Louis, MO, USA), and homogenized. The suspension was incubated at 95 °C for 5 min, DNA was purified and eluted in 200 μL of AE buffer and the samples obtained were stored at −20 °C. Finally, bacterial DNA from faecal samples was extracted and quantified by a real-time PCR, performed to evaluate Bifidobacteria levels. Briefly, PCR amplification and detection were performed on optical-grade 96-well plates, using the Applied Biosystems 7500 Real-Time PCR instrument (Applied Biosystems, Inc., Norwalk, CT, USA). The reaction mixture (25 μL) was composed of SensiMix SYBR Low-ROX (BIOLINE, Taunton, MA, USA), 500 nM primers for *Bifidobacterium* genus, and 2.5 μL of template DNA. A melting curve analysis was made after amplification, to distinguish target amplicons from aspecific non-target PCR products. Standard curves were made by using 10-fold dilutions of DNA, extracted from *Bifidobacterium breve*. All samples were analyzed in duplicate in two independent real-time PCR assays.

2.5. Statistical Analysis

A Wilcoxon test for paired samples and Pearson's correlation coefficient were applied for data analysis, using SPSS version 24 for Windows (IBM, New York, NY, USA). Graphics were done using GraphPad Prism software, version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Demographic and Clinical Characteristics of HIV-1-Positive Patients

All 35 HIV-1 infected individuals enrolled were Caucasian. The majority of our population was represented by males (94%), the median age of participants was 48 years old (IQR: 38–54) and the median duration of time from diagnosis was 14 years (IQR: 7–23). All included subjects had finished at least the primary course of school education.

Individuals enrolled in the study had been taking cART therapy for a median of 14 years (IQR: 8–19) and they had been on a stable and effective ARV regimen for at least 1 year at the time of inclusion (all participants showed plasma HIV RNA <37 copies/mL at enrollment). Antiretroviral therapy did not change and anti-inflammatory drugs were not used during the follow-up. The median value, in regard to cluster of differentiation 4 (CD4) nadir, was 250 cell/μL (IQR: 45–400) while the median value of the actual CD4 count was 566 cell/μL (IQR: 397–714).

The CNS Penetration-Effectiveness score (CPE) of ARV regimens included in the study was calculated according to the classification proposed by Letendere et al. and the median CPE score of our population was 7 (IQR: 7–8).

3.2. Correlations between Neuroinflammation and Neuropsychological Impairment

At baseline (T0), all participants underwent a lumbar puncture to evaluate their HIV-1 viral load and the extent of neuro-inflammation (determined through the concentration of neopterin) into CSF. All subjects enrolled in the study showed an HIV-RNA in CSF <37 copies/mL and the median concentration of neopterin in CSF was 23.4 nmol/L (10.5–65.2). Participants also showed negative polimerase-chain reaction (PCR) tests on CSF for CMV, EBV, HSV-1, HSV-2, VZV, HHV-8, BKV and JCV.

At baseline, all participants showed a normal performance on the Mini-Mental State Examination (MMSE) and no impairment on the instrumental activities of daily living (IADL) scale. Neuropsychological tests, administered at T0, with the aim of assessing the neurocognitive performance, showed that all subjects presented an altered result in at least one test exploring the executive functions, moreover most participants presented with a pathological impairment in at least two different domains.

In our population, we did not observe a correlations between the CPE score and neopterin concentration in CSF ($r = 0.220$; $p = 0.271$); the CPE score did not show any correlations with the results of any of the proposed neurocognitive tests either. On the contrary, at T0, neopterin was inversely correlated with the results for the following tests: forward Corsi Block Tapping Test ($r = -0.474$; $p = 0.004$), backward Corsi Block Tapping Test ($r = -0.468$; $p = 0.005$), forward Digit test ($r = -0.480$; $p = 0.004$) and Verbal Fluency test ($r = -0.361$; $p = 0.033$). Neopterin showed slight inverse correlations with Raven's Standard Progressive Matrices test ($r = -0.308$; $p = 0.071$), the time estimation during the Test of Weights and Measures Estimation ($r = -0.295$; $p = 0.085$) and the Test of Weights and Measures total score ($r = -0.294$; $p = 0.087$).

3.3. Results of Probiotic Supplementation: Reduction of Neuroinflammation and Recovery of Neuropsychological Impairment

A subgroup of nine subjects (intervention group), including the individuals presenting with the highest extent of neuro-inflammation from the 35 patients enrolled, underwent a 6 months course with a high dose of oral probiotics supplementation (the main characteristics of this subpopulation are shown in Table 1). The main characteristics of this subpopulation of nine people, compared with the control group of the remaining 26 subjects, are shown in Table 1.

Table 1. Sub-study population (supplemented with multi-strain probiotics) and control group main characteristics (expressed as median values; interquartile range is reported between brackets).

Characteristics	Probiotics Supplementation Group	Control Group	*p*-Value
N of subjects	9	26	
Males	9	24	
Females	0	2	
Age	45 (35–52.5)	43 (38.2–53)	0.097
Years from diagnosis	14 (5–19.5)	12.5 (7–23)	0.593
Years on ARV treatment	14 (6.5–16)	12.5 (7–20)	0.373
T CD4 nadir	180 cell/μL (40–438)	288 cell/μL (57–407)	0.678
T CD4 at enrollment	651 cell/μL (563–883)	526 cell/μL (340–663)	0.515
CPE score	7 (7–7.25)	7 (7–8)	0.527
HIV-RNA in CSF	<37 copies/mL	<37 copies/mL	-
Neopterin in CSF	34.14 nmol/L (22.5–65.2)	12.3 nmol/L (10.1–14.8)	0.008

Abbreviations. ARV: antiretroviral; CD4: cluster of differentiation 4; CPE: Central Nervous System Penetration Effectiveness; CSF: cerebrospinal fluid.

The median neopterin concentration in CSF at T0 in this subpopulation was 34.14 nmol/L (IQR: 22.53–65.2), showing no significant difference in comparison to the median value of the entire population ($p = 0.655$), but significantly higher than the median value of the control group ($p = 0.008$) Before probiotic supplementation (T0), we did not find significant differences in neurocognitive

performances between the two subgroups, despite the differences in neopterin levels. (Table 2). No statistically significant difference in CSF neopterin levels were found between the two groups ($p > 0.05$) at T0; higher levels were found in patients with the impairment of at least two neurocognitive domains, than in those with a single neurocognitive domain impairment.

Table 2. Baseline neurocognitive tests results (expressed as median values; interquartile range is reported between brackets).

Neurocognitive Tests	Probiotics Supplementation Group (T0)	Control Group (T0)	*p*-Value
Rey–Osterrieth Complex Figure immediate recall (*more is better*)	16.6 (15.9–17.8)	13.1 (8.5–22.0)	0.860
Rey–Osterrieth Complex Figure delayed recall (*more is better*)	15.5 (14.3–17.8)	11.6 (6.3–19.9)	0.280
Rey Auditory Verbal Learning Test immediate recall (*more is better*)	46.0 (29.4–47.4)	30.6 (27.9–40.0)	0.214
Rey Auditory Verbal Learning Test delayed recall (*more is better*)	9.2 (5.6–10.9)	5.2 (3.5–8.0)	0.360
Rey Auditory Verbal Learning Test recognition (*more is better*)	98.0 (90.0–100.0)	96.0 (92.0–98.0)	0.400
Verbal Fluency (*more is better*)	15.0 (13.7–16.0)	15.9 (13.9–18.0)	0.314
Phonological Verbal Fluency (*more is better*)	30.0 (23.6–39.2)	26.7 (21.6–35.1)	0.906
Semantic Verbal Fluency (*more is better*)	47.0 (33.5–57.5)	39.0 (33.0–42.0)	0.173
Visual Search Test (*more is better*)	46.2 (45.1–60.0)	46.7 (40.2–50.6)	0.374
Test of Weights and Measures Estimation—Time (*more is better*)	19.0 (13.0–23.5)	22.0 (19.0–24.0)	0.074
Test of Weights and Measures Estimation—Weight (*more is better*)	19.0 (14.0–20.5)	19.0 (16.5–21.0)	0.933
Test of Weights and Measures Estimation—Total (*more is better*)	38.0 (30.5–46.5)	40.0 (36.5–43.5)	0.594
Raven's Standard Progressive Matrices (*more is better*)	27.5 (22.6–31.6)	28.3 (25.7–31.8)	0.327
Verbal Span forward (*more is better*)	5.0 (3.5–5.7)	5.2 (4.9–6.0)	0.065
Verbal Span backward (*more is better*)	5.0 (4.0–5.0)	4.0 (3.0–4.5)	0.161
Corsi Block Tapping Test forward (*more is better*)	4.7 (4.0–5.2)	5.5 (4.7–6.0)	0.078
Corsi Block Tapping Test backward (*more is better*)	4.0 (3.0–4.0)	5.0 (4.0–6.0)	0.196
Aachener Aphasia Test (*more is better*)	9.0 (9.0–9.0)	9.0 (9.0–9.0)	1.000
Trail Making Test A (s) (*less is better*)	50.0 (44.0–62.0)	50.0 (41.0–67.0)	0.575
Trail Making Test B (s) (*less is better*)	115.0 (93.0–142.0)	97.0 (77.5–144.5)	0.086

At the end of supplementation (T6), in the intervention group, we observed that the results of viral replication in CSF remained stably suppressed (HIV-RNA in CSF resulted <37 copies/mL in all subjects at T6) and the neopterin concentration in CSF significantly decreased at T6 when compared to T0 values (T6 24.11 nmol/L vs. T0 34.14 nmol/L; $p = 0.011$). The results of the neuropsychological tests at T0 and T6 among participants who underwent probiotics supplementation are shown in Table 3; an improvement in overall neurocognitive performance was observed in the majority of our population, with a significant improvement revealed in multiple tests—immediate copy of the Rey–Osterrieth Complex Figure ($p = 0.0089$), delayed copy of the Rey–Osterrieth Complex Figure ($p = 0.0039$), immediate recall during the Rey Auditory Verbal Learning Test ($p = 0.027$), delayed recall during the Rey Auditory Verbal Learning Test ($p = 0.042$), time estimation during the Test of Time and Weights Estimation ($p = 0.043$), weight estimation during the Test of Time and Weights Estimation ($p = 0.035$), Phonological Verbal Fluency Test ($p = 0.027$), Trail Making Test A ($p = 0.0502$) and forward Corsi Block Tapping Test ($p = 0.057$). A direct correlation with neopterin concentration in CSF was observed at T6 with the results for the Trail Making Test B ($r = 0.741$; $p = 0.022$); in contrast an inverse correlation was observed between neopterin and the forward Corsi Block Tapping Test ($r = -0.793$; $p = 0.011$). A slight inverse correlation was also observed between Raven's Standard Progressive Matrices test and neopterin in CSF ($r = -0.644$; $p = 0.061$). CPE scores did not show any correlation with neopterin or any of the provided neurocognitive tests, at T6. No difference was observed in the CD4 count between T0 and T6 among these individuals (674 cell/μL vs. 682 cell/μL; $p = 0.959$).

Neurocognitive performance was also evaluated among the 26 individuals who did not undergo supplementation with probiotics; this was assessed by a second administration of the assay of neuropsychological tests at T6 (results are shown in Table 3). No difference between T0 and T6 was observed for neurocognitive performance among this group of participants.

Table 3. Neurocognitive tests results after supplementation with probiotics (expressed as median values; interquartile range is reported between brackets).

Performed Neurocognitive Tests	Probiotics Supplementation Group (T0 vs. T6)	Control Group (T0 vs. T6)	Probiotics Supplementation Group vs. Control Group (T6 vs.T6)
Rey–Osterrieth Complex Figure immediate recall (*more is better*)	16.6 vs. 22.0 ($p = 0.007$)	13.1 vs. 14.7 ($p = 0.603$)	22.0 (19.0–23.7) vs. 14.7 (8.8–20.3) ($p = 0.011$)
Rey-Osterrieth Complex Figure delayed recall (*more is better*)	15.5 vs. 22.4 ($p = 0.008$)	11.6 vs. 12.6 ($p = 0.369$)	22.4 (22.0–25.5) vs. 12.6 (5.7–19.1) ($p = 0.011$)
Rey Auditory Verbal Learning Test immediate recall (*more is better*)	46.0 vs. 53.0 ($p = 0.028$)	30.6 vs. 32.5 ($p = 0.619$)	53.0 (49.3–55.6) vs. 32.5 (28.7–37.5) ($p = 0.008$)
Rey Auditory Verbal Learning Test delayed recall (*more is better*)	9.2 vs. 12.0 ($p = 0.034$)	5.2 vs. 5.3 ($p = 0.241$)	12 .0 (10.7–13.8) vs. 5.3 (4.3–8.0) ($p = 0.008$)
Rey Auditory Verbal Learning Test recognition (*more is better*)	98.0 vs. 99.0 ($p = 0.176$)	96.0 vs. 96.0 ($p = 0.575$)	99.0 (97.0–100.0) vs. 96.0 (92.0–98.0) ($p = 0.013$)
Verbal Fluency (*more is better*)	15.0 vs. 15.9 ($p = 0.233$)	15.9 vs. 15.3 ($p = 0.152$)	15.9 (14.1–18.0) vs. 15.3 (13.7–16.8) ($p = 0.594$)
Phonological Verbal Fluency (*more is better*)	30.0 vs. 44.0 ($p = 0.028$)	26.7 vs. 25.9 ($p = 0.271$)	44.0 (42.5–45.0) vs. 25.9 (21.6–36.1) ($p = 0.021$)
Semantic Verbal Fluency (*more is better*)	47.0 vs. 49.0 ($p = 0.373$)	39 vs. 38.0 ($p = 0.396$)	49.0 (46.0–49.0) vs. 38.0 (33.5–43.5) ($p = 0.123$)

Table 3. *Cont.*

Performed Neurocognitive Tests	Probiotics Supplementation Group (T0 vs. T6)	Control Group (T0 vs. T6)	Probiotics Supplementation Group vs. Control Group (T6 vs.T6)
Visual Search Test (*more is better*)	46.2 vs. 49.0 (p = 0.859)	46.7 vs. 46.7 (p = 1.000)	49.0 (45.6–50.0) vs. 46.7 (40.2–50.6) (p = 0.722)
Test of Weights and Measures Estimation—Time (*more is better*)	19.0 vs. 23.0 (p = 0.038)	22.0 vs. 22.0 (p = 0.776)	23.0 (21.0–23.5) vs. 22.0 (18.0–25.0) (p = 0.512)
Test of Weights and Measures Estimation—Weight (*more is better*)	19.0 vs. 21.0 (p = 0.027)	19 vs. 20.0 (p = 0.843)	21.0 (20.5–23.5) vs. 20.0 (15.5–21.5) (p = 0.08)
Test of Weights and Measures Estimation—Total (*more is better*)	38.0 vs. 45.0 (p = 0.138)	40.0 vs. 40.0 (p = 0.776)	45.0 (41.5–46.0) vs. 40.0 (35.5–44.0) (p = 0.02)
Raven's Standard Progressive Matrices (*more is better*)	25.7 vs. 30.0 (p = 0.208)	28.3 vs. 28.3 (p = 0.939)	30.0 (28.5–33.5) vs. 28.3 (25.2–31.6) (p = 0.374)
Verbal Span forward (*more is better*)	5.0 vs. 5.0 (p = 0.121)	5.2 vs. 5.2 (p = 0.632)	5.0 (5.0–6.0) vs. 5.2 (4.6–6.0) (p = 0.551)
Verbal Span backward (*more is better*)	5.0 vs. 5.0 (p = 1.000)	4.0 vs. 4.0 (p = 0.344)	5.0 (4.0–5.0) vs. 4.0 (3.75–5.0) (p = 0.206)
Corsi Block Tapping Test forward (*more is better*)	4.7 vs. 5.2 (p = 0.049)	5.5 vs. 5.2 (p = 0.980)	5.2 (5.0–5.5) vs. 5.2 (5.0–6.0) (p = 0.888)
Corsi Block Tapping Test backward (*more is better*)	4.0 vs. 4.0 (p = 0.180)	5.0 vs. 5.0 (p = 0.317)	4.0 (4.0–4.5) vs. 5.0 (4.0–5.0) (p = 0.102)
Aachener Aphasia Test (*more is better*)	9.0 vs. 9.0 (p = 1.000)	9.0 vs. 9.0 (p = 1.000)	9.0 (9.0–9.0) vs. 9.0 (9.0–9.0) (p = 1.000)
Trail Making Test A (s) (*less is better*)	50.0 vs. 43.0 (p = 0.041)	50.0 vs. 51.0 (p = 0.747)	43.0 (39.0–53.0) vs. 51.0 (40.5–65.5) (p = 0.674)
Trail Making Test B (s) (*less is better*)	115.0 vs. 120.0 (p = 0.726)	97.0 vs. 98.0 (p = 0.279)	120.0 (76.0–138.0) vs. 98.0 (78.5–146.0) (p = 0.138)

3.4. Adherence to Probiotic Supplementation and Safety of the Treatment

Adherence to the probiotic supplementation was documented by the increase in Bifidobacteria spp. in fecal samples collected at T6, compared to their basal level (T0). No side effects were observed over the course of 6 months of treatment in all patients.

4. Discussion

In recent years, the interdependence between the microbiome, gut and brain has been highlighted. The disruption of the gut mucosa barrier and intestinal dysbiosis—both conditions commonly observed in HIV-1 infected patients—play a pivotal role in the pathogenesis of HIV infection [13] and several studies have shown the beneficial effects of probiotics on the intestinal barrier, systemic immune activation and tryptophan metabolism of on HIV-1 infected patients [14,15].

At T0, in the overall analysis of the 35 patients enrolled, we observed that higher neopterin levels in CSF were correlated with a poorer result in the neurocognitive tests. No correlation was observed between the CNS Penetration Effectiveness (CPE) score and the neopterin concentration in CSF, nor between the CPE score and neurocognitive performance, suggesting a possible low impact of cART on the treatment of neuroinflammation and neurocognitive impairment.

When we divided the enrolled patients into two groups (intervention group and control group) on the basis of their neuroinflammation levels, we observed that, at T0, both groups showed similar

results in neurocognitive performances (Table 2), despite their different neopterin concentrations observed in CSF; taken together, these data suggest that the levels of neuroinflammation are not the only determinant of the neurocognitive impairment in HIV-1 infected patients. On the other hand, to investigate the role of dysbiosis on neuroinflammation and cognitive performance, we assigned nine participants (with highest levels of neuroinflammation shown by their levels of neopterin in CSF, which were higher than the control group) from our study population, to start supplementation with oral probiotics for 6 months (interventional group).

At the end of the supplementation period, we observed a reduction in CNS immune activation and an improvement in cognitive performance in the intervention group, thus suggesting a possible role of probiotics in the treatment of the intrathecal immune activation and cognitive impairment of HIV infected individuals. In fact, at T6, the intervention group showed improved results in comparison to controls in the following tests: immediate recall of ROCF, delayed recall of ROCF, immediate recall of RAVLT, delayed recall of RAVLT, recognition of RAVLT, FAB, STEP weight and STEP total; improvements were also observed within the intervention group between T0 and T6, while, on the other hand, controls showed no advances (Table 3).

Also, the neopterin concentration decreased after supplementation with probiotics, but the reduction in the neopterin concentration cannot be explained by better suppression of HIV replication in CSF, given the fact that HIV-RNA results were undetectable in all participants at T0 and T6. On the other hand, the alteration of the gut mucosa barrier that occurs during HIV-1 infection allows a large amount of bacterial-derived products (such as lipopolysaccharides) to enter into the general circulation, thus causing systemic immune activation. Experimental evidence has shown that lipopolysaccharides can alter the blood-brain barrier permeability [16], possibly leading to leakage of the serum pro-inflammatory milieu into the CSF, precipitating local inflammation. In this sense, several studies have shown that the gut microbiota and its products play central regulatory roles in this bidirectional relationship between the enteric and the central nervous system and on gut-brain axis [17–22]. The administration of probiotics to correct dysbiosis reduces microbial translocation, systemic inflammation and, possibly, the subsequent blood-brain barrier permeability, thus exerting an ameliorative effect on intrathecal immune activation and cognitive function.

For these reasons, probiotic supplementation could represent an innovative therapeutic resource, enhancing the direct-indirect effects of the gut environment on the CNS, through the rebalance of the microbiota composition.

5. Conclusions

The results of this study were limited by the small sample size of patients analyzed and by the possible influence of the operator and repeated tests on neuropsychological performances. To minimize these practice effects, we used parallel/alternate forms of tests, but we are aware that it does not fully eliminate concerns about this topic. Further limits were the lack of CSF samples at T6 in the control group and of plasmatic/serological markers of immune activation. The significance of our results was restricted by the inclusion of only patients with ANI (Asymptomatic Neurocognitive Impairment). Moreover, a possible limitation is the role of ANI that is, at present, being debated.

Despite these limitations, justified by the complexity of the procedures and ethical issues, our findings suggest that supplementation with probiotics could represent a novel strategy to manage neurocognitive impairment in cART treated HIV-1 infected patients, but larger studies are needed prior to introducing this intervention into everyday clinical practice.

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Effect of a *Lactobacillus Salivarius* Probiotic on a Double-Species *Streptococcus Mutans* and *Candida Albicans* Caries Biofilm

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Abstract: The aim of the study was to evaluate the anti-cariogenic effects of *Lactobacillus salivarius* by reducing pathogenic species and biofilm mass in a double-species biofilm model. Coexistence of *S. mutans* with *C. albicans* can cause dental caries progression or recurrence of the disease in the future. Fifty-nine children with diagnosed early childhood caries (ECC) were recruited onto the study. The condition of the children's dentition was defined according to the World Health Organization guidelines. The participants were divided into children with initial enamel demineralization and children showing dentin damage. The study was performed on the *S. mutans* and *C. albicans* clinical strains, isolated from dental plaque of patients with ECC. The effect of a probiotic containing *Lactobacillus salivarius* on the ability of *S. mutans* and *C. albicans* to produce a double-species biofilm was investigated in an in vitro model. The biomass of the formed/non-degraded biofilm was analyzed on the basis of its crystal violet staining. The number of colonies of *S. mutans* and *C. albicans* (CFU/mL, colony forming units/mL) forming the biofilm was determined. Microorganism morphology in the biofilm was evaluated using a scanning electron microscope (SEM). In vitro analysis demonstrated that the presence of *S. mutans* increased the number of *C. albicans* colonies (CFU/mL); the double-species biofilm mass and hyphal forms produced in it by the yeast. *L. salivarius* inhibited the cariogenic biofilm formation of *C. albicans* and *S. mutans*. Under the influence of the probiotic; the biofilm mass and the number of *S. mutans*; *C. albicans* and *S. mutans* with *C. albicans* colonies in the biofilm was decreased. Moreover; it can be noted that after the addition of the probiotic; fungi did not form hyphae or germ tubes of pathogenic potential. These results suggest that *L. salivarius* can secrete intermediates capable of inhibiting the formation of cariogenic *S. mutans* and *C. albicans* biofilm; and may inhibit fungal morphological transformation and thereby reduce the pathogenicity of *C. albicans*; weakening its pathogenic potential. Further research is required to prove or disprove the long-term effects of the preparation and to achieve preventive methods.

Keywords: *Lactobacillus salivarius*; probiotic; *Candida albicans*; cariogenic biofilm; *Streptococcus mutans*

1. Introduction

Early childhood caries (ECC) is one of the most widespread infectious diseases associated with biofilm formation in children worldwide—in Poland, it is prevalent in over 85% of pre-school children (<6 years) [1,2]. Untreated, ECC can lead to rapid tooth damage, causing pain and dangerous systemic infections [3]. Despite preventive measures and dental interventions, the first signs of ECC create a high risk of future recurrences [4], generating health and economic burdens among people at risk.

As proposed by the FAO/WHO (Food and Agriculture Organization of the United Nations, World Health Organization) definition, probiotics are live microorganisms, not causing any adverse effects on the organism and provide health benefits when administered in appropriate amounts [5].

Currently, interest in probiotic usage, in the case of caries prophylaxis, is growing, but their clinical efficacy in disease prevention appears limited and controversial [6,7]. Research on the use of probiotics in caries focuses primarily on their mechanism of growth inhibition and dental plaque reduction, created by pioneer *Streptococcus mutans* (*S. mutans*) strains, which are the main etiologic agent of this disease [8]. Even in clinical trials, endpoints usually constitute indirect measurements and are mainly associated with a decrease in *S. mutans* in the saliva [9–11] or a reduction of dental plaque acidity [12]. They do not affect the improvement of tooth enamel mineralization or the inhibition of plaque formation, which seems to be more adequate in the context of permanent (residential) colonization of *S. mutans* at the site of developing caries lesions (in plaque but not saliva). Bacteria, such as *S. mutans* as well as *Candida* genus fungi, present in the saliva (suspended as planktonic forms) are not direct etiologic factors of oral diseases [13]. Saliva is a material that only contains the transient presence of cariogenic bacteria or opportunistic fungi of the *Candida* genus and mediates the invasion of pathogenic bacteria in inflammatory foci [14].

In several clinical trials, the administration of *Lactobacillus* genus (*L. reuteri*, *L. salivarius*, *L. rhamnosus*) probiotics has demonstrated a decrease in the intensity of caries in children [10,15,16]. However, certain literature data show the reverse effects of the administration of *Lactobacillus*-containing probiotics (including *L. rhamnosus*, *L. reuteri*, and *L. paracasei*), due to the lack of *S. mutans* inhibition [17–19].

Probiotics contain microorganisms that do not show negative effects on the body; instead, they affect the human microflora through various mechanisms. They compete with other bacteria for nutrients and binding sites to the medium, inhibit their growth by producing bacteriocins, and further stimulate the immune response of the host. This occurs through the possible control of cytokine over-expression [20–22]. Nevertheless, the use of probiotics in immunocompetent hosts seems controversial, because of the reported cases of generalized infections from probiotic strains [20,23].

Indeed, *Candida albicans* (*C. albicans*) and *S. mutans* coexist for early childhood caries (ECC) [24,25]. On one hand, *Candida*-derived β-1,3-glucans affect the structure of the exopolysaccharide matrix (EPS), while mannan and β-glucan provide binding sites for glucosyltransferase B (GtfB). *Candida albicans* occurs in 96% of children with caries (age: 6–12 years), but only in 24% of children without this disease [26]. Currently, no in vivo studies have been conducted to demonstrate mutual interactions between *S. mutans* and *C. albicans* in models similar to actual oral cavity conditions in children with caries.

Oral streptococci produce proteins anchored in the cell wall to facilitate binding to *C. albicans* [27]. There is a specific hyperadditive effect of *S. oralis* and *C. albicans* during the fungi supported streptococcal biofilm production on mucous membranes [28]. Thus, not only mutants, but also species, such as *C. albicans*, can be decisive in determining the cariogenicity of the formed biofilms [29]. This was confirmed by recent studies, which demonstrated that increased *Candida* was associated with reduced diversity of salivary microbiota and displacement of the microbial aggregate toward streptococci [30].

Probiotics aggravate or delay the colonization of pathogenic bacteria during biofilm formation [31,32]. The mechanism of coaggregation of *S. mutans* with other bacteria has been intensively investigated, but there are few studies evaluating the effect of probiotics on this process.

These focus mainly on the ability to inhibit mono-species biofilms or they evaluate the effects of probiotics only on salivary *S. mutants*, without evaluation of their relationships with an oral microbiome [33–35].

The limited number of studies on mutual interactions between clinical *S. mutans* and *C. albicans* strains in cariogenic biofilms and the effects of the probiotic *L. salivarius* on such interactions prompted us to address this problem.

The aim of the study was to evaluate the effect of a probiotic containing *Lactobacillus salivarius* on the mutual interactions of *S. mutans* and *C. albicans* as well as the ability to form a double-species biofilm, isolated from clinical strains, in an in vitro model.

2. Materials and Methods

2.1. Study Group

The examination was conducted according to the guidelines outlined in the Helsinki Declaration of 2008. The material was collected after the written consent of all participants (children and parents as their legal guardians). The Bioethics Committee of the Jagiellonian University in Krakow approved the study protocol (No. 122.6120.99.2016.).

The study was conducted from December 2016 to May 2017, involving a total of 59 pediatric subjects who had been screened/examined for study inclusion/exclusion by the University Dental Clinic, who were recruited by the Children's Dentistry Laboratory of Dental Clinics, Jagiellonian University, Krakow. Bacterial strains were isolated from plaque samples derived from those participants (n = 59, mean age: 4.54 $\pm$ 0.79 years) who were diagnosed with early childhood caries (ECC) of the deciduous teeth. ECC was only diagnosed by a clinical examination.

The condition of the children's dentition was determined in accordance with the guidelines of the World Health Organization for epidemiological studies on oral health, with the use of the International Caries Detection and Assessment System (ICDAS) classification. The study group was divided into two main groups: cavitated (where carious lesions were defined as cavity lesions in fissures and smooth surfaces with soft bottoms and walls) and non- cavitated (with white or brown enamel discolorations, but without enamel quantity damage, as well as undiluted enamel without cavities) [36,37].

The non-cavitated group (initial enamel demineralization, or white spots) corresponded to 1–2 in the ICDAS classification, whereas the cavitated group (dentine or cavitated damage) corresponded to 5–6 in the ICDAS classification [38]. Thirty participants qualified for the non-cavitated group, whereas 29 qualified for the group conventionally referred to as cavitated.

The exclusion criteria included: age below 2 years or over 6 years; inflammatory oral diseases, other oral diseases such as epithelial dysplasia, and periodontal pathology; and systemic illnesses, such as diabetes mellitus or hypertension. The use of antibiotics, anti-inflammatory drugs, or steroids, and a diet rich in supplements, such as vitamins or probiotics, in the past 5 months were also criteria for exclusion, along with partial or complete rejection of the dental examination by the child or their legal guardian. Plaque was evaluated, based on the simplified oral hygiene index (OHI-S index) [39].

2.2. Plaque Sampling Methods

Dental plaque was collected using dental probes after the patient's qualification. Each patient was instructed about how to prepare for the test. Prior to the decision about giving consent for the study, details of the study and the scope of application were explained by the investigator. The study protocol was conducted with respect for the religious values of the study participants. The plaque samples were collected in the morning, between 8 a.m. and 9 a.m., under fasting conditions, and before the clinical examination and brushing. Prior to plaque collection, each patient rinsed their mouth with deionized water. Plaque samples were then placed in tubes containing 0.5 mL of phosphate buffered saline (PBS), maintained anaerobically. The samples were transported within 2 h at 4 °C to the laboratory. Plaque samples were broken up in an ultrasonic homogenizer (Hielscher UP50H)

for 30 s at 25% amplitude and gently vortexed to give a homogeneous suspension. Cells were harvested in a logarithmic growth phase and washed 3 times with 40 mM potassium phosphate buffer (pH 7.0). Fifty µL of a homogenous microorganism suspension was used in the study for conventional culture methods, using a Sabouraud medium (Sabouraud Dextrose Agar, SDA) and a HLR-S (HL Ritz medium containing 40 g tryptic soy agar (TSA), 20% sucrose, 0.3 U/mL bacitracin, 1.75 µg/mL polymyxin B sulfate and 0.5 µg/mL crystal violet) selective medium, in three 10-fold dilutions, for each prepared sample. The material was incubated at 37 °C for 48 h under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2). Positive samples were selected when colony numbers >10,000 cells/mL. The morphological characteristics of individual colonies cultivated on the medium with sheep blood were evaluated, as was the type of hemolysis caused by these colonies. In addition, dental plaque dilutions were performed, and these were inoculated on the Sabouraud medium and on the HLR-S medium. Once grown, the colonies were counted via determination of CFU/mL (colony forming units/mL). The number of microorganisms present in a particular test sample was determined using the formula:

$$\text{CFU/mL} = \text{CFU} \times \text{dilution factor} \times 1 \text{ aliquot}$$

2.3. Characteristics of Isolated Species of Bacteria and Fungi

Pure *S. mutans* colonies were inoculated from the selective HLR-S to the Tryptic Soy Agar (TSA) with 5% sheep blood, as well as the Sabouraud medium, and incubated under the optimal conditions determined previously. The characteristic appearance of the colonies, including shape, form (single cells, pseudomycelium cells, or mycelium hyphae), hemolysis factor and other parameters that may create the phenotype were evaluated. Gram staining was performed every time as an element of a pre-differential diagnosis (gram-positive and gram-negative bacteria).

2.4. Phenotyping

The species of isolated *S. mutans* were determined using a commercial STREPTOtest24 bioassay routine test (Erba Lachema, Brno, Czech Republic) and the API 20C AUX test (bioMérieux, Warsaw, Poland) for *C. albicans*.

2.5. Preparation of Microbial Suspensions

S. mutans and *C. albicans*, isolated from children with ECC, were used in the study. Single *S. mutans* and *C. albicans* colonies were cultured for 8 h at 37 °C in the presence of 5% CO_2 in 4 mL of the Brain Heart Infusion medium (Merc, Darmstadt, Germany) and the Sabouraud liquid medium, with the addition of 5% sucrose, respectively. Bacteria and yeasts were harvested during the logarithmic growth phase, then washed twice with a 40 mM PBS (pH 7.0).

Microbial growth control was studied by flow cytometry (LSRII, BD Immunoassay Systems, San Jose, CA, USA). Cell conglomerates and doubles were discarded using a gated width-to-height spreading (FSC) and lateral scattering (SSC) strategy.

Bacterial and yeast suspensions were standardized to contain approximately 10^6 CFU/mL. This was performed by dilution overnight of a bacterial/yeast culture in 5 mL of PBS. The inoculum density was measured using a MicroSpeak dual densitometer and confirmed by counting single colonies after 24 h growth under the same conditions as those described for *S. mutans* (Brain Heart Infusion Agar BHI agar) and *C. albicans* (Sabouraud dextrose agar).

2.6. Biofilm Generation

Biofilm generation was determined using a widely accepted, microtiter plate type, where the biofilm grew on the bottom and the walls of the wells, or on the disks located in the plate wells [40]. Clinical strains of *Streptococcus mutans*, selected as a pathogenic factor of caries and *Candida albicans*, as a potential ECC etiologic factor, were isolated from children with caries and used to form single or double-species biofilms, as described below. Biofilm formation occurred on polystyrene discs,

placed vertically at the bottom of the 24-well microtiter plate wells, using sterile handles. Bacterial, fungal, and bacterial–fungal biofilm growth was investigated in the presence of a probiotic containing *L. salivarius* (HM6 Paradens). The crystal violet staining method was used to determine the biomass of the generated/degraded biofilm [41].

2.6.1. Mono-Species Biofilm

One hundred µL of a standardized bacterial suspension in a Brain Heart Infusion medium enriched with 5% sucrose of 1×10^6 CFU *S. mutans*/mL density was added to the wells of the microtiter plate. One hundred µL of a standardized fungal suspension in a Sabouraud liquid medium with 5% sucrose of 1×10^4 CFU *C. albicans*/mL density was added to the other wells. Plates were incubated for 90 min at 37 °C under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) to initiate the attachment of the microorganisms. Afterwards, the wells were washed twice with PBS solution. Next, 100 µL of FBS (Fetal Bovine Serum) was added to initiate biofilm formation, and the microplates were incubated for the next 2 h under microaerophilic conditions. Plates were then rinsed twice with PBS and 50 µL of a tested strain (HM6 Paradens) was added. Fifty µL of PBS (PBS Control), 50 µL of BHI + 5% sucrose (BHI Control), and 50 µL of Sabouraud + 5% sucrose (Sabouraud Control) were used as controls. For the biofilm growth and maintenance, 200 µL Sabouraud liquid medium with 5% sucrose or 200 µL of the BHI medium with 5% sucrose were added to *C. albicans* and *S. mutans* wells, respectively. Microplates were heated at 37 °C under microaerophilic conditions for 18, 20, 22, and 24 h (Figure 1).

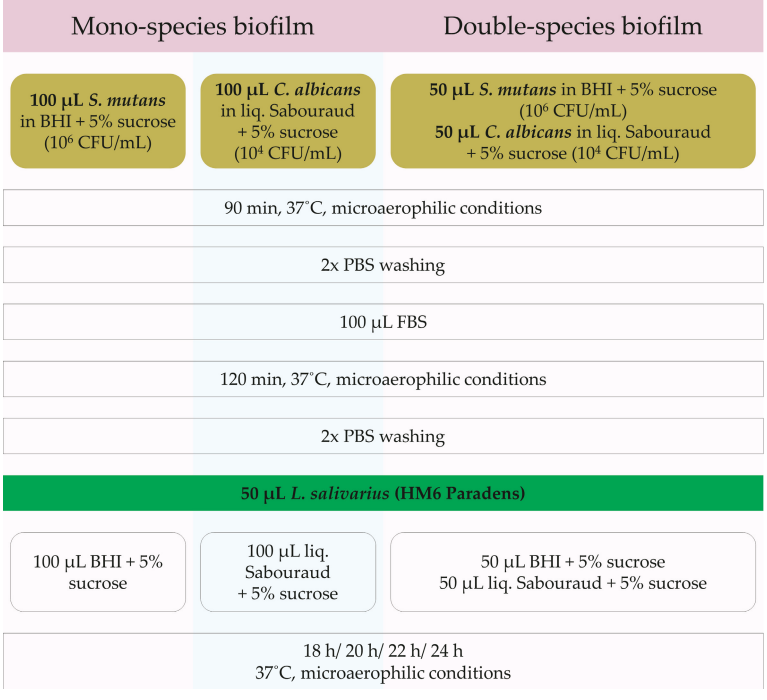

Figure 1. The protocol of biofilm generation and measurement. BHI: Brain Heart Infusion broth, CFU: colony forming units, liq: liquid, FBS: Fetal Bovine Serum, PBS: phosphate-buffered saline.

2.6.2. Double-Species Biofilm

In the case of double-species *S. mutans*/*C. albicans* biofilms, 50 µL of a standardized bacterial suspension in BHI medium enriched with 5% sucrose of 1×10^6 CFU *S. mutans*/mL density and 50 µL of a standardized fungal suspension in a Sabouraud liquid medium with 5% sucrose of 1×10^4 CFU *C. albicans*/mL density were added to the wells. Plates were incubated for 90 min at 37 °C under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) to initiate the attachment of the microorganisms. Afterwards, the wells were washed twice with PBS solution. Next, 100 µL FBS (Fetal Bovine Serum) was added to initiate biofilm formation, and the microplates were incubated for the next 2 h under microaerophilic conditions. Plates were then rinsed twice with PBS and 50 µL of a tested strain (HM6 Paradens) was added. Fifty µL of PBS (PBS Control), 50 µL of BHI + 5% sucrose (BHI Control), and 50 µL of Sabouraud + 5% sucrose (Sabouraud Control) were used as controls. For the *C. albicans*/*S. mutans* biofilm growth, 100 µL Sabouraud liquid medium with 5% sucrose and 100 µL of the BHI medium with 5% sucrose were added to the wells containing both *C. albicans* and *S. mutans*. Microplates were heated at 37 °C under microaerophilic conditions for 18, 20, 22, and 24 h (Figure 1).

Microorganism proportions in the wells were similar to those found in saliva samples in children with ECC. The organisms were grown without interruption, to allow generation and formation of biofilm, for 18, 20, 22 and 24 h, until the end of the experimental period.

2.7. Bacterial Enumeration (CFU/mL) in Biofilms

The biofilms were washed thrice with phosphate-buffered saline at different time points (after 18, 20, 22, 24 h of incubation). The biofilm generated at the bottom of the well was removed by blending in an ultrasonic homogenizer (Hielscher UP50H, Teltow, Germany) for 20 s at 25% amplitude. Serial dilutions of the resultant solution were prepared and seeded in amounts of 100 µL on Sabouraud agar for *C. albicans* and mitis salivarius-bacitracin agar with sucrose MSBS (containing bacitracin and sucrose) for *S. mutans*, and then their growth was promoted for the next 48 h under microaerophilic conditions. The colony forming units (CFU/mL) were indicated. The protocol was performed in triplicate.

2.8. Biofilm Mass Determination

The mass of any formed biofilm was determined at different time points (after 18, 20, 22, 24 h of growth) via the crystal violet method. The formed biofilm was fixed in methanol (99+%, Sigma–Aldrich, Poznan, Poland) for 20 min. Then, the supernatants were discarded and the plates were air dried. Next, 125 µL of crystal violet (CV, 0.1%) solution was pipetted to microtiter plate wells. CV excess was removed by 3-fold PBS washing. The bounded stain was released with 200 µL of 95% ethanol (Sigma–Aldrich, Poznan, Poland). Subsequently, the contents of the wells were pipetted and 125 µL of the suspension was carried out to the new plate. The biomass of the generated biofilm was determined through its measured absorbance, using the standard curve at the maximum wavelength (λmax) = 540 nm. The protocol was performed at 20 to 25 °C twice at different times. The biofilm generation curve was plotted.

2.9. Scanning Electron Microscopic Analysis of Biofilm

Microbial biofilms were grown on 13 mm diameter round basic slides (Agar Scientific, Stansted, UK) in the wells of a 24-well plate, according to the protocol described above.

The slides were then stabilized in 1 mL of a 2.5% glutaraldehyde for 1 h and dehydrated in serial dilutions (50, 70, 80, 90, 95, 100% *v/v*) of ethanol for 20 min. Subsequently, the slides were immersed in 100% ethanol for 1 h. The slides were air-dried for one day and then transferred to copper disks and dusted with gold (160 s, 40 mA). The samples were analyzed using a scanning electron microscope (JEOL JSM-35CF, SEM, Jeol, Japan) at 20–25 kV in the Laboratory of the Otolaryngology

Clinic, University Hospital, Krakow. The protocol was conducted at the above-mentioned time points in triplicate.

2.10. Statistical Methods

A statistical analysis was performed using R 3.2.3 (R Development Core Team, 2009). The ages of the children from the two subgroups were compared using Fisher's exact test (because of the low expected numbers in the contingency table). The Shapiro–Wilk test was used to verify the normality of the data. Non-parametric analyses were conducted. Data are demonstrated as median and range. To compare CFU (log-transformed) and the optical density (OD) between *S. mutans*, *C. albicans*, and the co-culture, the Kruskal–Wallis test was performed. Dunn's test (post-hoc test) was performed to determine which of the 3 groups actually differed.

The paired Wilcoxon's test was used to compare CFU (log-transformed) and the optical density (OD), before and after *Lactobacillus salivarius* (HM6 Paradens) administration. Spearman's coefficient of correlation was used to evaluate the relationship between colony-forming units (log-transformed) and the optical density ($p < 0.05$ indicated a statistically significant result).

3. Results

3.1. Study Design

During the study, bacterial and fungal strains were isolated from the plaque of children ($n = 59$, mean age: 4.54 ± 0.79 years) who were diagnosed with ECC of deciduous teeth.

Participants were assigned to two subgroups: children with early enamel deamination (white spots), defined as "non-cavitated" ($n = 30$; 1–2 in the ICDAS code); and children with dentin damage, assigned to the "cavitated" group ($n = 29$; 5–6 in the ICDAS code) [38]. Thirty participants, including 11 girls (4.91 ± 1.04 year/o) and 19 boys (4.47 ± 0.61 year/o), were allotted to the "non-cavitated" group, while 29 children to the "cavitated" one (14 girls aged 4.43 ± 0.94 years and 15 boys aged 4.47 ± 0.64 years). The form of caries did not depend on age, as shown in Table 1.

Table 1. Division of study groups according to patient age.

Examined Groups	Age: 3 Years ($n = 6$)	Age: 4 Years ($n = 20$)	Age: 5 Years ($n = 28$)	Age: 6 Years ($n = 5$)	p *
	n	*n*	*n*	*n*	
Non-cavitated	2	11	13	4	0.489
Cavitated	4	9	15	1	

* Fisher's exact test (because of the low numbers in the table).

3.2. Morphological Characterization of Isolated Species of Bacteria and Fungi

Interactions among *C. albicans* and *S. mutans* groups can create the biofilm demonstrated in Figure 2A–C. The isolated species of bacteria and fungi are listed in Table 2.

Table 2. Frequency of isolation of *S. mutans* and yeasts in the studied groups of patients.

Microorganisms	Girls ($n = 25$)	Boys ($n = 34$)	Total ($n = 59$)
	n	*n*	*n*
C. albicans	12	18	30
S. mutans	13	16	29

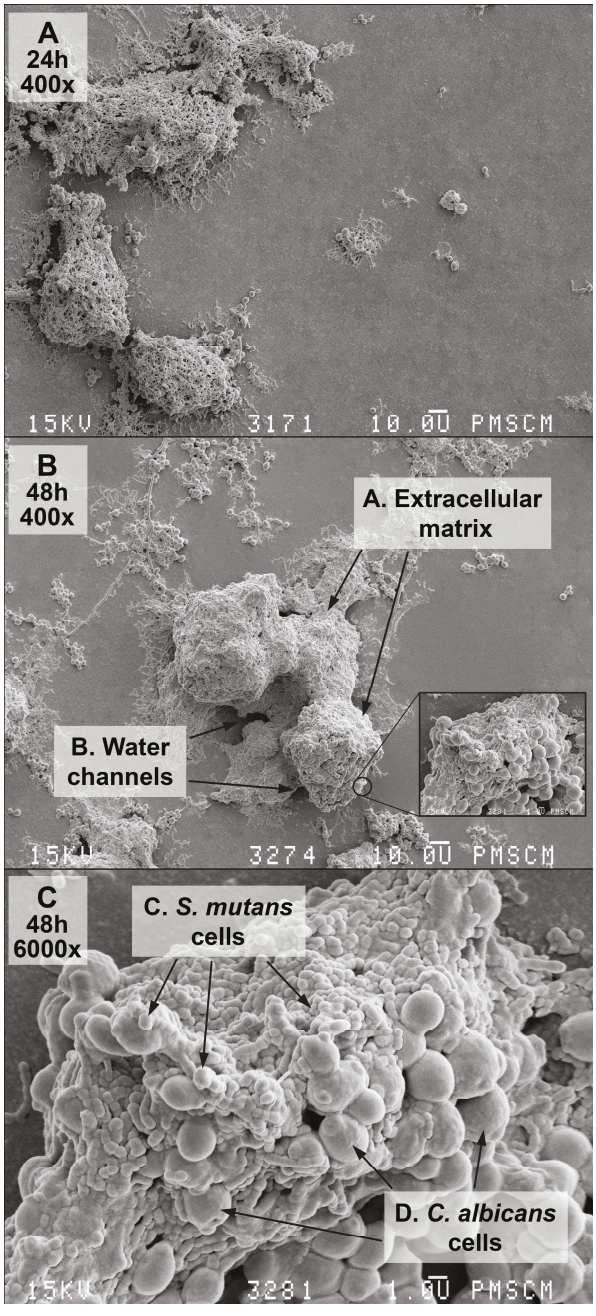

Figure 2. Scanning electron microscopy (SEM). (**A**–**C**) images of the double-species biofilm formed by *C. albicans* and *S. mutans*, after 24 (**A**) and 48 (**B**,**C**) h of biofilm formation. Culture was maintained at 37 °C, pH 7.0 and pCO_2 5%, in bovine serum as a medium additive promoting the growth of the culture in the presence of a sucrose substrate (5%). Original magnification: 400× and 6000×.

3.3. Analysis of Biofilm before and after Incubation with L. salivarius Probiotic

Differences in the colony forming units (CFU/mL) and the total biofilm mass of microorganisms (*C. albicans*, *S. mutans*, and the co-culture) at 18, 20, 22, and 24 h were statistically significant (Figure 3).

The inhibitory effect of *Lactobacillus salivarius* (HM6 Paradens) on biofilm generation by *S. mutans*, yeasts, and the co-culture is shown in Figures 3 and 4, and the scanning electron microscope images (Figures 5B,D,F 6–7 and 8B,D,F) after 24 h biofilm formation. The photographs show morphologically different fungal colonies and bacterial cells in single and co-cultures, before and after the administration of *Lactobacillus salivarius*.

The quantities of microorganisms' (*S. mutans*, *C. albicans*, and the co-culture) logCFU/mL forming biofilms at 18, 20, 22, and 24 h were statistically lower after the administration of a probiotic in all analyzed groups (Figures 3, 5 and 6). The *p* value was statistically significant for 18–24 h points.

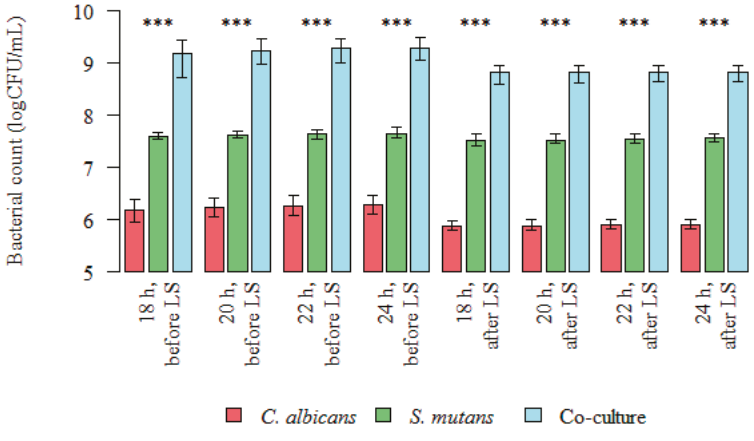

Figure 3. Intergroup differences between bacterial count (logCFU/mL), before and after *Lactobacillus salivarius* (HM6 Paradens, LS) administration. The Kruskal–Wallis test was used for dependent (repeated) measurements; $p < 0.001$; *** Dunn's test (post-hoc test).

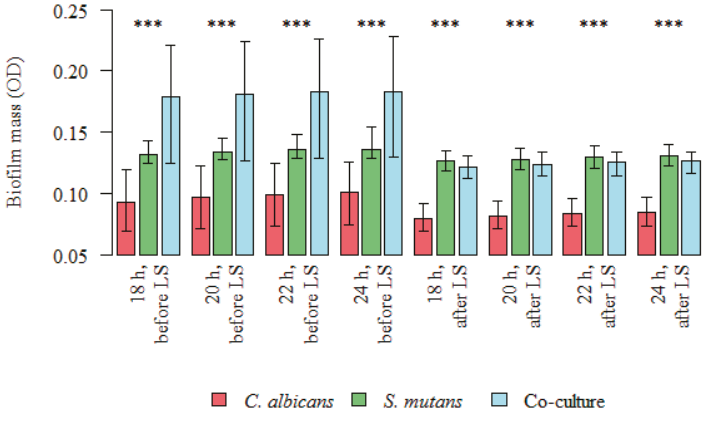

Figure 4. Intergroup differences between biofilm mass (OD), before and after *Lactobacillus salivarius* (HM6 Paradens, LS) administration. Kruskal–Wallis test was used for dependent (repeated) measurements; $p < 0.05$; *** Dunn's test (post-hoc test).

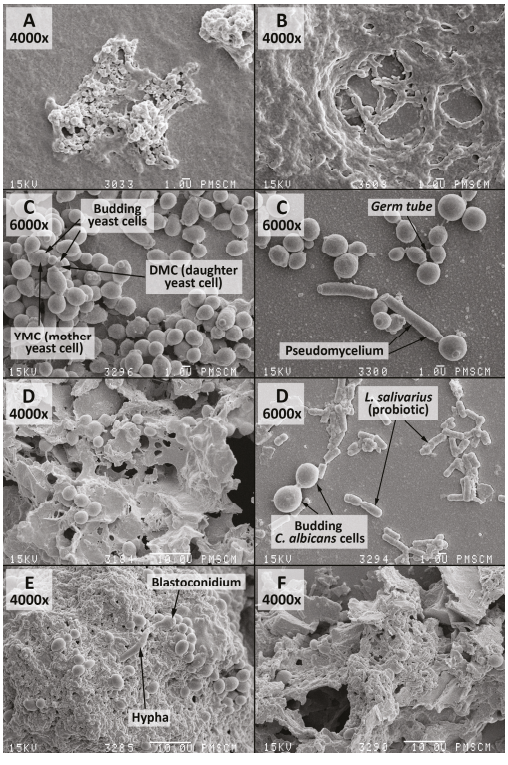

Figure 5. SEM images of the mono-species biofilm, generated by *S. mutans*, *C. albicans* and the double-species oral streptococci/yeasts biofilm—untreated and treated with the *Lactobacillus salivarius* (HM6 Paradens)—after 24 h of biofilm formation. (**A**) The 14 h *S. mutans* biofilm formed on a flat agar surface (Agar Scientific, Stansted, UK). *S. mutans* adheres to the polystyrene surface, mainly using a sucrose-dependent mechanism; (**B**) The 24 h *S. mutans* biofilm formed on a flat agar surface; visible polymeric Extracellular Matrix (ECM), which has an open architecture with nutrient channels, and other properties; (**C**) The 24 h *C. albicans* biofilm formed on a flat agar surface (Agar Scientific, Stansted, UK). *C. albicans* adheres to the polystyrene surface, mainly using mycelial forms, visible pseudohyphae, budding yeast, and the so-called germ tube, considered to be the key features of the pathogenicity of the fungus. The culture was maintained at 36 °C, pH 7.0 and pCO_2 5%, in bovine serum as a medium additive promoting growth of the culture in the presence of a sucrose substrate (5%); (**D**) The 24 h *C. albicans* biofilm formed on a flat agar surface (Agar Scientific, Stansted, UK) under the influence of *Lactobacillus salivarius* (HM6 Paradens). There was no clear, compact structure for the *C. albicans* biofilm and single loosely located budding cells. Other morphological forms of yeasts were invisible; (**E**) The 24 h double-species oral streptococci/yeasts biofilm formed on a flat agar surface (Agar Scientific, Stansted, UK). There was an apparent change in the *C. albicans* morphotype in the *S. mutans* common culture and visible pleomorphic forms were true hyphae and, blastoconidia, which in the mixed culture also produce mycelial forms, whose role is related to damage to immune cells (macrophages), leading to microorganism invasion. There was abundant extracellular matrix between cells and covering bacterial and yeast cells. Bacterial cells were visible in chains adhering to yeast cells and wrapped around them; (**F**) The 24 h double-species *S. mutans*/*C. albicans* biofilm formed on a flat agar surface (Agar Scientific, Stansted, UK) under the influence of *Lactobacillus salivarius* (HM6 Paradens). There was no clear, compact *S. mutans*/*C. albicans* biofilm structure or single loosely located budding cells. Other morphological forms of *C. albicans* were invisible. There was no visible extracellular matrix. Original magnification: 4000× and 6000×.

The median (range) bacterial count log(CFU/mL) value for single-species *S. mutans* biofilm after 24 h was 7.643 (7.568–7.756) and was significantly higher than the bacterial count log(CFU/mL) after probiotic administration: 7.505 (7.415–7.623) (Kruskal–Wallis test for dependent (repeated) measurements; $p < 0.05$) (Figures 3 and 6).

The median (range) bacterial count log(CFU/mL) value for single-species *C. albicans* biofilm after 24 h was 6.279 (6.114–6.462) and was significantly higher than the bacterial count log(CFU/mL) after probiotic administration: 5.900 (5.833–5.991) (Kruskal–Wallis test for dependent (repeated) measurements; $p < 0.05$) (Figures 3 and 6).

The median (range) bacteria/fungi count log(CFU/mL) value for double-species *S. mutans*/*C. albicans* biofilm after 24 h was 9.267 (9.041–9.477) and was significantly higher than the log(CFU/mL) after probiotic administration: 8.816 (8.633–8.940) (Kruskal–Wallis test for dependent (repeated) measurements; $p < 0.05$) (Figures 3 and 6).

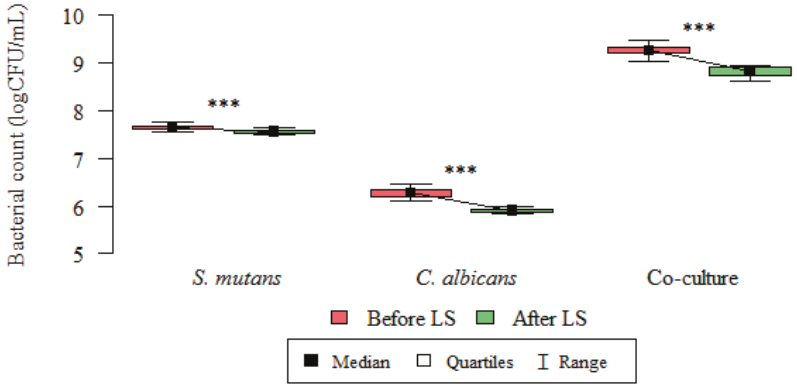

Figure 6. *S. mutans*, *C. albicans*, and oral streptococci/yeast biofilm formation: changes in microorganism count (logCFU/mL), before and after administration of *Lactobacillus salivarius* (HM6 Paradens) after 24 h. Wilcoxon's test was used for dependent (repeated) measurements (paired Wilcoxon's test; $p < 0.05$). Data are represented as median ± 1–3 quartiles for the three experiments. *** Indicates statistically significant difference compared to untreated biofilms ($p < 0.001$).

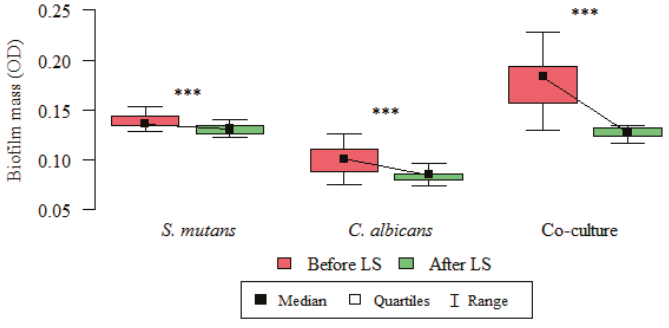

Figure 7. *S. mutans*, *C. albicans*, and oral streptococci/yeast biofilm formation: changes in optical density (OD) of biofilm mass, before and after administration of *Lactobacillus salivarius* (HM6 Paradens) after 24 h. Wilcoxon's test was used for dependent (repeated) measurements (paired Wilcoxon's test; $p < 0.05$). Data are represented as median ± 1–3 quartiles for three experiments. *** Indicates statistically significant difference compared to untreated biofilms ($p < 0.001$).

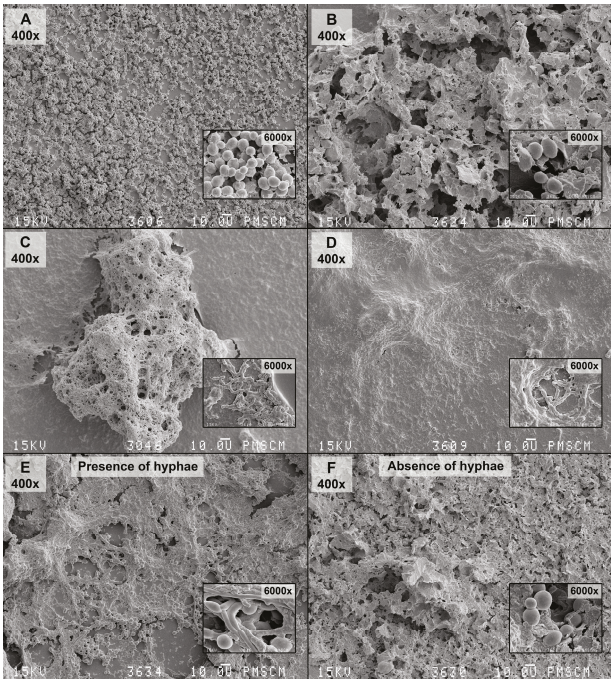

Figure 8. SEM images of the mono-species biofilm generated by *C. albicans*, *S. mutans* and the double-species oral streptococci/yeasts biofilm—untreated and treated with the *Lactobacillus salivarius* (HM6 Paradens)—after 24 h of biofilm formation. (**A**) The 24 h *C. albicans* biofilm formed on a flat agar surface (Agar Scientific, Stansted, UK); (**B**) The 24 h *C. albicans* biofilm formed on a flat agar surface treated with the *Lactobacillus salivarius* (HM6 Paradens); (**C**) The 24 h *S. mutans* biofilm formed on a flat agar surface (Agar Scientific, UK); (**D**) The 24 h *S. mutans* biofilm formed on a flat agar surface treated with the *Lactobacillus salivarius* (HM6 Paradens); (**E**) Co-culture oral streptococci/yeasts biofilm—untreated with the *Lactobacillus salivarius* (HM6 Paradens)—after 24 h of biofilm formation; (**F**) Co-culture oral streptococci/yeasts biofilm—treated with the *Lactobacillus salivarius* (HM6 Paradens)—after 24 h of biofilm formation. The Culture was maintained at 36 °C, pH 7.0 and pCO_2 5%, in bovine serum as a medium additive promoting growth of the culture in the presence of a sucrose substrate (5%). The occurrence of a co-culture biofilm at this stage may depend on the *C. albicans* morphotypes showing a twofold nature: buds and *C. albicans* hyphae may colonize mucous membranes and constitute physiological microflora (commensal) or may lead to infection under favorable conditions (opportunistic pathogens). There was no clear, compact structure for the *C. albicans* biofilm and single loosely located budding cells. Other pathological forms of yeasts are invisible. There was an apparent change in the *C. albicans* morphotype in the *S. mutans* common culture and visible pleomorphic forms were true hyphae, blastoconidia, which in the mixed culture also produce mycelial forms, whose role is related to damage to immune cells (macrophages) leading to microorganism invasion. There was abundant extracellular matrix between cells and covering bacterial and yeast cells. Bacterial cells were visible in chains adhering to yeast cells and wrapped around them. There was no clear, compact *S. mutans*/*C. albicans* biofilm structure or single loosely located budding cells. Other morphological forms of *C. albicans* were invisible. There was no visible extracellular matrix (**D**,**F**), which is formed by *S. mutans* alone and *S. mutans* with *C. albicans* treated with the *Lactobacillus salivarius* (**C**,**E**). Original magnification: 400×, 4000× and 6000×.

3.4. Analysis of Formed Biofilm Mass before and after Incubation with L. salivarius Probiotic

The total biomass (OD) of microorganisms (*S. mutans*, *C. albicans*, and the co-culture) producing a biofilm at 18, 20, 22, and 24 h were statistically lower after the administration of the probiotic in all the analyzed groups. The *p* value was statistically significant for all time points (18–24 h) (Table 3).

The inhibitory effect of *L. salivarius* (HM6 Paradens) on biofilm generation by *S. mutans*, yeasts, and the co-culture is shown in Table 3 and Figures 4 and 7 after a 24 h biofilm formation.

The mean biofilm mass (OD) value for single-species *S. mutans* biofilm after 24 h was 0.139 ± 0.007 and was significantly higher than the OD value after probiotic administration: 0.131 ± 0.004 (paired Wilcoxon's test; $p < 0.001$) (Figures 4 and 7).

The mean biofilm mass (OD) value for single-species *C. albicans* biofilm after 24 h was 0.100 ± 0.013 and was significantly higher than the OD value after probiotic administration: 0.084 ± 0.006 (paired Wilcoxon's test; $p < 0.001$) (Figures 4 and 7).

The mean biofilm mass OD value for double-species *S. mutans*/*C. albicans* biofilm after 24 h were 0.176 ± 0.024 and was significantly higher than the OD value after probiotic administration: 0.127 ± 0.005 (paired Wilcoxon's test; $p < 0.001$) (Figures 4 and 7).

Table 3. *S. mutans*, *C. albicans*, and oral streptococci/yeast biofilm formation: total biomass (OD) evaluation before and after administration of *Lactobacillus salivarius* (HM6 Paradens, LS) at individual time points.

Species	Time	Test	*n*	Mean	SD	Median	Min	Max	Q1	Q3	*p* ***
S. mutans	18 h	Before LS	29	0.134	0.005	0.132	0.125	0.143	0.130	0.138	<0.001
		After LS	29	0.127	0.005	0.127	0.119	0.135	0.122	0.130	
	20 h	Before LS	29	0.136	0.005	0.134	0.128	0.145	0.132	0.141	<0.001
		After LS	29	0.128	0.005	0.128	0.120	0.137	0.124	0.132	
	22 h	Before LS	29	0.138	0.006	0.136	0.129	0.148	0.134	0.143	<0.001
		After LS	29	0.130	0.005	0.130	0.121	0.139	0.126	0.134	
	24 h	Before LS	29	0.139	0.007	0.136	0.129	0.154	0.135	0.144	<0.001
		After LS	29	0.131	0.004	0.131	0.123	0.140	0.126	0.134	
C. albicans	18 h	Before LS	30	0.095	0.013	0.094	0.070	0.120	0.084	0.106	<0.001
		After LS	30	0.078	0.006	0.080	0.070	0.092	0.074	0.082	
	20 h	Before LS	30	0.098	0.013	0.097	0.072	0.123	0.086	0.108	<0.001
		After LS	30	0.081	0.006	0.082	0.072	0.094	0.076	0.084	
	22 h	Before LS	30	0.100	0.013	0.100	0.074	0.125	0.088	0.110	<0.001
		After LS	30	0.083	0.006	0.084	0.074	0.096	0.078	0.086	
	24 h	Before LS	30	0.100	0.013	0.101	0.075	0.126	0.089	0.111	<0.001
		After LS	30	0.084	0.006	0.085	0.074	0.097	0.080	0.086	
S. mutans*/*C. albicans	18 h	Before LS	29	0.171	0.024	0.179	0.125	0.221	0.151	0.191	<0.001
		After LS	29	0.123	0.006	0.122	0.112	0.131	0.118	0.129	
	20 h	Before LS	29	0.173	0.024	0.181	0.127	0.224	0.154	0.193	<0.001
		After LS	29	0.125	0.006	0.124	0.114	0.134	0.120	0.130	
	22 h	Before LS	29	0.175	0.024	0.183	0.129	0.226	0.156	0.195	<0.001
		After LS	29	0.127	0.005	0.126	0.115	0.134	0.123	0.132	
	24 h	Before LS	29	0.176	0.024	0.184	0.130	0.228	0.157	0.194	<0.001
		After LS	29	0.127	0.005	0.127	0.117	0.134	0.123	0.132	

Wilcoxon's test was used for dependent (repeated) measurements (paired Wilcoxon's test; * $p < 0.05$, *** $p < 0.001$). SD, standard deviation.

3.5. Relationship between the Colony Forming Unit Log(CFU/mL) and the Biofilm Mass (OD) before and after Lactobacillus salivarius Administration

A correlation was noted between the microorganisms forming a biofilm (log(CFU/mL) and their mass (optical density) at the considered time points (Figure 9). After the administration of *Lactobacillus salivarius* (HM6 Paradens), the correlation between colony forming units and the *S. mutans*, the biofilm mass (OD) was lower or not present at all (Figure 10 and Table 4). For the *C. albicans* and

S. mutans/*C. albicans* biofilms, no correlation between log(CFU/mL) and biofilm biomass (OD) was observed (Table 4).

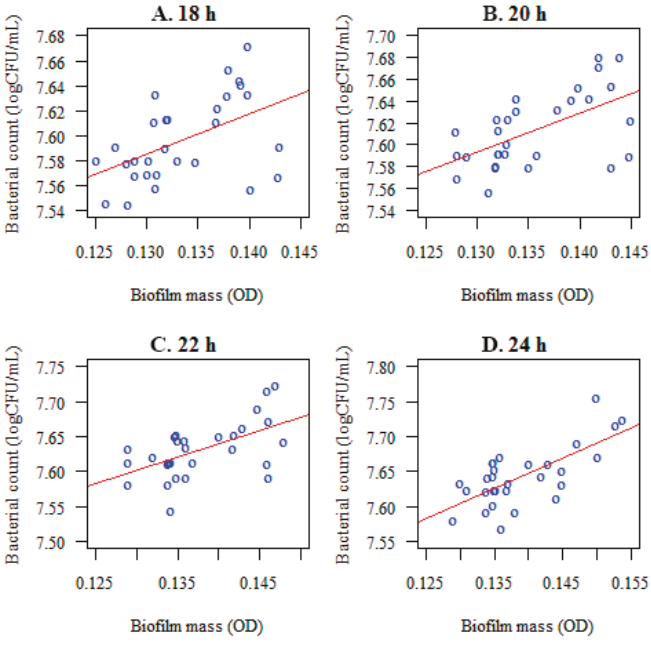

Figure 9. Correlations between the number of microorganisms forming a biofilm (*S. mutans*) (log(CFU/mL) and the optical density (biofilm mass) after 18 (**A**), 20 (**B**), 22 (**C**), and 24 (**D**) h of incubation, before *Lactobacillus salivarius* probiotic administration.

Table 4. Correlations between the number of microorganisms forming a biofilm (*S. mutans*, *C. albicans*, and *S. mutans*/*C. albicans*) and the optical density (biofilm mass, OD) at different time points, before and after *Lactobacillus salivarius* (HM6 Paradens, LS) administration.

Species	Incubation Time	Before LS		After LS	
		r	*p*	*r*	*p*
S. mutans	18 h	0.495	0.006	0.371	0.048
	20 h	0.534	0.003	0.379	0.043
	22 h	0.500	0.006	0.502	0.005
	24 h	0.553	0.002	0.473	0.009
C. albicans	18 h	0.920	<0.001	0.136	0.473
	20 h	0.918	<0.001	0.128	0.927
	22 h	0.931	<0.001	0.127	0.888
	24 h	0.935	<0.001	0.135	0.853
S. mutans/*C. albicans*	18 h	0.764	<0.001	0.181	0.337
	20 h	0.769	<0.001	0.191	0.393
	22 h	0.766	<0.001	0.200	0.459
	24 h	0.842	<0.001	0.192	0.628

The observed correlation was statistically significant throughout the biofilm generation ($p < 0.05$). The following connection was positive: the higher the number of colony forming units of tested microorganisms the higher optical density (biofilm mass). The strongest associations were reported after 24 h of biofilm generation (Table 4).

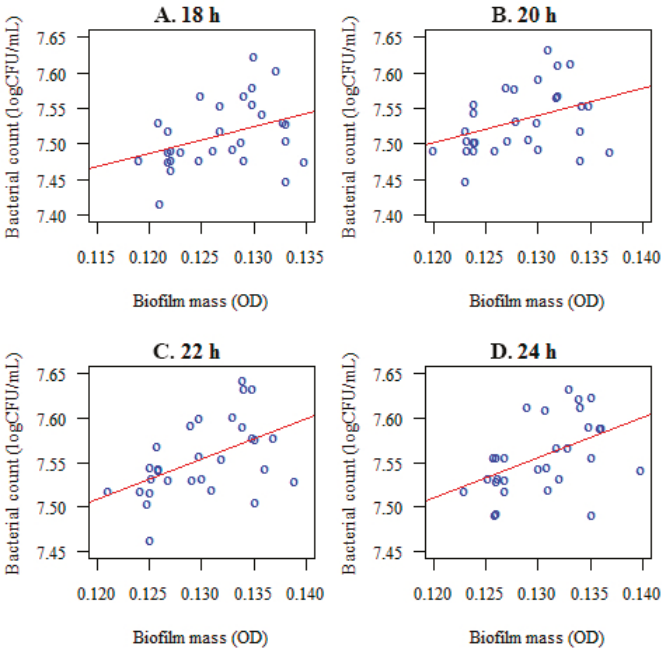

Figure 10. Correlations between the number of microorganisms forming biofilm (*S. mutans*) colony forming units and the optical density (the biofilm mass) at different time points ((**A**)–18 h; (**B**)–20 h; (**C**)–22 h; (**D**)–24 h) after *Lactobacillus salivarius* (HM6 Paradens) administration.

4. Discussion

The formation of biofilm on the surface of the teeth is a major factor in the development of early childhood caries (ECC) [1]. Studies have shown that bacteria and fungi present in biofilms may communicate with each other by sending extracellular signaling molecules or physical intercellular interactions to support the formation and development of cariogenic biofilms that contribute to the progression and recurrence of ECC [42].

The multifactorial etiology of the disease makes it necessary to perform a complex evaluation. The main causes include mutual host-microbial interactions, changes in mixed biofilms (reflecting both the richness of the microorganisms of the oral cavity and its conditions), and external factors initiating continuous pH changes in dental plaque. Stress factors associated with a reduction in the pH of the oral environment are responsible for the decreasing diversity of bacterial species that form oral biofilms. This results in minimization of metabolic activity (particularly, pyruvate kinase as the key enzyme for the entire process) and structural damage to the cell membrane, proteins, and DNA [43,44]. Hence, there are few species of microorganisms able to have an active metabolism under the above-mentioned conditions [45]. Dental plaque is associated with oral conditions associated with a drop in pH, resulting in the dominance of acid-producing strains connected to the development of caries. In physiological conditions, saprophytic/probiotic bacteria maintain the microbial balance and do not allow pathogenic flora to overgrow. The present paper describes the effect of a probiotic containing *L. salivarius* (HM6 Paradens) on a double-species biofilm of *S. mutans*/*C. albicans* as a new approach to maintaining the interspecies balance in the oral cavity and an additional method for supporting existing caries prevention methods.

A number of clinical studies have indicated that daily intake of *Lactobacillus*-derived dairy products can reduce caries, improve overall health, and reduce the need for antibiotic use in preschool

children. However, not all children eat dairy products equally rich in probiotic substances. In addition, the impact of probiotics on oral health and caries inhibition in children is not entirely clear; further, their effects appear to be short-lived and are often directed exclusively at the reduction of cariogenic *S. mutans*, without affecting other microbial species colonizing the oral cavity that have been confirmed to contribute to caries development.

As a result of advanced technology, the number of documented microorganism species involved in the development of caries is increasing. There are literature data indicating that, in addition to *S. mutans* and *C. albicans*, fungi are involved in the carious process and are detectable in large amounts in the plaque and saliva of children with ECC [1,26,45–47].

Bacterial–fungal infections are common in humans, as part of the natural physiological flora. Under favorable conditions, microorganisms that have been thus far regarded as saprophytes incapable of inducing human diseases and have become an etiological factor in a number of diseases [3,48]. *C. albicans*, as a main representative of a fungal microbiome, is present in the mouth, in the mucous membranes, dentures, and orthodontic devices [49,50]. However, a number of observations have indicated that interactions between yeasts and oral streptococci can also occur on cleansed surfaces of enamel, dentine, or on the surface of dental plaque [51,52], particularly in the presence of nutrients, such as sucrose [53,54].

We demonstrated that in the presence of a probiotic, containing inactivated *L. salivarius* (HM6 Paradens), biofilm formation was different from that in a double-species model, without the use of the probiotic. After a suitable culture time, there was no observable mixed *S. mutans* and *C. albicans* biofilm formation. Single aggregates of yeasts and streptococci did not produce a common structure, as in the double-species model. This suggests that *L. salivarius* perhaps competes with *S. mutans* for nutrient substrates and does not allow these bacteria to consume them, along with inhibiting the aggregation of oral streptococci and yeasts, thus resulting in the lack of a double-species biofilm structure. It is necessary to carry out studies that would either provide or deprive biofilms of certain nutrients, in order to confirm the hypothesis of a possible mechanism.

In this study, CFU/mL results showed a higher number of *C. albicans* in mixed *C. albicans*/*S. mutans* biofilms, compared to single-species *C. albicans* biofilms, indicating that *S. mutans* stimulate *C. albicans* growth. These results coincide with those of Júnia Oliveira Barbos et al. and Tomé et al. [55,56]. Although the molecular mechanism for these behaviors is undefined, He et al. [57] confirmed that the interaction between *S. mutans* and *C. albicans* is related to the upregulation of most carbohydrate-transport-linked genes and metabolic processes. The presence of *C. albicans* enhances the expression of 393 genes in *S. mutans* in the double-species biofilm, as compared to the single *S. mutans* biofilm. Molecular studies have shown that the coexistence of *S. mutans* with *C. albicans* affects the use of carbohydrates by *S. mutans*. Furthermore, a co-culture with *C. albicans* changes the transcription of *S. mutans* signal transduction genes (comC and ciaRH) associated with its condition and virulence [3]. Occurrence of a biofilm at this stage may depend on the *C. albicans* morphotypes showing a twofold nature: buds and *C. albicans* hyphae may colonize mucous membranes and constitute physiological microflora (commensal) or may lead to infection under favorable conditions (opportunistic pathogens). These data provide extensive evidence for bacterial–fungal interactions that may progress to dental caries or recurrence of the disease in the future.

During initial adhesion of pioneer colonizers, such as *S. mutans*, to an enamel surface, there is increased activation of glucosyltransferases (Gtfs), particularly GtfB. These, in turn, become the binding sites of secondary colonizers, such as *C. albicans* and *S. mutans* [58]. Fungi, occurring in a hyphal form [59], exhibit better adhesion to the surface than blastospores. However, these effects are not as strong as when *C. albicans* is present in association with *S. mutans* [24], as stated in the literature. This is probably related to increased levels of proteolytic enzymes, i.e., aspartyl proteinases (Saps) of *C. albicans*, which increase mutual interspecies interactions. In particular, Sap1–5 dominate in dental biofilms and can be a crucial factor in ECC development [60,61]. These, and a number

of other, yet unknown, unique effects enhance the adhesion properties of both microorganisms for teeth colonization.

In terms of the double-species *S. mutans*/*C. albicans* biofilm, one can note that fungi occurring in the form of hyphae and blastoconidia are much better at biofilm-producing than in the absence of these forms, under the action of the *L. salivarius* probiotic (HM6 Paradens), as seen in scanning electron microscope images (Figure 4C,D). This formulation can act as a proteolytic enzyme inhibitor, i.e., aspartyl proteases (Saps) of *C. albicans*, which are considered to be the main contributor to interspecies interactions. The above observation is quite innovative, since research on *S. mutans* and *C. albicans* biofilms has confirmed that *S. mutans* stimulates *C. albicans* to grow as biofilms in vitro. However, it has been thought so far that the bacteria inhibit the formation of hypha by yeast [51]. In our studies on clinical strains from children with childhood caries, the opposite effect can be observed. In SEM samples, double-species biofilms were plentiful in hyphae formed by fungi, which was not noticeable after a probiotic application. When evaluating the fungal morphology, we noticed that *L. salivarius* inhibited the formation of germ tubes and pseudomycelium when added to the *S. mutans* and *C. albicans* common culture. These observations indicate that this probiotic may produce signaling molecules or indirectly inhibit the generation of double-species biofilm by inhibiting *S. mutans*. Without the use of a probiotic, *S. mutans* supported the cariogenicity of the biofilm. In the presence of HM6, *C. albicans* mycelium was not formed in a mixed biofilm. The present model seems interesting, due to the fact that, thus far, it has been presumed that it is *S. mutans* in the *C. albicans* mixed biofilm that sends signaling molecules, such as mutanobactin A, that naturally inhibit *C. albicans hyphae*, thus preventing the development of cariogenic biofilms.

This results in increased production of the EPS biofilm matrix and a specific hyperadditive effect that enhances the biofilm virulence. *C. albicans* dominates in the competition for unique eco-niches, such as teeth gaps and fractures [62], and, using its natural ability for thigmotropism, penetrates deep into the open dental canals. Owing to increased penetration of hard-to-reach places, such as tooth roots, *C. albicans* leads to aggressive caries and contributes to rapid progression of the disease [63,64]. It is necessary to further study the mechanisms of multispecies interactions of yeasts and oral streptococci in the biofilms during which EPS growth and the activation of metabolic pathways has a significant impact on the aggressiveness of the formed structure and the rapid progression of the disease. The results of this study explain why current caries prophylaxis, based only on *S. mutans* identification, is not as effective as screening methods for diagnosing children at risk for early caries. Research should be conducted on the mutual relationships between potentially interacting microorganisms (pathogenic and non-pathogenic). An approach, based on the current state of knowledge on caries, increases the chances of understanding the pathogenesis of this disease and may, therefore, lead to new ways of prevention. Then, it will be possible to design potential modulators for the development and progression of diseases, such as caries.

The studies on the *S. mutans*/*C. albicans* interactions cited earlier in this work concerned biofilm models using in vitro reference strains. The presented model uses clinical strains and shows the variability that clinical strains may exhibit, even within the same species. The same strain does not necessarily induce a hostile response to disease development. Calculation of the pathogenic effect for microorganisms appears to be estimative and despite an inoculum-dependent effect, there are still other limitations, such as the occurrence of variable conditions of infection [65]. Comparisons of virulence among microorganisms provide evidence that any action aimed at eliminating its factors should be undertaken with caution, given that any change in the host–microbial relationship can alter the pathogenic potential of microorganisms.

Although in vivo studies seem to be much more reflective of the real situation, they are becoming increasingly limited due to ethical reasons.

Application of an in vivo model, using potentially cariogenic clinical strains, and evaluation of the effect of *Lactobacillus salivarius* (HM6 Paradens) probiotic on *C. albicans*/*S. mutans* co-culture biofilm were the objectives of this study.

This observation demonstrates that a probiotic containing thermally inactivated *L. salivarius* strains inhibited the ability of the strains to form a common structure between oral streptococci and yeasts. The examined strains were derived from the dental biofilm of children with early childhood caries. The evaluation of the generated biofilm under the influence of the test compound showed a decrease in both the grown colonies and the biofilm biomass, along with reduced cross-linking of the biofilm structure. These results coincide with those of Wu et al. [66], Ahmed et al. [67], and Nishihara et al. [15].

The obtained results are of interest because a number of studies using probiotics appear to be controversial, particularly as probiotics that inhibit the growth of *S. mutans* monocultures do not necessarily reduce the cariogenicity of multi-species biofilms [68], where *S. mutans* is only one component among a number of other plaque-forming species [69]. Therefore, it seems more appropriate to study the effect of probiotics on the relationships between the different biofilm-forming species and the reduction of cariogenic *S. mutans*. In addition, the above data on the inhibition of biofilm formation, not only of *S. mutans* but also by the *S. mutans*/*C. albicans* co-culture, indicate that the action of the *Lactobacillus salivarius* (HM6 Paradens) is very promising. It meets the modern definition of caries as a multifactorial disease [70], which is dependent on a number of species of microorganisms forming biofilms and not just on selected single species of bacteria [71]. Furthermore, the findings support the assumption of Koo and Bowen [72], who proposed the possibility of including anti-fungal (anti-*Candida*) therapy for ECC.

Although there are many published studies showing the mutual interactions of fungi with bacteria, including *S. mutans*, most of the mechanisms responsible for these processes remain unclear. Recently, several key findings have emerged that characterize the molecules involved in the *S. mutans*/*C. albicans* interactions. However, these models do not take into account the factors connected to the host and pathogenicity of the strains depending on the host environment. It is known that under certain conditions, bacteria or fungi acquire features that condition their pathogenicity [65]. Our model has the advantage of taking into account clinical strains from the source of infection (dental plaque from children with caries), which have developed their virulence in response to the environment where they exist. This is confirmed by the results on interactions between *S. mutans* and *C. albicans* clinical strains, which produce pathogenic factors such as mycelia and germ tubes in the co-culture. This is constrast to the results obtained by other researchers (using standard strains), where such factors were not present. International research uses advanced technology, but such technology is not always or everywhere accessible and applicable. Thus, the observations obtained during the presented studies are unique because they show changes in the level of fungal morphogenesis associated with the production of mycelium. In clinical practice, this is a manifestation of *C. albicans* pathogenesis, associated with an active infection, which is severe caries. Continuing the observed relationships, in the future we would like to evaluate hyphal growth factor HWP1, or ALS1 and ALS3, which, as seen in studies by Ellepola et al. [42], may be a potential mechanism of mycelial growth in *S. mutans*/*C. albicans* co-cultures. We plan to verify how available nutrients, except sucrose, modify the above-mentioned feature. This new discovery may explain the mutual roles of *S. mutans* and *C. albicans* in cariogenic biofilm formation and provide a point of action for the creation or utilization of molecules with antineoplastic potential. The results of the study on the inhibition of cariogenic *S. mutans*/*C. albicans* biofilm are also a prerequisite for further research on this probiotic in in vivo studies. In addition, the multifunctional nature of biofilm development and resistance to conventional treatment encourage the use of probiotics, which are intended to protect native microflora by interfering with microorganisms accumulated in the biofilm structure.

Probiotic bacteria, such as *L. salivarius*, colonize the oral cavity of naturally born children [73]. Their protective role may be based on the stimulation of the host's immune system to produce antibodies and immunoglobulins [74]. The results of the potential use of probiotics in caries are very encouraging. Our study, although a small section of probiotic research, encourages us to take further steps to establish a consensus on the use of probiotics in the prevention of oral diseases, such as dental

caries. The research is important, as the available databases have only a few clinical studies with strong scientific evidence proving the effectiveness of probiotics in ECC counteraction [64].

5. Conclusions

Research on the use of probiotics in the prevention of oral diseases, such as dental caries, has opened up new opportunities for establishing a balance between diet and oral health. Studies show that probiotic bacteria given in any form are safe for humans and can provide a good complement to daily oral hygiene. Administering probiotics based on the occurrence of a natural comorbid flora in our organism is the future of biotherapeutic research, as these bacteria are ideally adapted to the human microbiome and occupy the supreme position while displacing potentially pathogenic species. Probiotic supplementation to a daily balanced diet may be a strategy for preventing caries or other oral infectious diseases in children.

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In Vitro Fermentation Patterns of Rice Bran Components by Human Gut Microbiota

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Abstract: Whole grain rice is a rich source of fiber, nutrients, and phytochemicals that may promote gastrointestinal health, but such beneficial components are typically removed with the bran during polishing. Soluble feruloylated arabinoxylan oligosaccharides (FAXO) and polyphenols (RBPP) isolated from rice bran are hypothesized to have positive impacts on human gut microbiota through a prebiotic function. Using an in vitro human fecal fermentation bioassay, FAXO and RBPP treatments were assessed for short-chain fatty acids (SCFA) production patterns and by evaluating their impacts on the phylogentic composition of human gut microbiota by 16S rRNA gene sequencing. Fresh fecal samples collected from healthy adults (n = 10, 5 males, 5 females) were diluted with anaerobic medium. Each sample received five treatments: CTRL (no substrates), FOS (fructooligosaccharides), FAXO, RBPP, and MIX (FAXO with RBPP). Samples were incubated at 37 °C and an aliquot was withdrawn at 0, 4, 8, 12, and 24 h Results showed that SCFA production was significantly increased with FAXO and was comparable to fermentation with FOS, a well-established prebiotic. RBPP did not increase SCFA productions, and no significant differences in total SCFA production were observed between FAXO and MIX, indicating that RBPP does not modify FAXO fermentation. Changes in microbiota population were found in FAXO treatment, especially in *Bacteroides*, *Prevotella*, and *Dorea* populations, indicating that FAXO might modulate microbiota profiles. RBPP and MIX increased *Faecalibacterium*, specifically *F. prausnitzii*. Combined FAXO and RBPP fermentation increased abundance of butyrogenic bacteria, *Coprococcus* and *Roseburia*, suggesting some interactive activity. Results from this study support the potential for FAXO and RBPP from rice bran to promote colon health through a prebiotic function.

Keywords: feruloylated arabinoxylan oligosaccharides; rice bran polyphenols; short-chain fatty acids; gut microbiota; prebiotic; colon health

1. Introduction

Interactions of human gut microbiota and non-digested dietary components play important roles in health and disease [1,2]. This involves a variety of metabolic functions, including energy harvest and storage. Furthermore, the gut microbiota interacts with the host immune system, providing signals to promote the maturation of immune cells and the normal development of immune functions [3]. Factors affecting gut microbial populations includes mode of birth, age and diet [2]. In recent years, there has been a growing interest in whole grain rice and functional rice bran products [4,5]

that reach the colon, such as prebiotics, dietary fiber, and other nondigested dietary components, such as polyphenolics. There they modulate beneficial gut microbiota, supporting production of short-chain fatty acids (SCFA), which have been proved to confer positive colonic health benefits [6]. Bran components and their fermentative products may also contribute to sustaining the epithelial barrier function and associated innate immunity [7,8].

A dietary prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit" ([9], p. 493). Only a few non-digestible oligosaccharides are established prebiotics, including fructooligosaccharides (FOS) and galactooligosaccharides (GOS). These compounds have been reported to stimulate *Bifidobacterium* and *Lactobacillus* population, which are considered beneficial bacteria and are often targets for dietary intervention [10]. Recently, the prebiotic definition was expanded to include non-carbohydrate substances and alternative application sites [9].

The non-starch polysaccharide arabinoxylan (AX) present in cereal bran fiber, and non-digestible oligosaccharides derived from it, have been proposed to be candidate prebiotics since they appear to confer a degree of fermentation selectivity [11]. Arabinoxylan oligosaccharides isolated from cereal bran such as wheat, rye, and corn have shown to exert prebiotic-like properties in that they may pass through the upper gastrointestinal tract undigested to the colon where they are hydrolyzed and subsequently fermented by gut microbiota to produce SCFA [12,13].

Ferulic acid is a phenolic acid that is covalently bound to AX (attached by O5 ester bond to some arabinofuranosides) and can form intermolecular cross-links that immobilize AX within the insoluble fiber matrix [14,15]. Feruloylated arabinoxylan oligosaccharides (FAXO) for biofunctional studies are isolated from cereal bran by autohydrolytic [16], chemical [17], and enzymatic [18] methods. FAXO may provide increased bioavailability for ferulic acid in the colon environment, where microbial esterases can release them, providing anti-inflammatory and other effects associated with its high antioxidant potential [19–22]. Functional studies of FAXO have focused on those isolated from wheat and maize brans, while there is considerable variation in AX fine structure between cereal species and likely varieties within species [15,16,23].

Polyphenols and their derived products can also positively affect the intestinal ecology [24,25]. In vitro fermentation of grape seed flavanol fractions showed the changes of the gut microbiota composition by selectively inhibiting pathogen growth and stimulate the growth of beneficial bacteria, thus influencing the microbiota composition [26]. A randomized, controlled, double-blind, crossover intervention study with cocoa flavanols showed selective stimulation of beneficial gut microbiota in humans [27].

Feruloylated arabinoxylan and polyphenols are abundant in whole grain rice and pigmented rice bran [28,29]. However, these components are largely removed from rice during polishing [4]. Rice bran FAXO and polyphenols (RBPP) have not been studied for their potential colon health promoting properties, particularly prebiotic activities. Furthermore, there is potential for synergistic activities between FAXO and RBPP in improving colonic health. Therefore, the objectives of the present study with rice bran FAXO and RBPP were to determine fermentative SCFA production patterns by human gut microbiota and their ability to stimulate beneficial human gut microbiota populations.

2. Materials and Methods

2.1. Substrates and Standards

Feruloylated arabinoxylan oligosaccharides were isolated from multiple batches of rice bran fiber hydrolysed in hot trifluoracetic acid essentially as described by Saulnier [17], except that the majority of material was treated with 100 mM TFA for 1 h. The pooled soluble material recovered from Amberlite XAD-4 resin was analysed for ferulic acid and neutral monosaccharide contents, which provided an approximate molar ratio for Fer:Ara:Xyl:Gal:Glu of 1:1:2.2:0.2:2. Rice bran polyphenols fraction (RBPP, 50% ethanol fraction) was isolated from red rice bran (IITA 119, PI 458466) as previously described [29]. Fructooligosaccharides (FOS) were purchased from Megazyme International Ireland

Ltd. (Bray Business Park, Wicklow, Ireland). All materials were tightly sealed and stored at −20 °C until use. SCFA standards including acetic acid, propionic acid, and butyric acid were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Subjects, Dietary Records, and Fecal Sample Collection

The study protocol was approved by University of Arkansas Institutional Review Board (IRB #13-09-080). All participants were recruited from the University of Arkansas (Fayetteville, AR, USA) and surrounding area. Thirty-two volunteers (15 males and 17 females), 21–45 years of age, participated in a screening session to sign a consent form and a screening form. Only participants who were generally healthy ($18.5 \leq$ body mass index (BMI) < 25) with no digestive diseases, non-smokers, not currently taking any medications, and had not taken antibiotics in the last 6 months were recruited. During screening sessions, height and weight of subjects were recorded for body mass index calculation. Medical history and bowel movement habits were also recorded to confirm eligibility of participants. Ten eligible subjects (5 males and 5 females) were selected for continuing the study. No other previous studies with rice FAXO and RBPP are available in order to perform a power analysis. Each participant received a stool collection kit (Commode Specimen Collection System; Fisher Scientific, Pittsburgh, PA, USA) one or two days before the day of experiment. Subjects were instructed to deliver a tightly sealed fecal sample within one hour of defecation. Fecal samples were immediately transferred to an anaerobic chamber upon delivery to perform the experiment.

2.3. In Vitro Fermentation

In vitro fermentation of substrates with the fecal inocula was carried out following the method described by Yang et al. [30]. In short, 50 mg each of FAXO, RBPP, their combination (MIX-FAXO with RBPP), and FOS used as control were mixed in 10 mL of sterile fermentation medium consisting of (per liter) peptone (2 g; Fisher Scientific), yeast extract (2 g; Alfa Aesar, Ward Hill, MA, USA), bile salts (0.5 g; Oxoid, Hampshire, UK), $NaHCO_3$ (2 g), NaCl (0.1 g), K_2HPO_4 (0.08 g), $MgSO_4 \cdot 7H_2O$ (0.01 g), $CaCl_2 \cdot 6H_2O$ (0.01 g), L-cysteine hydrochloride (0.5 g; Sigma-Aldrich, St. Louis, MO, USA), bovine hemin (50 mg; Sigma-Aldrich), Tween 80 (2 mL), vitamin K (10 μL; Sigma-Aldrich), and 0.025% (wt/vol) resazurin solution (4 mL). Fecal slurry was prepared by vortexing 1.0 g of fecal sample with 10 mL of sterile phosphate-buffered saline until fully suspended then filtering through four layers of cotton gauge. Test tubes containing fermentation medium and treatments were then inoculated with 0.2 mL of fecal slurry. All steps for fermentation were conducted in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA). Test tubes were then capped, tightly sealed, and vortexed for 5 seconds to mix. Subsequently, test tubes were transferred to the incubator set at 37 °C. Immediately before incubation, 1.5 mL of the mixture was taken out from each test tube using a sterile syringe for time point 0 h into a 2-mL centrifuge tube containing 0.1 mL of 2 M KOH stop solution. Subsequent aliquots were obtained in the same manner at 4, 8, 12, and 24 h and stored at −80 °C until analysis.

2.4. Short-Chain Fatty Acid Analysis

Fermentation samples were thawed at room temperature and mixed with a vortex mixer. An amount of 225 μL was withdrawn from each aliquot and was combined with 25 μL of a mixture containing 5% meta-phosphoric acid and 5% copper sulfate with 50 mM 4-methyl-valeric acid added as an internal standard. After 10 min of reaction time, the mixture was centrifuged for 2 min at 12,000× *g*. A supernatant of 200 μL was transferred into a labelled tube and stored at −20 °C until analysis.

SCFA standards were prepared using 1:2 serial dilution with the stock solution containing 10% *v*/*v* of each SCFA (acetic acid, propionic acid, and butyric acid) in Milli-Q water. SCFA standards were also treated with the same mixture containing meta-phosphoric acid, copper sulfate, and 4-methyl-valeric acid as with fermentation samples.

SCFA contents in samples were measured quantitatively using a gas chromatograph with flame ionization detection (Shimadzu Corp., Kyoto, Japan) equipped with a BP21 fused silica capillary column (SGE Analytical Science/Trajan, Pflugerville, TX, USA; 30 mm × 0.25 mm, 25 μm). Temperature ramp was as following: 4 °C/min from 100 °C (2 min) to 120 °C (1 min), then 3 °C/min until 150 °C. In addition, 1 μL of treated sample (thawed and homogenized) was injected in split mode (30:1). Nitrogen was used as a carrier gas. Data were recorded and processed using the integrated Shimadzu database. Concentrations of acetic acid, propionic acid, and butyric acid were determined using a standard curve of each SCFA.

2.5. DNA Extraction and Sequence Analysis

Bacterial proliferation capability of each treatment was assessed by DNA sequencing analysis of samples at time point 24 h. Bacterial DNA was extracted from sample aliquots using QIAamp Fast DNA Stool Mini Kit (Qiagen, Gaithersburg, MD, USA). DNA concentrations were measured using NanoDrop 1000 (Thermo Fisher Scientific, Waltham, WA, USA). All samples were diluted with DNase- and RNase-free water to achieve concentrations of 10 ng/μL. DNA samples were then mixed with AccuPrime Pfx SuperMix (Thermo Fisher Scientific, Waltham, WA, USA) and primers and were amplified via polymerase chain reaction (PCR) using Eppendorf Mastercycler pro S (Eppendorf, Hamburg, Germany). Amplification of DNA samples were confirmed by agarose gel electrophoresis. Amplified DNA samples were normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, WA, USA) before pooling to make DNA sample library. Sequencing based on 16S-rRNA V4 region was performed using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) with the method developed by Kozich et al. [31]. Raw sequencing data acquired from Illumina BaseSpace were processed with a bioinformatics tool QIIME (Quantitative Insights into Microbial Ecology) pipeline (version 1.9.0) [32].

2.6. Statistical Analyses

All statistical analyses were carried out by JMP software (version 12; SAS Institute, Cary, NC, USA), using one-way ANOVA for comparing three or more data sets or paired t-test for comparing two data sets. A Tukey test was performed to correct for multiple comparisons. Data are presented as Mean ± SEM (Standard Error of Mean) unless specified as standard deviation (SD). Statistically significance was accepted at $p < 0.05$.

3. Results

3.1. Subject Characteristics

In the present study, 10 subjects (5 males, 5 females) were recruited. Participant information including age, height, weight, and body mass index (BMI) is shown in Table 1. BMI of all subjects were within normal range (19.6–24.6).

Table 1. Subject characteristics.

Measurements	All (n = 10)	Male (n = 5)	Female (n = 5)
Age (year)	25.8 ± 4.7	26.0 ± 5.1	25.6 ± 4.2
Height (m)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.0
Weight (kg)	66.6 ± 7.3	69.8 ± 7.5	63.5 ± 5.8
Body mass index (kg/m^2)	22.8 ± 2.0	23.1 ± 1.8	22.5 ± 2.3

Values are expressed as mean ± SD (standard deviation).

3.2. Short-Chain Fatty Acid Analysis

No significant differences in total and individual SCFA production were observed between males and females. SCFA concentrations were measured at time points from 0 h to 24 h (Table 2). FAXO appeared to be a preferred substrate by the microbiota as evidenced by the increase in total SCFA. Compared to FOS, a widely-recognized prebiotic, total SCFA production of FAXO was very comparable at later time points of 12 h and 24 h as no significant differences were found. However, at time point 4 h and 8 h, SCFA production of FOS was significantly higher compared to FAXO ($p < 0.05$), indicating different fermentation patterns of FOS and FAXO. FAXO appeared to have slower and steadier fermentation rates throughout the incubation period compared to FOS, which were rapidly fermented in the beginning (time point 4 h and 8 h) but then slowed down over time.

As expected for saccharolytic fermentation, RBPP was not a significant source of SCFA. Furthermore, there was no significant difference in total SCFA production between FAXO and MIX (FAXO with RBPP) at any time points, indicating that RBPP or its potential metabolic products did not affect SCFA production from FAXO.

Table 2. Total production of SCFA (Short-Chain Fatty Acid) during in vitro fermentation with human fecal samples.

Time Point (h)	Total SCFA (mM)			
	FOS	**FAXO**	**RBPP**	**MIX**
0	1.5 ± 0.3^{a}	1.5 ± 0.3^{a}	0.6 ± 0.2^{a}	1.3 ± 0.3^{a}
4	4.0 ± 0.8^{a}	2.4 ± 0.7^{b}	0.3 ± 0.1^{c}	2.9 ± 1.0^{ab}
8	10.2 ± 1.1^{a}	6.7 ± 1.8^{b}	0.0 ± 0.2^{c}	4.9 ± 1.7^{b}
12	9.9 ± 1.1^{a}	10.5 ± 2.7^{a}	0.1 ± 0.2^{b}	9.3 ± 3.1^{a}
24	9.3 ± 1.1^{a}	14.0 ± 3.6^{a}	0.8 ± 0.7^{b}	11.7 ± 3.2^{a}

Values are expressed as mean ± SEM. Treatments with different superscripts within the same row are significantly different ($p < 0.05$). FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenols, MIX: mixture of FAXO and RBPP.

Individual SCFA production (acetate, propionate, and butyrate) was also investigated (Figure 1). Acetate (Figure 1A) showed to be the predominant SCFA produced as its concentration at time point 24 h is 3–4 times higher compared to propionate and butyrate (Figure 1B,C), which were produced at similar levels. Acetate production of all treatments also exhibited similar trends as observed in total SCFA production. In particular, acetate production of FOS were significantly higher than that of FAXO at time point 4 h and 8 h but not at time point 12 h and 24 h. However, for propionate and butyrate production, no significant differences were found at time point 4 h and 8 h when comparing FOS and FAXO. No additive effects between FAXO and RBPP were observed as there was no significant difference between FAXO and MIX at any time point for all three individual SCFA.

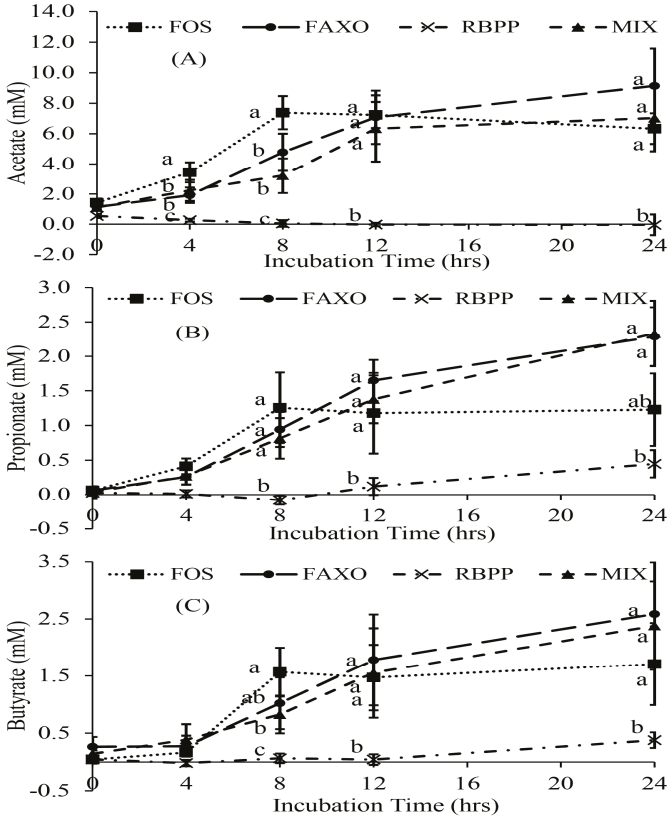

Figure 1. Individual SCFA (Short-Chain Fatty Acid) production during in vitro fermentation. (**A**) Acetate production; (**B**) propionate production; (**C**) butyrate production. Data are expressed as mean + SEM. Different letters at the same incubation time denote significant difference ($p < 0.05$). FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenols, MIX: mixture of FAXO and RBPP.

3.3. Microbiota Analysis

The 16S rRNA sequencing data were analyzed to investigate the changes in microbiota composition after 24 h incubation with FAXO, RBPP, and MIX compared with CTRL (control, no substrate) and FOS. At phylum level, four major phyla were identified in all samples including Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Figure 2). After 24 h of incubation, different substrates appeared to be able to modulate the microbiota composition significantly. FAXO appeared to increase Bacteroidetes and decrease Firmicutes abundance significantly compared to CTRL ($p < 0.05$). Proteobacteria population was also suppressed significantly in FAXO compared to CTRL ($p < 0.05$). RBPP alone and in combination with FAXO (MIX) only affected the Proteobacteria population as evidenced by a significant decrease in the abundance of this phylum compared to CTRL ($p < 0.05$). However, RBPP seemed to decrease Proteobacteria to a lesser extent compared to FAXO. On the other hand, FOS, which is used as a positive control, showed a significant increase in Actinobacteria population and a significant decrease in Proteobacteria compared to CTRL ($p < 0.05$). However, no significant changes were found in the abundance of Bacteroidetes and Firmicutes with FOS treatment compared to CTRL.

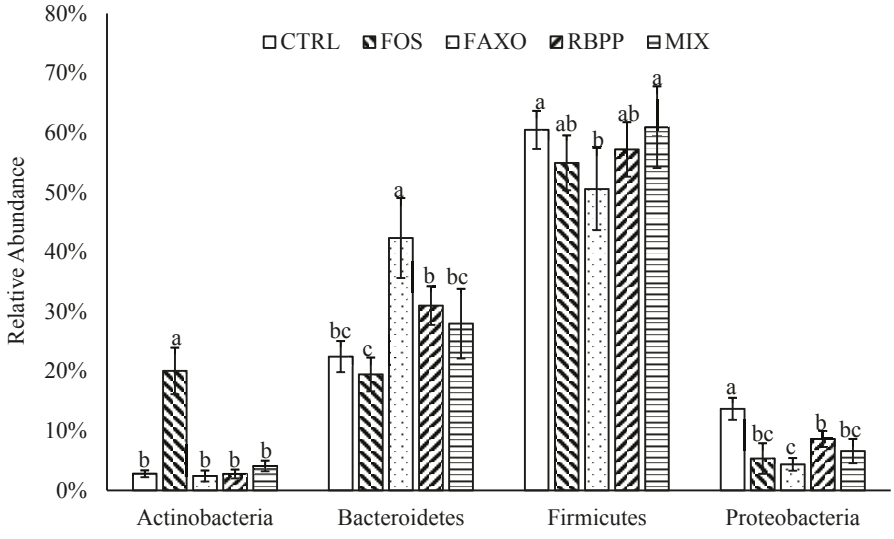

Figure 2. Microbiota composition at phylum level after 24 h incubation with different substrates. Data are expressed as mean ± SEM. Different letters in the same phylum denote significant difference ($p < 0.05$). CTRL: no substrate, FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenols, MIX: mixture of FAXO and RBPP.

Relative abundance of representative genera was also investigated. Results showed that FOS increased *Bifidobacterium* abundance dramatically, which indicated FOS were utilized by *Bifidobacterium* sp. ($p < 0.05$) (Figure 3A). An increase in *Lactobacillus* was observed; however, it was not significant compared to CTRL (Figure 3B). No significant changes were observed in *Bifidobacterium* and *Lactobacillus* with FAXO treatment. Similarly, RBPP and MIX did not appear to significantly affect the abundance of *Bifidobacterium* and *Lactobacillus*. Overall, FAXO, RBPP, and MIX did not seem to exert any effects on *Bifidobacterium* and *Lactobacillus*, two genera that are often targets for prebiotic action as they are commonly associated with many health benefits. However, considering other genera, significant differences were observed. Specifically, *Bacteroides* was increased significantly in FAXO and RBPP compared to both CTRL and FOS ($p < 0.05$) (Figure 3C). MIX treatment, however, did not affect *Bacteroides* abundance. FAXO also appeared to increase *Prevotella* abundance significantly compared to CTRL ($p < 0.05$) (Figure 3D). Population of *Dorea* was also affected by FOS, FAXO, and RBPP with significant decreases compared to CTRL ($p < 0.05$) (Figure 3E). In MIX, no significant difference in *Dorea* were found compared to CTRL. *Akkermansia*, a mucus-degrading genus that has gained significant attention in recent years because of its correlation to gut health, was also detected. However, no significant differences in *Akkermansia* population were found between treatments. The average abundance of *Akkermansia* was between 0.20% and 0.41% for all treatments. *Faecalibacterium*, a butyrate-producing genus, was increased significantly in abundance with RBPP and MIX compared to CTRL and FOS ($p < 0.05$) (Figure 3F). FAXO also seemed to increase *Faecalibacterium*, but no significant difference was observed. Taken together, these results suggested that, although FAXO and RBPP did not alter *Bifidobacterium* and *Lactobacillus*, they appeared to be able to modulate other gut bacteria populations that could also play important roles in host health.

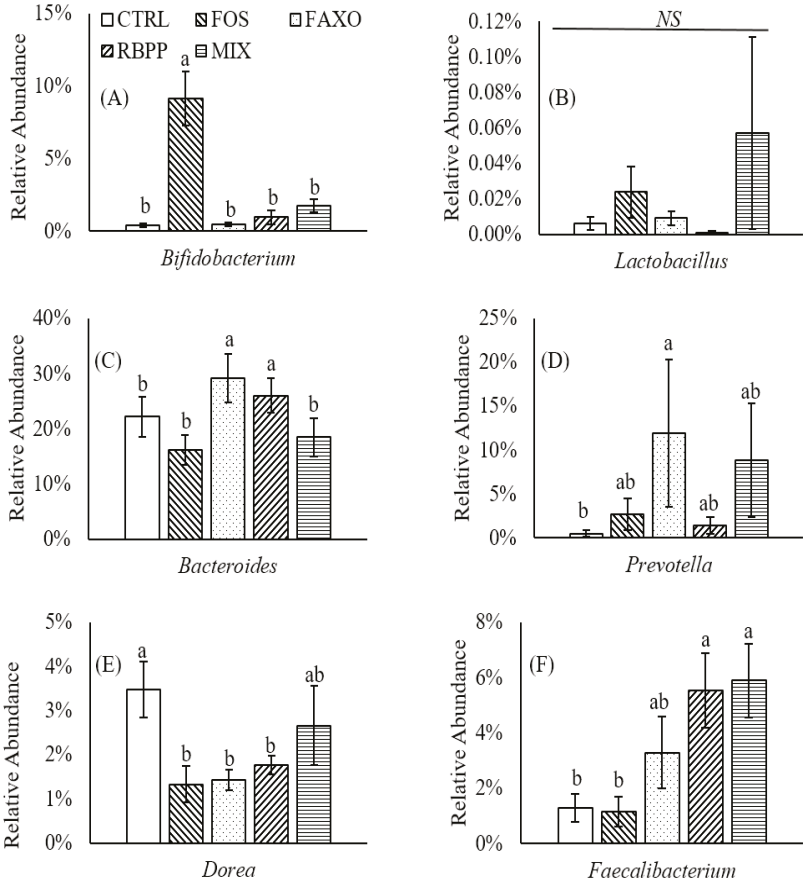

Figure 3. Change in abundance of different genera after 24 h incubation with different substrates. (**A**) *Bifidobacterium*; (**B**) *Lactobacillus*; (**C**) *Bacteroides*; (**D**) *Prevotella*; (**E**) *Dorea*; (**F**) *Faecalibacterium*. Data are expressed as mean ± SEM. Different letters denote significant difference ($p < 0.05$). CTRL: no substrate, FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenols, MIX: mixture of FAXO and RBPP.

3.4. Relationship of Human Gut Microbiota and SCFA Production

The production of butyrate has received much attention for its anti-inflammatory and anti-neoplastic effects on colonocytes [33–35]. Butyrate is not only produced directly from carbohydrate sources by butyrate-producing bacteria, but it can also be produced from acetate. As reported by Duncan et al. [36], butyrate could be converted from acetate by butyrogenic bacteria including *Coprococcus sp.*, *Roseburia sp.*, and *Faecalibacterium prausnitzii*. The abundance of these bacteria groups was also assessed to evaluate the relationship of gut microbiota and SCFA production (Figure 4). Combined effects by FAXO and RBPP were observed in *Coprococcus* and *Roseburia* as evidenced by a significant increase in MIX compared to CTRL in these two genera ($p < 0.05$) while both FAXO and RBPP did not differ from CTRL (Figure 4A,B). *F. prausnitzii*, the most abundant species in *Faecalibacterium* genus, also exhibited similar trends as in its genus shown above. Comparing to CTRL, both RBPP and MIX appeared to increase the abundance of this particular species significantly ($p < 0.05$) (Figure 4C).

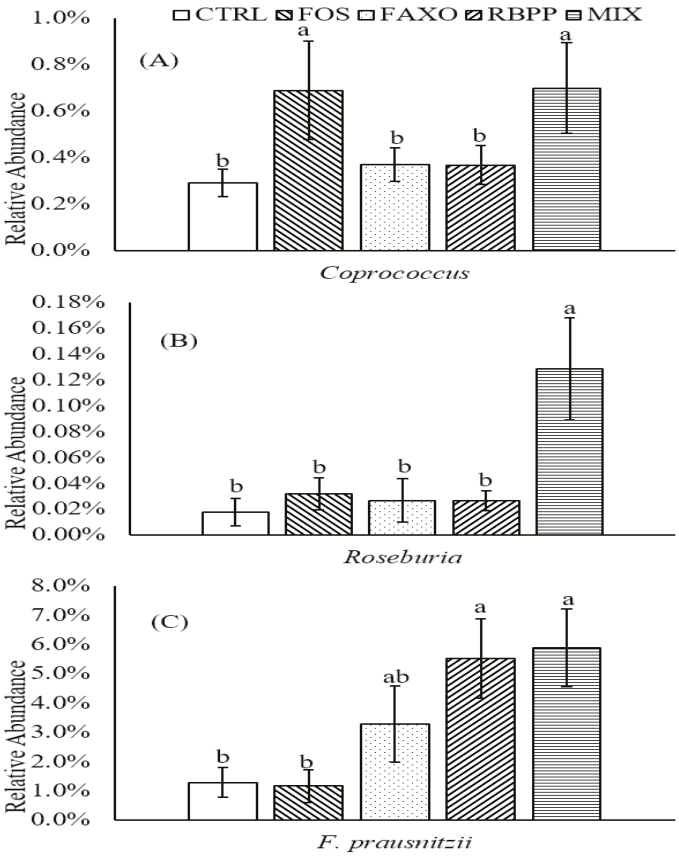

Figure 4. Changes in abundance of different butyrogenic bacteria after 24 h incubation with different substrates. (**A**) *Coprococcus*; (**B**) *Roseburia*; (**C**) *F. prausnitzii*. Data are expressed as mean ± SEM. Different letters denote significant difference ($p < 0.05$). CTRL: no substrate, FOS: fructooligosaccharides, FAXO: feroloylated arabinoxylan oligosaccharides, RBPP: rice bran polyphenols, MIX: mixture of FAXO and RBPP.

4. Discussion

In recent years, cereal bran AX and FAXO derived from them, have gained considerable interest in nutrition science for their prebiotic-like activities [37–39]. Metabolism of dietary prebiotics by gut microbiota results in production of SCFA and a shift in the composition colonic microbiota that is associated with improved health.

In the present study, FAXO isolated from rice bran were shown to be fermented by gut microbiota to produce SCFA. The fermentation patterns of FAXO were characterized by slower rates at time point 4 h and 8 h and faster rates at later time points compared to FOS. Rumpagaporn et al. [39] also reported similar findings in fermentation patterns of cereal arabinoxylans isolated from wheat, corn, sorghum, and rice compared to FOS (a positive control). As described in their study, fecal samples were collected from three healthy subjects and pooled. An amount of 50 mg of each substrate and 1 mL of pooled fecal slurry were used for the experiment. Arabinoxylans from different bran sources including corn and sorghum and hydrolyzed arabinoxylan products including corn, wheat, and rice were used in that study.

Total SCFA production in hydrolyzed rice bran arabinoxylan treatment were shown to have significantly higher levels compared to FOS at time point 24 h. In the present study, although total SCFA of FAXO was higher than that of FOS, no significant differences were found at time point 24 h when comparing FAXO and FOS.

Acetate was the major SCFA produced during fermentation of rice FAXO. Similar findings were also reported in wheat arabinoxylans [40], arabinoxylan oligosaccharides from brewery spent grain [41] and hydrolyzed rice arabinoxylans [39]. Acetate production of FAXO and FOS also showed similar patterns as in total SCFA production in this study. In colonic fermentation, acetate is considered the primary SCFA and is often used to monitor colonic events. In the colon, unlike propionate and butyrate, acetate is less metabolized and is readily absorbed. The presence of acetate also decreases colonic pH, which results in increased bio-availability of calcium and magnesium and inhibition of pathogenic bacteria [42,43]. In addition, acetate can be converted to butyrate. Two mechanisms that have been reported for the production of butyrate in the colon are acetate utilization and lactate fermentation [44].

The propionate production of FAXO was also comparable to FOS in this study. Other studies have shown that arabinoxylans produced relatively high propionate [38,39]. Rumpagaporn et al. [39] also reported that propionate concentration in rice arabinoxylans was significantly higher compared to FOS at time point 24 h. In this study, propionate production of FAXO at 24 h tended to be higher compared to FOS; however, the differences were not significant. After produced by gut bacteria, propionate is absorbed into bloodstream and transported to liver [43]. Propionic acid production has been shown to have beneficial health effects including lowering glucose-induced insulin secretion in isolated pancreatic islet cells of rats [45] and anti-proliferative effects on liver cancer cells [46].

Among all SCFA, butyrate has been of greatest interest due to its protective effects of colonocytes against cancer [34,35]. The combination of butyrate and mevastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, synergistically inhibited growth of colon cancer cells [47]. FOS has been known for the ability to increase butyrate production, hence the butyrogenic effects [48–50]. Fermentation of arabinoxylans generated lesser butyrate compared to FOS in a study by Rumpagaporn et al. [39]. However, in the present study, no significant differences were found between FAXO and FOS. Butyrate was the preferred energy source for colonocytes and inhibited the growth of colonic carcinoma cells [51]. As discussed above, the production of butyrate also comes from the conversion of acetate. Therefore, the production of each individual SCFA depends on other SCFA and SCFA concentrations in anaerobic fermentation can be changed interdependently.

Besides SCFA production upon fermentation, another criterion that a prebiotic must meet is the ability to selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being. *Bifidobacteria* and *lactobacilli* were thought to be the targets of prebiotic effects since they have been the focus of research, especially *bifidobacteria* as they are more abundant in human gut microbiota than *lactobacilli*. However, in recent years, many studies have reported the potential health effects of different groups of bacteria other than *bifidobacteria* and *lactobacilli*.

FAXO appeared to be a relatively selective substrate as demonstrated in several studies. Pure cultures of different *Bifidobacterium*, *Bacteroides*, and *Lactobacillus* species were shown to efficiently utilize FAXO [52–54]. Another study conducted by Vardakou et al. [55] found that in vitro fermentation of arabinoxylan oligosaccharides significantly raised *Bifidobacterium* and reduced *Bacteroides* levels. In the present study, FAXO did not stimulate the growth of *Bifidobacterium* and *Lactobacillus* genus. However, when assessing populations of other bacteria genera, FAXO might be able confer certain positive health effects by modulating other genera. Specifically, FAXO increased *Bacteroides* and *Prevotella* abundance, while reducing *Dorea* abundance.

Bacteroides is one of the most abundant genera in the gut microbiota. Aside from its correlation with increased propionate production, *Bacteroides* has also been shown to have protective effects against the invasion of exogenous bacteria in the colon by producing antagonic substances including bacteriocins [56]. This bacterial characteristic might play an important role in establishing and maintaining the intestinal ecosystem.

It was also interesting to see an increase in *Prevotella* sp. with FAXO. This genera is often associated with people with diets high in carbohydrates and fiber [57]. However, the variation between subjects in FAXO is quite large and comparing to FOS, FAXO did not differ significantly (Figure 3D). A study conducted by De Filippo et al. [58] revealed the differences in changes in gut microbiota of European and African children when solid food was introduced. European children's diet was rich in fat and low in fiber while African children's diet was rich in fiber and low in fat and animal proteins. During the breast-milk feeding period, no significant differences in gut microbiota were found between two groups of children. However, when solid food was introduced, differences in bacteria populations were observed. There was a significant enrichment of *Prevotella* genus in gut microbiota of African children compared to that of European counterparts. The differences were explained by the ability to produce cellulases and xylanases of this genus. Therefore, an increase in *Prevotella* with FAXO was expected.

Compared to other genera, *Dorea* was far less studied. However, correlation between *Dorea* population and disease has been demonstrated. Specifically, it has been shown that irritable bowel syndrome (IBS) was characterized by an increase in *Dorea* population and a decrease in *Bifidobacterium* and *Faecalibacterium* [59,60]. These findings suggest that there might be a link between gut microbiota and IBS, which could potentially be used for therapeutic treatments.

As discussed above, some bacteria are also capable of converting acetate to butyrate, namely *Coprococcus*, *Roseburia*, *R. intestinalis*, and *Faecalibacterium prausnitzii* [32]. In the present study, RBPP and MIX increased the abundance of a butyrate producing genus, *Faecalibacterium prausnitzii*. In addition, it is interesting that, while both FAXO and RBPP did not appear to modulate *Coprococcus* and *Roseburia* abundance, MIX treatment showed significant increases in both genera, especially in *Roseburia*, where FOS also did not affect its population. These results suggested that there might be synergistic effects between FAXO and RBPP in modulating these gut microbiota. Although these butyrogenic bacteria were increased in abundance, no significant differences were observed in neither acetate production nor butyrate production between FAXO and MIX.

The study was conducted using an in vitro anaerobic fermentation model, which is commonly used for first assessment of impacts of various compounds on metabolic activities of gut microbiota. However, this method certainly has its limitations. First of all, since it is a closed system, metabolites produced are constrained by amounts of substrates used. Second, the end-products accumulated during the fermentation period could alter the conditions of fermentation environment and affect the formation of certain metabolites. Third, in vitro methods do not fully replicate in vivo intestinal conditions, which affects the in vivo relevance of the study. Moreover, due to limited availability of the FAXO substrate, only 10 subjects were recruited for the study. The small sample size could reduce the statistical power and undermine the treatment effects.

The present study investigated the fermentibility of FAXO and RBPP and the changes in gut microbiota during fermentation with these components. The results demonstrated that FAXO had different fermentation patterns compared to FOS. FAXO were also found to be able to modulate several bacteria populations that could contribute to overall host health.

5. Conclusions

Total SCFA produced in vitro from rice bran FAXO by gut microbiota fermentation was comparable with the established prebiotic FOS, while the overall fermentation pattern from FAXO was different. Furthermore, FAXO showed distinctive modulating effects on microbiota phylogenetic composition profiles. A stimulatory effect was also observed for mixed FAXO and RBPP in modulating certain butyrate-producing bacteria. This study warrants further investigation of these rice bran components to confirm the prebiotic-like properties and any cooperative activity for modulating the gut microbiota.

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Symptom Severity Following Rifaximin and the Probiotic VSL#3 in Patients with Chronic Pelvic Pain Syndrome (Due to Inflammatory Prostatitis) Plus Irritable Bowel Syndrome

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Abstract: This study investigated the effects of long-term treatment with rifaximin and the probiotic VSL#3 on uro-genital and gastrointestinal symptoms in patients with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) plus diarrhoea-predominant irritable bowel syndrome (D-IBS) compared with patients with D-IBS alone. Eighty-five patients with CP/CPPS (45 with subtype IIIa and 40 with IIIb) plus D-IBS according to the Rome III criteria and an aged-matched control-group of patients with D-IBS alone (n = 75) received rifaximin and VSL#3. The primary endpoints were the response rates of IBS and CP/CPPS symptoms, assessed respectively through Irritable Bowel Syndrome Severity Scoring System (IBS-SSS) and The National Institute of Health Chronic Prostatitis Symptom Index (NIH-CPSI), and performed at the start of therapy (V0) and three months after (V3). In IIIa prostatitis patients, the total NIH-CPSI scores significantly ($p < 0.05$) decreased from a baseline mean value of 21.2 to 14.5 at V3 , as did all subscales, and in the IIIb the total NIH-CPSI score also significantly decreased (from 17.4 to 15.1). Patients with IBS alone showed no significant differences in NIH-CPSI score. At V3, significantly greater improvement in the IBS-SSS and responder rate were found in IIIa patients. Our results were explained through a better individual response at V3 in IIIa prostatitis of urinary and gastrointestinal symptoms, while mean leukocyte counts on expressed prostate secretion (EPS) after prostate massage significantly lowered only in IIIa cases.

Keywords: chronic pelvic pain syndrome; irritable bowel syndrome; irritable bowel syndrome-severity scoring system; rifaximin; probiotic VSL#3

1. Introduction

Over the past decade, the emerging insights of studies on the concomitant presence of some urologic chronic pelvic pain syndromes (UCPPS) (chronic pelvic pain, interstitial cystitis, painful bladder syndrome, prostatitis syndromes (PS), and vulvodynia) and non-urological associated syndromes (fibromyalgia, chronic fatigue syndrome, and irritable bowel syndrome (IBS)) have attracted significant interest by the Multidisciplinary Approach to the Study of Chronic Pelvic Pain research network established by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK),

with the aim of better understanding and evaluating visceral pain and lower urinary tract symptoms associated with UCPPS, and more systemic contributions to the pathophysiology of these disabling syndromes [1].

In particular, PS and IBS are functional, somatoform disorders with a high worldwide prevalence estimated at 11–16% [2,3] and 10–20% [4,5], respectively. Recently, we observed the simultaneous presence of PS and IBS in 30.2% and 31.8% of patients screened by andrologists and gastroenterologists, respectively [6,7], which is in agreement with other reports [8,9]. We found that patients with PS plus IBS also had a significantly higher frequency of chronic bacterial prostatitis (CBP) and lower frequency of non-inflammatory prostatitis (IIIb category) compared with patients with PS alone. The frequency of inflammatory prostatitis (IIIa category) had similar results [6].

PS and IBS are both characterised by a multifactorial pathogenesis, and these conditions are defined on the basis of clinical presentation rather than clear diagnostic markers or findings.

In fact, in terms of pathogenetic aspects, IBS includes disorders of the intestinal barrier, motility, secretion, visceral sensitivity, and interactions between psychological and psychosocial factors [10,11], while chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is defined as "urologic pain or discomfort in the pelvic region, associated with urinary symptoms and/or sexual dysfunction, lasting for at least three of the previous six months" in the absence of any identifiable pathology such as cancer, culturable infection, or anatomic abnormalities, often accompanied by "associated negative cognitive, behavioural, sexual, or emotional consequences" [12].

On the other hand, in terms of diagnostic aspects, the diagnosis of IBS requires the administration of the Rome III questionnaire, while PS is identified through the National Institute of Health's Chronic Prostatitis Symptom Index (NIH-CPSI).

The main diagnostics of the Rome III criteria as established by international professional organisations are based on exclusion criteria and the occurrence and rate of symptoms. In particular, IBS is defined by the Rome III criteria as an abdominal pain or discomfort, often associated with defecation, and with at least two of the following features: altered frequency or consistency, and/or passage of stools, and/or associated feelings of abdominal distension or bloating (10). The symptoms have to be present for at least three months and evidence for an organic underlying cause must be excluded to establish the diagnosis [10,13].

The severity of IBS symptoms are usually measured by using one of the following qualitative instruments: the IBS Severity Scoring System (IBS-SSS), the Bristol Stool Form Scale (BSFS), and the Gastrointestinal Symptom Rating Scale modified for use in patients with IBS (GSRS-IBS) [14–16].

On the other hand, the NIH-CPSI, considered the first validated tool for assessing symptom severity in PS, has been proposed for quantifying signs and symptoms and their impact on a patient's quality of life [17,18].

Despite intensive study over the past decade, clinical trials have failed to identify effective therapies for IBS or CP/CPPS. The efficacy of some probiotics and non-systemic antibiotics (e.g., rifaximin), mainly in infectious IBS, is supported by various evidences [19].

The theoretical basis for simultaneous treatment of the genitourinary and gastrointestinal tract has become more compelling given the evidence of overlapping innervations of the colon and bladder, and the influence of inflammation in one organ on the other [20]. This is why we aimed in the present study to ascertain whether uro-genital and gastrointestinal symptoms of men with CP/CPPS plus IBS may benefit from this three-month therapeutic combination, along with encouragement by recent results of long-term treatment with rifaximin and the probiotic VSL#3, which was effective in lowering the progression of prostatitis into more complicated forms of male accessory gland infections in infertile patients with bacteriologically cured CBP plus IBS [21].

The primary endpoints were the response rate of D-IBS and CP/CPPS symptoms, respectively assessed by using the IBS-SSS (taking a cut-off IBS-SSS reduction level of 50 points as an improvement) [15] and the total scores of the NIH-CPSI, considering as a minimum a six-point reduction in the total NIH-CPSI score [18].

2. Materials and Methods

This observational study was conducted at the Andrology and Endocrinology Unit Clinic, Policlinic University of Catania (Catania, Italy), between January 2011 and January 2013. Eighty-five selected male outpatients (median age: 30 years, range: 23–44 years) with a confirmed diagnosis of CP/CPSS plus D-IBS (Rome III criteria) were enrolled in this study, and 75 patients (median age 35 years, range 32–45 years) screened in the same period, affected by IBS alone, served as the control group. The diagnosis of CP/CPPS and D-IBS was made 20–60 months before the patients were included in this study. The protocol was approved by the internal Institutional Review Board of the University of Catania and an informed written consent was obtained from each man. All patients and controls underwent collection of their clinical history, administration of Rome III and NIH-CPSI questionnaires for IBS and prostatitis, respectively, and a physical examination.

Inclusion criteria of patients and the control group were, respectively, diagnosis of CP/CPSS plus D-IBS and IBS alone. Exclusion criteria were as follows: (1) history of chronic bacterial prostatitis (NIH type II) with a positive bacteriological finding at spermculture or at the Meares-Stamey four-glass test [22]; (2) subjects suffering from chronic or acute illness that could interfere with the study, who were taking medications that could interfere in the study (including anti-inflammatory drugs, proton pump inhibitors (PPIs), antidepressants, anti-diarrhoeal, prokinetics, and antispasmodic agents), and who consumed antibiotics or probiotics in the four weeks prior to entering the study; (3) obesity (defined as a body mass index (BMI) greater than or equal to 30 kg/m^2); (4) subjects affected by major concomitant diseases, with known anatomical abnormalities of the urinary tract or with evidence of other urological diseases, and with residual urine volume >50 mL resulting from bladder outlet obstruction; (5) patients with a history of gastrointestinal bleeding or duodenal or gastric ulcers [23]; and (6) patients that use VSL3 or other probiotic formulations, herbal medicines, or prostatitis treatments [24–28].

2.1. Diagnostic Rome III Criteria for IBS

This diagnosis was specifically based on the presence of abdominal pain or discomfort for at least three months in the previous six months, with two or more of the following symptoms: pain improved after defecation, symptoms associated with a change in frequency of stool, and symptoms associated with a change in stool appearance. A simple 10-point objective questionnaire based on the Rome III IBS module was used [18,23]. The presence of a diarrhoea-predominant IBS was found if patients had loose, mushy, or watery stools in the last three months, with no hard or lumpy stools ((question 9 = 0) and (question 10 > 0)) [18,23].

2.2. Diagnostic Symptoms Suggestive of CP/CPPS

The symptoms of CP/CPPS were evaluated by the NIH-CPSI questionnaire included for at least three months during the six months before the study, according to the European Association of Urology (EAU) guidelines [29]: pain or discomfort in the pubic or bladder area, perineum, testis, or at the tip of the penis not related to urination; ejaculatory pain; pain or burning during urination, incomplete emptying, and urinary frequency. Their NIH-CPSI pain sub-score was >8 (moderate to severe) [30].

A patient was assigned to the NIH category of the IIIa group if negative bacteriological findings were revealed by the Meares-Stamey four-glass test [31] and the white blood cell (WBC) count in the expressed prostrate secretion (EPS) was equal to or greater than 10 per high power field (HPF) or the WBC count in the VB3 was equal to or greater than 5 per HPF [32]. Conversely, a patient was assigned to the IIIb group if the WBC count in the EPS was less than 10 per HPF or the WBC count in the VB3 was less than 5 per HPF [32].

2.3. All Patients Completed the Treatment

Treatment Plan

At time-point V0 (visit zero), all patients and control groups were prescribed treatment with rifaximin, a non-absorbable antibiotic (200 mg, 2 tablets bid) (Normix®, Alpha Wassermann, Alanno, PE, Italy) for seven days per month for three months followed by a probiotic combination VSL#3 (450×10^9 CFU/day, one small envelope) (VSL#3®, Ferring SpA, Milan, Italy).

The probiotic VSL#3 is a mixture of eight different species of Gram-positive bacteria, namely *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacteria longum*, *Bifidobacteria infantis*, and *Bifidobacteria breve*. The probiotic combination VSL#3 was chosen because it has many interesting properties (reduction in inflammation, antioxidant capacity, and potential benefit for the treatment of IBS; effective in lowering the progression of prostatitis into more complicated forms of male accessory gland infections in infertile patients with bacteriologically cured CBP plus IBS), both in vitro and in vivo that may account for its clinical efficacy [13,21].

2.4. Assessment of Symptoms

A follow-up visit, including assessment of symptoms of IBS and CP/CPPS through specific validated questionnaires and urological visits, was performed three months (V3) after the start of therapy (V0).

D-IBS: To monitor D-IBS symptoms and changes, all three groups were asked to register their symptoms weekly using the IBS-SSS questionnaire [16], which includes five items on a 0–100 mm visual analogue scale with total scores ranging from 0 to 500 mm: severity of abdominal pain (Question 1), frequency of abdominal pain (Question 2), severity of abdominal distension (Question 3), dissatisfaction with bowel habits (Question 4), and interference with quality of life (Question 5). This score classifies subjects as having no symptoms (<75), mild (75–174), moderate (175–300), or severe IBS (>300). The primary endpoint was a cut-off IBS-SSS reduction level of 50 points was considered to be an improvement [15]. Secondary endpoints were expressed as individual symptoms of IBS.

CP/CPPS: CP/CPPS symptoms were assessed at V0 and V3 through the NIH-CPSI score. The primary endpoint was a minimum six-point reduction in the total NIH-CPSI score, because it was considered as a clinically appreciable improvement of CP/CPPS symptoms [7]. Secondary endpoints were expressed as individual symptoms of CP/CPPS (NIH-CPSI subscale values); WBC on EPS after prostate massage.

2.5. Statistical Analysis

The software SPSS 9.0 for Windows (Chicago, IL, USA) was used for statistical evaluation. Quantitative data were expressed as median and range, and qualitative data were expressed as percentages throughout the study. Intragroup differences in NIH-CPSI or IBS-SSS questionnaire scores before/after therapy were analysed using Wilcoxon's signed rank test. Mann-Whitney U tests were used for analyses that compared different groups. A statistically significant difference was accepted when the p value was lower than 0.05.

3. Results

The demographic and baseline characteristics of the participants are shown in Table 1. Age and time since diagnosis were similar in patients with CP/CPPS (prostatitis IIIa and IIIb subtypes) plus D-IBS or D-IBS alone (Table 1). All patients in our study had a normal BMI, which was similar in all three groups. Furthermore, the mean leukocyte counts on EPS after prostate massage were significant ($p < 0.05$) in NIH category IIIa patients plus D-IBS > IIIb patients plus D-IBS > D-IBS alone (Table 1). All patients and controls completed the treatment as planned.

Table 1. Baseline characteristics of patients with chronic prostatitis (Type IIIa or IIIb) plus irritable bowel syndrome (IBS), or with IBS alone.

	Categories		
	Type IIIa Plus IBS	**Type IIIb Plus IBS**	**IBS Alone**
Patients (*n*)	45	40	75
Age (years)	30 (23–44)	29 (24–44)	30 (23–44)
BMI (kg/m^2)	23 (19–28)	22 (20–28)	23 (21–28)
Time since diagnosis (months)	32 (20–60)	34 (22–58)	34 (24–50)
WBC on EPS after prostate massage	12 $^{*,\circ}$ (10–15)	7 † (4–10)	4 (2–6)

Irritable bowel syndrome = IBS; BMI = body mass index. WBC = white blood cells; EPS = expressed prostate secretion. Values were expressed as mean and range (in parentheses); * $p < 0.01$ vs. matched values of patients with IBS alone; $^{\circ}$ $p < 0.05$ vs. matched values of patients with prostatitis type IIIb; † $p < 0.05$ vs. patients with IBS alone.

However, the total NIH-CPSI scores significantly ($p < 0.05$) decreased in IIIa patients from a baseline (V0) mean value of 21.2 to 14.5 at V3, as did all subscales (pain, urinary, quality of life), and the total NIH-CPSI score significantly decreased in IIIb patients (from 17.4 to 15.1). In contrast, patients with IBS alone did not show any significant differences in the NIH-CPSI score (total and subscales) (Table 2).

Furthermore, 49.4% of patients (42 out of 85) showed clinical improvement (in terms of a six-point or more reduction in total NIH-CPSI score), with a significant difference between the response rate of NIH category IIIa and IIIb, since a six-point or more reduction in total NIH-CPSI score was found respectively in 71% or 25% of these categories.

Regarding gastrointestinal symptoms, at V0 patients affected by the IIIa inflammatory sub-category of CP/CPPS generally exhibited not statistically significant higher IBS-SSS (mean 298.4, range 180–410) than that registered in IIIb patients (270.0, range 163–388) or the control group (262.5, range 156–397) (Table 2). At V3, the IBS-SSS was significantly reduced in patients with IIIa prostatitis (mean 192.5, range 117–246) compared to that observed in IIIb prostatitis (mean 198.5, range 135–265) or controls (mean 204.0, range 129–266). In patients with IIIa prostatitis, the significant improvement from baseline for IBS-SSS was associated with a responder rate (in terms of decline >50 point) of 77.7% (35 out of 45 patients), significantly higher than the rate values found in IIIb patients (32.5%; 13 out of 40 patients).

Furthermore, regarding the secondary endpoints of the study, we also registered a better individual response in IIIa prostatitis (compared with IIIb prostatitis plus D-IBS or D-IBS alone) of urinary and gastrointestinal symptoms, and mean leukocyte counts on EPS after prostate massage were significantly lowered (from 12 to 7) in IIIa cases only ($p < 0.05$).

Table 2. Intragroup and intergroup analysis of National Institute of Health Chronic Prostatitis Symptom Index NIH-CPSI score and gastrointestinal symptoms in patients with chronic prostatitis (Type IIIa or IIIb plus IBS, or with IBS alone assessed before the treatment (V0) and three months afterward (V3).

	Categories					
	Type IIIa Plus IBS		Type IIIb Plus IBS		IBS Alone	
Study timepoint	V0 (n = 45)	V3 (n = 45)	V0 (n = 40)	V3 (n = 40)	V0 (n = 75)	V3 (n = 75)
Outcomes related to CP-CPPS						
Primary outcome						
NIH-CPSI responder rate (≥6 point decline) No./total No. (%)	NA	32/45 (71.1)	NA	10/40 (25)	NA	NA
Secondary outcomes						
WBC on EPS after prostate massage	12 *,° (10–15)	7 * (5–9)	7 † (4–10)	6 (4–9)	4 (2–6)	4 (2–6)
Prostatitis symptoms (NIH-CPSI score)						
Total score	21.2 (15–24)	14.5 * (9–19)	17.4 ° (10–21)	15.1 (13–18)	12.0 (6–14)	7.5 (3–10)
Pain subscale	11.9 (8–15)	8.5 * (5–11)	9.8 (8–11)	8.7 (7–11)	5.5 (4–7)	3.0 (2–5)
Urinary subscale	4.5 (3–6)	2.5 * (0–3)	3.6 (2–5)	3 (2–5)	3.5 (1–5)	2.0 (1–3)
Quality of life subscale	4.8 (3–7)	3.2 * (2–5)	4.0 (3–6)	3.4 (2–6)	4.0 (3–6)	2.5 (1–4)
Outcomes related to D-IBS						
Primary outcomes						
Mean IBS severity score	298.4 (180–410)	192.5 * (117–246)	270.0 (163–388)	198.5 (135–265)	262.5 (156–397)	204.7 (129–266)
IBSS responder rate (>50-point decline) No./total No. (%)	NA	35/45 (77.7)	NA	13/40 (32.5)	NA	NA
Secondary outcomes						
Gastrointestinal symptoms						
Abdominal pain	48.6 (25–63)	25.5 * (10–35)	40.5 ° (21–65)	28.5 * (12–40)	38.5 ° (18–58)	30.5 * (11–43)
Frequency of abdominal pain	50 (28–75)	35.0 * (30–55)	48.5 ° (30–70)	38.5 * (22–60)	45.0 ° (35–70)	35.0 * (30–67)
Abdominal distension/bloating	44.5 (35–74)	25.5 * (18–35)	39 (30–67)	28.5 * (15–40)	42 (30–85)	28.5 * (15–38)
Dissatisfaction with bowel habits	78.0 (62–94)	50.5 * (40–74)	75.0 (66–100)	55.0 (40–82)	75.0 (60–95)	55.0 (35–78)
Interference with quality of life	77.0 (60–105)	38.0 * (22–47)	68.0 (15–87)	48.0 (15–25)	66.0 (15–89)	55.7 (33–77)

Irritable bowel syndrome = IBS; National Institute of Health Chronic Prostatitis Symptom Index (NIH-CPSI); No. = number; IBS Severity Scoring System = IBS-SSS; Values were expressed as mean and range (in parentheses); NA = not applicable; * $p < 0.05$ vs. pre-treatment matched values; ° $p < 0.05$ vs. matched values of patients with prostatitis type IIIa; † $p < 0.05$ vs. patients with IBS alone.

Compliance

All patients completed the treatment. No serious adverse events were reported in the study groups.

4. Discussion

Comorbidities are common in IBS and can affect both the gastrointestinal tract, including functional chest pain, heartburn, dyspepsia, and/or abdominal pain [33], and other systems with pain-related disorders, including migraine headache, fibromyalgia, and chronic pelvic pain [34].

In our study, we observed a significant decrease of the total NIH-CPSI and in pain, urinary disorder, and quality of life subscales in IIIa patients and in total NIH-CPS score in IIIb patients; in contrast, no significant differences were observed in patients with IBS alone.

Recently, we showed that PS and IBS co-exist in an elevated percentage (31.2%) of patients who seek medical advice for PS or IBS in andrological or gastroenterological settings, respectively [21]. These patients also had severer urinary (total score and pain subscale) and gastrointestinal symptoms, with significantly higher scores in the NIH-CPSI and Rome III questionnaires compared with patients with PS or IBS alone [21]. These results are in agreement with other reports [8,9].

Despite intensive study over the past decade, clinical trials have failed to identify effective therapies.

In patients with IBS, although probiotics had beneficial effects on global IBS, abdominal pain, bloating, and flatulence scores [35], when considered as a whole, meta-analyses are difficult due to the heterogeneity of the studies of probiotics in IBS and the use of different bacterial strains and different mixtures of these strains, as well as different dosages [36]. Rifaximin was approved in 2015 for the treatment of IBS with diarrhoea, and after two weeks of treatment can result in symptom improvement that persists $\geq$12 weeks post-treatment [19]; it is by far the best-studied antibiotic in IBS, and it has several appealing properties (gut-specificity; predominant effect intraluminal, limited systemic availability; it does not significantly alter the microbiota of the GI tract; it does not tend to cause diarrhoea or other superinfections such as *C. difficile*). Rifaximin proved more effective than a placebo for global symptoms and bloating in IBS patients [37].

In patients with IBS, the mechanisms of action of rifaximin, beyond direct bactericidal effects, include decrement of host pro-inflammatory responses to bacterial products in patients with IBS, and has antibiotic efficacy against isolates derived from patients with small intestinal bacterial overgrowth [19,38–40].

On the other hand, patients with CP/CPPS traditionally receive empirical treatment, with the most common in clinical practice including antimicrobial agents and alpha-adrenergic receptor antagonists [32,38,41], or combination, multimodal therapy [39,42].

Our results fulfilled both primary outcomes, since we demonstrated that, in patients with diarrhoea-predominant IBS plus CP/CPPS, the administration of rifaximin followed by VSL#3 for a period of three months registered a reduction of $\geq$six points of the total NIH-CPSI score in 71.1% of patients with NIH IIIa prostatitis plus D-IBS. Notably, this value is higher than the placebo effect of ~64%, demonstrated in long-term studies [7]. At the same time, patients with IIIa prostatitis achieved as a result of therapy a significant improvement from baseline for IBS-SSS; this was associated with an IBS-SSS responder rate (in terms of decline >50 points) of 77.7%, significantly higher than the rate values found in IIIb patients (32.5%).

Results of the secondary endpoints were also noticeable, showing a better individual response at V3 in IIIa prostatitis (compared with IIIb prostatitis plus D-IBS or D-IBS alone) of urinary and gastrointestinal symptoms, and significant reduction of mean leukocyte counts on EPS after prostate massage in IIIa cases only.

The explanation of a better response on combined therapy in our study might be the complexity of IBS symptoms and natural disease course, requiring medications that might be effective against specific symptoms. These results suggest that patients with CP/CPPS plus D-IBS may have similar underlying pathophysiology, but they differ in severity.

The mechanisms that are not fully understood involve a genetic predisposition in the presence of a complex interaction of colonic cells, imbalance between commensal and pathogen bacteria of the gut microbiome, and local low-grade inflammation associated with IBS with abnormal immune function, gastrointestinal motility, and brain-gut interactions [40,41]. The resultant products of altered gut fermentation, termed 'the fermentome', can exist in the gaseous phase and are recognisable by volatile organic compounds (VOC) analysis in urine, breath, and faeces [41,43].

In patients with D-IBS burdened by the presence of comorbidities such as inflammatory prostatitis, we hypothesise that more severe urinary and intestinal symptoms in these patients may be secondary to an increased presence of VOC facilitated in part by increased intestinal permeability altered in certain gut diseases [43]. In this regard, ultrasound evaluation has recently revealed a significantly higher frequency of dilatation of the prostatic venous plexus (anatomical space between the posterior wall of the prostate and the anterior wall of the rectum) in patients with CBP plus IBS (75%) compared with patients with CBP alone (10%) [44].

Therefore, in our clinical model of patients, our adopted therapy (rifaximin followed by probiotic VSL#3) may have improved the urinary and gastrointestinal symptoms pattern through a likely reduction of the fermentome and VOC, at least in urine and faeces.

Although 49.4% of the patients (42 out of 85) showed clinical improvement in our study, there was a significant difference between the IIIa and IIIb groups, since a six-point or more reduction in total NIH-CPSI score was found respectively in 71% and 25%.

This huge difference may reflect the heterogeneous aspect of CP/CPPS mainly within subjects of category IIIb, which in a contemporary concept has a clinical presentation that is not prostate-specific but incorporates a phenotyping system with a varying number of positive domains among the following six: urinary, psychosocial, organ-specific, infection, neurologic/systemic, and tenderness (UPOINT), and a European study modified the clinical phenotyping system with an additional sexual dysfunction domain (UPOINTS) [45,46].

In May 2016, the new diagnostic criteria for functional bowel and anorectal disorders were defined in Rome IV criteria. The changes in criteria and new research findings might influence not only pathophisiological factors but also future treatments. Further studies are required to investigate these disorders [47,48].

5. Conclusions

The mechanisms of both commensal and pathogenic bacteria interaction with colonic cells and prostatitis are not fully understood. The treatment with rifaximin followed to probiotics reduces the urinary and gastrointestinal symptoms with a good compliance. Further studies with higher numbers of participants are necessary to confirm these results.

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Effect of a Partially Hydrolysed Whey Infant Formula Supplemented with Starch and *Lactobacillus reuteri* DSM 17938 on Regurgitation and Gastric Motility

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Abstract: Functional regurgitation (FR) is common in early infancy and represents a major drain on healthcare resources. This double-blind, randomized controlled trial investigated the effects of a formula containing partially hydrolysed, 100% whey protein, starch and *Lactobacillus reuteri* (DSM 17938) on gastric emptying rate (GErate) and regurgitation frequency in infants with FR. Enrolled infants were randomly allocated to receive either the test formula or a standard starter formula for four weeks. Ultrasound GErate assessment was performed at baseline (week 0) and at week 4; the number of regurgitations, feed volumes and potential adverse events were recorded in a daily diary. Eighty infants aged four weeks to five months were enrolled; 72 (test group = 37; control group = 35) completed the study. Compared to controls, the test group showed greater percentage changes in GErate (12.3% vs. 9.1%, $p < 0.01$). Mean daily regurgitations decreased from 7.4 (0.8) at week 0 to 2.6 (1.0) at week 4 in the test group and from 7.5 (1.0) to 5.3 (1.0) in controls (between-group difference, $p < 0.0001$). Compared to a standard formula, a starch-thickened partially hydrolysed whey protein formula supplemented with *Lactobacillus reuteri* is more effective in decreasing the frequency of regurgitation and improving GErate, and can be of benefit to infants with FR.

Keywords: infant formula; regurgitation; gastric emptying; *Lactobacillus reuteri*; partially hydrolysed whey; starch

1. Introduction

Regurgitation is defined as the backflow of gastric contents into the pharynx or mouth [1]. Due to multiple physiological predisposing factors (i.e., predominantly supine position, liquid meals, loose gastro-oesophageal junction), this condition is particularly common in early infancy: more than 50% of infants between three and four months of age experience daily regurgitation, and about 20% have ≥4 episodes of regurgitation per day [2].

Different from gastro-oesophageal reflux disease (GERD), functional regurgitation (FR) is not associated with troublesome complications such as poor growth, inflammatory esophagitis or respiratory symptoms [3,4]. However, it can become a major cause of parental anxiety [5], leading to an increased number of visits to paediatricians: nearly 1 in 5 parents has sought help for frequent regurgitation [6,7]. Moreover, despite their favourable prognosis, infants with FR not infrequently

undergo extensive interventions, such as multiple dietary changes and pharmacological treatments, which may or may not be of benefit [8].

The most important non-pharmacological approaches for infants with FR are parental reassurance, avoidance of overfeeding and dietary interventions [3,9]. The use of anti-reflux formulas thickened with starch, guar gum, or locust bean gum may reduce the number of regurgitation episodes [10,11]. Partially hydrolysed protein formulas have also been shown to decrease the volume and frequency of regurgitation in infants [12]; among the possible mechanisms of action, an improvement of gastric emptying has been proposed. Probiotic supplementation with *Lactobacillus reuteri* has also been associated with an improved gastric motility in both animal and human studies [13,14]. In a study from 2014, we demonstrated the efficacy of oral supplementation with this probiotic strain in preventing FR in healthy term newborns [15]. Nevertheless, the effectiveness of the combination of the above therapeutic approaches on reflux frequency and gastric motility has not been evaluated yet. Given that abnormalities of one or more of the three physiologic processes—namely, oesophageal motility, lower oesophageal sphincter function, and gastric motility—can contribute to FR [4], the aim of the present study was to evaluate the efficacy of a formula containing partially hydrolysed whey protein, additional starch, and the probiotic *L. reuteri* in reducing regurgitation frequency and improving gastric emptying in infants with FR.

2. Methods

2.1. Study Population

This randomized, double-blind, controlled trial was conducted between 1 January 2014 and 28 February 2015 in the Paediatric Gastroenterology Clinic (Department of Paediatrics) of the University of Bari Aldo Moro (Bari, Italy), and in a Paediatric Primary Care Clinic in Naples (Italy).

Infants referred to these clinics were eligible for the study if they were full-term, appropriate for gestational age, exclusively formula-fed, aged between four weeks and five months at the time of recruitment and if they fulfilled the Rome III criteria for FR diagnosis on the basis of retrospective reports from parents or caregivers (i.e., episodes of gastro-oesophageal reflux in the absence of nausea, hematemesis, aspiration, apnea, failure to thrive, difficulty in feeding or swallowing, or abnormal posture for at least one week) [16].

Currently, there is no validated diagnostic questionnaire for infants or toddlers with functional gastrointestinal diseases, unlike that for children and adolescents (Questionnaire on Paediatric Gastrointestinal Symptoms—Rome III Version [QPGS-RIII]) [16,17]. Hence, we translated the Rome III diagnostic criteria for infants and toddlers into a series of questions on signs and symptoms that would be easily understood by parents. Most responses were either Likert-type scales or categorical. Moreover, to assess their own symptoms experienced during the previous four weeks [18], the infants' parents completed the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire for adults.

Exclusion criteria included congenital malformations, diseases or syndromes that could affect normal growth, cow's milk protein allergy and any other chronic or allergic disease, treatment with antibiotics, proton pump inhibitors, H2 antagonists or antacids. In order to avoid possible confounding influences, infants receiving probiotic supplementation or formula supplemented with prebiotics and/or probiotics at the time of enrolment were also ruled out.

Infants assessed for eligibility were exclusively formula-fed and received the same enteral feeding regimen and feed volumes until randomization.

This study was conducted in conformity with the principles and regulations of the Helsinki Declaration. Written, informed consent was obtained from the children's parents/legal guardians, who were fully informed of the nature and purpose of the study. The study protocol was approved by the Ethical Institutional Review Board of Institutional Ethics Committee of Bari University Hospital and is registered in the Protocol Registration System Clinical Trial.gov (ClinicalTrials.gov Identifier: NCT01956682).

2.2. Study Design

Enrolled infants were assigned consecutive numbers, starting with the lowest available, and were randomly allocated to the control or experimental group using a computer-generated randomization list. Both parents and physicians were blinded to the group assignment. The control group received a commercially available starter formula that included 70% whey protein and 30% casein, providing 1.85 g of protein per 100 kcal (NAN 1, Nestle Nutrition, Vevey, Switzerland). The test formula was a commercially available (NAN A.R., Nestle Nutrition, Vevey, Switzerland) partially hydrolysed 100% whey formula thickened with starch, providing 1.9 g protein per 100 kcal, and supplemented with a mixture of potato, corn starch (4 g/100 kcal) and *Lactobacillus reuteri* DSM 17938 (2.8×10^6 CFU/g powder). Infants in both groups were fed the assigned formula in standardised amounts according to their weight, age and appetite for four weeks.

2.3. Outcome Evaluation

In order to analyse in depth the role of gastric motility in the pathophysiology of FR, the primary aim of this study was the evaluation of gastric emptying rate (GErate). GErate was evaluated at baseline (week 0) and at the end of the study period (week 4) by means of a real-time apparatus (Image Point HX, Hewlett Packard Company, Palo Alto, CA, USA) equipped with a 3.5 MHz linear probe. The probe was placed at the level of the trans-pyloric plane to allow a simultaneous visualization of gastric antrum, superior mesenteric vein and aorta. The antral measurements were taken from the outer profile of the wall. Since the cross section of the gastric antrum, corresponding to the sagittal plane passing through the superior mesenteric vein, is elliptical in shape, its area can be calculated by measuring the longitudinal (L) and anteroposterior (AP) diameters and applying the formula for calculating the area of an ellipse (π L $\times$ AP/4) [19]. Antral cross-sectional plane area was used as a proxy for gastric content volume, and measurements were done before and immediately after the end of the test meal (time 0) and at 30, 60, 90, and 120 minutes after the meal. GErate was expressed as the percent reduction in antral cross sectional area at time 0 and 120 min after meal ingestion (GErate = ((antral area time 0 min − antral area 120 min)/antral area time 0) $\times$ 100) [20]. The percentage difference between baseline GErate values and those recorded at the end of week 4 was then calculated and used for statistical analysis.

Secondary outcomes were the frequency of regurgitation episodes, growth rates (weight, length, and head circumference, measured at week 0 and 4), and formula intakes. Parents/caregivers were thus instructed to record symptoms (e.g., the number of regurgitations per day, and possible related interventions), feed volumes, administration of any food other than the study formula, and potential adverse events in a structured daily diary, which was returned to the study investigators at the end of the experimental period.

2.4. Statistical Analysis

The sample size calculation was based upon the assumption that an improvement in GErate would be expected in 75% of infants receiving the test formula and in 15% of those receiving the control formula. On this basis, a minimum of 30 infants per group was required to achieve an alpha error of 0.05 and a beta error of 0.2. Assuming a dropout rate of 25%, the target enrolment goal was 40 infants per group; recruitment was stopped when this goal was reached.

Data were first analysed using simple descriptive statistics of centrality and dispersion. Regurgitation frequency was calculated as the daily number of regurgitation episodes averaged over the previous seven-day period. To evaluate the distribution of the daily number of regurgitations, a kurtosis test (asymmetry) was performed. The variable was normally distributed at baseline and week 1 but not at other time points, thus at weeks 2, 3 and 4 it was normalized with squaring. Mean values of the normally distributed and normalized variables, including anthropometric parameters, were compared using the Student *t*-test for unpaired samples. An ANOVA model for repeated measures

was used to evaluate differences in regurgitation frequency between groups. Differences in gastric emptying features, which were not normally distributed, were evaluated using the Mann–Whitney rank sum test. A per-protocol (PP) analysis was performed.

For all tests, a p-value < 0.05 was considered statistically significant. The software package used for the statistical analysis was STATA (STATA version 4.0 Statistical Software, Stata Corporation Houston, TX, USA).

3. Results

A total of 80 infants were enrolled and underwent randomization (40 in the test group and 40 in the control group). As shown in the enrolment flow chart (Figure 1), eight of these were lost to follow-up and were thus excluded from the analysis due to incomplete data, whereas 72 completed the trial (37 in the test group and 35 in the control group) and were included in the per-protocol analysis.

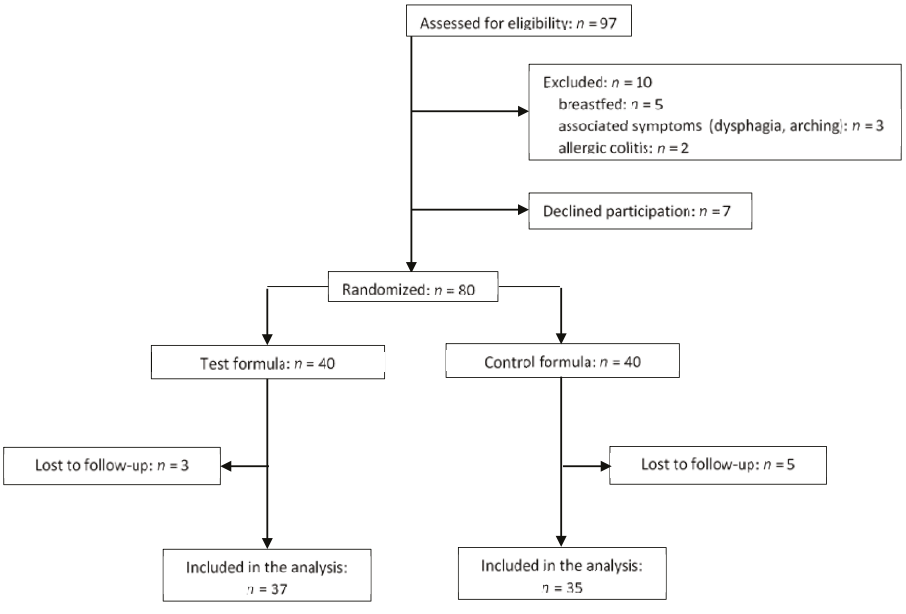

Figure 1. Flow diagram of study enrolment, allocation to the study groups and study dropout.

At baseline, infants were, on average, 60 days old, weighed 5.6 kg, and had approximately seven episodes of regurgitation per day. No baseline differences in fasting antral areas (Table 1), GErate (test group: median −54.9% [5th percentile = −75.6%, 95th percentile = −44.2%]; control group: median −55.3% [5th percentile = −85.2%, 95th percentile = −44.3%]), age, anthropometric parameters (Table 2) and regurgitation frequency (Figure 2) were seen between the study groups.

With regard to gastric motility parameters (Table 1), at the end of the intervention period the median fasting antral area was significantly reduced in infants receiving the test formula compared to controls (3.5 cm^2 (5th percentile = 2.0; 95th percentile = 4.6 cm^2) vs. 4.6 cm^2 (5th percentile = 2.4; 95th percentile = 6.0), $p = 0.01$). When compared to baseline, median fasting antral areas at week 4 were increased in both groups, consistent with the infants' growth.

Moreover, infants fed on the test formula showed a significantly higher GErate percentage change between week 0 and week 4 compared to controls (median 12.3% (5th percentile = −3.9%, 95th percentile = 22.0%) vs. 9.1% (5th percentile = −27.0%; 95th percentile = 25.5%), $p < 0.01$). Of note,

the 5th percentile of GErate percent change at week 4 was noticeably more negative in the controls compared to the test group (−27% vs. −3.9%), thus suggesting a better gastric motility in the latter.

Infants receiving the test formula showed a significant reduction in the frequency of daily regurgitations compared to the control group (Figure 2). In particular, the mean daily number of regurgitations decreased from 7.4 (standard deviation (SD) = 0.8) at baseline to 2.6 (SD, 1.0; 95% CI = 2.2–2.9) at week 4 in the test group and from 7.5 (SD 1.0) to 5.3 (SD 1.0; 95% CI = 5.0–5.6) in the control group (between-group difference, $p < 0.0001$). No difference in body weight or in the other anthropometric parameters was seen between the two groups at the end of the trial (Table 2). Mean formula intakes were also similar in the test (742 mL/day) and the control (738 mL/day) groups. No adverse events related to both the study formulas were reported.

Table 1. Gastric emptying parameters, expressed as median (5th and 95th percentile) at baseline (week 0) and at the end of the study (week 4) in the test and control groups.

	Test (n = 37)		Control (n = 35)		p-Value
	Week 0	Week 4	Week 0	Week 4	
Fasting antral area, cm^2	2.7 (2.0, 3.1)	3.5 (2.0–4.6)	2.7 (1.4, 3.1)	4.6 (2.4, 6.0)	0.01 [a]
GErate percent change from week 0 to week 4, %	12.3 (−3.9, 22.0)		9.1 (−27.0, 25.5)		<0.01 [b]

GErate: gastric emptying rate. [a] p-value for between-groups difference at the end of the study. [b] p-value for between-group difference in GErate percentage change.

Table 2. Age and anthropometric measures (mean ± standard deviation) at baseline (week 0) and at the end of the study (week 4) in the test and control groups.

	Test (n = 37)		Control (n = 35)		p-Value [a]
	Week 0	Week 4	Week 0	Week 4	
Age, days	59 ± 8.2	92 ± 4.3	60 ± 5.3	93 ± 3.3	NS
Weight, g	5590 ± 631	6280 ± 391	5670 ± 739	6320 ± 239	NS
Length, cm	53.7 ± 1.8	58.1 ± 0.8	54.1 ± 1.5	57.7 ± 1.1	NS
Head circumference, cm	40.8 ± 1.3	42.3 ± 0.3	39.7 ± 1.1	41.8 ± 0.7	NS

NS = not significant ($p > 0.05$). [a] p-values for between-group difference.

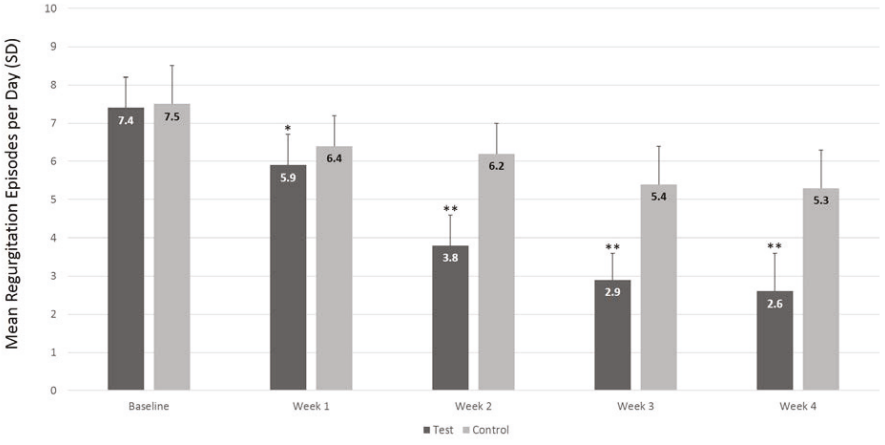

Figure 2. Mean number of daily regurgitation episodes by week in the test and control groups. Repeated measures ANOVA showed a significant difference between groups ($p < 0.0001$) over the entire study period. Means at each time point were compared using Student t-test for unpaired samples. * $p = 0.0094$; ** $p < 0.0001$.

4. Discussion

According to the present results, a starch-thickened, partially hydrolysed starter formula supplemented with the probiotic *L. reuteri* leads to a significant improvement in gastric motility and regurgitation frequency in infants diagnosed with FR.

Functional abnormalities of oesophageal, gastric and enteric nervous system are known to play a contributing role in the multifactorial pathogenesis of regurgitation [21]. By activating the stretch receptors adjacent to the gastro-oesophageal junction, an excessive gastric distension ensuing from an impaired visceral motility has been previously proposed as representing a significant trigger stimulus for transient lower oesophageal sphincter relaxations [22,23], which are among the main pathophysiological mechanisms underlying gastro-oesophageal reflux in the paediatric population [24]. On this basis, the lower fasting antral areas and the higher GErate percentage changes observed in infants fed the test formula might have contributed to the reduction in regurgitation frequency in the present study.

The molecular and physiological pathways through which gut microbiota can influence intestinal motility are far from fully understood. Nevertheless, following recent evidence [25], it is reasonable to postulate that gut neuromuscular apparatus, (i.e., enteric neurons, interstitial cells of Cajal and smooth muscle cells) can act as a potential mediator for the effects that probiotics exert beyond the intestine, on central and autonomic nervous system. The probiotic strain used in this study has been previously shown to significantly improve gastric motility in rodents and preterm infants [13,14,26]; moreover, daily supplementation with *L. reuteri* DSM 17989 has been associated with a lower reported incidence of functional gastrointestinal disorders in term infants at 3 months of life [15]. Similarly, the present results showed an increased GErate delta in infants fed a partially hydrolysed, starch-thickened formula supplemented with *L. reuteri* DSM 17938, thus suggesting that the beneficial effects of this strain can be preserved if added to infant formulas.

When compared to standard formulas, hydrolysed protein formulas have proved to accelerate feeding advancement, to reduce gastrointestinal transit time and to subsequently increase stool frequency [27,28], while their effect on gastric emptying still remains controversial [29,30] and possibly dependent on the extent of hydrolysis [31,32]. According to current literature, partially hydrolysed formulas (PHFs) may offer a useful alternative to intact protein formulas in the dietary management of common functional gastrointestinal symptoms in early infanthood [33,34]. Moreover, a thickened PHF has been shown to significantly reduce the number and volume of regurgitations in infants with FR when compared to a standard, thickened one [12]. The thickened PHF tested in the present study proved to be more effective than a non-thickened, standard formula in reducing the frequency of regurgitations and in improving GErate. However, larger targeted randomized trials are needed to better investigate the exact role of partial protein hydrolysis on regurgitations and gastric emptying in term infants with FR.

To the best of our knowledge, this is the first study aimed at evaluating the combined impact of three different anti-regurgitation dietary strategies, whose effectiveness on gastro-oesophageal reflux has been previously analysed separately; however, a number of limitations need to be acknowledged. First, the data on regurgitation frequency relied on the accuracy of reports from parents and caregivers. Moreover, although the test formula proved to be overall effective, the design of the present study did not allow us to assess the exact contribution of each dietary strategy in improving GErate and reducing the frequency of regurgitations. However, the present results support their synergic beneficial effect on FR. Furthermore, the positive effects observed in this study might be further enhanced by adding other probiotic strains [35], prebiotics or human milk oligosaccharides to the combination tested [36]. Finally, due to the four-week duration of the trial, no conclusions can be drawn on possible long-term effects of the test formula.

As suggested by a recent retrospective study [37], according to which the highest incidence of functional abdominal pain was seen in adolescents who had been affected by colic and regurgitation during the neonatal period, the importance of an appropriate anti-regurgitation treatment in early

infancy extends beyond this period. In this regard, further research on the long-term impact of infanthood functional gastrointestinal disorders, focusing not only on their pathogenesis and treatment but also on the development of effective preventive approaches, would be of interest.

5. Conclusions

The use of a starch-thickened, partially hydrolysed infant formula supplemented with the probiotic L. reuteri effectively decreases the daily frequency of regurgitation and significantly enhances gastric emptying in infants affected by FR. Targeted studies are needed to shed light on the exact mechanisms through which each component of this formula exerts its beneficial effects, and to evaluate long-term data on efficacy and safety.

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Author Contributions: Flavia Indrio: designed the study, analysed and interpreted the results, developed the first draft of the manuscript, and approved the final manuscript as submitted; Giuseppe Riezzo: interpreted the ultrasound results and approved the final manuscript as submitted; Silvia Martini and Luigi Corvaglia: coordinated and supervised data collection, and approved the final manuscript as submitted; Maria Ficarella Paola Giordano and Maria Paola Miolla: recruit the patient collect the data analysed data from parental diaries and approved the final manuscript as submitted; Ruggiero Francavilla: assisted in data interpretation, reviewed and revised the manuscript, and approved the final manuscript as submitted.

Conflicts of Interest: Infant formula for the study was supplied by the Nestlé company. The Nestlé Company provided no other funding for the study and had no role in the design of the study. FI serve as a speaker for NNI. The authors declare that they have received no individual funding from Nestlé company, including conference attendance in the past five years.

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Probiotic Supplementation in Preterm: Feeding Intolerance and Hospital Cost

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Abstract: We hypothesized that giving the probiotic strain *Lactobacillus reuteri* (*L. reuteri*) DSM 17938 to preterm, formula-fed infants would prevent an early traumatic intestinal inflammatory insult modulating intestinal cytokine profile and reducing the onset of feeding intolerance. Newborn were randomly allocated during the first 48 h of life to receive either daily probiotic (10^8 colony forming units (CFUs) of *L. reuteri* DSM 17938) or placebo for one month. All the newborns underwent to gastric ultrasound for the measurement of gastric emptying time. Fecal samples were collected for the evaluation of fecal cytokines. Clinical data on feeding intolerance and weight gain were collected. The costs of hospital stays were calculated. The results showed that the newborns receiving *L. reuteri* DSM 17938 had a significant decrease in the number of days needed to reach full enteral feeding ($p < 0.01$), days of hospital stay ($p < 0.01$), and days of antibiotic treatment ($p < 0.01$). Statistically significant differences were observed in pattern of fecal cytokine profiles. The anti-inflammatory cytokine interleukin (IL)-10, was increased in newborns receiving *L. reuteri* DSM 17938. Pro-inflammatory cytokines: IL-17, IL-8, and tumor necrosis factor (TNF)-alpha levels were increased in newborns given placebo. Differences in the gastric emptying and fasting antral area (FAA) were also observed. Our study demonstrates an effective role for *L. reuteri* DSM 17938 supplementation in preventing feeding intolerance and improving gut motor and immune function development in bottle-fed stable preterm newborns. Another benefit from the use of probiotics is the reducing cost for the Health Care service.

Keywords: preterm newborn; feeding intolerance; probiotic

1. Introduction

During the third trimester of pregnancy and in the first days after birth, important processes of intestinal maturation take place. Although anatomical differentiation of the human gut is usually achieved within 20 weeks of gestation, the functional maturation of the gastrointestinal tract occurs later and requires organized peristalsis and coordinated sucking and swallowing reflexes that are

not established until 29–30 weeks and 32–34 weeks of gestations, respectively [1]. Sensory-motor gastrointestinal functions are strictly related to the infant's immune system, which plays a crucial role in modulating appropriate and non-exaggerated responses to luminal antigens. This fundamental enteric function, known as "oral tolerance" is based on the interaction between the luminal content (microbiota, food antigens, and other molecules), the intestinal epithelium, and the tolerogenic dendritic cells (DCs) from mesenteric lymph nodes of the gut associated lymphoid tissue (GALT) [2], and is associated with specific cytokine patterns. It has been suggested that the early composition of the intestinal microbiota at birth can influence the correct ontogenesis of the gut barrier, and motor and immune function through a complex neuroendocrine cross-talk [3,4]. Consequences of prematurity like antibiotic usage, feeding type, and being located in neonatal intensive care unit (NICU) may cause an intestinal dysbiosis that affects the intestinal integrity and disrupts the delicate balance between intestinal microbiota and the immune system of premature infants. An aberrant microbial colonization pattern might contribute to the development of an early traumatic inflammatory insult on the gut-brain axis with short- and long-term consequences on gastrointestinal well-being [5].

Early colonization of the gastrointestinal tract with a probiotic may contribute to the neonatal tolerance, as breast-feeding does, resulting in correct gut ontogenesis [6–9]. Besides, it has been demonstrated that *L. reuteri* DSM 17938 significantly reduced intestinal mucosal levels of IL-8 and interferon (IFN)-γ when newborn rat pups were fed formula containing lipopolysaccharide (LPS) $\pm$ *L. reuteri*. *L. reuteri* DSM 17938 was able to significantly reduce the intestinal histological damage produced by LPS plus cow milk formula in the same animal model. Even cow milk formula feeding without LPS produced a mild gut inflammation, evidenced by elevated mucosal IFN-γ and IL-13 levels, and that process could be suppressed by the strain 17938 [10].

In this framework, we hypothesized that giving the probiotic strain *L. reuteri* DSM 17938 to preterm, formula-fed infants would prevent an early traumatic intestinal inflammatory insult modulating intestinal cytokine profile and would reduce the onset of feeding intolerance acting also on gastrointestinal motility.

2. Methods

A randomized, double-blinded, clinical, and placebo-controlled trial, was conducted in two Italian neonatal intensive care units (NICUs), Brindisi and Crotone, from January 2011 to November 2012. Preterm neonates (gestational age <37 weeks), within the first 48 h of life: adequate for gestational age weight, formula fed, Apgar score at 5 min >7, <24 h of age, hemodynamically stable, with absence of congenital malformations, inborn errors of metabolism, proven sepsis, or infections at birth were included. The ethical committees of each participating institution approved the study protocol. All preterm newborns were screened at birth and the parents of eligible newborns gave signed consent. Trial registered ClinicalTrials.gov (National Institutes of Health) with the number NCT00985816.

2.1. Randomization and Interventions

Preterm newborns were randomly assigned to receive a *L. reuteri* DSM 17938 supplementation or placebo by the use of a computer-generated randomization scheme. The study personnel, health care workers, and parents were masked to the study group allocation. The active study product consisted of freeze-dried *L. reuteri* DSM 17938 suspended in a mixture of pharmaceutical grade sunflower and medium-chain triglyceride oils supplied in a dark bottle fitted with a dropper cap and an individual number indicating the randomization sequence. Five drops of the formulation, delivering a dose of 1×10^8 colony-forming units (CFUs) of *L. reuteri* DSM 17938, were administered to infants in the probiotic group each day regardless of whether enteric feeds were started and until 30 days of life. The placebo consisted of an identical formulation of oils supplied in an identical bottle and was administered following the same protocol as that described for the probiotic group. There were no differences in smell or taste between the two formulations. Analysis of total Lactobacillus counts was performed in our laboratory on three randomly selected bottles from separate batches to ensure the

viability of the live bacteria. Each bottle of 10 mL, containing at least 45 dosages, was confirmed to conform with the stated content of *L. reuteri* of at least 4.5×10^9. The study products were stored refrigerated, which keeps the live content at a stable level.

L. reuteri DSM 17938 has the ability to colonize the entire human gastrointestinal tract with a blood safety profile similar to *L. reuteri* ATCC 55730 (*L. reuteri* DSM 17938 is daughter strain of *L. reuteri ATCC 55730*). Its colonization is only temporary, and genome annotation did not reveal any further gene or gene cluster known to be involved in virulence or antibiotic resistance [11].

Both the *L. reuteri* DSM 17938 and placebo were manufactured and donated by BioGaia AB (Stockholm, Sweden). The viability and purity of the packaging data was guaranteed by the certificate of analysis provided from the manufacturer for each of the batches. In order to minimize differences in feeding and nutrition practices among participating NICUs, the preterm newborns were all exclusively bottle-fed with the same preterm standard formula. Minimal Enteral Feeding was started on day 1 or 2 with small amounts of enteral feedings of formula at intakes of 10–20 mL/kg/day. The limit of tolerance for increasing formula amount (10–20 mL/kg/day) was set at 50% of previous feeds on the assessment of pre-feed gastric residual volumes. Parenteral nutrition, when needed, was started on the second day of life following a standardized protocol.

2.2. Symptoms and Data Evaluation

Anthropometrical parameters, occurrence of adverse reactions, time to regain birth weight, time taken to reach full enteral feeding, duration of antibiotic treatment, days of hospital stay, and stool frequency were recorded. In order to perform quantification of fecal cytokines and fecal calprotectin, a stool sample was obtained at the end of supplementation (30 days of life). Fecal interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-17, TNFα, and fecal calprotectin were evaluated. Briefly, for measurement of fecal IL, TNF, and calprotectin, fecal samples were taken from the diaper with a sterile plastic spoon. Samples were stored in a sterile screw cap tube at 2–8 °C for a maximum of 7 days. Samples were extracted and diluted 1:50 with an incubation buffer. The homogenate was microcentrifuged for 5 min at 10,000× *g*, and the supernatant was stored at −20 °C until analysis. The concentrations of fecal cytokines were determined in each fecal sample photometrically with commercially available enzyme-linked immunosorbent assay (ELISA) kits (IL-1β, IL-6, IL-8, IL-10, IL-17, and TNFα (IDK® TNFα), ELISA (Immunodiagnostik, Bensheim, Germany), according to the instructions provided by the manufacturer. Calprotectin levels were measured by ELISA using the Calprotectin-MPR8/14 kit (Buhlmann, Basel, Switzerland). All of the concentrations quantified were adjusted to the wet weight of the individual fecal sample.

Scintigraphy is considered the "gold standard" among methods to evaluate gastric emptying in clinical practice [12]. Scintigraphy allows a complete and physiologic study of the motor function of the stomach. However, it is an expensive technique and specialized personnel from nuclear medicine are needed to perform a scintigraphic study. Moreover, scintigraphy induces significant radiation emission [13], and as result it has limited application in newborns and children, and does not allow repetitive measurements in a short period of time. In comparison, the study of gastric emptying time by ultrasound is a non-invasive method and can be used for research or practical purposes to evaluate gastric emptying in healthy adults, children, and newborns, and in those with gastrointestinal motility disorders. On considering these factors, an ultrasound gastric emptying examination was performed on day 30 after birth, according to a previously reported procedure [14,15]. Lastly, a cost-analysis assessment during NICU hospitalization was performed.

2.3. Outcome Measures

The primary outcome was the assessment of *L. reuteri* DSM 17938 supplementation on feeding tolerance through evaluation of cytokine fecal profile, clinical parameters, and ultrasound measurement during the first month of life. The secondary outcome was to evaluate the costs of supplementation, calculated based on duration of hospitalization.

2.4. Sample Size

To calculate the minimum sample size, we used previous published data [7] about the reduction of average time of gastric emptying; we hypothesized a reduction of 10% of the average time and we used an alpha of 0.05 and a power of 0.9. Using Stata Software, we estimated a required sample size of 20 for each group.

We repeated the sample size calculation using, as the main outcome, the reduction of the cytokine fecal profile and setting an alpha of 0.05 and a power of 0.9. In this case, the required sample was around 30. We hypothesized a drop-out rate of 20%; then, we planned to enroll 72 subjects.

2.5. Data Analysis

Clinical and functional gastrointestinal variables were considered prospectively. The mean and standard deviation of each quantitative parameter were calculated and means were compared by unpaired Student's *t*-test, after establishing the normal distribution of variables by Bartlett's test. Significance was set at $p < 0.05$. To evaluate the costs of the hospitalizations, we used the Diagnosis Related Group (DRG) rate as reported on the Official website of Italian Ministry of Health for DRG 388 (prematurity without major risks).

3. Results

A total of 72 preterm newborns were eligible. Twelve newborns were excluded from analysis because of parental refusal to participate (three neonates), withdrawal from the study (five neonates), or maternal desire to breastfeed (three neonates). The need for exclusive bottle-feeding should be considered the most important covert/clearly expressed cause of high drop-out of these infants. However, the response rate was 83.3% (60/72), higher than that expected (80%). A total of 60 preterm newborns were randomly assigned to *L. reuteri* DSM 17938 or to the placebo group. Clinical and demographic characteristics of the infants at birth are shown in Table 1. No significant differences were observed between the two groups at baseline. The newborns receiving *L. reuteri* DSM 17938 had a significant decrease in the number of days needed to reach full enteral feeding ($p < 0.01$), days of hospital stay ($p < 0.01$), and days of antibiotic treatment ($p < 0.01$) compared with those given placebo. Further, significant differences were found in time to regain birth weight, which was reduced in the *L. reuteri* DSM 17938 supplemented group ($p < 0.05$), and in body weight at the end of the study, which was lower in the placebo group ($p < 0.05$), Table 2. Multiple statistically significant differences were observed in the pattern of fecal cytokine profiles between the two groups. The anti-inflammatory cytokine IL-10, which is involved in immune tolerance, was increased in newborns receiving *L. reuteri* DSM 17938. Pro-inflammatory cytokines: IL-17, IL-8, and TNFα levels were increased in newborns given placebo. The newborns receiving *L. reuteri* DSM 17938 had a significant decrease in calprotectin level compared with the placebo group. The IL-1β was increased in the group treated with *L. reuteri* DSM 17938. IL-6 was increased in newborns receiving *L. reuteri* DSM 17938, but the difference was not statistically significant (Table 3). Regarding gastric emptying, significant differences in the half-emptying (T1/2) time and fasting antral area (FAA) were reported, confirming the improved motility pattern in the probiotic administration group (Table 4). There were no adverse events related to the trial in the *L. reuteri* DSM 17938 supplemented babies.

Table 1. Clinical and demographic data at baseline.

Total *n* = 60	***L. reuteri* DSM 17938 (*n* = 30)**	**Placebo (*n* = 30)**	***p***
Gestational age	30.2 ± 1.2	30.1 ± 1.2	n.s.
Gender (M/F)	15/15	16/14	n.s.
Delivery (VD/CD)	4/26	5/25	n.s.
Birth weight (g)	1471.5 ± 455.1	1406.6 ± 536.4	n.s.

Gender: M/F = Male/Female; Delivery: vaginal delivery (VD)/cesarean delivery (CD). Non significant (n.s.)

Table 2. Clinical results at the end of the study.

Clinical Parameter	*L. reuteri* DSM 17938	Placebo	*p*
Time taken to reach full enteral feeding (day)	4.2 ± 1.1	7.5 ± 3.2	<0.01
Day of hospitalization (day)	13.4 ± 2.2	22.4 ± 3.2	<0.01
Duration of antibiotic treatment (day)	4.2 ± 4.3	12.5 ± 7.2	<0.01
Time to regain birth weight (day)	6.4 ± 1.6	7.3 ± 1.3	<0.05
Weight at the end of the study (g)	1955.3 ± 653.4	1737.6 ± 512	<0.05
Stool frequency (*n*/day on the last week)	2.5 ± 0.7	2.8 ± 0.9	<0.05

Table 3. Fecal cytokines.

Group	IL-1β pg/mL	IL-8 pg/mL	IL-10 pg/mL	IL-17 pg/mL	Calprotectin µg/g	TNFα pg/mL	IL-6 pg/mL
LR	57.4 ± 73.3	56.7 ± 72.4	6.3 ± 3.2	6.5 ± 1.9	246.6 ± 78.4	8.0 ± 3.1	3.2 ± 2.8
Placebo	17.1 ± 16.7	197.3 ± 222.1	4.2 ± 1.7	8.8 ± 3.5	323.9 ± 111.7	12.7 ± 7.7	2.9 ± 1.7
p	0.04	0.04	0.02	0.02	0.01	0.01	n.s.

LR = *L. reuteri* DSM 17938; IL = interleukin; TNF = tumor necrosis factor.

Table 4. Gastric emptying parameters at the end of the study.

Parameter	*L. reuteri* DSM 17938	Placebo	*p*
T1/2 (Half-emptying time) (min)	73.8 ± 7.5	80.4. ± 6.1	0.0004
Fasting antral area (cm^2)	0.6 ± 0.2	0.8 ± 0.3	0.009

The cost of treatment saved by the reduction of hospitalization by giving *L. reuteri* DSM 17938 amounted to 2043 Euros per infant.

4. Discussion

L. reuteri DSM 17938 supplementation in preterm newborns improves intestinal motility and changes the cytokine profile in stools. Our study underlines the potential beneficial effects of *L. reuteri* DSM 17938 supplementation on clinical and functional variables related to maturation of gastrointestinal function. In particular, it shows that oral supplementation with *L. reuteri* DSM 17938 improves feeding tolerance in preterm newborns with clinical effects on growth, hospitalization, and antibiotic treatment.

Primary colonization of the gut can be considered an important step in the development of intestinal functions and the transferal of the microbiota at birth from maternal vaginal and intestinal flora to the newborn gut is fundamental [16]. The continuous and complex cross-talk between the gut and its microbial content is a normal part of development and plays a crucial role in the ability to distinguish harmless bacterial and food antigens from potentially dangerous antigens. This function requires a sophisticated system that is responsive to a wide variety of microbial and food antigens that transit or populate the gut [17].

An imbalance of normal intestinal microbiota, or the host response to such an imbalance are thought to be involved in the pathogenesis of several intestinal diseases [18]. The beneficial effect of probiotic supplementation on feeding intolerance and immunomodulation has been reported in several studies [4]. A recent study by Rojas [19] reported feeding intolerance episodes and duration of hospitalization significantly lower in preterm infants <1500 g exposed to *L. reuteri* DSM 17938 in comparison to placebo exposed infants. Different from the study by Rojas, our newborns supplemented with probiotic reached the full enteral feeding in a shorter time.

The specific mechanism of probiotic supplementation on gastrointestinal function is not yet clear. Functional components of the human gastrointestinal tract do not evolve simultaneously and it has been shown that a reduced or an abnormal microbial colonization during the first months of life would

provoke a slower postnatal maturation of epithelial cell barrier functions, of neuronal route, and of the immunomodulation system of the GALT [20]. This aberrant development of gut functions could finally lead to mucosal inflammation and play a pivotal role in the development of feeding intolerance [21] or other diseases later in life. In an animal model, *L. reuteri* DSM 17938 significantly reduced intestinal mucosal IL and interferon levels, and such reduction corresponded to a reduction in the low grade histological damage induced by cow milk formula feeding [10].

In agreement with these data, in our study, early probiotic supplementation induced a decrease in fecal pro-inflammatory cytokines, IL-17, IL-8, and TNFα, and an increase in the fecal anti-inflammatory cytokine IL-10. Our data suggest a potential anti-inflammatory effect of probiotic with a shift in the tolerogenic mechanism on naïve CD4+ T cells that suppress the expression of T effector cells (Th1 and Th2) and stimulate the expansion of regulatory T cells (Tregs). Furthermore, significant changes in IL-1β and IL-6 levels were found in preterms given probiotic compared to placebo, and previous studies in animals have shown that these two cytokines can have excitatory and neuromodulatory roles in the myenteric plexus, stimulating gastrointestinal motility [22,23]. Also, fecal calprotectin, a well-known marker of gut inflammation, was reduced in the infants supplemented with *L. reuteri* DSM 17938. The modulation of fecal calprotectin by this specific probiotic strain has been reported in the literature [24,25].

Another important aspect is the growing evidence suggesting that the intestinal microbiota may be emitting and receiving a multiplicity of signals to and from the brain, thus playing a critical role in the modulation of the gut-brain axis [26,27]. An immature ontogenesis of this bidirectional interrelationship between the enteric microbiota and the nervous system could affect the pathophysiology of feeding intolerance [28]. In this context, the strength of contemporary action on the motility and immunity of the intestine could result in a better functionality of the whole intestinal function. Our study demonstrates that gastric motility was improved in preterm infants given *L. reuteri* DSM 17398, as shown by the significantly increased gastric emptying time, and the significantly reduced FAA. The clinical counterpart of such improved gastric activity is the decreased gastric residual in preterm infants given *L. reuteri* DSM 17938 and the consequent earlier achievement of full enteral feeding and the faster regain to birth weight compared to the placebo group. This bacterial strain has already been used in a pediatric population [29], and a recent paper [30] showed that LR DSM 17938 increased both colonic migrating motor complex frequency and velocity in an animal model. The authors, based upon the effects of LR DSM 17938 on the adult mouse colon, speculated that this approach may help to screen and identify the therapeutic effect of LR DSM 17938 on constipation and, generally, to correlate the given effect of the probiotic on the enteric nervous system with the action on GI motility.

5. Conclusions

Our study demonstrates an effective role for *L. reuteri* DSM 17938 supplementation in preventing feeding intolerance and improving gut motor and immune function development in bottle-fed stable preterm newborns. The physiological mechanisms underlying these effects may involve changes in cytokine inflammatory patterns. Finally, in light of our cost-analysis assessment, another benefit from the use of this probiotic is in the form of reductions in the costs for the Health Care service.

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Author Contributions: Flavia Indrio: conceptualized and designed the study, interpreted the results, drafted, reviewed and revised the manuscript, and approved the final manuscript; Giuseppe Riezzo: calculated and interpreted all the gastric emptying parameters, reviewed and revised the manuscript, and approved the final manuscript; Silvio Tafuri: conducted and interpreted the statistical analyses, drafted the initial manuscript, reviewed and revised the manuscript, and approved the final manuscript; Barbara Carlucci, Maria Ficarella,

and Massimo Bisceglia: participated in the clinical study, recruited the newborns, reviewed and approved the final manuscript; Lorenzo Polimeno: coordinated and supervised the laboratory analyses, interpreted the results, reviewed and revised the manuscript, and approved the final manuscript; Ruggiero Francavilla: supervised the clinical study, reviewed and approved the final manuscript.

Conflicts of Interest: BioGaia AB, Sweden supported this study by kindly providing active study product and placebo. BioGaia AB had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript. Flavia Indrio (FI) and Ruggiero Francavilla (RF) serve as speakers for Biogaia. The other authors disclose no conflicts of interest.

Abbreviations

DCs	Dendritic cells
GALT	Gut associated lymphoid tissue
NICU	Neonatal intensive care unit
CFU	Colony forming unit
IL	Interleukin

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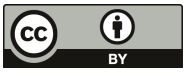

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Article

Modulation of Gut Microbiota of Overweight Mice by Agavins and Their Association with Body Weight Loss

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Abstract: Agavins consumption has led to accelerated body weight loss in mice. We investigated the changes on cecal microbiota and short-chain fatty acids (SCFA) associated with body weight loss in overweight mice. Firstly, mice were fed with standard (ST5) or high-fat (HF5) diet for five weeks. Secondly, overweight mice were shifted to standard diet alone (HF-ST10) or supplemented with agavins (HF-ST + A10) or oligofructose (HF-ST + O10), for five more weeks. Cecal contents were collected before and after supplementation to determine microbiota and SCFA concentrations. At the end of first phase, HF5 mice showed a significant increase of body weight, which was associated with reduction of cecal microbiota diversity (PD whole tree; non-parametric *t* test, $p < 0.05$), increased Firmicutes/Bacteroidetes ratio and reduced SCFA concentrations (*t* test, $p < 0.05$). After diet shifting, HF-ST10 normalized its microbiota, increased its diversity, and SCFA levels, whereas agavins (HF-ST + A10) or oligofructose (HF-ST + O10) led to partial microbiota restoration, with normalization of the Firmicutes/Bacteroides ratio, as well as higher SCFA levels ($p < 0.1$). Moreover, agavins noticeably enriched *Klebsiella* and *Citrobacter* (LDA > 3.0); this enrichment has not been reported previously under a prebiotic treatment. In conclusion, agavins or oligofructose modulated cecal microbiota composition, reduced the extent of diversity, and increased SCFA. Furthermore, identification of bacteria enriched by agavins opens opportunities to explore new probiotics.

Keywords: agavins; prebiotics; microbiota; overweight; body weight loss; short chain fatty acids

1. Introduction

Agavins are branched neo-fructans found in *Agave* plants, which contain a mixture of β(2-1) and β(2-6) linkages [1,2]. The degree of polymerization (DP) and the chemical structure of agavins become more complex as the plant ages. Plants from two to four years old have a high content of agavins with low DP and simpler chemical structures, while plants from five to seven years old contain a large proportion of high-DP agavins and highly-complex chemical structures [3].

Agavins act as prebiotics inducing benefits to host health by providing specific changes in the composition and/or activity of the gut microbiota [4]. Due to their structural complexity, endogenous gastrointestinal enzymes cannot degrade agavins during their passage through the stomach and the small intestine; so they reach both the cecum and colon, where they are fermented by saccharolytic microbiota present in these sites, producing short chain fatty acids (SCFA), mostly acetate, propionate, and butyrate. SCFA are very important because they reduce body weight gain, through G-protein-coupled receptors (GPRs), influencing the secretion of hormones involved in

appetite control [5–7]. In addition, SCFA increment through agavins fermentation in both cecum and gut induces a pH drop; which might change the intestinal microbiota structure [8,9].

On the other hand, earlier investigations showed that mice fed with standard or high-fat diets with agavins of low DP led to body weight loss [10–12]. However, the microbial mechanisms remain unclear [12,13]. New molecular techniques that enable analysis of non-cultivable bacteria are starting to be applied in studies investigating the impact of prebiotics on the cecal microbiota. For example, investigations examined the effects of oligofructose (linear fructans) on cecal microbiota using the 16S rRNA gene sequencing technique, showed that the intake of oligofructose in mice not only stimulated the growth of *bifidobacteria* and *lactobacilli*, but also increased other bacteria such as *Streptococcus*, *Clostridium*, *Enterococcus*, *Olsenella*, *Akkermansia*, and *Allobaculum* [14,15]. The abundance of specific taxa, such as *Bifidobacterium* spp. and *Akkermansia muciniphila* has been negatively associated with inflammation in adipose tissue, circulating glucose, leptin, triglycerides, and insulin [16], whereas the enrichment of *Allobaculum* has been associated with body weight loss in obese mice [17]. On the other hand, Firmicutes and Bacteroidetes are usually the most abundant members of the cecal microbiota; however, the ratio of these bacterial groups can change over time or by different factors, such as age, environment, or diet, and especially those with a high fat content [18–20].

In the present work we used agavins from four-year-old *Agave tequilana* plants containing a high proportion of short DP fructans, and studied the response on the microbiota of mice, continuing our previous study on prebiotic supplementation in overweight mice [21]. Here we present changes of cecal microbiota after a diet shift and agavins supplementation, and the possibility of their association with body weight loss in overweight mice. Our hypothesis was that agavins supplementation might improve the host health, through the enrichment of probiotic bacteria, in relation to the diet shift alone.

To our knowledge, this is the first report on the global effects of a diet shift and agavins supplementation on the cecal microbiota composition through a 16S rRNA analysis in overweight mice. Finally, agavins (branched fructans) effects were compared to oligofructose (linear fructans), which was used as a positive control to evaluate the cecal microbiota changes.

2. Materials and Methods

2.1. Animals and Diets

Forty-two male C57BL/6 mice (12 weeks old at the beginning of the experiment were obtained from the Universidad Autonoma Metropolitana, Mexico City, Mexico) and housed in a temperature and humidity controlled room with a 12 h light-dark cycles. Mice were maintained in individual cages since water intake containing the fructans was measured every day. The animals were subject to a two-phase trial, the first to gain weight and the second to lose weight (Supplementary Figure S1). In the first phase mice were fed with standard (n = 12; 5053 Lab Diet, St. Louis, MO, USA) or high-fat diets (n = 30; 58Y1 Test Diet, St. Louis, MO, USA) for five weeks. The standard diet (5053 Lab Diet) contained 62.4% calories from carbohydrates (28.6% starch, 3.24% sucrose, 1.34% lactose, 0.24% fructose, and 0.19% glucose), 24.5% from proteins, and 13.1% from fat. The high-fat diet (58Y1 Test Diet) had 20.3% calories from carbohydrates (16.15% maltodextrin, 8.85% sucrose, and 6.46% powdered cellulose), 18.1% from proteins, and 61.6% from fat (31.7% lard and 3.2% soybean oil). In the second phase, healthy control mice were kept with the standard diet (ST-ST10; n = 8), and the overweight mice were shifted to the standard diet alone (HF-ST10; n = 8) or supplemented with agavins (HF-ST + A10; n = 8) or oligofructose (HF-ST + O10; n = 8) for five more weeks. Food and water were provided ad libitum throughout the experiment.

Mice experiments were conducted according to the Mexican Norm NOM-062-ZOO-1999 and approved by the Institutional Care and Use of Laboratory Animals Committee from Cinvestav-Mexico (CICUAL; protocol number 0091-14).

2.2. Agavins and Oligofructose Fructans

Agavins from four-year-old *Agave tequilana* Weber blue variety plants were extracted and purified in our laboratory and presented an average DP of 8 [21]. Oligofructose was bought from Megafarma® (Mexico City, Mexico) and possess an average DP of 5. Agavins and oligofructose were added in the water at a concentration of 0.38 g/mouse/day [15,22].

2.3. gDNA Extraction

Cecal contents were collected before and after the fructans supplementation (at five and 10 weeks, respectively). At the end of first and second experimental phase, mice were anaesthetized with a 60 mg/kg intraperitoneal dose of sodium pentobarbital and the gastrointestinal tract was exposed for cecum removal. Cecal content was snap frozen in liquid nitrogen and stored at −70 °C until their use. Genomic DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep (Irvine, CA, USA), following the manufacturer's instructions. The concentration and purity of DNA were evaluated using a Nanodrop spectrophotometer. Extracted DNA was stored at −20 °C until its use.

2.4. PCR Amplification of the V4 Region of the Bacterial 16S rRNA Gene

To assess microbial composition, the V4 region of the bacterial 16S *rRNA* gene was amplified with barcoded fusion primers (F515/R806) [23]. PCR reactions were carried out in triplicate, 25 μL reactions with 5 μM forward and reverse primers, 2 μL template DNA, and 1X of HotMasterMix (5 PRIME, Gaithersburg, MD, USA). Thermal cycling of PCR reactions consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, and extension at 72 °C for 90 s, with a final extension of 10 min at 72 °C.

2.5. Amplicon Quantitation, Pooling, and Sequencing

DNA concentration for each amplicon was measured using the Quant-iT PicoGreen dsDNA reagent and kit (Thermo Scientific, Waltham, MA, USA). Assays were carried out using 2 μL of cleaned PCR product in a total reaction volume of 200 μL in black, 96-well microtiter plates. Fluorescence was measured on a BioTek Synergy HT plate reader using the 480/520-nm excitation/emission filter pair. Following quantitation, cleaned amplicons were combined in equimolar ratios into a single tube. The final concentration of the pooled DNA was determined using the Qubit high-sensitivity dsDNA assay (Invitrogen, Carlsbad, CA, USA). Sequencing was carried out on the Illumina MiSeq platform at New York University.

2.6. Sequence Analysis

Sequences were processed and analyzed in the QIIME software package (Quantitative Insights Into Microbial Ecology, v1.8.0, La Jolla, CA, USA) following the pipeline described by Caporaso et al. [24]. Sequences were removed from the analysis if they were <200 or >350 nt in length, had a mean quality score < 20, contained ambiguous characters, contained an uncorrectable barcode, or did not contain the primer sequence. Remaining sequences were assigned to samples by examining the 12-nt barcode. Similar sequences were clustered into operational taxonomic units (OTUs) using the open reference method. Taxonomic assignments for each OTU were made using the Greengenes database (May 2013) with a minimum identity of 97%. Finally, an OTU table was used to generate relative abundance plots and to calculate alpha and beta diversity (alpha diversity refers to the diversity within each sample, and beta diversity refers to patterns of similarities and differences among samples). All communities were rarefied up to 6525 reads per sample to calculate the bacterial diversity.

The raw sequences supporting the results of this article are available in the NCBI Sequence Read Archive repository under accession no. SRX1532779.

2.7. LEfSe Analysis

Linear discriminant analysis effect size (LEfSe) was used to detect significant changes in relative abundance of microbial taxa between overweight mice fed with the standard diet and fructans supplements. Briefly, LEfSe is an algorithm for applying 16S *rRNA* gene datasets to detect bacterial organisms that are differentially abundant between two or more microbial environments [25]. LEfSe first identifies features that are significantly different among biological classes using the non-parametric factorial Kruskal-Wallis ran-sum test, and then LEfSe utilizes linear discriminant analysis (LDA) to estimate the effect of each differentially-abundant feature.

2.8. SCFA and pH Determinations

A weight of 0.05 g of homogenized cecal content was placed in a conic tube. The pH was measured directly in the cecal sample through insertion of a microelectrode (PHR-146, Lazar Research Laboratories Inc., Los Angeles, CA, USA) in the tube. The pH value was read when stability was achieved; after each reading, the microelectrode was removed and rinsed with distilled water. SCFA analysis was carried out in the same sample using gas chromatography and flame ionization detection (GC-FID) [26]. Briefly, 0.3 mL of Milli-Q water was added to the tube with cecal content. The solution was acidified with 0.05 mL of H_2SO_4 and SCFA were extracted by shaking with 0.6 mL of diethylether and subsequent centrifugation at 10,000× *g* for 30 s. One microliter of the ether phase was injected directly onto a Nukol™ capillary column (30 m × 0.32 mm; Supelco, Bellefonte, PA, USA) using an injector temperature of 180 °C and nitrogen as the carrier gas. The column temperature was initially 80 °C, then increased to 120 °C at 15 °C/min and kept at this temperature for 10 min, following an increment to 200 °C at 10 °C/min and remaining at this temperature for 10 min. The detector temperature was 230 °C. The identification and quantification of the SCFA were carried out using the retention times and calibration curves for each acid, respectively.

2.9. Statistical Analysis

Results are presented as mean ± SEM. Differences between ST5 and HF5 groups were assessed by Student's *t* test. Differences between the diets were determined using a one-way ANOVA followed by Bonferroni's multiple comparison tests. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

3. Results

To assess the impact of agavins on cecal microbiota of overweight mice, we sequenced V4 amplicons of 16S rRNA genes. After trimming, assembly, and quality filtering, we obtained a total of 635,054 sequence reads from 42 samples using a MiSeq sequencing platform. The average sequence read was 15,041 ± 1561 per sample (Supplementary Table S1).

The results of present work showed that the mouse cecal microbiota was greatly dominated by three phyla (Firmicutes, Bacteroidetes, and Proteobacteria) with six other minor phyla (Tenericutes, Actinobacteria, Cyanobacteria, Defferribacteres, Verrucomicrobia, and TM7; Supplementary Figure S2).

3.1. High-Fat Diet Induced Overweight and Altered Microbial Diversity and Composition

At the end of the first phase trial, after a high-fat diet consumption for five weeks, HF5 mice showed a significant increase in body gain weight (reaching overweight levels [27]) in relation to the ST5 group (7.26 ± 0.54 g vs. 2.22 ± 0.23 g, respectively; *t* test, $p < 0.001$; Figure 1A); which was associated with a substantial loss of bacterial alpha diversity in cecum of HF5 mice, compared to the standard diet control group ST5 (438 ± 61 vs. 774 ± 75, the number of observed species, respectively; non-parametric *t* test, $p < 0.05$; Figure 1B). In addition, a clear separation of bacterial structures between mice fed with the high-fat or standard diet was observed (weighted UniFrac distances; PERMANOVA, $p < 0.05$; Figure 1C).

On the other hand, HF5 mice were characterized by the increased relative abundance in approximately 41% of Proteobacteria (Helicobacteraceae and Desulfovibrionaceae families, including the *Bilophila* genus) and decreased in about of 17% the Firmicutes (Ruminococcaceae, Lactobacillaceae, Erysipelotrichaceae, Lachnospiraceae, and Dehalobacteriaceae families, including the genera *Lactobacillus*, *Coprobacillus*, *Allobaculum*, *Roseburia*, and *Dehalobacterium*) and approximately 25% the Bacteroidetes (S24_7 and Prevotellaceae families including *Prevotella* genus) (LDA > 3.0; Figure 1D and Supplementary Figure S3), with an increase of the Firmicutes/Bacteroidetes ratio (1.78 vs. 1.17 for HF5 and ST5, respectively; *t* test, $p < 0.05$; Figure 1E).

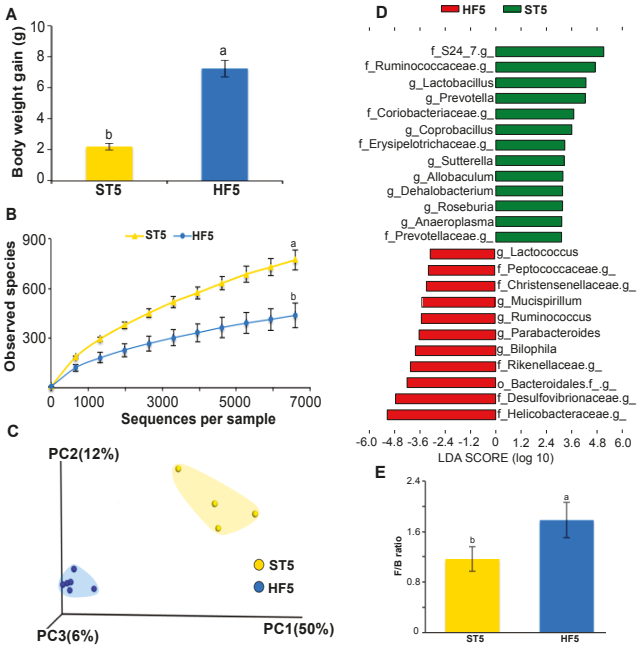

Figure 1. High-fat diet consumption for five weeks induced overweight and modified the cecal microbiota composition of mice. Body weight gain (**A**); bacterial alpha diversity in cecum according to diet (**B**); principal coordinate analysis (PCoA) plot of cecal communities (**C**); linear discriminant analysis showing the differentially-overrepresented genera between mice fed with standard and high-fat diets (**D**); and the effect of the diet on the Firmicutes/Bacteroidetes ratio (**E**). Treatments with different superscript letters indicate significant differences (*t* test, $p < 0.05$).

High-fat diet consumption not only induced alterations in the body weight and composition of the cecal microbiota, but also changed the biochemical environment and microbiota activity in the cecum of HF5 mice which showed a significant reduction of SCFA levels and an increment of pH in the cecal content in relation to the standard diet (ST5) group (*t* test, $p < 0.05$; Table 1).

Table 1. The effect of high-fat diet intake for five weeks on short-chain fatty acid concentration and pH in the cecal content of mice.

Group	Acetic Acid *	Propionic Acid *	Butyric Acid *	pH
ST5	15.13 ± 0.92^{a}	3.85 ± 0.20^{a}	4.54 ± 0.33^{a}	7.60 ± 0.12^{b}
HF5	5.43 ± 0.65^{b}	1.96 ± 0.11^{b}	1.72 ± 0.09^{b}	8.18 ± 0.06^{a}

ST5: mice fed with a standard diet; HF5: mice fed with a high-fat diet. Data are shown as mean ± SEM. Means with different letters (a,b) indicate significant differences (*t* test, $p < 0.05$). * µmoles/g of wet weight.

3.2. Diet Shift Induced Body Weight Loss and Restored Altered Microbial Diversity in Overweight Mice

At the end of the second phase, after overweight mice were switched for five weeks to the standard diet, HF-ST10 mice exhibited a body weight loss (Figure 2A). In addition, the return to standard diet in all cases—with or without prebiotic supplementation—decreased the Firmicutes/Bacteroidetes ratio from 1.78, observed in HF5, to 1.64, 1.52, and 0.75 for HF-ST10, HF-ST10 + A, and HF-ST + O, respectively (Bonferroni's test, $p < 0.05$; Figure 2B), as well as the relative abundance of Proteobacteria in approximately 34% (Helicobacteraceae and Desulfovibrionaceae families; Figure 2C).

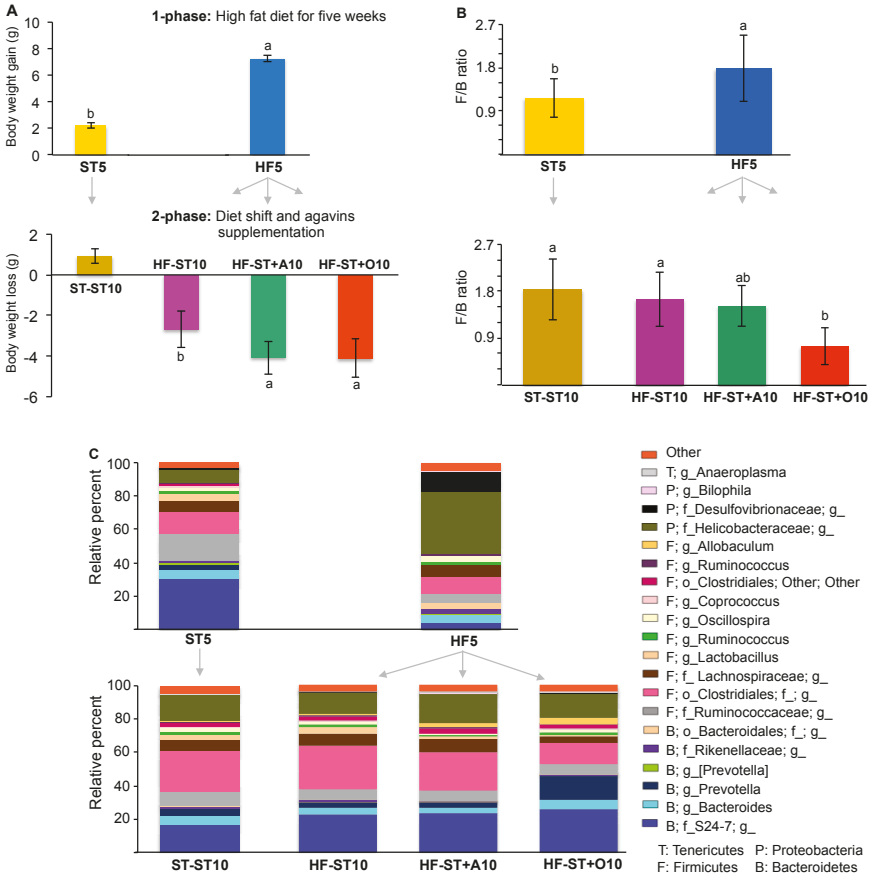

Figure 2. The effect of the diet shift and prebiotic supplementation on body weight loss and cecal microbiota composition in overweight mice. Body weight loss (**A**); Firmicutes/Bacteroidetes (F/B) ratio after the switch to a standard diet alone, or supplemented with agavins or oligofructose (**B**); differences in relative abundance of bacterial taxa in cecum according to diet group (**C**). Each taxon representing >1% of the average relative abundance in study groups is indicated by a different color.

HF-ST10 mice were characterized by a complete restoration of bacterial alpha diversity in relation to the HF5 group (771 ± 115 vs. 438 ± 61, the number of observed species, respectively; non-parametric *t* test, $p > 0.05$; Figure 3A), as well as the bacterial community structures in cecum, compared to the standard diet (ST-ST10) group (weighted UniFrac distances; PERMANOVA, $p > 0.05$; Figure 3B).

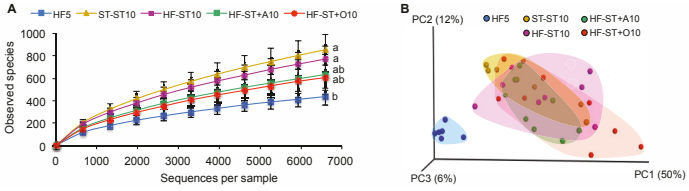

Figure 3. The effect of the diet shift and prebiotic supplementation on the cecal microbiota composition of overweight mice. Bacterial alpha diversity in cecum according to diet group (**A**); treatments with different superscript letters indicate significant differences (Bonferroni's test, $p < 0.05$). Principal coordinate analysis (PCoA) plot of cecal communities (**B**). Weighted UniFrac distances were used to evaluate beta diversity.

Furthermore, the HF-ST10 group showed a drastic change on the cecal microbiota composition in relation to the HF5 group, increasing the abundance of the genera *Lactobacillus*, *Prevotella*, *Allobaculum*, *Anaeroplasma*, *Blautia*, *Coprobacillus*, *Dehalobacterium*, *Candidatus Arthromitus*, *Sutterella*, and *Adlercreutzia* and decreased *Lactococcus*, *Acinetobacter*, *Anaerotruncus*, *AF12*, *Mucispirillum*, *Parabacteroides*, and *Bilophila* (LDA > 3.0; Figures 2C and 4A).

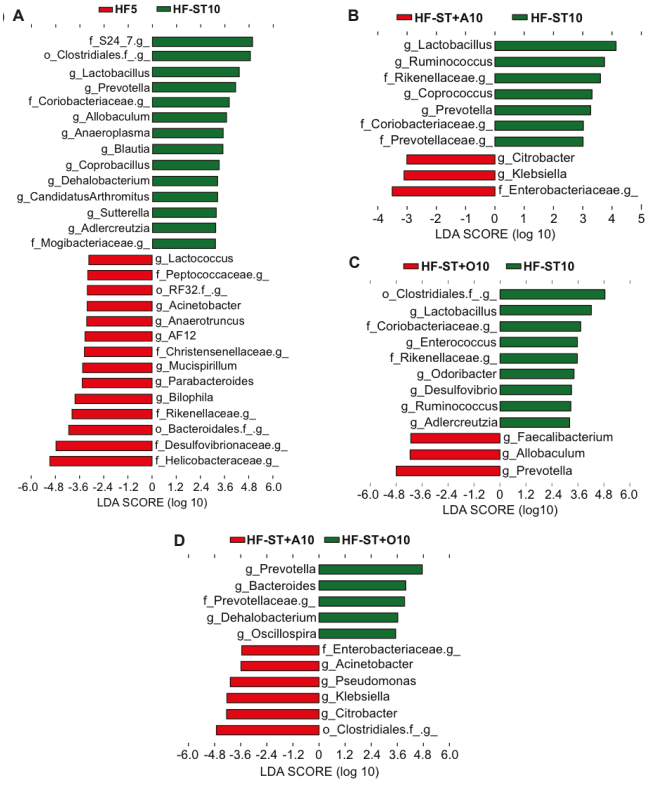

Figure 4. Linear discriminant analysis showing the differentially-overrepresented genera between overweight mice fed with: a high-fat diet and the diet shift (**A**); the diet shift and agavins supplement (**B**); the diet shift and oligofructose supplement (**C**); and agavins and oligofructose supplements (**D**). LDA effect size (3.0-fold) was used to determine the significant biomarkers.

Moreover, the diet shift led HF-ST10 mice to recover the biochemical environment and microbiota activity in the cecum, displaying similar SCFA concentration and pH values in relation to the standard diet (ST-ST10) group (*t* test, $p > 0.05$; Table 2).

Table 2. The effect of the diet shift and agavins supplementation on short-chain fatty acid concentration and pH in the cecal content of overweight mice.

Group	Acetic Acid *	Propionic Acid *	Butyric Acid *	pH
ST-ST10	20.73 ± 2.07^{c}	$6.07 \pm 0.60^{a,b}$	$6.53 \pm 0.60^{a,b}$	7.65 ± 0.07^{a}
HF-ST10	26.68 ± 0.69^{b}	6.48 ± 0.26^{b}	6.16 ± 0.39^{b}	7.26 ± 0.08^{a}
HF-ST + A10	34.27 ± 1.77^{a}	7.16 ± 0.44^{a}	7.51 ± 0.46^{a}	6.92 ± 0.03^{b}
HF-ST + O10	34.89 ± 1.85^{a}	7.73 ± 0.29^{a}	6.95 ± 0.40^{a}	6.79 ± 0.06^{a}

ST-ST10: healthy mice fed with the standard diet for ten weeks. HF-ST10: overweight mice switched for five weeks to the standard diet alone; or supplemented with agavins (HF-ST + A10) or oligofructose (HF-ST + O10). Data are shown as mean ± SEM. Means with different letters (a,b,c) indicate significant differences (Bonferroni's test, $p < 0.1$). * µmoles/g of wet weight.

3.3. Effects of Prebiotic Supplementation on Cecal Microbiota in Overweight Mice

In contrast, to standard diet alone, overweight mice that were shifted to the standard diet and received any prebiotic treatments (agavins or oligofructose) exhibited an accelerated body weight loss (Figure 2A), as well as a partial restoration of the cecal diversity in relation to the HF5 group (635 ± 177 and 607 ± 117, the number of observed species for HF-ST + A and HF-ST + O, respectively, vs. 438 ± 75, the number of observed species; non-parametric *t* test, $p < 0.05$; Figure 3A). Alpha bacterial diversity was not significantly different between mice fed agavins (branched fructans) or oligofructose (linear fructans). Interestingly, bacterial community structures from the agavins and oligofructose supplementation were not different to those from the unsupplemented standard diet (non-parametric *t* test, $p < 0.001$; Figure 3B and Supplementary Figure S4A). Noticeably, the HF-ST + O10 group showed the highest dispersion along the PC1 axis in relation to HF-ST + A10 and ST-ST10 groups (non-parametric *t* test, $p < 0.01$; Figure 3B and Supplementary Figure S4B). In addition, the cecal microbiota of the agavins-supplemented diet group (HF-ST + A10) was more similar to the standard diet groups (ST-ST10 or HF-ST10) compared to the oligofructose (HF-ST + O10) (weighted UniFrac distance; *t* test, $p < 0.05$; Supplementary Figure S4C,D).

On the other hand, the supplementation of agavins (HF-ST + A10) or oligofructose (HF-ST + O10) was associated with different communities: in relation to HF-ST10 group, agavins increased two genera (*Citrobacter* and *Klebsiella*) and decreased four genera (*Lactobacillus*, *Ruminococcus*, *Prevotella*, and *Coprococcus*), while oligofructose increased three genera (*Prevotella*, *Faecalibacterium* and *Allobaculum*) and decreased six genera (*Lactobacillus*, *Enterococcus*, *Odoribacter*, *Adlercreutzia*, *Desulfovibrio*, and *Ruminococcus*). In relation to oligofructose, agavins increased *Citrobacter*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*, and decreased *Prevotella*, *Bacteroides*, *Dehalobacterium*, and *Oscillospira* (LDA > 3.0; Figures 2C and 4B–D).

In the same way as the diet shift alone, prebiotic (agavins or oligofructose) supplementation modified not only the cecal microbiota composition, but also the microbiota activity. However, supplementation with agavins (HF-ST + A10) or oligofructose (HF-ST + O10) significantly increased the concentration of acetic, propionic, and butyric acids with a noticeable reduction of pH in the cecal content in relation to non-supplemented controls (ST-ST10 and HF-ST10; Bonferroni's test, $p < 0.1$; Table 2).

4. Discussion

We previously reported that agavins supplementation to a standard diet reverted the metabolic syndrome (including body weight loss) induced by high-fat diet consumption [21]. However, we do not know that the changes originated with agavins consumption on the gut microbiota, which could be

associated with this effect. Therefore, the present study describes the changes in the cecal microbiota, SCFA production, and pH values associated with body weight loss in overweight mice.

High-fat diet consumption for five weeks significantly increased the body weight gain of mice, and also led to a substantial decrease of bacterial diversity in the cecal microbiota (Figures 2A and 3A); which is consistent with the effects of fat in reducing diversity, as previously reported [28–30].

Firmicutes and Bacteroidetes are the most abundant members of the cecal microbiota, however, the ratio of these bacterial groups can change over time or by different factors, such environment and diet (especially those with a high fat content) [18–20]. An increase of the ratio of Firmicutes/Bacteroidetes was seen in overweight mice, which has been associated with obesity [18,20].

We found that a high-fat diet enriched *Bilophila*, a genus that include some opportunistic pathogens (for example *B. wadsworthia* [31]). Microbial changes under a high fat diet reduced cecal SCFA concentrations and increased pH, which is consistent with altered microbial metabolic activity, as previously reported [32].

Supplementation with agavins or oligofructose showed an accelerated body weight loss with partially restored the cecal microbiota diversity (Figures 2A and 3A), as well as an increase in the SCFA concentrations and acidic pH (Table 2). This might be mediated by selected supplement addition for specific bacterial taxa that tolerate a more acidic pH [33], since the direct effect of probiotic supplements on the microbiota have not been demonstrated [9,14,34]. Moreover, acetic acid suppresses appetite [35] and propionate and butyrate acids modulate hormones, such as GLP-1 and PYY, involved in satiety [5–7], and this mechanism might also contribute to body weight loss.

Weight loss was associated with a decrease in the Firmicutes/Bacteroidetes ratio, as in previous reports [18,36], and the effect is due, in part, to a reduction of caloric intake in fructans-supplemented mice [21]. Taxa associated with greater body weight loss included *Klebsiella* and *Citrobacter* (Enterobacteriaceae; Figure 4B). Enterobacteriaceae have also been reported to increase during weight loss in obese mice [37] and humans [38,39]. Other bacteria enriched by supplementation with oligofructose included *Prevotella*, *Allobaculum*, and *Faecalibacterium* genera (Figure 4C). Similarly, a previous study has reported an association between *Allobaculum* and a reduction of body weight in obese mice [17].

Interestingly, agavins and oligofructose supplementation led to the highest cecum SCFA, despite of structural differences between these fructans. However, fructan structure and the degree of polymerization were associated with differences in the bacteria genera enriched by agavins (branched) or oligofructose (linear). In relation to supplementation with oligofructose, agavins supplementation enriched *Citrobacter*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*, and decreased *Prevotella*, *Bacteroides*, *Dehalobacterium*, and *Oscillospira* (Figure 4D). Nevertheless, both supplements shared the physiological response of accelerating body weight loss perhaps due to functional redundancy of the gut microbiota [40].

5. Conclusions

In conclusion, diet supplementation of agavins restored microbiota diversity depleted by a high-fat diet, reduced the Firmicutes/Bacteroidetes ratio, enriched members of the Enterobacteriaceae, and increased the SCFA concentration in cecum, which could induce an accelerated weight loss in mice. These results could provide novel insight to develop a new supplementary strategy using agavins to modulate gut microbiota in overweight or obese individuals, which might have positive consequences on body weight loss. Furthermore, the enrichment of members of Enterobacteriaceae has not been reported previously under a prebiotic supplement, which opens opportunities to explore new probiotics.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/9/821/s1, Figure S1: Experimental design, Figure S2: Relative average abundance of bacterial phyla in the cecal microbiota of mice by diet, Figure S3: Differences in relative abundance of bacterial taxa in cecum between mice fed with

a high-fat diet or standard diet for five weeks, Figure S4: Weighted UniFrac distances according to diet group, Table S1: Sequencing yield and operational taxonomic units obtained through MiSeq sequencing analysis.

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Author Contributions: A.H.-G. and M.G.L. had the idea, designed the study, and performed the mouse experiments. H.S. and A.H.-G. performed the analysis of microbiota sequence data. A.H.-G., H.S., and M.G.L. contributed to the discussion of the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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Short Note

The Efficacy of *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 Probiotic Treatment in Infants with Rotavirus Infection

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Abstract: A total of 57 infants hospitalized with rotavirus disease were included in this study. The children were randomly divided into the study's two treatment groups: three days of the oral administration of (i) a probiotics formula containing both *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 ($N = 28$); or (ii) a placebo (probiotic-free skim milk, $N = 29$) and the standard therapy for diarrhea. There were no differences in age, sex, or blood characteristics between the two groups. When the 57 cases completed the protocol, the duration of the patients' diarrhea was significantly shorter in the probiotics group (4.38 ± 1.29, $N = 28$) than the placebo group (5.61 ± 1.23, $N = 29$), with a p-value of 0.001. Symptoms such as duration of fever ($p = 0.119$), frequency of diarrhea ($p = 0.119$), and frequency of vomiting ($p = 0.331$) tended to be ameliorated by the probiotic treatment; however, differences were not statistically significant between the two groups. There were no serious, adverse events and no differences in the frequency of adverse events in both groups.

Keywords: probiotics; rotavirus; *Bifidobacterium*; *Lactobacillus*

1. Introduction

Diarrhea-associated deaths in children under five years old in developing countries have been a major cause of childhood mortality [1]. These illnesses are caused by multiple factors, including infections by pathogenic microorganisms, viruses, and parasites [2]. Among the many acute diarrheal diseases, infections caused by rotavirus may be more fatal in infants than in adults [3]. Global reports show that most babies and toddlers are infected with rotavirus by the age of five [4]. This causes serious problems in developing and/or low-income countries (e.g., South Asian and sub-Saharan African countries), and hundreds of thousands of babies are killed by rotavirus annually [5]. Recently, the developments of rotavirus vaccines (e.g., RotaTeg and Rotarix) have dramatically reduced the number of outbreaks in many countries and were proven safe; however, concerns remain regarding the cost of the rotavirus vaccines and their limited effectiveness in some cases [6]. Accordingly, supported therapeutic methods that are compatible with common rotavirus medical treatments and effectively relieve its symptoms should be developed.

A number of studies have identified the effect of several probiotic species (e.g., *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Propionibacterium*, *Saccharomyces* and *Streptococcus*) in the treatment and prevention of intestinal infections [7]. These probiotic bacteria have been shown to inhibit intestinal disease [8–11]. *Bifidobacterium* and *Lactobacillus* spp. are the most common bacteria and are considered the most beneficial probiotic organisms [12].

Although multiple probiotic microorganisms could be utilized in rotavirus treatments, some studies have not identified any significant therapeutic effects; therefore, the underlying mechanisms of the therapeutic effects of probiotics in humans are still unclear [13]. Studies have shown that some probiotic bacteria have little or no statistically significant effect on rotavirus [14,15]. Moreover, we can deduce that the effect of probiotics may vary based on the type of microorganism administered to the host. We aim to determine the efficacy of a commercially available probiotic product containing two probiotic cell types, i.e., *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031, in infants and/or toddlers with rotavirus-associated symthoms.

2. Materials and Methods

Design: All participants' guardians completed written, informed consent forms prior to the clinical experiment. All patients were recruited and classified from the inpatient Department of Pediatrics at Yonsei University Hospital in Seoul, Korea. This double-blind, randomized, and placebo-controlled clinical study tests the efficacy of probiotics formula to ameliorate the pathological symptoms in children hospitalized with rotavirus infections. The criteria applied to the experimental subjects are as follows: nine- to 16-month-old male and female infants were diagnosed as infected with rotavirus via a latex agglutination test. A total of 57 infants hospitalized with rotavirus infection were enrolled in this study. 28 patients were assigned to the probiotics treatment group, and the remaining 29 patients were assigned to the placebo group. The probiotics group was fed probiotic formula containing *B. longum* BORI and *L. acidophilus* AD031.

Diet and probiotic microorganisms: The probiotic powder contained two lyophilized probiotic species. Each probiotic packet contained 20 billion CFU/g of *B. longum* BORI and two billion CFU/g of *L. acidophilus* AD031 in powder form. The probiotics-free skim milk powder (placebo packet) was not visually distinguishable from the composite probiotic packet. Both the probiotic and placebo packets were supplied by BIFIDO Co., Ltd. (Hongchun, Korea). Each participant consumed the packets (i) twice a day (ii) for a total of three days (iii) within 10 min of each meal.

Statistical analysis: Paired *t*-tests were performed to assess the quantitative changes in the symptoms of rotavirus infection: duration of fever, frequency of diarrhea, frequency of vomiting, and duration of diarrhea before and after the study period in both groups. Results were considered statistically significant when the *p*-values were < 0.05.

3. Results and Discussion

A total of 57 infants hospitalized with rotavirus infection were enrolled in this study. Twenty-eight patients were randomly assigned to the probiotics group and 29 to the placebo group. The probiotics group was fed a probiotic formula containing *B. longum* BORI and *L. acidophilus* AD031. There were no differences in the age, sex, or blood characteristics of the two groups. The experimental outcomes are summarized in Table 1. The probiotics group showed a slightly reduced duration of fever ($p = 0.119$), frequency of diarrhea ($p = 0.119$), and frequency of vomiting ($p = 0.331$) compared to the placebo group; however, these differences were not significant. By contrast, the duration of diarrhea during the three-day treatment showed a significant difference between the probiotics group (4.38 ± 1.29) and the placebo group (5.61 ± 1.23) with a *p*-value of 0.001 (Table 1). There were no serious, adverse events and no difference in the frequency of adverse events in both groups.

Table 1. Duration and frequency of rotavirus-associated symptoms in patients treated with probiotics and placebo.

Symptoms		Condition		*p*-Value
Category	**Parameter**	**Placebo (*N* = 29)**	**Probiotics (*N* = 28)**	
Duration	Fever	4.32 ± 1.94	3.66 ± 1.14	0.119
(Days)	*Diarrhea*	*5.61 ± 1.23*	*4.38 ± 1.29*	*0.001*
Frequency	Vomiting	1.82 ± 0.94	1.55 ± 1.12	0.119
(Times/Day)	Diarrhea	2.64 ± 0.73	2.38 ± 0.49	0.331

The probiotic formula containing *B. longum* BORI and *L. acidophilus* AD031 utilized in this work is likely be an effective adjuvant to relieve acute diarrhea caused by rotavirus. Several studies showed that various strains of probiotic bacteria, such as *L. reuteri* and *L. rhamnosus*, were effective in managing acute diarrhea caused by rotavirus in toddlers. In the present experiment, the efficacy of *B. longum* BORI and *L. acidophilus* AD031 probiotic products was tested. Our rationale for the *L. acidophilus* and *B. longum* combination was based on the general microbial composition, which shows a predominance of *Lactobacillus* sp. in the small intestine and *Bifidobacterium* sp. in the large intestine (among a variety of beneficial bacteria present in healthy human subjects). Eighteen of 23 clinical trials of probiotic formulas resulted in mitigating acute diarrhea, and the reduction of the duration of diarrhea in the studies' probiotics treatment group was reported to be 0.5 to 1.5 days [16]. The duration of diarrhea may vary depending on a child's health status, diet, and prescribed medication. Our study demonstrated a statistically significant diarrhea reduction of 1.2 days. The efficacy of probiotics is strain-specific, so this may be due to the use of different strains in different studies. Basu et al. [17] conducted a clinical study with 10^7 CFU/day LGG and concluded that it was not effective, but when they performed the same study again [18] with 10^{10} and 10^{12} CFU/day LGG, they concluded that a higher concentration of LGG administration in acute diarrhea patients was effective in reducing the diarrhea frequency, diarrhea period, and hospitalization period. Fang et al. [19] reported a dose-dependent effect of *Lb. rhamnosus* on fecal rotavirus concentration and suggested 6×10^8 CFU/day as the minimal effective dose, which was similar to the data of Guanidalin [20], who concluded that at least 10 billion cells/day was necessary. Dubay [21] also applied the commercially available probiotic formula (VSL#3, CD Pharma India, New Delhi, India) to mitigate acute diarrhea, which showed a more rapid recovery compared to the control group and decreased the necessity of electrolyte treatments. In contrast to the positive results mentioned above, a probiotic formula containing 10^9 CFU/day of *B. lactis* and 10^8 CFU/day of *S. thermophilus* failed to decrease the duration of rotavirus diarrhea [22]. These contrasting results suggest that further clinical experiments are necessary in order to understand the scientific basis of the efficacy of probiotics and its relation to a number of criteria the strain of probiotics, the type of rotavirus, the severity of the symptoms, the ages and races of the children, etc. Further study using animal models also should be considered since the experimental conditions in this model can be better controlled [23–27].

4. Conclusions

The results of the present study demonstrated that a probiotic formula containing *Bifidobacterium longum* BORI and *Lactobacillus acidophilus* AD031 reduced the duration of rotavirus diarrhea in young Korean children.

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Author Contributions: Myeong Soo Park and Bin Kwon designed the experiment under the supervision of Geun Eog Ji. Myeong Soo Park and Bin Kwon performed the research. Myeong Soo Park, Seockmo Ku and Geun Eog Ji analyzed the data and wrote the manuscript. All authors were involved in the revision and editing of this work.

Conflicts of Interest: The authors declare no conflict of interest.

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In Vitro and In Vivo Evaluation of *Lactobacillus delbrueckii* subsp. *bulgaricus* KLDS1.0207 for the Alleviative Effect on Lead Toxicity

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Abstract: Lead (Pb) is a toxic contaminating heavy metal that can cause a variety of hazardous effects to both humans and animals. In the present study, *Lactobacillus delbrueckii* subsp. *bulgaricus* KLDS1.0207 (*L. bulgaricus* KLDS1.0207), which has a remarkable Pb binding capacity and Pb tolerance, was selected for further study. It was observed that the thermodynamic and kinetic model of *L. bulgaricus* KLDS1.0207 Pb binding respectively fit with the Langmuir–Freundlich model and the pseudo second-order kinetic model. Scanning electron microscopy and energy dispersive spectroscopy analysis disclosed that the cell surfaces were covered with Pb and that carbon and oxygen elements were chiefly involved in Pb binding. Combined with Fourier transform infrared spectroscopy analysis, it was revealed that the carboxyl, phosphoryl, hydroxyl, amino and amide groups were the main functional groups involved in the Pb adsorption. The protective effects of *L. bulgaricus* KLDS1.0207 against acute Pb toxicity in mice was evaluated by prevention and therapy groups, the results in vivo showed that *L. bulgaricus* KLDS1.0207 treatment could reduce mortality rates, effectively increase Pb levels in the feces, alleviate tissue Pb enrichment, improve the antioxidant index in the liver and kidney, and relieve renal pathological damage. Our findings show that *L. bulgaricus* KLDS1.0207 can be used as a potential probiotic against acute Pb toxicity.

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus*; lead toxicity; adsorption; antioxidative activity

1. Introduction

Lead (Pb) is a toxic contaminating heavy metal, with no constructive biological role, that remains a public health concern. The key sources of Pb in the human environment are diet, cosmetics, Pb-based paint, soil and dust from Pb-contaminated paint, gasoline, mining, and industrial activity [1–3]. Pb can be inhaled, as it can enter the atmosphere through industrial burning, smelting, and the emissions of vehicles with leaded gasoline. Pb can also enter drinking water through water supply pipes. Consumption of contaminated food and water are also potent sources of Pb exposure and toxicity. Pb is immensely toxic even at low concentrations, and mainly accumulates in bones, the brain [4,5], the liver, kidneys [6] and muscles causing several serious disorders, such as oxidative stress [6,7], carcinogenesis [8], disruption of calcium homeostasis, degenerative changes, nervous disorders [9,10], and sickness and tissue diseases, predominantly in children [11]. The half-life of Pb in blood plasma

is 27 days, in blood is 35 days, in the brain it is about two years, and in bone it may persist for decades [12,13]. Oxidative stress is an imbalance between reactive oxygen species (ROS) and cellular antioxidant systems. Many studies propose oxidative stress as one of the significant mechanisms of the toxic effects of Pb [5,14,15].

The standard treatment for heavy metal poisoning is chelation therapy with the most commonly used chelating agents being $CaNa_2EDTA$ and Meso-2,3-dimercaptosuccinic acid (DMSA). However, adverse effects, including renal injury, malaise, nausea, vomiting, skin reactions [16,17] and progressive deficiencies of copper, zinc and other essential trace nutrients, which are an indispensable part of the body's antioxidant defenses [18]. Therefore, nontoxic natural alternatives of chelating agents have been studied in recent years.

It has been reported that some lactic acid bacteria (LAB) strains, including *L. rhamnosus*, *L. plantarum*, and *Leuconostoc mesenteroides*, are capable of binding and removing heavy metals, such as silver, cadmium and lead in vitro [19–21]. Furthermore, various recent studies have revealed that LAB can effectively protect against metal-induced oxidative stress through antioxidation in vivo [22–24]. Based on this, LAB may serve as a candidate to relieve the symptoms of heavy metal toxicity and their fermented products can be used as dietary fortification against heavy metal poisoning.

The aim of the present study was to select a novel probiotic LAB strain with high Pb-binding capacity and investigate different possible doses to treat acute Pb-induced tissue damage through biochemical enzyme analyses and histopathological studies. This study provides useful information on the utilization of LAB to aid recovery from Pb poisoning.

2. Materials and Methods

2.1. Chemicals and Reagents

Kits used to measure the levels of malondialdehyde (MDA; Njjcbio A003-1 kit), total superoxide dismutase (T-SOD; Njjcbio A001-1 kit), glutathione (GSH; Njjcbio A006-2kit), GSH peroxidase (GSH-PX; Njjcbio A005 kit), aspartate aminotransferase (AST; Njjcbio C010-2 kit), alanine aminotransferase (ALT; Njjcbio C009-2 kit) and a protein quantification kit, rapid (Njjcbio A045-2,) were procured from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Lead dinitrate, lead acetate and other analytical reagents were purchased from the Tianli Chemical Reagent Company (Tianjin, China).

2.2. Bacterial Strain and Culture

L. rhamnosus KLDS1.0205, KLDS1.0911 and KLDS1.0912, *L. bulgaricus* KLDS1.0207 and KLDS1.9201, *L. plantarum* KLDS1.0386 and KLDS1.0344, *L. acidophilus* KLDS1.1003, *L. helveticus* KLDS1.0903 and *L. casei* KLDS1.0351 were isolated from traditional dairy products in Sinkiang Province, China and identified by API 50CH strips and 16S rRNA gene similarity analysis. These LAB strains were stored at the Key Laboratory of Dairy Science (KLDS), Ministry of Education, China. All strains were anaerobically incubated in De Man, Rogosa and Sharpe (MRS) broth (Hopebio Company, Qingdao, China) at 37 °C for 18 h and were sub-cultured twice prior to the experiment.

2.3. Estimation of Pb Binding and Pb Tolerance

The Pb binding ability of 10 strains was analyzed as previously described with little modification [25]. All strains were incubated for 18 h and the cultured biomass was centrifuged at 8000 rpm for 20 m. The centrifuged mass was washed twice with ultrapure water to obtain cell pellets. The bacterial concentration was adjusted to 1 g/L (wet weight) using ultrapure water containing 50 mg/L lead dinitrate and samples were then incubated at 37 °C for 24 h (pH 6.0). The samples were centrifuged at 8000 rpm for 20 m and the residual Pb concentrations of the supernatants were measured by flame atomic absorption spectrophotometry (Spectra AA 220; Varian, Palo Alto, CA, USA). The metal removal efficiency based on mass balance was calculated using the following equation:

$$\text{Removal}(\%) = \frac{C_i - C_e}{C_i} \times 100\%$$

where, C_i and C_e are the initial Pb concentration and residual Pb concentration after removal, respectively.

The Pb tolerance of each strain was determined by the minimum inhibitory concentration (MIC) approach [26]. MRS agar medium containing 50 to 1000 mg/L lead dinitrate solution was prepared, and 10 μL of cultured LAB strain was spotted on the MRS agar medium at an inoculum level of 1×10^9 CFU/mL. LAB growth was recorded after cultivation at 37 °C for 48 h. The minimum concentration of Pb that completely inhibited LAB growth was considered as the MIC in this study.

2.4. Equilibrium Isotherm and Kinetic Study

The equilibrium isotherm was performed as previously reported [27], and the harvested cell pellets were suspended in ultrapure water containing 5 to 75 mg/L lead dinitrate to give a final bacterial concentration of 1 g/L (dry weight). The Pb binding assay was then conducted with an initial pH of 6.0 and the equilibrium content of Pb bound by the bacterium was expressed as follows:

$$q_e(\text{mg metal/g biosorbent}) = \frac{C_i - C_e}{m/V} \quad (1)$$

where, C_i and C_e are the initial Pb concentration and residual Pb concentration after removal, respectively; and m/V = 1 g/L.

The Langmuir, Freundlich and Langmuir–Freundlich models were used to determine the sorption equilibrium between the biosorbent and metal ions. Isotherm constants for the three models were obtained by non-linear regression methods [28,29].

The kinetic study was conducted as previously described [27], the harvested cell pellets were suspended in ultrapure water containing 50 mg/L lead dinitrate to give a final bacterial concentration of 1 g/L (dry weight). The Pb binding assay was then conducted with an initial pH of 6.0 and the concentration of Pb in the supernatant was detected at different time intervals up to 240 m.

The pseudo first and second-order rate equations were used in the kinetic model of Pb biosorption with the integrated form of the pseudo first-order model as follows:

$$\lg \frac{q_e}{q_e - q_t} = \frac{k_1}{2.303} t$$

where q_t is adsorption capacity; and time t and k_1 are first-order rate constants. k_1 can be determined from the slope of the plot of $\lg \frac{q_e}{q_e - q_t}$ vs. t.

The pseudo second-order integrate equation is expressed as:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{1}{q_e} t$$

where k_2 can be determined from the interception of the linearized plot of t/q_t vs. t.

2.5. Scanning Electron Microscopy (SEM) Analysis

The samples for SEM observation were prepared as described earlier [30]. The untreated harvested cell pellets and those treated with Pb (50 mg/L) were fixed by 2.5% glutaraldehyde (*v*/*v*) at 4 °C for 1.5 h, and washed thrice with phosphate buffer solution. Supernatants were discarded and the cell pellets treated with different concentrations (50%, 70%, 90% and 100%) of alcohol as well as a mixture of alcohol and t-butanol (1:1) to wash cells successively. Finally, the cell pellets were eluted with plain t-butanol. The specimens were then put in a freeze-drier for 4 h and sputter-coated with gold. The scanning and photography were performed using SEM with an energy dispersive spectrometer (EDS).

2.6. Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

The untreated harvested cell pellets and those treated with Pb (50 mg/L) were lyophilized and mixed with KBr powder as KBr discs. Discs containing 2% (*w*/*w*) of finely ground powder of each sample were prepared. The FT-IR technique was employed to characterize the changes in the functional groups on the untreated cell pellets and those treated with Pb.

2.7. In Vivo Protective Potential of L. bulgaricus KLDS1.0207 Against Acute Pb Toxicity

2.7.1. Animals and Experimental Design

A total of 72 female BALB/c mice (6–7 weeks old, weighing 16 to 20 g) were purchased from the Vital River Laboratory Animal Technology Company (Beijing, China) and housed in a room under controlled environmental conditions at 25 °C and with a 12-h light/dark cycle. All the mice were put into plastic cages for one week before the begin of the experiments. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Northeast Agricultural University under the approved protocol number Specific pathogen free rodent management (SRM)-06.

Mice were fed with standard commercial pellets and water ad libitum and were randomly divided into two major groups. The prevention and therapy groups were divided into three subgroups and six subgroups, respectively. Each subgroup had a total of eight BALB/c mice. The details of the administration are shown in Table 1. On the basis of Azar's studies [31], the oral dose of lead acetate [$(CH_3COO)_2Pb{\cdot}3H_2O$] was 100 mg/kg body weight. The dose of DMSA used in this study was 50 mg/kg/day [17,32].

Table 1. Experimental design of the prevention and therapy groups.

Groups	Treatment on the Indicated Day(s) for the Following:	
Prevention groups	1–14 days	15 day
Pre-control (*n* = 8)	SM	PW
Pre-Pb (*n* = 8)	SM	Pb
Pre-LAB (*n* = 8)	SM + LAB	Pb
Therapy groups	1 day	2–15 days
Control (*n* = 8)	PW	SM
Pb (*n* = 8)	Pb	SM
High dose (*n* = 8)	Pb	SM + LAB
Medial dose (*n* = 8)	Pb	SM + LAB
Low dose (*n* = 8)	Pb	SM + LAB
Drug (*n* = 8)	Pb	DMSA(50 mg/kg/day)

PW, 0.4 mL plain water. SM, 0.4 mL skim milk. Pb, [$(CH_3COO)_2Pb{\cdot}3H_2O$] at 2 mg in 0.4 mL plain water. SM + LAB, in the Pre-LAB group, 1×10^9 CFU/mL *L. bulgaricus* KLDS1.0207 in 0.4 mL skim milk. SM + LAB in the high, medial and low dose group, 1×10^{10}, 1×10^9 and 1×10^8 CFU/mL *L. bulgaricus* KLDS1.0207 in 0.4 mL skim milk, respectively. Meso-2,3-dimercaptosuccinic acid (DMSA), DMSA at 1 g in 0.4 mL 5% sodium bicarbonate solution. All modes of administration were oral.

All of the therapy groups had their feces collected every week to determine Pb concentration. The animals were then sacrificed under ether anesthesia, and blood samples were collected with Pb-free needles, and all tissue samples were obtained and kept at −80 °C for biochemical assays and estimation of the Pb concentration. Some kidney samples were put into 10% neutral formalin to analyze their pathology.

2.7.2. Determination of Pb in Blood, Feces and Tissue

Samples were digested in concentrated HNO_3 by using a microwave digestion system. Pb concentration in the livers, kidneys, blood, and feces was determined by a graphite furnace atomic absorption spectrophotometer.

2.7.3. Biochemical Assays

The levels of MDA and GSH and the activities of T-SOD and GSH-Px in the mouse kidneys and livers, and the activities of ALT and AST in the mouse serum were measured by using the assay kit in accordance with the recommendations of the manufacturer.

2.7.4. Histopathological Studies

The kidneys were fixed in 10% neutral formalin for 48 h. Samples were then embedded in paraffin, sliced into 5 μm thickness and stained with hematoxylin-eosin (H&E) for examination by light microscopy.

2.8. Statistical Analysis

All values are expressed as the mean ± standard deviation (SD). A minimum of three independent experiments were carried out for each assay. The statistical significance of data comparisons was determined using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Pb Biosorption and Pb Tolerance of LAB Strains

The Pb-binding abilities of the 10 strains are presented in Figure 1. While the initial Pb concentration was 50 mg/L, the range of Pb removal by the tested strains was from 22.47% to 79.18%. *L. bulgaricus* KLDS1.0207 showed the best binding ability among the 10 strains. Moreover, *L. bulgaricus* KLDS1.0207 had the highest MIC value (>1000 mg/L) (Table 2). Based on these characteristics, *L. bulgaricus* KLDS1.0207 was selected as a candidate for the further study of its biosorption mechanism and in vivo assays.

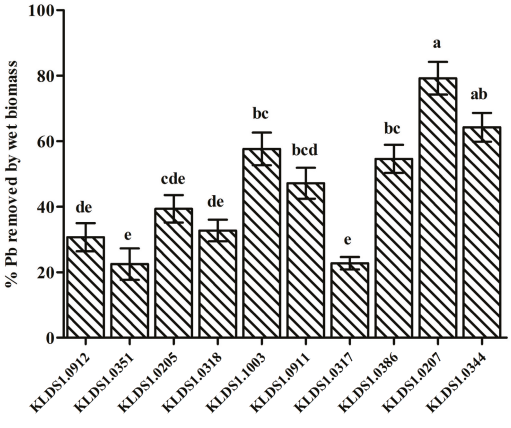

Figure 1. The Pb-binding ability of the tested lactic acid bacteria (LAB) strains when incubated with an initial Pb concentration of 50 mg/L. Values are mean ± SD of three determinations. Significant differences ($p < 0.05$) among the strains are indicated with different letters above the graphical bars. KLDS1.0205, KLDS1.0911 and KLDS1.0912 are *L. rhamnosus*. KLDS1.0207 and KLDS1.9201 are *L. bulgaricus*. KLDS1.0386 and KLDS1.0344 are *L. plantarum*. KLDS1.1003 is *L. acidophilus*. KLDS1.0903 is *L. helveticus*. KLDS1.0351 is *L. casei*.

Table 2. Pb tolerance of the tested LAB strains.

Strains	Minimum Inhibitory Concentration for Pb (mg/L)
L. bulgaricus KLDS1.0207	>1000
L. bulgaricus KLDS1.9201	200
L. helveticus KLDS1.0903	350
L. acidophilus KLDS1.1003	300
L. plantarum KLDS1.0386	150
L. plantarum KLDS1.0344	450
L. rhamnosus KLDS1.0205	100
L. rhamnosus KLDS1.0911	50
L. rhamnosus KLDS1.0912	400
L. casei KLDS1.0351	200

3.2. Biosorption Isotherms and Kinetic Models

As indicated in Figure 2, the Pb concentration in the solution was positively correlated with the Pb-binding ability of *L. bulgaricus* KLDS1.0207. The data of different isotherm models, including Langmuir, Freundnlich and Langmuir–Freundlich, are listed in Table S1, respectively. The values of the coefficients of correlation (R^2) illustrated that the Langmuir–Freundlich model (R^2 = 0.9820) best suited our experimental data.

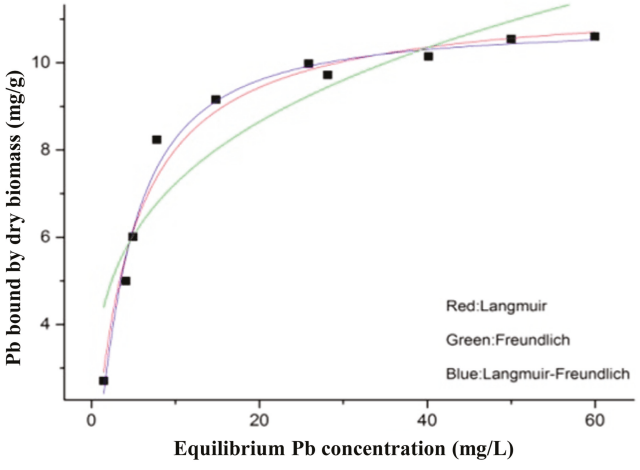

Figure 2. Adsorption isotherm of Pb binding by *L. bulgaricus* KLDS1.0207.

As shown in Figure 3, the biosorption of Pb onto *L. bulgaricus* KLDS1.0207 was efficient, because the binding process was nearly completed in 60 m. The rate kinetics of the reaction adopted pseudo first-order kinetic model (R^2 = 0.9665) for first 15 m (Figure S1A). Further analysis showed that the pseudo second-order kinetic model (R^2 = 0.9916) was far better at explaining the biosorption of Pb on the surface of *L. bulgaricus* KLDS1.0207 for the complete process (Figure S1B).

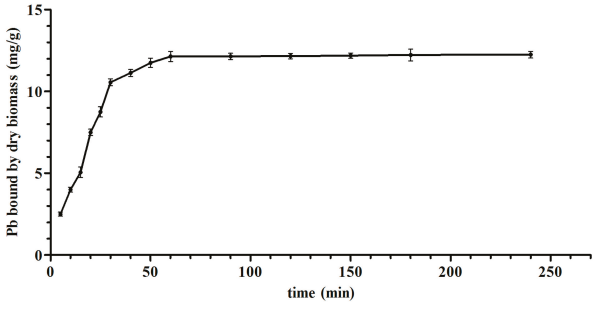

Figure 3. Pb binding of *L. bulgaricus* KLDS1.0207 at different time points. Values are mean ± standard deviation (SD) of three determinations.

3.3. Electron Microscopy Analysis

The SEM micrographs of *L. bulgaricus* KLDS1.0207 after the treatment with Pb indicated that many light clusters of metal precipitates were localized but did not cover the cell surface evenly (Figure 4B). However, no light precipitates were found on the surface of untreated cell pellets (Figure 4A). The EDS analysis confirmed that the presence of Pb resulted in the light precipitates (Figure 4C, Table S2).

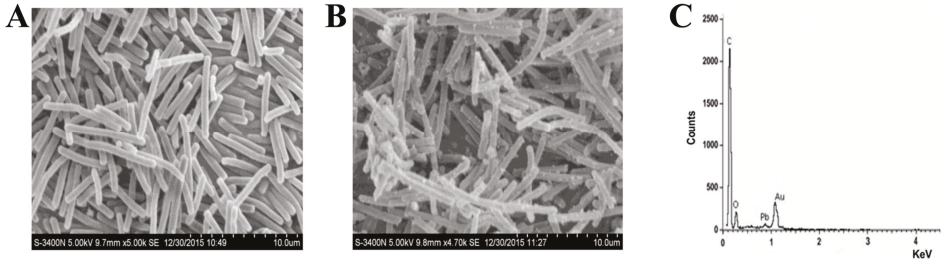

Figure 4. Scanning electron micrographs of *L. bulgaricus* KLDS1.0207 untreated and treated with Pb (50 mg/L): (**A**) Untreated biomass; (**B**) Biomass after lead binding; and (**C**) Energy dispersive spectrometer (EDS) analysis of biomass after Pb binding. Scale bar = 10.0 μm.

3.4. FT-IR Analysis

The FT-IR spectrums of untreated *L. bulgaricus* KLDS1.0207 and that treated with Pb (50 mg/L) are shown in Figure 5. The marked shift to a strong wave number at 3500–3200 cm^{-1} may be due to the interaction of the –NH group of amide and −OH group of alcohol-phenol with Pb. In the 1720–1700 cm^{-1} region, a C=O stretching vibration of carboxylic acid was observed. The amine II band (1400–1410 cm^{-1}) is related to a combination of the NH in-plane bending mode with the stretching of the C−N peptide bond. The disappearance of the peak at 1250–1150 cm^{-1} indicated that the P=O and O−H or C−O stretching vibrations of polysaccharides could play a role in Pb biosorption. These constituents can be combined with Pb by the main functional carboxyl, phosphoryl, hydroxyl, amino, and amide groups.

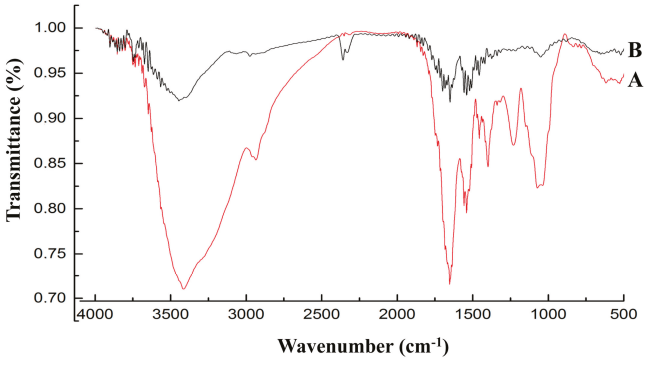

Figure 5. Fourier transform infrared spectrums of *L. bulgaricus* KLDS1.0207 untreated and treated with Pb (50 mg/L): (**A**) Untreated biomass; and (**B**) Biomass after Pb binding.

3.5. Mortality and Viscera Index Analysis

The survival rates of BALB/c mice are presented in Table 3. No mortality was recorded in the pre-control group and the mortality of pre-LAB group (1/8) was lower than the pre-Pb group (2/8). The mortality in the low dose and medial dose group were 1/8, no death was recorded in the high dose and drug group, indicating that the high dose group played a role in mitigating Pb toxicity.

In the viscera indices, the pre-Pb group was significantly different ($p < 0.05$) from the pre-control group, suggesting that some pathological changes may be present in the pre-Pb group. There was no significant difference between the pre-LAB group and the pre-control group, which may be due in part to the presence of *L. bulgaricus* KLDS1.0207. The high dose and Pb group were significantly different in the liver body ratio ($p < 0.05$), but no significant difference in the kidney body ratio was found between the high dose and control group. This was dissimilar from the low dose, medial dose and drug group, which may imply that the high dose group had a better protection against Pb exposure than the drug group as the long-term intake DMSA may have resulted in some damage to the liver and kidneys.

Table 3. The mortality and the viscera indices of different groups.

Groups	Mortality	Liver Body Ratio	Kidney Body Ratio
Pre-control	0/8	3.80 ± 0.25^{b}	1.17 ± 0.03^{bc}
Pre-Pb	2/8	3.28 ± 0.22^{a}	1.06 ± 0.04^{a}
Pre-LAB	1/8	3.65 ± 0.23^{ab}	1.16 ± 0.05^{bc}
Control	0/8	4.95 ± 0.31^{d}	1.42 ± 0.06^{d}
Pb	2/8	3.33 ± 0.14^{a}	1.11 ± 0.04^{ab}
High dose	0/8	4.47 ± 0.27^{c}	1.40 ± 0.03^{d}
Medial dose	1/8	3.43 ± 0.19^{ab}	1.31 ± 0.03^{c}
Low dose	1/8	3.37 ± 0.28^{ab}	1.21 ± 0.04^{c}
Drug	0/8	3.39 ± 0.15^{ab}	1.23 ± 0.07^{c}

Values are mean ± SD. Significant differences ($p < 0.05$) among different groups are indicated with different superscript letters.

3.6. Pb Levels in Feces and Tissues

The changes in the Pb levels in the feces of the mice in the therapy groups are presented in Table 4. In the first week, in comparison to the Pb group, the fecal Pb levels of all treated groups increased significantly ($p < 0.05$). No dose–response relationship by the administration of *L. bulgaricus* KLDS1.0207 was observed. During the second week, the excretion amounts of Pb in the Pb group and

treated groups were lower than the first week, indicating that *L. bulgaricus* KLDS1.0207 and DMSA were effective against acute Pb toxicity in the first week.

Table 4. Effects of *L. bulgaricus* KLDS1.0207 on Pb level in feces under different time.

Groups	Pb (μg/g)	
	First Week	Second Week
Control	0.16 ± 0.03^{a}	0.13 ± 0.04^{a}
Pb	23.15 ± 1.35^{b}	0.40 ± 0.13^{b}
High dose	31.15 ± 2.32^{c}	0.65 ± 0.14^{bc}
Medial dose	29.48 ± 1.12^{c}	0.52 ± 0.10^{bc}
Low dose	28.81 ± 1.87^{c}	0.41 ± 0.18^{b}
Drug	42.81 ± 2.43^{d}	0.70 ± 0.16^{c}

Values are mean ± SD. Significant differences ($p < 0.05$) among different groups are indicated with different superscript letters.

The Pb concentrations of the blood, liver and kidneys in the pre-LAB group were significantly lower than the pre-Pb group ($p < 0.05$). In the blood and kidneys, the decreasing Pb levels in all dose groups were significantly different from the values obtained for the Pb group ($p < 0.05$), whereas no significant differences were observed in the liver. This implies that *L. bulgaricus* KLDS1.0207 is more efficient in the kidney than in the liver (Table 5).

Table 5. Effects of *L. bulgaricus* KLDS1.0207 on Pb levels in the blood and tissue of mice.

Groups	Blood (μg/L)	Liver (μg/g)	Kidney (μg/g)
Pre-control	0.32 ± 0.02^{a}	0.15 ± 0.02^{a}	0.17 ± 0.03^{a}
Pre-Pb	383.30 ± 23.12^{f}	1.51 ± 0.04^{e}	1.72 ± 0.07^{g}
Pre-LAB	344.03 ± 24.32^{e}	1.13 ± 0.09^{d}	1.25 ± 0.06^{f}
Control	0.43 ± 0.05^{a}	0.12 ± 0.04^{a}	0.16 ± 0.04^{a}
Pb	302.20 ± 25.32^{d}	0.46 ± 0.08^{c}	0.66 ± 0.08^{e}
High dose	234.12 ± 10.18^{c}	0.35 ± 0.07^{bc}	0.45 ± 0.06^{bc}
Medial dose	248.01 ± 7.54^{c}	0.39 ± 0.05^{c}	0.54 ± 0.04^{cd}
Low dose	258.33 ± 8.13^{c}	0.43 ± 0.07^{c}	0.63 ± 0.05^{de}
Drug	176.14 ± 6.17^{b}	0.27 ± 0.06^{b}	0.36 ± 0.08^{b}

Values are mean ± SD. Significant differences ($p < 0.05$) among different groups are indicated with different superscript letters.

3.7. Activity of Antioxidant Enzymes

The levels of antioxidant capacity in the liver and kidney of mice are shown in Figure 6. In both prevention and therapy groups, acute Pb exposure induced a remarkable increase in the levels of MDA and a marked decrease in the levels of GSH, GSH-PX and T-SOD in the liver. All *L. bulgaricus* KLDS1.0207 administrations had significant protective effects on the antioxidant capacity ($p < 0.05$). Particularly, the high dose group was significantly different with the low dose, medial dose and drug group, indicating that the high dose of *L. bulgaricus* KLDS1.0207 could more efficiently relieve oxidative stress in the liver. Generally, in the kidney, there were significant differences in MDA, GSH and GSH-PX levels in all dose group ($p < 0.05$), however, a significant increase in T-SOD levels was only observed in the high dose group. The MDA levels of the pre-LAB group showed non-significant decrease trends compared with the pre-Pb group. There was a decreasing pattern in the MDA level of all dose groups ($p < 0.05$). In contrast, the drug group was significantly higher than the Pb group in the MDA level ($p < 0.05$), indicating that the drug group had a significantly adverse effect on the kidney. In all, the high dose of *L. bulgaricus* KLDS1.0207 could serve as an effective antioxidant in the liver and kidney.

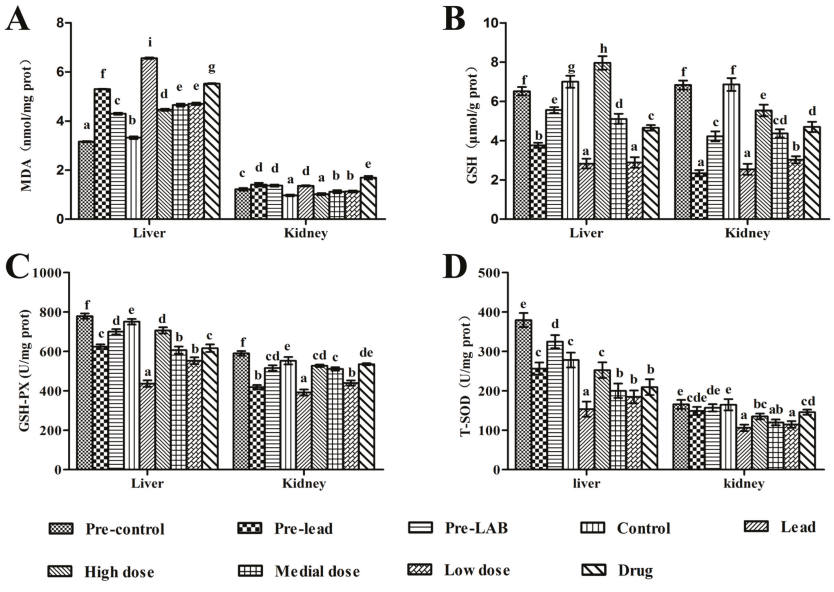

Figure 6. Effects of *L. bulgaricus* KLDS1.0207 on Pb-induced variations of antioxidant capacity in the liver and kidney of mice: (**A**): Malondialdehyde (MDA); (**B**): Glutathione (GSH); (**C**): Glutathione peroxidase (GSH-PX); and (**D**): Total superoxide dismutase (T-SOD). Values are mean ± SD. Significant differences ($p < 0.05$) among the groups are indicated with different letters above the graphical bars.

The activities of marker enzymes in the serum of mice are shown in Table 6. Although no significant difference in the activity of ALT was observed in the prevention group, a significant decrease ($p < 0.05$) in the AST/ALT ratio between the pre-LAB group and the pre-Pb group was noticed. In the therapy group, the AST/ALT ratio in all treated groups was significantly reduced ($p < 0.05$), especially the high dose group showed the lowest AST/ALT ratio.

Table 6. Effects of *L. bulgaricus* KLDS1.0207 on Pb-induced variations in the activity of marker enzymes in the serum of mice.

Groups	ALT (U/L)	AST (U/L)	AST/ALT
Pre-control	33.56 ± 2.13^{a}	55.87 ± 3.01^{a}	1.67 ± 0.02^{a}
Pre-Pb	40.74 ± 1.76^{c}	84.23 ± 3.32^{c}	2.07 ± 0.01^{b}
Pre-LAB	38.67 ± 2.38^{bc}	65.89 ± 4.21^{b}	1.70 ± 0.00^{a}
Control	35.39 ± 3.01^{ab}	100.23 ± 4.54^{d}	2.84 ± 0.11^{c}
Pb	46.19 ± 2.88^{d}	228.12 ± 5.65^{h}	4.95 ± 0.19^{g}
High dose	34.23 ± 1.47^{a}	123.33 ± 4.25^{e}	3.60 ± 0.03^{d}
Medial dose	37.87 ± 2.13^{abc}	153.33 ± 4.02^{f}	4.05 ± 0.12^{e}
Low dose	40.02 ± 2.42^{c}	167.88 ± 5.23^{g}	4.20 ± 0.12^{ef}
Drug	33.87 ± 2.12^{a}	146.46 ± 4.37^{f}	4.33 ± 0.14^{f}

Values are mean ± SD. Significant differences ($p < 0.05$) among different groups are indicated with different superscript letters. AST, aspartate aminotransferase; and ALT, alanine aminotransferase.

3.8. Histopathology

In the control groups (Figure 7A,D), normal kidney histomorphology was apparent. In the pre-Pb group (Figure 7B), glomeruli were hyperemic, the glomerular volume became significantly bigger and few glomeruli were missed, some renal tubular epithelial cells showed swelling, which was

alleviated in the pre-LAB group (Figure 7C). The symptoms of all treated groups (Figure 7F–I) including inflammatory cells, swelled tubular epithelial cells and granular degeneration and glomerular hyperemic were ameliorated to some extent when compared to the Pb group (Figure 7E).

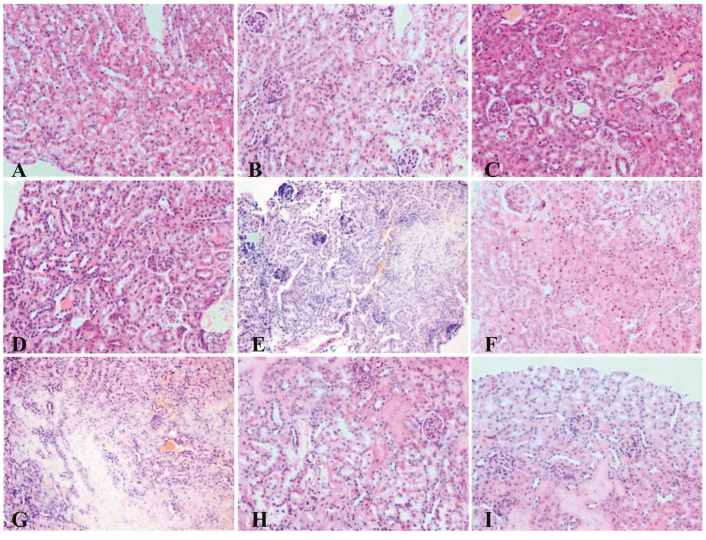

Figure 7. Representative photomicrographs of renal tissue of mice (hematoxylin-eosin (H&E) staining; magnification ×400): (**A**) Normal appearance of the renal tissue of mice in the control prevention group; (**B**) Renal tissue of mice in the pre-lead group; (**C**) Renal tissue of mice in the pre-LAB group; (**A**) Renal tissue of mice in the control therapy group; (**B**) Renal tissue of mice in the lead-only therapy group; (**C**) Renal tissue of mice in the high-dose therapy group; (**D**) Renal tissue of mice in the medial-dose group; (**E**) Renal tissue of mice in the low-dose group; and (**F**) Renal tissue of mice in the drug group.

4. Discussion

Pb is a prevalent environmental pollutant with no beneficial biological role. It adversely induces oxidative stress damage to the host. Many researches have reported that LAB can not only sequester Pb, but also relieve the oxidative damage [1,2]. Screening LAB strains against Pb toxicity should take a number of properties into consideration. First, LAB strains must show high Pb-binding ability, enabling them to bind Pb before the intestinal absorption of Pb by the host. Second, LAB strains should exhibit high resistance to Pb to avoid it being poisoned. Furthermore, to perform Pb removal in the gastrointestinal tract, it is necessary for the screened strains to remain viable in high concentrations of bile and stomach acids. In the present study, *L. bulgaricus* KLDS1.0207 showed the best binding aptitude among 10 strains (Figure 1) and had a remarkable tolerance to Pb (Table 2). In addition, *L. bulgaricus* KLDS1.0207 showed good resistance to simulated gastrointestinal tract conditions (data not shown). Based on these properties, *L. bulgaricus* KLDS1.0207 was selected as a potential strain for the further study of its Pb biosorption mechanism and in vivo assays.

Various mechanisms of metal biosorption, including adsorption, ion exchange, complexation, chelation and microprecipitation have been proposed because of differences in the bacterial structures among the species [33]. The cell walls of the gram-positive bacteria consist of peptidoglycans, teichoic acids, proteins and polysaccharides [34,35]. These contents also contain negatively charged functional groups, which serve as the primary sites of metal ion sorption on a bacterium surface [36]. The phosphate and carboxyl group present in peptidoglycans and teichoic acids are the primary sites of metal ion binding on the surface of the bacterial cell [37]. SEM micrographs and EDS analysis

(Figure 4) confirmed that the added toxic Pb metal was localized on the cell surface evenly. A previous study has reported a similar phenomenon in *L. mesenteroides* after Pb binding [21], this phenomenon may be related to the passive physicochemical adsorption mechanism.

By analyzing the FT-IR spectrum (Figure 5), it was able to be speculated that the functional groups (carboxyl, phosphoryl, hydroxyl, amino and amide) of biological macromolecules (fatty acids, polysaccharides, S-layer proteins and teichoic acid) bind Pb through complexation, ion exchange and physical adsorption (electrostatic attraction). These predictions were consistent with the previous assertion that the carboxyl, hydroxyl and amide groups were involved in Pb uptake [38–40]. It may be necessary in future studies to elucidate the effects of the specific groups in this mechanism.

The thermodynamic and kinetic models of *L. bulgaricus* KLDS1.0207 binding Pb fit with the Langmuir–Freundlich model and pseudo second-order kinetic model, respectively (Figure 2 and Figure S1), this is in line with the cadmium binding characterization of an acidophilic bacterium [27], indicating that adsorption (physical and chemical) is a complex process.

As *L. bulgaricus* KLDS1.0207 had an excellent Pb-binding capacity, the protective effects of it against acute Pb toxicity in mice was evaluated using prevention and therapy groups. In the present study, mice were orally given a lead acetate solution of 100 mg/kg bodyweight to stimulate acute Pb toxicity, followed by treatment with different concentrations of *L. bulgaricus* KLDS1.0207 for two weeks. Our study showed that the high dose therapy group had a higher ability to increase fecal excretion of Pb than low dose groups (Table 4). Similar results were observed for the other *Lactobacillus* strains, which could modulate intestinal heavy metal absorption in mice by increasing fecal heavy metal excretion [22,23,41].

The high concentration of Pb in different tissues was associated with increased oxidative reaction, which might be responsible, at least in part, for Pb-induced toxic effects [42]. Several studies have reported a possible link between oxidative stress and the disruption of metal ion homeostasis [43–45]. Our results showed that *L. bulgaricus* KLDS1.0207 lowered Pb-induced oxidative stress and facilitated a protective role in reducing the lipid peroxide by decreasing MDA concentration and improving other antioxidants, such as T-SOD, GSH and GSH-Px in the liver and kidneys (Figure 6).

Changes in the ALT and AST levels are often used for liver pathological examination, and AST/ALT is an important biochemical indicator [46,47]. ALT mainly exists in the liver cell plasma. When liver cell damage is lower, changes in liver-cell-membrane permeability elevate ALT levels in the blood. On the other hand, increased AST blood levels are a result of severely damaged liver cells. It thus follows that higher AST/ALT ratios imply more severe liver cell damage. The treatment of *L. bulgaricus* KLDS1.0207 could improve the ALT and AST levels (Table 6).

Compared with the dose groups, the drug group was able to increase Pb excretion. Nonetheless, the drug DMSA has been shown in a previous study to have some adverse effects, including depletion of zinc and copper in the body [17]. In all, the results from the present study have shown that the most effective dose in the therapy groups was 1×10^{10} CFU/mL of *L. bulgaricus* KLDS1.0207 in 0.4 mL skim milk. This was similar to the findings of earlier studies that 2×10^{10} CFU/mL of *L. plantarum* CCFM8246 (0.2 mL) and 1×10^{9} Colony-Forming Units (CFU) of *L. plantarum* CCFM8610 (0.5 mL) were effective against copper and cadmium toxicity in mice, respectively [22,41]. These effective LAB strains can be freeze-dried or spray-dried to obtain the strain powders for the practical administration. Interestingly, LAB strains such as *L. plantarum* and *L. rhamnosus* can improve the absorption and bioavailability of several trace elements in animals [48,49]. Thus, it is very significant to exploit LAB as a heavy metal removal agent added to food or feed.

5. Conclusions

L. bulgaricus KLDS1.0207 had high Pb biosorption and Pb tolerance in vitro. The adsorption process of Pb was complex and efficient by the main functional groups, including the carboxyl, phosphoryl, hydroxyl, amino and amide groups. *L. bulgaricus* KLDS1.0207 facilitated Pb detoxication in vivo by increasing Pb levels in the feces, alleviated tissue Pb enrichment, improved the antioxidant

index, and relieved renal pathological damages. Therefore, *L. bulgaricus* KLDS1.0207 may be used as a novel probiotic candidate against acute Pb toxicity.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/8/845/s1, Figure S1: Kinetic models of the Pb binding by *L. bulgaricus* KLDS1.0207. (A) Pseudo first-order kinetic model of the Pb binding by *L. bulgaricus* KLDS1.0207 in the first 15 m. (B) Pseudo second-order kinetic model of the Pb binding by *L. bulgaricus* KLDS1.0207. Table S1: Adsorption constants derived from simulations with different isotherm models. Table S2: Quantitative analysis of EDS.

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Author Contributions: B.L., F.L., S.Y. and G.H. conceived the study and designed the project. B.L., D.J. and S.Y. performed the experiment, analyzed the data and drafted the manuscript. S.E.E. and Z.M. helped to revise the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest: The authors hereby declare that, there was no conflict of interest in the present study.

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Dose-Dependent Prebiotic Effect of Lactulose in a Computer-Controlled In Vitro Model of the Human Large Intestine

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Abstract: Lactulose, a disaccharide of galactose and fructose, used as a laxative or ammonia-lowering drug and as a functional food ingredient, enhances growth of *Bifidobacterium* and *Lactobacillus* at clinically relevant dosages. The prebiotic effect of subclinical dosages of Lactulose, however, remains to be elucidated. This study analyses changes in the microbiota and their metabolites after a 5 days Lactulose treatment using the TIM-2 system, a computer-controlled model of the proximal large intestine representing a complex, high density, metabolically active, anaerobic microbiota of human origin. Subclinical dosages of 2–5 g Lactulose were used. While 2 g Lactulose already increased the short-chain fatty acid levels of the intestinal content, 5 g Lactulose were required daily for 5 days in this study to exert the full beneficial prebiotic effect consisting of higher bacterial counts of *Bifidobacterium*, *Lactobacillus*, and *Anaerostipes*, a rise in acetate, butyrate and lactate, as well as a decrease in branched-chain fatty acids, pH (suggested by an increase in NaOH usage), and ammonia.

Keywords: Lactulose; microbial fermentation; *Bifidobacteria*; lactobacilli; *Anaerostipes*; butyrate; ammonia

1. Introduction

Lactulose is a synthetic disaccharide consisting of galactose and fructose. It is indicated for the symptomatic treatment of constipation at doses of 10 to 30 g and the treatment of portal systemic encephalopathy at doses of 60 to 100 g. Furthermore, lower dosages than 10 g Lactulose are used as a functional food ingredient. Lactulose is neither digested nor absorbed from the upper gastrointestinal tract. Once it reaches the colon it is anaerobically fermented by the microbiota and serves as a prebiotic substrate by increasing the count of *Bifidobacterium*, *Lactobacillus*, and bacterial metabolites like short-chain fatty acids (SCFA) [1,2]. Hence, Lactulose can be characterized as a non-digestible carbohydrate with fiber-like effects. In healthy individuals, daily dosages of 10 g Lactulose already exerted beneficial effects on the human microbiota [3], as did even lower dosages like 4 g [4] and 3 g [5]. This raises the potential for the use of subclinical dosages of Lactulose, which do not have a strong

laxative effect, exclusively for prebiotic effects. The minimal dose of Lactulose required for a prebiotic effect, however, remains to be determined.

The above mentioned studies used different durations of treatments and different subject populations which limits the interpretation of dose-dependency of the prebiotic effect of Lactulose. Furthermore, direct effects of substances on the metabolic activity of the microbiota are difficult to determine in humans due to the substantial absorption and metabolism of products like short-chain fatty acids in the gut [6]. This drawback can be circumvented using the TIM-2 system, a computer-controlled model of the proximal large intestine representing a complex, high density, metabolically active, anaerobic microbiota of human origin. Thus, we aimed at the evaluation of the prebiotic effect of different dosages of Lactulose under controlled experimental conditions in the TIM-2 system.

In former studies with this system, daily 10 g Lactulose administration for 48 h led to a change in the SCFA ratio mainly based on an increase in acetate and a decrease in butyrate [2], which was most probably due to an increase in acetate producing *Bifidobacterium* and *Lactobacillus* species. When 7.5 g Lactulose were administered for 72 h to fecal samples of lean volunteers in this system, elevated levels of SCFA were observed in the Lactulose group as well and this dose of Lactulose mainly stimulated the growth of *Bifidobacterium* as well as *Alistipes* spp., *Parabacteroides* spp., *Parasutterella* spp., and *Anaerostipes* spp. [1]. No effect on *Lactobacillus* was reported in this group.

The current study is aimed at comparing daily dosages of 2 to 5 g Lactulose for 120 h in the TIM-2 system. In this study, we show that even 2 g Lactulose leads to an increase in SCFA, mainly reflected by acetate, while 3 or more grams of Lactulose are required to also observe an increase in butyrate. At 4 g Lactulose, the reduction in ammonia was as prominent as at a dose of 5 g, whereas an increase in relative abundance of *Bifidobacterium*, *Lactobacillus* and *Anaerostipes* was most evident in the 5 g Lactulose group. Our results show that a maximum daily dose of 5 g Lactulose for five days was associated with the full pattern of beneficial prebiotic effects on the intestinal microbiota.

2. Materials and Methods

2.1. Test Product

The product administered in this study was Laevolac® (Fresenius Kabi Austria GmbH, Linz, Austria), an oral solution containing 670 mg/mL lactulose. Experiments without the addition of Laevolac® served as negative control.

2.2. Intestinal Conditions of the TIM-2 System

The TNO Intestinal Model (TIM-2) is a dynamic in vitro model of the proximal colon that has been previously published [1,2,7,8]. The TIM-2 system was inoculated with a dense and highly metabolically active colon microbiota of human origin. In the system the following standardized conditions were simulated: body temperature; pH in the lumen of the proximal colon (pH 5.8); anaerobiosis; delivery of a substrate from the 'ileum' (SIEM; Standardized Ileum Efflux Medium); mixing and transport of the intestinal contents; absorption of water and absorption of fermentation products, metabolites and other low molecular weight compounds (via a semipermeable membrane inside the colon model).

SIEM simulates material passing the ileocecal valve in humans, or in other words material reaching the colon. SIEM was prepared as described previously [2,7–9] and contained the major non-digestible carbohydrates (pectin, xylan, arabinogalactan, amylopectin, starch) found in a normal western diet as well as protein (bactopepton, casein), ox-bile, Tween 80 as well as vitamins and minerals. SIEM does not require pre-digestion and was added to the system at a speed of 2.5 mL/h. The speed of the dialysis liquid was 1.5 mL/min. During the experiment, the intestinal contents are mixed continuously by the peristaltic movements of the TIM-2 system. In order to simulate the transit of the chime from proximal to distal colon, 25 mL of the lumen is removed every 24 h and discarded.

Prior to the performance of each experiment the secretion fluids and dialysis solutions were prepared freshly, the pH electrodes calibrated, new membrane units installed and the system was

inoculated (one day before the start of the test period) with a standardized microbiota of human origin. This standardized microbiota was prepared as described [7] using fecal donations from a group of 4 healthy volunteers (1 male, 3 females, age 38.8 ± 3.9 years; BMI (body mass index) 24.2 ± 1.5 kg/m^2). Individuals provided signed informed consent prior to participation, were non-smokers and had not used antibiotics, prebiotics, probiotics or laxatives within 1 month before the donation.

At the start of the adaptation period, the TIM-2 system was inoculated with approximately 30 mL of the standardized microbiota and 80 mL dialysis fluid. The microbiota was allowed to adapt to the model conditions and SIEM for 16 h. After the adaptation period the 120 h test period started, in which test product was added to TIM-2 in a daily dose.

2.3. Addition of Test Product

The test product was added to the system at daily doses of 2 g, 3 g, 4 g, and 5 g Lactulose, mixed in the SIEM, which was added throughout the entire test period. Each dose was studied in duplicate (n = 2), while the control experiment was carried out as quadruplicate (n = 4). The test period of the TIM-2 experiments lasted 120 h (5 consecutive days).

2.4. Sampling from TIM-2

Metabolites like the short-chain fatty acids, branched-chain fatty acids (BCFA), ammonia and lactate produced in TIM-2 were continuously removed from the lumen by a semipermeable membrane unit. This dialysate was collected at the start of the test period and after 24, 48, 72, 96, and 120 h. Volumes were measured and samples were taken from the dialysate.

Luminal samples taken at the beginning and end of the experiment (t = 0 h and t = 120 h) were used to investigate the composition of the microbiota. The samples were snap frozen in liquid nitrogen and stored at ≤−72 °C until analysis.

2.5. Sodium Hydroxide Usage (pH)

The pH was kept at pH 5.8 by automatic titration with 2 M NaOH, the consumption of NaOH was monitored.

2.6. Short-Chain Fatty Acids and Branched-Chain Fatty Acids

The dialysate and lumen fractions of TIM-2 were analyzed with gas chromatography for SCFA (acetate, propionate and butyrate) and BCFA (iso-butyric acid and iso-valeric acid).

For SCFA/BCFA, samples were prepared and analyzed as described previously [10]. Briefly, dialysate samples were directly used, lumen samples were centrifuged (12,000 rpm at 4 °C for 10 min). A mixture of formic acid (20%), methanol, and 2-ethyl butyric acid (internal standard, 2 mg/mL in methanol) was added to the supernatant. A 3 µL sample with a split ratio of 75.0 was injected on a GC-column (ZB-5HT inferno, ID 0.52 mm, film thickness 0.10 um; Zebron; Phenomenex, Utrecht, The Netherlands) in a Shimadzu GC-2014 gas chromatograph (Shimadzu Europe, Duisburg, Germany).

2.7. Lactate and Ammonia

Samples for lactate and ammonia analysis were centrifuged as described above. In the clear supernatant, both L- and D-lactate were determined enzymatically (based on Boehringer, UV-method, Cat. No. 1112821035, Roche Diagnostics, West Sussex, UK). Ammonia was determined based on the Berthelot reaction [11] in which ammonia first reacts with alkaline phenol and then with sodium hypochlorite to form indophenol blue. In the currently used method, due to its toxicity, phenol was replaced with salicylic acid.

2.8. 16S rDNA Amplicon Sequencing

The bacterial population in the TIM-2 samples was analyzed using Next Generation sequencing. Total DNA from the collected TIM-2 lumen samples at the start (t = 0 h) and at the end (t = 120 h) of the experiments was isolated as described [12] with some minor adjustments: The samples were initially mixed with 250 µL lysis buffer (Agowa, Berlin, Germany), 250 µL zirconium beads (0.1 mm), and 200 µL phenol, before being introduced to a Bead Beater (BioSpec Products, Bartlesville, OK, USA) for twice 2 min. To determine the recovery of bacterial DNA from the samples, a quantitative polymerase chain reaction (qPCR) using primers specific for the bacterial 16S rRNA gene was used. Changes in the microbiota composition were analyzed by using mass V4 16S rDNA amplicon sequencing. For 16S rDNA amplicon sequencing of the V4 hypervariable region, 100 pg of DNA was amplified as described [13] using 30 amplification cycles, applying F533/R806 primers [14]. Primers included Illumina adapters and a unique 8-nt sample index sequence key [13]. Amplicon yield, integrity and size was analyzed on a Fragment Analyzer (Advanced Analytical Technologies, Inc., Heidelberg, Germany). The amplicon libraries were pooled in equimolar amounts and purified using agarose gel electrophoresis and subsequent the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Paired-end sequencing of amplicons was conducted on the Illumina MiSeq platform (Illumina, Eindhoven, The Netherlands).

Processing of the sequencing data was done using the Mothur pipeline. The differences between the two bacterial community profiles were identified by using the LEfSe (Linear Discriminant Analysis Effect Size) analysis [15]. The method is based on categorical non-parametric hypothesis test and Linear Discriminant Analysis (LDA) which is a mathematical technique to characterize the difference between classes. This is a method for metagenomic biomarker discovery and therefore it allows finding organisms that significantly can describe the differences between two microbial communities. For this a cut-off level of relative abundance of individual genera was included with 0.01% of total sequences. In the analysis, the different test conditions were each (as replicate) compared to the control experiments. This shows which genus became significantly more or less abundant as a consequence of a test product condition compared to the control.

2.9. Statistical Analysis

Due to the amount of experimental replicates (n = 2), no statistics were performed. Instead, mean values of the experiments were compared to mean values of the control experiments.

3. Results

3.1. Sodium Hydroxide Usage

During fermentation of carbohydrates the microbiota produces acidic metabolites (for example SCFA and lactate), therefore the use of NaOH during the experiments indicates the activity of the microbiota fermenting the SIEM plus the test product which were added to the TIM-2 system.

The addition of Lactulose in different doses in the test period showed an increased use of NaOH during the TIM-2 experiments as compared to the control as shown in Figure 1. The dose effect was clearly visible in the NaOH consumption as it was highest for the 5 g dose and decreasing per dose to the 2 g dose. The usage of NaOH was 303 ± 28 mL, 250 ± 19 mL, 214 ± 7 mL and 154 ± 9 mL for the experiments with 5 g, 4 g, 3 g and 2 g Lactulose, respectively.

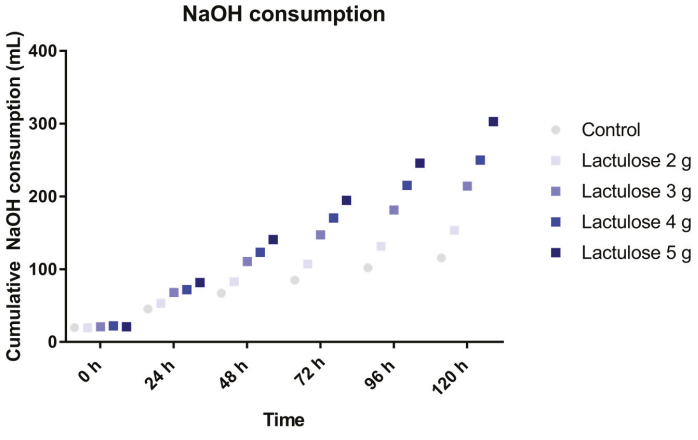


Figure 1. Sodium hydroxide cosumption during TIM-2 runs (mean of $n = 2$ (Lactulose dosages) or $n = 4$ (control)) with different dosages of Lactulose. Values at the start of the test period are on average 20.76 mL due to NaOH consumption during the pre-incubation period. All data points shown at the proximity of the individual time points indicated at the *X*-axis belong to these specific time points.

3.2. SCFA Production

Figure 2a shows the cumulative total SCFA (acetate, propionate and butyrate) production over time during the 120 h test period in TIM-2. The different Lactulose doses all show a higher SCFA production as compared to the control, with the highest SCFA production for the highest Lactulose dose of 5 g per day. The mean amounts of total SCFA produced for the increasing dose of Lactulose is 451 ± 3 mmol (2 g), 399 ± 21 mmol (3 g), 427 ± 76 mmol (4 g), and 471 ± 12 mmol (5 g), for the respective daily doses of Lactulose, respectively, compared to 332 ± 34 mmol (control).

The production profiles from each of the different SCFA measured (Figure 2b; acetate, Figure 2c; propionate and Figure 2d; butyrate), indicate that the acetate is the predominantly produced SCFA. The propionate production (Figure 2c) in the TIM-2 experiments with the higher doses (5, 4 and 3 g) of Lactulose was lower compared to the control. Whereas Lactulose tended to slightly increase butyrate production (Figure 2d) compared to the control as shown for the 3, 4 and 5 g dose (~80 mmol at $t = 120$ h).

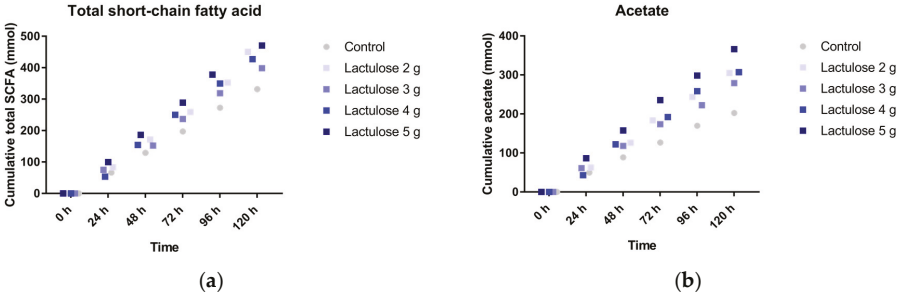
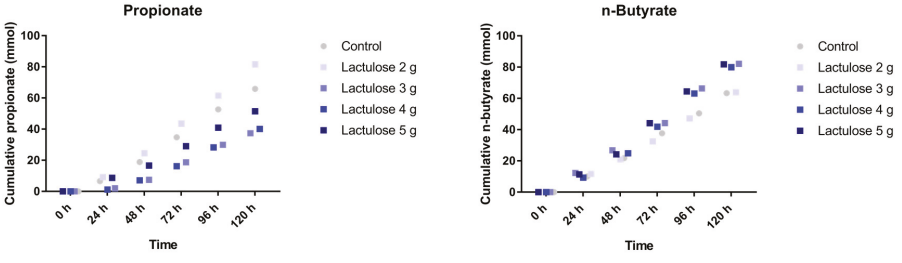


Figure 2. *Cont.*



(**c**) (**d**)

Figure 2. Production of (**a**) total short chain fatty acids (SCFA); (**b**) acetate; (**c**) propionate; and (**d**) butyrate in TIM-2 runs (mean of $n = 2$ (Lactulose dosages) or $n = 4$ (control)) with different dosages of Lactulose. Values at the start of the test period were set to zero. All data points shown at the proximity of the individual time points indicated at the *X*-axis belong to these specific time points.

3.3. BCFA Production

The total production of BCFA in 120 h is shown in Figure 3. The BCFA production was reduced in the experiment when a higher amount of Lactulose was added to the TIM-2 system. BCFA production was 8.4 ± 4.2 mmol (control), 4.6 ± 2.2 mmol (2 g Lactulose), 2.8 ± 0.8 mmol (3 g Lactulose), 2.3 ± 0.7 mmol (4 g Lactulose), and 1.5 ± 0.2 mmol (5 g Lactulose).

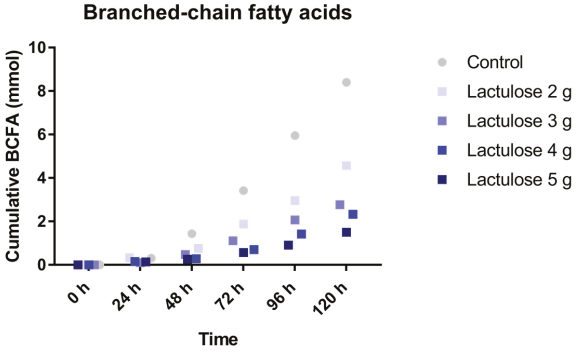


Figure 3. Cumulative branched-chain fatty acids (BCFA) (iso-butyrate and iso-valerate) production over time during the 120 h test period in TIM-2 runs (mean of $n = 2$ (Lactulose dosages) or $n = 4$ (control)). All data points shown at the proximity of the individual time points indicated at the *X*-axis belong to these specific time points.

3.4. Lactate

The cumulative amount of lactate (Figure 4) produced in the experiment with the lowest Lactulose dose (2 g per day, 3.8 ± 1.9 mmol) was similar to the control (5.8 ± 2.2 mmol). For the different doses of Lactulose, particularly at the highest dose, much higher amounts of lactate are formed during Lactulose fermentation. The amount of lactate produced appears to be dose-dependent. The highest lactate production was observed for the two highest Lactulose doses of 4 and 5 g per day. Lactate production was 17.9 ± 10.0 mmol (3 g), 60.7 ± 36.2 mmol (4 g), 55.7 ± 31.0 mmol (5 g) for the daily Lactulose dose, respectively.

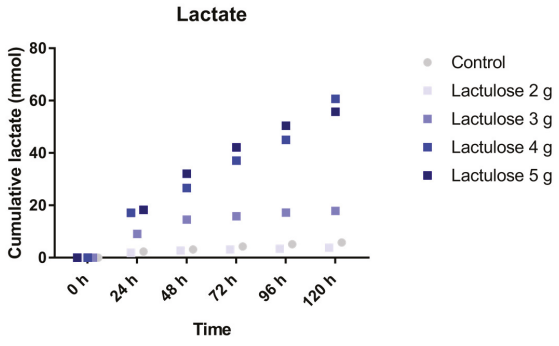


Figure 4. Cumulative lactate production over time during the 120 h test period in TIM-2 runs (mean of $n = 2$ (Lactulose dosages) or $n = 4$ (control)). All data points shown at the proximity of the individual time points indicated at the *X*-axis belong to these specific time points.

3.5. Ammonia

The cumulative (total) amount of ammonia was measured for each of the test condition as shown in Figure 5. The mean ammonia production with the different Lactulose dosages decreased with rising doses of Lactulose. Ammonia production for the different test conditions was 87.0 ± 27.9 mmol (control), 68.8 ± 4.7 mmol (2 g Lactulose), 54.5 ± 5.8 mmol (3 g Lactulose), 28.3 ± 12.6 mmol (4 g Lactulose), and 30.5 ± 5.1 mmol (5 g Lactulose).

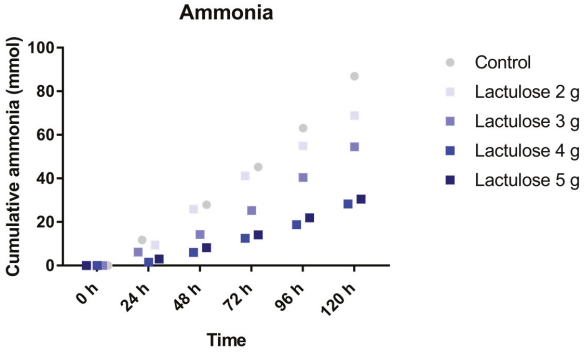


Figure 5. Cumulative ammonia production over time during the 120 h test period in TIM-2 runs (mean of $n = 2$ (Lactulose dosages) or $n = 4$ (control)). All data points shown at the proximity of the individual time points indicated at the *X*-axis belong to these specific time points.

3.6. Microbiota Composition

Analysis with mass V4 16S rDNA amplicon sequencing resulted in an overview of the relative abundance of the different bacterial genera present in the microbiota lumen samples collected from the TIM-2 experiments after 120 h exposure to the different test conditions. The genus level abundance change for the control and test conditions after 120 h in TIM-2 is shown in Table 1.

Table 1. Relative change of bacterial genera after 120 h fermentation experiments in TIM-2 (n = 2).

Genus	Relative Abundance (%)	Lactulose 2 g	Lactulose 3 g	Lactulose 4 g	Lactulose 5 g
Growth enhancement					
Bifidobacterium	19.8	0.5	2.62	2.37	7.96
Lactobacillus	6.7	0.86	1.86	2.84	1.98
Blautia	6.1	1.61	1.99	1.89	1.52
unclassified_Ruminococcaceae	1.6	0.54	1.88	1.23	1.11
Collinsella	0.8	0.71	5.26	7.41	3.11
Allisonella	0.3	0.66	1.38	1.46	1.06
unclassified_Clostridiales	0.3	0.74	1.01	1.27	1.14
unclassified_Erysipelotrichaceae	0.2	12	372	352	323
Clostridium_XI	0.1	0.8	1.2	1.6	2.4
unclassified_Bacteria	0.08	0	2	2	2
Methanobrevibacter	0.07	0.31	3.13	4.46	1.05
Ruminococcus2	0.06	1.36	8.36	14.73	6.18
Anaerostipes	0.03	>6	>5.5	>37	>39.5
Butyricococcus	0.02	0	2.8	2.2	9.8
Olsenella	0.01	4.86	6	11.43	7.71
unclassified_Coriobacteriaceae	0.01	1	6	3	9
Growth reduction					
Prevotella	25.9	1.35	0.95	0.84	0.25
Clostridium sensu stricto	3.9	0.14	0.33	0.62	2.05
Ruminococcus	2.7	0.18	0.11	0.03	0.03
Bacteroides	1.6	0	0.01	0.01	0.03
Weissella	1.3	0.99	0.38	0.24	1.4
Dialister	1.2	0.76	0.35	0.23	0.25
Acinetobacter	0.6	0	0	0	0
Escherichia/Shigella	0.5	0	0	0.55	2.18
Enterobacter	0.3	0.32	0.31	0.78	0.45
Peptoniphilus	0.3	0.19	0.15	0.3	0.55
Paraprevotella	0.3	0	0.09	0.1	0.4
unclassified_Enterobacteriaceae	0.2	0.58	0.12	0.62	0.16
Oscillobacter	0.2	0.12	0.05	0.04	0.21
Clostridium XIVa	0.2	0.14	0.22	0.71	0.63
Sutterella	0.2	0.09	0.32	0.53	0.28
Parabacteroides	0.1	0	0	0	0
unclassified_Prevotellaceae	0.1	0.26	0.58	0.76	0.64
Methanosphera	0.08	0.49	0.23	0.3	0.19
Succiniclasticum	0.06	0.06	0.02	0.13	0.03
Shewanella	0.06	0.15	0.31	0.92	6.62
unclassified_Clostridiales	0.05	0.03	0.23	0.03	0.56
unclassified_Firmicutes	0.05	0	0	0	0
Pseudomonas	0.04	0	0	0	0
Atopobium	0.02	0.05	0.18	0.36	0.32
Clostridium_IV	0.01	0.33	0	0	0
Alistipes	0.01	0	0	0	0
Finegoldia	0.01	0	0	0.27	2.53
No clear dose-dependent effect					
Enterococcus	14.0	0.27	0.7	1.53	4.51
Faecalibacterium	5.9	1.15	0.91	1.26	0.86
Dorea	0.9	0.41	0.94	1.24	0.61
unclassified_Lachnospiraceae	0.5	0.45	0.39	1.79	0.51
Staphylococcus	0.4	n/d	n/d	>0.5	n/d
Streptococcus	0.3	n/d	n/d	>0.5	>8
Moraxella	0.3	n/d	n/d	n/d	n/d
Anaerococcus	0.3	0.05	0.89	3.07	31.96
Roseburia	0.3	3.08	0.08	12.85	5.31
Gemmiger	0.3	0.13	0.39	2.08	0.18
Coprococcus	0.1	0.45	0.27	3.19	4.39
Halomonas	0.1	0.17	0.67	1.08	7
Corynebacterium	0.1	n/d	n/d	>0.5	n/d
Subdoligranulum	0.06	0.26	1.13	0.65	0
Lachnospira	0.06	1.38	0.03	0.74	1.69
Clostridium_XIVb	0.03	2	0	0.1	1.14
Catenibacterium	0.02	n/d	n/d	>2.5	>84
unclassified_Bacteroides	0.02	2	0	10	24
Sporobacter	0.01	n/d	n/d	n/d	n/d
Slackia	0.01	>0.5	n/d	>3	>2
Akkermansia	0.01	n/d	n/d	n/d	n/d

The ratio between the mean of the two runs of each Lactulose dosage and the control mean was calculated. Depicted are genera with a relative abundance of $\geq$0.01%. A value equal to 1 indicates no change, a value of >1 indicates an increase, a value of <1 indicates a decrease of the microbial genera. If the respective bacterial genus was not detected in control, but in the Lactulose groups, this is indicated with the prefix ">" in front of the value, whereas a value of zero states that the respective bacterium was below the detection limit in the Lactulose, but not in the control group. Bacterial genera below the detection limit in both the control and the Lactulose group are indicated with "n/d" for "not detected". "Growth enhancement" and "growth reduction" refers to values > or <1, respectively, in at least 3 consecutive dosages of the 4 dose groups.

The increase in the absolute bacterial count of the two genera Bifidobacterium and Lactobacillus is represented in form of a detailed heat map in Figure 6, in addition to the heat map for all analyzed species (Table 2) and the diagram for phylum shifting (Figure 7).

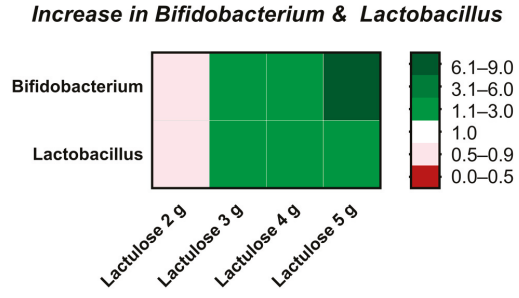


Figure 6. Heat map depicting fold changes of the increase in Bifidobacterium and Lactobacillus at different Lactulose doses compared to control after the 120 h test period in TIM-2 runs. A value equal to 1 (white) indicates no change, a value of >1 (green) indicates an increase, a value of <1 (red) indicates a decrease of the microbial genera.

Table 2. The heatmap indicates the average relative number n of the different bacterial genera in the microbiota in the 5 different lactulose conditions 0 g, 2 g, 3 g, 4 g, and 5 g, respectively, after 120 h of exposure in TIM-2. The increasing reddish gradient color indicates the increase in the dominant genera in a particular sample ($n > 1000$), the yellowish gradient color indicates the genera that are present at an intermediate level ($9 < n < 1000$) while the increasing greenish gradient color represents the genera that are becoming more marginally present in the microbiota ($n < 10$).

Genus	Lactulose 0 g	Lactulose 2 g	Lactulose 3 g	Lactulose 4 g	Lactulose 5 g
Prevotella	12,069	16,305	11,493	10,138	3072
Bifidobacterium	1468	733	3842	3474	11,689
Enterococcus	272	75	191	416	1225
Lactobacillus	135	116	251	384	267
Blautia	1934	3112	3849	3654	2940
Faecalibacterium	1941	2227	1766	2448	1660
Clostridium_sensu_stricto	50	7	17	31	103
Ruminococcus	2535	457	267	80	65
Bacteroides	660	3	7	7	17
unclassified_Ruminococcaceae	581	315	1094	715	647
Weissella	40	40	15	10	56
Dialister	740	566	256	167	184
Dorea	415	169	390	515	251
Collinsella	70	50	368	519	218
Acinetobacter	1	0	0	0	0
unclassified_Lachnospiraceae	298	134	117	534	151
Escherichia/Shigella	3	0	0	2	6
Staphylococcus	0	0	0	1	0
Moraxella	0	0	0	0	0
Enterobacter	43	14	13	33	19
Anaerococcus	29	2	26	88	911
Peptoniphilus	178	35	28	54	99
Paraprevotella	43	0	4	5	17
Roseburia	7	20	1	84	35
Allisonella	53	35	73	77	56
Streptococcus	0	0	0	1	8
Gemmiger	162	21	63	338	29
unclassified_Clostridiales	128	95	129	163	147
unclassified_Enterobacteriaceae	63	36	8	39	10
Oscillibacter	286	34	15	11	60
Clostridium_XlVa	153	22	34	109	96
Sutterella	47	4	15	25	13
unclassified_Erysipelotrichaceae	1	6	186	176	162

Table 2. *Cont.*

Genus	Lactulose 0 g	Lactulose 2 g	Lactulose 3 g	Lactulose 4 g	Lactulose 5 g
Coprococcus	30	14	8	95	131
Parabacteroides	4	0	0	0	0
Halomonas	6	1	4	7	42
Corynebacterium	0	0	0	1	0
Clostridium_XI	1	1	2	2	3
unclassified_Prevotellaceae	69	18	41	53	44
Methanosphaera	13	7	3	4	3
unclassified_Bacteria	0	0	1	1	1
Methanobrevibacter	20	6	61	87	21
Ruminococcus2	6	8	46	81	34
Subdoligranulum	29	8	33	19	0
Lachnospira	20	27	1	15	33
Succiniclasticum	79	5	2	11	2
Shewanella	3	1	1	3	22
unclassified_Clostridia	15	1	4	1	9
unclassified_Firmicutes	3	0	0	0	0
Pseudomonas	0	0	0	0	0
Anaerostipes	0	6	6	37	40
Clostridium_XlVb	5	11	0	1	6
Butyricicoccus	3	0	7	6	25
Catenibacterium	0	0	0	3	84
unclassified_Bacteroidetes	0	1	0	3	6
Atopobium	11	1	2	4	4
Sporobacter	0	0	0	0	0
Clostridium_IV	2	1	0	0	0
Olsenella	2	9	11	20	14
unclassified_Coriobacteriaceae	1	1	3	2	5
Alistipes	0	0	0	0	0
Finegoldia	4	0	0	1	10
Slackia	0	1	0	3	2
Akkermansia	0	0	0	0	0

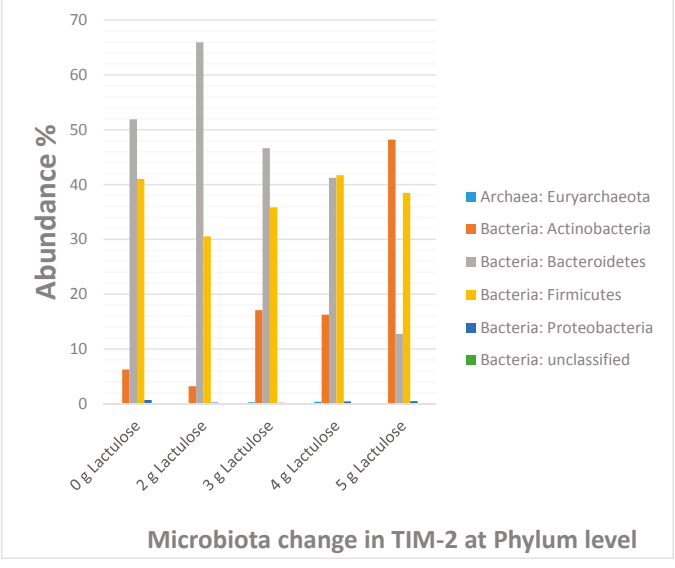


Figure 7. Column diagram of the bacterial distribution in percentage at the phylum level in control (0 g Lactulose) and different Lactulose dosages, 2 g, 3 g, 4 g and 5 g respectively. Apart from the 2 g lactulose data, an increase is observed with respect to the phylum *Actinobacteria* while a decrease is observed with respect to the phylum *Bacteroidetes*. The relative abundance of the *Firmicutes* is affected to a lesser extent.

4. Discussion

This study showed that low dosages of Lactulose have an effect on the gut microbiota after 5 days of treatment in an in vitro model of the human proximal colon depicted by an increase in NaOH consumption suggesting a decrease in pH, decreased production of ammonia and BCFA as well as an increase in acetate, butyrate and lactate. With regard to dose-dependency, even dosages as low as 2 g Lactulose lead to a rise in SCFA, mainly acetate, and a correlating acidification of the intestinal content. At this dose, however, a slight decrease rather than an increase in the bifidobacteria or lactobacilli was observed with our methods compared to the control, indicating that the rise in SCFA is based on bacteria other than *Bifidobacterium* and *Lactobacillus* here.

An increase in the relative amount of bifidobacteria and lactobacilli started at a dose of 3 g/day Lactulose. Moreover, at this dose, elevated levels of butyrate and a decrease in branched-chain fatty acids was observed after five days of treatment. Similar results have been described before, when healthy individuals treated with 3 g/day Lactulose for 2 weeks showed an enhanced growth of *Bifidobacterium*, but not *Lactobacillus* [5]. Neither short-chain or branched-chain fatty acids nor *Anaerostipes* were investigated in this trial [5].

When the TIM-2 system was inoculated with 4 g Lactulose per day, an additional increase in lactate and a marked decrease in ammonia levels were detected. When this dose was applied for three weeks to healthy humans, the increase in *Bifidobacterium* as observed with 3 g/day was again confirmed [4]. In addition, these volunteers showed a non-significant trend to higher levels of acetate, butyrate and lactate as well as a decrease in propionate and ammonia [4]. *Anaerostipes* was not analysed in this trial.

In our study, the growth support of *Bifidobacterium* was most prominent at 5 g Lactulose per day, as was the increase in acetate levels, while the other effects like increase in butyrate were comparable to the 4 g dose. Bifidobacteria produce acetate and thus may be the basis for the high acetate levels observed in this dose group. A dose of 5 g Lactulose per day was administered for 10 days to healthy volunteers [4], however, neither the production of SCFA or ammonia nor the microbiota were analyzed in this dose groups. Therefore for the time being clinical trials administering 5 g Lactulose per day to healthy human volunteers are still missing for a comparison of these results to the in vivo situation.

In the TIM-2 system, a dose of 10 g Lactulose led to lower butyrate levels after 48 h of treatment [2], suggesting a shift in bacteria to non-butyrate producing microbiota with accelerating dose of Lactulose. In healthy volunteers treated with 10 g/day Lactulose for 6 weeks, an increase in fecal *Bifidobacterium* was observed, while SCFA were not analyzed [3]. In another study, a higher dose of 20 g/day Lactulose for 4 weeks indeed led to decreased fecal butyrate levels [16].

These results indicate that 5 g/day Lactulose after 5 days of treatment leads to a balanced growth enhancement of *Bifidobacterium*, *Lactobacillus* and *Anaerostipes*, resulting in an increase in the metabolites acetate, butyrate and lactate as well as higher NaOH consumption, while ammonia and BCFA decrease. Although higher levels of butyrate and lactate as well as the decreased levels of ammonia are comparable to the 4 g/day dose, due to the most prominent increase in *Bifidobacterium* accompanied with a further increase in acetate the 5 g/day dose is considered superior to the lower Lactulose dosages. Higher dosages of Lactulose than 5 g/day bear the risk of decreased butyrate levels due to a one-sided increase in non-butyrate producing bacteria.

The effect of high dosages of Lactulose on butyrate levels has been the subject of recent debate [1,2,16]. In our experimental setting 3, 4 and 5 g of Lactulose led to higher butyrate levels after 96 and 120 h. This is most probably due to an increase in butyrate producing *Anaerostipes* [17] at these dosages of Lactulose. Consistent with these high butyrate levels, lactate, which can be fermented to butyrate [18], was increased as well. In contrast, a decrease in butyrate was observed in another study with the TIM-2 system [2]. These studies investigated a higher dosage of Lactulose (10 g/day) for a different time period (7 days in vivo followed by 48 h in vitro or 48 h in vitro without in vivo pre-treatment). Interestingly, the butyrate levels of healthy volunteers treated for 7 days with 10 g/day Lactulose in this trial did not differ when analyzed directly after sampling, as did the samples from

individuals without Lactulose pre-treatment after in vitro incubation with Lactulose for 48 h [2]. The fecal samples of the group pre-treated with Lactulose for 7 days, however, showed decreased levels of butyrate after 48 h in vitro incubation [2], indicating a dose and time dependency of this butyrate reducing effect of Lactulose. This is in line with the study of Ballongue and coworkers showing that 10 g/day of Lactulose lead to a decrease in butyrate levels in healthy individuals after 4 weeks [16]. In constipated patients, however, where this dose of Lactulose is mainly administered, no decrease in fecal butyrate levels was observed in patients treated for 4 weeks [19]. The reduction of butyrate levels seems therefore to occur mainly in healthy individuals, but not in constipated patients. In our study, focusing on evaluating the prebiotic effect of subclinical dosages of Lactulose, an increase and no decrease in butyrate levels was observed after 5 days of treatment, which is corroborated by a clinical trial investigating a low dose (4 g/day) of Lactulose for 3 weeks still leading to an increase in butyrate levels [4]. We therefore conclude that the administration of 5 g/day Lactulose is prebiotically beneficial in healthy individuals for up to 3 weeks, while a longer period of application will have to be evaluated in clinical trials in the future.

In contrast to the butyrate levels the propionate production with Lactulose only showed an increase after addition of 2 g Lactulose, while 3 and 4 g Lactulose reduced the amount of propionate. A reduction, though not as extensive as with 3 and 4 g Lactulose, was also seen after addition of 5 g Lactulose. As for the butyrate levels, which may be based on the higher levels of *Anaerostipes*, this notion might be explained by the composition of the respective microbiota. For example, *Prevotella*, a propionate producing bacterium [20] with a relative abundance of more than 25% in our study, is slightly increased in abundance in the 2 g Lactulose groups, while the addition of 3 to 5 g Lactulose led to decreased levels of *Prevotella*. The slight increase in propionate in the 5 g Lactulose group compared to the 3 and 4 g Lactulose group might be accounted for by a strong increase in levels of other propionate producing bacteria in this dose group, like *Halomonas* [21], compensating for the propionate loss due to lower numbers of *Prevotella*.

Similarly, the increase in lactate, which is most prominent in the 4 and 5 g dose groups and surprisingly less extensive in the 5 g dose group compared to the 4 g dose group on day 5, can be explained by the composition of the microbiota. Lactate is the major product of lactic acid bacteria, including lactobacilli, bifidobacteria, enterococci, and streptococci [22]. Some of the lactate producing bacteria like lactobacilli slightly decreased in abundance in the 5 g group compared to the 4 g group and could account for the slight drop in lactate levels observed in this group at the fifth day of treatment. In addition some lactate-utilizing bacteria like *Shewanella* [23] or *Escherichia* [24] increased in the 5 g group and could contribute to this phenomenon.

BCFA are the product of protein degradation by proteolytic bacteria [25]. Relative abundance of such bacteria like *Bacteroides* is reduced after administration of Lactulose, a notion which might account for the lower levels of BCFA in the Lactulose groups.

In addition to healthy volunteers and constipated patients both benefiting from the prebiotic effect of Lactulose, this compound is also administered to patients with hepatic encephalopathy. The rationale for the treatment is the reduction of intestinal absorbable ammonia levels in order to decrease the total ammonia burden in these patients. Control of ammonia levels, is, however, also important for healthy individuals, as excess ammonia has been shown to change the morphology and metabolism of intestinal cells and reduce their lifespan [26]. In our study, the reduction of intestinal ammonia load was most prominent at dosages of 4 or 5 g Lactulose after 72, 96, and 120 h. In another experiment using the TIM-2 system, 7.5 g Lactulose for 3 days led to the lower ammonia levels (36 mmol) compared to apple fiber (65 mmol), sugar beet pectin (74 mmol) or galacto-oligosaccharides (44 mmol) in the stool of lean subjects [1]. These values are comparable to those observed in our study after 120 h with 4 or 5 g Lactulose, indicating that at least in the TIM-2 system an increase in Lactulose above 4 g/day does not lead to further improvement of the ammonia levels. In human healthy volunteers a daily dose of 3 g Lactulose for 14 days did not lead to significant improvement of total fecal ammonia [5]. This study, however, did not use the determination of a shift from urinary

to fecal ^{15}N-excretion, the current state of the art method [27]. Such a shift was observed in healthy individuals treated with a daily dose of 20 g Lactulose for 4 weeks [28], but lower dosages have not yet been investigated. Thus, the ammonia lowering effect of a lower dose of Lactulose like 4 or 5 g remains to be determined in a clinical trial.

In conclusion, the results of this study clearly demonstrate a dose-dependent increasing positive effect of 2 to 5 g Lactulose on the intestinal flora and its metabolic activity. Starting with elevated levels of SCFA at low dosages (2 to 3 g), Lactulose further expands its beneficial impact on microbial composition and metabolism at higher dosages (4 up to 5 g). According to this experimental setting a dose of 5 g Lactulose per day is supposed to exert beneficial clinical effects like increase in *Bifidobacterium*, *Lactobacillus*, *Anaerostipes*, butyrate, acetate and lactate after 5 days of treatment. These results remain to be confirmed in a human study with healthy volunteers.

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Conflicts of Interest: M.B., D.B., S.S.-G., A.K., B.G., A.K.-K. and J.S. are employees of Fresenius Kabi, the founding sponsor of this study.

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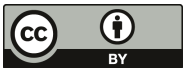

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Administration of Inulin-Supplemented Gluten-Free Diet Modified Calcium Absorption and Caecal Microbiota in Rats in a Calcium-Dependent Manner

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Abstract: In coeliac disease (CD), the risk of adverse calcium balance and reduced bone density is induced mainly by the disease, but also by a gluten-free diet (GFD), the only accepted CD therapy. Prebiotics through the beneficial impact on intestinal microbiota may stimulate calcium (Ca) absorption. In the present study, we hypothesised that the dietary inulin in GFD would influence positively the intestinal microbiota, and by that will stimulate the absorption of calcium (Ca), especially in the conditions of Ca malnutrition. In a six-weeks nutritional experiment on growing a significant ($p < 0.05$) luminal acidification, decrease in ammonia concentration and stimulation of short chain fatty acids formation indicated inulin-mediated beneficial effects on the caecal microbiota. However, the effect of inulin on characteristics of intestinal microbiota and mineral utilization depended on the dietary Ca intake from GFDs. Inulin stimulated bifidobacteria, in particular *B. animalis* species, only if a recommended amount of Ca was provided. Most benefits to mineral utilization from inulin consumption were seen in rats fed Ca-restricted GFD where it increased the relative Ca absorption. Administration of inulin to a GFDs could be a promising dietary strategy for beneficial modulation of intestinal ecosystem and by that for the improvement the Ca absorption.

Keywords: prebiotics; gluten-free bread; intestinal microbiota; SCFA; calcium balance

1. Introduction

Calcium (Ca) is an important macroelement of the human body and in majority it is deposited in bones providing the structural integrity of the skeleton. Ca homeostasis is precisely controlled with coordinated action of processes such as absorption in the intestine, reabsorption from the kidney and exchange from bones. The intestinal Ca absorption is essential to ensure the appropriate concentrations of intra- and extracellular calcium fluids without bone depletion [1]. On the other side, bones have a metabolic function since Ca is continuously exchanged between bone and blood, and can be released from bone to maintain extracellular calcium concentrations, regardless of intake.

Calcium requirements vary throughout an individual's life, with greater needs during the periods of rapid growth in childhood and adolescence [2]. Bone density increases until the end of puberty, when it reaches its peak value. If a normal peak bone mass is not achieved, the individual is at a higher risk for developing osteoporosis; thus, the amount of bone accrued during the paediatric years is an important predictor of an individual's future resistance to fractures [3]. Nevertheless, even

an adequate dietary Ca intake may not ensure a proper calcium balance. Besides the amount, its absorption is a critical factor determining Ca bioavailability for bone development and maintenance. In some cases, an adverse calcium balance is observed that may result from a poor intestine absorption caused by infection, inflammation or pathology in the intestine morphology. Among the group of the calcium deficiency risk there are individuals suffering from chronic intestinal diseases, including coeliac disease (CD).

In CD, defined as a permanent gluten (mixture of proteins found in wheat, rye, and barley) intolerance with genetic etiology [4], a chronic intestinal inflammation and malabsorption of calcium and vitamin D are observed that together with general malnutrition affects negatively bone health [5]. Recently, a persistent villus atrophy has been associated with serious sequelae, including osteoporotic fractures [6]. Several studies have demonstrated a low bone mineral density (BMD) both in children and adults with CD [7,8]. Additionally, a risk for less-than-optimal peak bone mass acquisition and a retarded growth in CD children is observed. These adverse alterations are mainly induced by the underlying disease, but, to a certain degree, also by a gluten-free diet (GFD) [9,10], which is the only accepted therapy for CD [11]. Above 80% of CD children adhering to a GFD consume lower than recommended amounts of Ca [12,13] and vitamin D, which could be due to the reduced nutritional quality of a GFD [14,15].

Nutrition plays an important role in proper bones mineralization [16]. To reduce a risk of osteoporosis, an increased calcium intake is proposed as one of the most effective strategy. However, when Ca intake remains inadequate, an improvement of Ca absorption becomes an important method of Ca balance restoration and bone health improvement. Among the dietary compounds, prebiotics are widely studied functional ingredients which can enhance the mineral absorption and bone properties [17,18]. Inulin, a polydisperse carbohydrate material consisting mainly of beta (2-1) fructosyl-fructose links, is an example of prebiotic naturally occurring in tubers, bulbs and tuberous roots of several edible fruits and vegetables [19]. Inulin and other inulin-type fructans (ITFs) are resistant to digestion in the small intestine and undergo fermentation in the large intestine, resulting in short-chain fatty acid (SCFA) production. On the other hand, they stimulate growth and/or activity of selected commensal bacteria, including the health-promoting bifidobacteria and lactobacilli [20]. Several animal studies have shown that the acidification of the gut environment resulting from bacteria fermentation of ITFs enhanced calcium and magnesium absorption and bone mineralization [21–23] nevertheless, the human studies have shown contentious results [24–26]. In the present study, we hypothesised that the dietary administration of inulin would influence positively the intestinal microbiota, and by that will stimulate the absorption of Ca from colon and caecum of rats, especially in the conditions of Ca malnutrition. To verify this hypothesis, a 6-weeks nutritional experiment was performed aimed to assess the impact of dietary inulin on gastrointestinal tract parameters, characteristics and activity of gut microbiota (SCFAs, PSCFAs, microbial enzymes), and mineral utilization in growing rats fed GFD with reduced Ca content, established as an experimental model that in some extend may correspond to a dietary conditions observed in many paediatric CD patients treated with GFD.

2. Materials and Methods

2.1. Composition of Experimental Gluten-Free Diets

The experimental GFDs were composed mainly of gluten-free breads with Ca content recommended for rats (O) or gluten-free breads with reduced (R) Ca content, supplemented or not supplemented with inulin (Frutafit HD, Hortimex, Konin, Poland) (Table 1). Gluten-free breads were baked in the laboratory conditions [27], dried (at RT/for 24 h) and ground into a powder. The only source of dietary Ca were Ca salts applied in gluten-free breads, in particular calcium caseinate (Lacpol Company, Murowana Goślina, Poland) and calcium citrate (Hortimex, Konin, Poland). All experimental GFDs were additionally supplemented with DL-Methionine, soybean oil ("Kruszwica" SA Company, Kruszwica, Poland), a Ca-free mineral mix and a vitamin mix (Table 1).

The energy density of each GFD was calculated by multiplying the amount of each macronutrient (Table S1) by the corresponding conversion factor according to FAO recommendations [28].

Table 1. Experimental gluten-free diets.

	O	R	OI	RI
Bread ingredients as diet compounds (%)				
Corn starch	48.4	49.8	40.4	41.8
Potato starch	11.7	11.7	11.7	11.7
Pectin	3.0	3.0	3.0	3.0
Sugar	3.7	3.7	3.7	3.7
Salt	1.0	1.0	1.0	1.0
Sunflower oil	1.7	1.7	1.7	1.7
Yeast	3.7	3.7	3.7	3.7
CAS *	12.0	12.0	12.0	12.0
CIT &	2.0	0.6	2.0	0.6
Inulin #	0.0	0.0	8.0	8.0
Other diet compounds (%)				
DL-Methionine	0.3	0.3	0.3	0.3
Soya oil	8.0	8.0	8.0	8.0
Ca-free mineral mix †	3.5	3.5	3.5	3.5
Vitamin mix §	1.0	1.0	1.0	1.0
Energy density (kcal/g)	4.01	4.00	3.82	3.80

O—GFD of recommended Ca content; OI—GFD of recommended Ca content with inulin; R—GFD of reduced Ca content; RI—GFD of reduced Ca content with inulin, * Calcium caseinate (contained: 92.8% protein, 2.06% fat, 4.01% ash, 5.12% moisture), & Calcium citrate (E 333(iii); contained 21.98 ppm of Ca), # Frutafit HD; contained 99.5% carbohydrates ($\geq$90% inulin, $\leq$10% fructose, glucose, sacchcrose), † Mineral mix AIN-93G-MX without Ca [29], § Vitamin mix AIN-93G-VM [29].

2.2. Animals

The study was conducted on 32 male growing Wistar rats (aged 4 weeks) with similar initial body weight (103 $\pm$ 4 g). All experimental protocols applied in the study were approved by the Institutional Laboratory Animal Care and Use Committee (Olsztyn, Poland, Permit Number: 15/2007/N). The experiment was conducted in compliance with European guidelines for the care and use of laboratory animals.

2.3. Experimental Design

Our approach was conducted in the growing rats model that in some extend may resemble children who are characterised by a greater Ca need to achieve the peak bone mass and bone strength during growth. The experimental model of dietary Ca shortage was established to mimic the conditions of insufficient dietary Ca intake that may occur in paediatric CD patients treated with GFD. Rats were divided into four experimental groups (8 per group): O-fed GFD with the recommended Ca content; OI-fed GFD with the recommended Ca content and inulin; R-fed GFD with reduced Ca content; and RI-fed GFD with reduced Ca content and inulin. The animals were housed individually in metabolic plastic cages under standard conditions at a temperature of 21–22 °C and a relative air humidity of 50–70% with intensive room ventilation and a 12-h lighting regiment. For 6 weeks, rats of each group were fed *ad libitum* one of the four experimental diets, with continuous access to distilled water.

2.4. Analysis of Food Intake, Body Weight Gain and Minerals Bioavailability

The food intake and body weight gains of individual rats were recorded daily throughout the study. The bioavailability of Ca, Mg and P, denoted by coefficients of apparent absorption and retention, was analysed in a nutritional experiment. After the preliminary period (10 days), faeces and urine were collected daily for 5 consecutive days from all rats kept in balance cages (Tecniplast Spa, Buguggiate,

Italy). The content of Ca, Mg and P in diets, as well as in faeces and urine collected in the balance period was determined by atomic absorption spectroscopy (AAS). The coefficient of apparent absorption was calculated as the difference between the intake of minerals and their quantity excreted with faeces, and it was expressed in relative (%) values. The coefficient of apparent retention was calculated as the difference between the intake of minerals and their quantity excreted with faeces and urine, and expressed in relative (%) values.

2.5. Sampling Procedures

At the end of the experiment, rats were anaesthetised with sodium pentobarbitone according to euthanasia guidelines for experimental animals (50 mg/kg body weight) [30]. Blood samples were collected from the tail vein, placed in plastic tubes and left for 1 h at room temperature to aggregate erythrocytes. After laparotomy, the small intestine, caecum and colon with the contents were collected and weighed. The pH of the small intestinal, caecal and colonic digesta was measured immediately (ca. 10 min), directly in segments (model 301 pH meter; Hanna Instruments, Vila do Conde, Portugal). Samples of fresh caecal digesta were used for an immediate determination of ammonia content, dry mass, bacterial enzyme activity and SCFA content, while the rest of the caecal digesta was transferred to tubes and stored at −70 °C for the molecular characteristics of microbiota. Duodenal, caecal and colonic walls were flushed clean with ice-cold physiological saline, blotted on filter paper and weighed for tissue mass. The blood was centrifuged for 15 min at 2500× *g* and 4 °C, and the obtained serum was then stored at −70 °C until minerals analysis.

2.6. Analytical Procedures

2.6.1. Ammonia and Dry Matter (DM) Content

The content of ammonia, which was extracted from the caecal digesta and trapped in a solution of boric acid, was quantified by direct titration with sulphuric acid acc. to Hofirek & Haas method [31]. The dry matter (DM) content of caecal digesta was determined at 105 °C.

2.6.2. Short Chain Fatty Acids

The concentrations of SCFAs in samples of caecal digesta samples were determined by gas chromatography (Shimadzu GC-14A, Kyoto, Japan) according to a previously described method [32]. Briefly, caecal digesta samples (0.2 g) were mixed with 0.2 mL formic acid, diluted with deionised water and centrifuged at 7211× *g* for 10 min. The supernatant was loaded onto a 2.5 m × 2.6 mm glass column, containing 10% SP–1200/1% H_3PO_4 on 80/100 Chromosorb W AW (Supelco, Bellefonte, PA, USA). Column temperature was 110 °C; the temperature of the flame ionisation detector (FID) was 180 °C, and the injection temperature was 195 °C. The caecal SCFA pool was calculated as the concentration of SCFA in the caecum (μmol/g) multiplied by the weight of caecal contents (g), and it was expressed in μmol per 100 g of body mass. The concentrations of caecal putrefactive SCFAs (PSCFAs) were calculated as the total content of isobutyric, isovaleric and valeric acid. All SCFAs analyses were performed in duplicate.

2.6.3. Activity of Bacterial Enzymes

The activity of selected bacterial enzymes in the caecal digesta was measured based on the rates of *p*- and *o*-nitrophenol release from their nitrophenyl glucosides according to a previously described method [32]. The activity of α- and β-glucosidases, α- and β-galactosidases, and β-glucuronidase was expressed in μmol of the product synthesised per min (unit) per gram of digesta in a fresh caecal sample.

2.6.4. Microbiota Characteristics with PCR-DGGE

DNA was extracted from ca. 0.1 g of the caecal digesta using the GeneMATRIX Bacterial and Yeast Genomic DNA Purification kit (Eurx, Gdańsk, Poland) and the bead-beating method according to the manufacturer's protocol. Variable regions of 16S rRNA gene were amplified with the use of universal, group- and genus-specific primer sets (Table S2), and were subsequently separated in polyacrylamide gels with a denaturing gradient of formamide and urea (Table S3) as described by Markiewicz et al. [33]. The profiles of total bacteria (TB), *Lactobacillus* (LAC), *Bacteroides* (BPP), and the *C. leptum* group (Clept) were determined. Amplifications were carried out in the C1000 thermal cycler (Bio-Rad, Warsaw, Poland) in a total volume of 30 µL comprising of a reaction buffer (3 µL), of JumpStart Taq DNA polymerase (1.25 U, Sigma, Poznań, Poland), $MgCl_2$ (Table S1), dNTP (200 µM), the template DNA (2 µL), filled up with sterile deionized water to 30 µL. The reaction program included: one cycle at 95 °C for 5 min, 35 cycles at 95 °C for 20 s, annealing temperature (Table S1) for 20 s, 72 °C for 20 s and the final cycle at 72 °C for 20 min. The PCR product (20 µL) was separated in 8%, polyacrylamide gel (acrylamide/bisacrylamide 37.5:1) in the 0.5× TAE buffer at 60 °C. Denaturing gradients and electrophoresis conditions are shown in Table S2. Gels were stained with SybrGreen I dye (Sigma) in the 1× TAE buffer according to the supplier's recommendations and photographed under UV light (Gel Doc™ XR, Bio-Rad). Selected DGGE bands were cut out from a gel with a sterile scalpel and incubated overnight in 40 µL of the TE buffer at 4 °C. Two microlitres of the aliquots were used as a template in the re-amplification reactions which were conducted under the previously described conditions. The re-amplified DNA was purified using the GeneMATRIX PCR/DNA Clean-Up Purification Kit (Eurx). The DNA was commercially sequenced by Genomed (Warsaw, Poland). The obtained sequences were identified using a BLASTn tool.

2.6.5. Quantification of Caecal Microbiota by Real-Time PCR

Real-time PCR method with the use of universal and group- and genus-specific primers was performed according to the procedure described by Fotschki et al. [34]. Briefly, a reference standard containing DNA isolated from known number of intestinal bacteria cells was prepared. DNA was isolated by the method described in above (point 2.6.4) from a total of 3.33×10^9 bacterial cells consisting of 3.18×10^8 cells of *Bacteroides*, 3.99×10^7 of the *Clostridium leptum* group (clostridial cluster IV), 1.44×10^9 of *Bifidobacterium*, 1×10^9 of *Enterococcus*, and 5.35×10^8 of *Lactobacillus* cells. Decimal dilutions of the DNA standard were used to plot a standard curve with each primer pair used (Table S4). Amplifications were performed in the iQ5 real-time PCR system (Bio-Rad) in a total volume of 25 µL (12.5 µL of SYBR Green Jump-Start Taq ReadyMix (Sigma), 1 µL of 10-fold diluted DNA, 200 µM of each primer, and PCR-grade water). The temperature program included 1 cycle of 95 °C for 3 min and 35 cycles of 95 °C for 20 s, primer annealing temperature (Table S4) for 30 s, and 72 °C for 30 s with signal acquisition. After each run a melting curve was prepared to confirm the specificity of amplicons. Samples were run in duplicate. The obtained values were normalized according to the dilution and weight of the sample. The results were expressed as log10 of the number of cells per gram of wet weight of a sample.

2.6.6. Mineral Concentration

Mineral concentrations in the biological materials were measured by flame (air—acetylene burner) atomic absorption spectrometry method (AAS) using an atomic absorption spectrophotometer (iCE 3000 SERIES, Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an autosampler and the appropriate cathode lamp operating at the resonance line of the analysed bioelements (Ca: 422.7 nm; Mg: 285.2 nm). Before analysis, the samples were wet-digested with a mixture (9:1; *v*/*v*) of concentrated nitric acid (65% HNO_3; Merck, Darmstadt, Germany) and hydrochloric acid (30% HCl; Merck) using a microwave system (Multiwave, Anton Paar GmbH, Graz, Austria). Calcium concentration was validated by adding a solution of lanthanum (III) chloride hydrate ($LaCl_3 \times 7H_2O$; Merck, Germany)

to all samples in sufficient amounts to obtain 0.5% concentration of La^{3+}. Phosphorus content was determined by the colorimetric molybdate method with hydroquinone (POCH S. A., Gliwice, Poland) and sodium (IV) sulphate (POCH S. A., Gliwice, Poland). Absorbance was measured using the VIS 6000 Spectrophotometer (KRÜSS–OPTRONIC, Hamburg, Germany) at λ = 610 nm. The concentrations of Ca, Mg and P were automatically read from a calibration curve (Ca: range 0.5–4.0 μg/mL; Mg: range 0.05–0.8 μg/mL; P: range 0.4–2.0 μg/mL) prepared with the AAS standard solution of Ca, Mg and P, respectively (J.T.Baker® Chemicals, Avantor, Center Valley, PA, USA) and expressed as mM/L or mg/g. The analyses were repeated (N = 8) for analytical quality control.

2.7. Statistic Analysis

The physiological responses of the treated animals were expressed by a mean of 8 values with standard deviation (±SD). The calculations were performed in STATISTICA 6.0 (StatSoft Corp., Kraków, Poland) software. Two-way ANOVA was performed to assess the effect of Ca level (recommended or reduced; Ca), the effects of dietary inulin (diets with and without inulin; I), and the interactions between the investigated factors (Ca × I). When significant treatment effects were found in the ANOVA, the post-hoc comparisons were performed using the Duncan's multiple range test. The data were checked for normality before statistical analyses. Differences $p < 0.05$ were considered significant.

The frequency of bacterial taxa (*Olsenella*) in the microbiota was compared with the Fisher's exact test performed in STATISTICA 6.0 (StatSoft Corp.). Differences were regarded as statistically significant at $p < 0.05$. DGGE banding profiles were processed using the BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Gels were normalized to one sample (as an external standard) run for each gel set. Profile similarities were calculated using the Pearson's product-moment correlation coefficient, and dendrograms were constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. The similarity matrices obtained during analyses of DGGE patterns of eubacteria, *Bacteroides*, *C. leptum* group and *Lactobacillus* were used for composite data set analysis to calculate average profile similarities. The generated similarity matrix was used to develop a multidimensional scaling (MDS) diagram [35].

3. Results

3.1. Effect of a Gluten-Free Diet Enriched with Inulin on a Daily Food Intake, Body Weight Gain and Gastrointestinal Tract Parameters

The intake of dietary Ca (recommended or restricted) and the addition of inulin to experimental GF diets had no significant effect ($p > 0.05$) on the daily food intake or body weight gains (BWG) of animals during the 6 weeks study (Table 2). However, these dietary factors influenced the majority of intestinal parameters, both alone and in combination. A two-way ANOVA revealed that independently of dietary Ca intake, the experimental GFDs containing inulin significantly increased the relative weight of the small intestine and the acidity of digesta in this segment of the gastrointestinal tract ($p < 0.05$ in both cases). The interaction between dietary Ca intake and inulin significantly influenced the weight of the examined tissues and accumulated digesta in both analysed segments of the large intestine ($p < 0.001$ for the caecum and $p < 0.05$ for the colon, respectively). The DM content of caecal digesta and the concentration of caecal ammonia were inulin-dependent, and a significant reduction in DM content and ammonia concentration ($p = 0.0346$ and $p < 0.001$, respectively) was noted in groups fed a GFD containing inulin (Table 2). The significant acidification of the caecal and colonic environment was attributed to both Ca intake ($p = 0.0019$ and $p < 0.001$, respectively) and inulin intake ($p < 0.001$ in both cases).

Table 2. Daily food intake, body weight (BW) gain and gastrointestinal tract parameters in rats fed experimental GF diets *.

	Diets				Ca Effect	Inulin Effect	Ca × I
	O	R	OI	RI			
Daily food intake (g/animal)	13.90 ± 1.22	13.15 ± 1.56	13.83 ± 0.54	13.26 ± 0.95	0.0688	0.7049	0.9054
Daily BW gain (g/animal)	4.55 ± 0.45	4.61 ± 0.53	4.42 ± 0.30	4.38 ± 0.34	0.9477	0.2312	0.7594
Small intestinal parameters							
Weight (g/100 g BW)	3.10 ± 0.13	2.86 ± 0.19	3.26 ± 0.32	3.19 ± 0.34	0.0983	0.0139	0.3633
pH of small intestinal digesta	7.03 ± 0.22	7.02 ± 0.18	6.86 ± 0.20	6.67 ± 0.24	0.1991	0.0015	0.2282
Caecum parameters							
Weight of tissue (g/100 g BW)	0.320 ± 0.03 ^{c}	0.332 ± 0.03 ^{c}	0.436 ± 0.04 ^{b}	0.587 ± 0.08 ^{a}	<0.001	<0.001	<0.001
Weight of digesta (g/100 g BW)	1.124 ± 0.198 ^{b}	1.112 ± 0.202 ^{b}	1.441 ± 0.240 ^{b}	3.110 ± 0.694 ^{a}	<0.001	<0.001	<0.001
DM content of digesta (%)	16.79 ± 1.34	15.65 ± 1.42	15.50 ± 1.33	14.79 ± 1.40	0.0671	0.0346	0.6537
Ammonia (mg/g digesta)	0.191 ± 0.026	0.183 ± 0.018	0.141 ± 0.016	0.154 ± 0.022	0.7069	<0.001	0.1704
pH of digesta	6.79 ± 0.13	6.53 ± 0.18	6.18 ± 0.24	5.83 ± 0.39	0.0019	<0.001	0.5949
Colonic parameters							
Weight of tissue (g/100 g BW)	0.531 ± 0.072 ^{a,b}	0.467 ± 0.042 ^{b}	0.535 ± 0.055 ^{a,b}	0.621 ± 0.094 ^{a}	0.6517	0.0028	0.0044
Weight of digesta (g/100 g BW)	0.519 ± 0.108 ^{b}	0.389 ± 0.153 ^{c}	0.570 ± 0.095 ^{a,b}	0.658 ± 0.078 ^{a}	0.5933	<0.001	0.0104
pH of digesta	6.71 ± 0.186	6.11 ± 0.178	6.11 ± 0.168	5.66 ± 0.132	<0.001	<0.001	0.2046

Values are expressed as means ± standard deviation. * O, group fed GFD with the recommended calcium content; R, group fed GFD with restricted calcium content; OI, group fed GFD with the recommended calcium content and inulin; RI, group fed GFD with restricted calcium content and inulin. BW, body weight. ^{a,b,c} Mean values in rows with different superscript letters are significantly different ($p < 0.05$). The differences between groups O, R, OI and RI groups are indicated with superscripts only when Ca × I interactions were statistically significant ($p < 0.05$).

3.2. Effect of a Gluten-Free Diet Enriched with Inulin on the Concentration of Short Chain Fatty Acids (SCFAs), Putrefactive SCFAs (PSCFAs) and Their Profiles in the Caecal Digesta

The results of two-way ANOVA revealed that the total concentration of SCFAs in the caecal digesta was significantly ($p < 0.01$) affected by the interaction between both dietary variables, Ca intake and inulin intake (Table 3). These resulted mainly from changes in the concentration of straight SCFAs. The experimental GFD with the recommended Ca content and inulin stimulated the formation of propionate and butyrate, whereas the opposite effect was observed when Ca intake was limited. The concentration of putrefactive SCFAs (PSCFAs) in the caecal digesta, determined as the sum of *iso*-butyric, *iso*-valeric and valeric acids, was significantly ($p < 0.05$) reduced by restricted intake of dietary Ca and administration of inulin. The interaction between Ca and inulin intake significantly affected ($p < 0.05$) the profiles of three major SCFAs (Table 3). Both insufficient intake of dietary Ca and inulin supplementation significantly reduced the ratio of acetic acid to total SCFAs, but increased the ratio of propionate to total SCFAs.

Table 3. The concentration of short chain fatty acids (SCFAs), putrefactive SCFAs (PSCFAs) and their profiles in the caecal digesta of rats fed experimental GFDs *.

	Diets				Ca Effect	Inulin Effect	Ca × I
	O	R	OI	RI			
	SCFAs (μM/g digesta)						
Acetic	55.17 ± 7.19 ^{b}	53.22 ± 9.46 ^{b}	58.49 ± 8.56 ^{a}	35.14 ± 8.94 ^{c}	<0.001	0.0216	0.0015
Propionic	16.30 ± 1.39 ^{c}	28.51 ± 6.15 ^{b}	39.91 ± 7.65 ^{a}	27.57 ± 7.48 ^{b}	0.9768	<0.001	<0.001
Iso-butyric	0.41 ± 0.22	0.17 ± 0.07	0.29 ± 0.13	0.05 ± 0.03	<0.001	0.0187	0.9336
Butyric	6.09 ± 2.02 ^{b}	5.68 ± 0.72 ^{b}	8.92 ± 2.79 ^{a}	2.31 ± 1.64 ^{c}	<0.001	0.6953	<0.001
Iso-valeric	0.70 ± 0.21	0.44 ± 0.17	0.25 ± 0.13	0.14 ± 0.06	0.0016	<0.001	0.1738
Valeric	1.28 ± 0.24	0.67 ± 0.14	0.64 ± 0.18	0.05 ± 0.02	<0.001	<0.001	0.5737
PSCFAs	2.39 ± 0.50	1.28 ± 0.21	1.19 ± 0.29	0.23 ± 0.08	<0.001	<0.001	0.4801
Total SCFAs	79.95 ± 9.43 ^{b}	88.69 ± 13.98 ^{b}	108.50 ± 13.95 ^{a}	65.31 ± 17.43 ^{c}	0.0017	0.6097	<0.001
	C2:C3:C4 profile (%)						
C2	69 ^{a}	60 ^{b}	54 ^{c}	54 ^{c}	<0.001	<0.001	<0.001
C3	21 ^{c}	32 ^{b}	37 ^{b}	42 ^{a}	<0.001	<0.001	0.0070
C4	8 ^{a}	6 ^{b}	8 ^{a}	4 ^{c}	<0.001	0.1097	0.0086

Values are expressed as means ± standard deviation. * O, group fed GFD with the recommended calcium content; R, group fed GFD with restricted calcium content; OI, group fed GFD with the recommended calcium content and inulin; RI, group fed GFD with restricted calcium content and inulin. BW, body weight. ^{a,b,c} Mean values in rows with different superscript letters are significantly different ($p < 0.05$). The differences between groups O, R, OI and RI groups are indicated with superscripts only when Ca × I interactions were statistically significant ($p < 0.05$).

3.3. Effect of a Gluten-Free Diet Enriched with Inulin on the Activity of Bacterial Enzymes in the Caecal Digesta

The activity of bacterial enzymes α- and β-glucosidase, α- and β-galactosidase, and β-glucuronidase in the caecum was analysed at the end of the 6-weeks study (Table 4). The results of a two-way ANOVA indicate that analysed variables, a dietary calcium intake and inulin intake stimulated significantly the activity of α-glucosidase and β-galactosidase in an independent manner (no interaction). While, their interaction (Ca × I) had a significant ($p < 0.001$) effect on activity of β-glucosidase and β-glucuronidase. The activity of bacterial β-glucuronidase was effectively reduced by lower dietary Ca intake as well as by inulin in the experimental diets ($p < 0.001$ for both variables).

Table 4. The activity of bacterial enzymes in the caecal digesta of rats fed the experimental GFDs *.

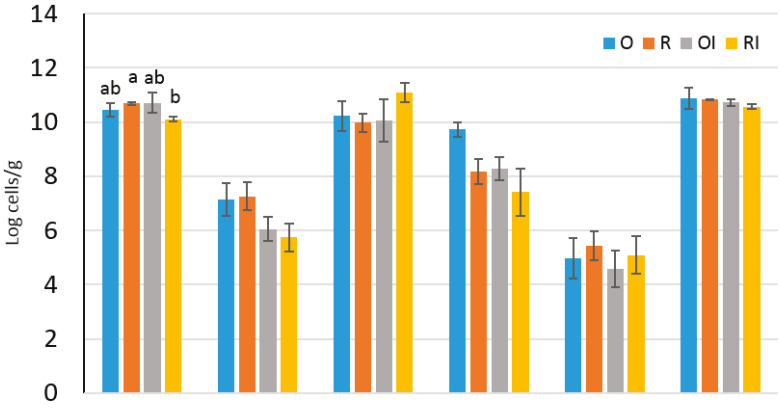
	Diets				Ca Effect	Inulin Effect	Ca × I
	O	R	OI	RI			
α-Glucosidase (μmol/h/g)	13.86 ± 7.28	31.80 ± 13.76	29.74 ± 10.55	40.72 ± 10.99	<0.001	0.0032	0.3737
β-Glucosidase (μmol/h/g)	4.94 ± 1.49 ^{b}	7.53 ± 1.30 ^{a}	6.90 ± 1.74 ^{a}	3.82 ± 0.71 ^{b}	0.6205	0.0803	<0.001
α-Galactosidase (μmol/h/g)	7.86 ± 1.71	4.48 ± 0.92	8.22 ± 3.58	7.84 ± 4.92	0.1066	0.1096	0.1939
β-Galactosidase (μmol/h/g)	27.37 ± 8.85	44.19 ± 10.08	48.99 ± 19.22	56.56 ± 19.59	0.0319	0.0039	0.3995
β-Glucuronidase (μmol/h/g)	18.82 ± 6.14 ^{a}	4.77 ± 1.98 ^{b}	5.67 ± 2.71 ^{b}	1.85 ± 0.98 ^{c}	<0.001	<0.001	<0.001

Values are expressed as means ± standard deviation. * O, group fed GFD with the recommended calcium content; R, group fed GFD with restricted calcium content; OI, group fed GFD with the recommended calcium content and inulin; RI, group fed GFD with restricted calcium content and inulin. BW, body weight. ^{a,b,c} Mean values in rows with different superscript letters are significantly different ($p < 0.05$). The differences between groups O, R, OI and RI groups are indicated with superscripts only when Ca × I interactions were statistically significant ($p < 0.05$).

3.4. Effect of a Gluten-Free Diet Enriched with Inulin on the Quantitative Profile of Caecal Microbiota

An analysis of the quantitative profile of caecal microbiota revealed that neither restricted Ca intake nor inulin supplementation influenced the total bacteria number (TBN) and *Enterococcus* (Figure 1). Inulin administration affected *Lactobacillus* counts ($p = 0.001$) independently of dietary Ca intake, whereas both restricted Ca intake and inulin intake lowered the counts of bacteria of *Clostridium*

leptum group (Figure 1) in an independent manner (no interaction). The *Bifidobacterium* count tended ($p = 0.053$) to be increased by Ca × I interaction, whereas *Bacteroides-Prevotella-Porphyromonas* counts were significantly affected by an interaction between the two investigated factors ($p < 0.01$).



	P value (2-factor ANOVA)					
Calcium (C)	0.185	0.776	0.296	0.003	0.225	0.311
Inulin (I)	0.265	0.001	0.206	0.005	0.349	0.059
Interaction C×I	0.008	0.495	0.053	0.312	0.967	0.787

Figure 1. Counts of *Bacteroides-Prevotella-Porphyromonas* (BPP), *Lactobacillus* (LAC), *Bifidobacterium* (BIF), *Clostridium leptum* group (Clept), *Enterococcus* (ECC) and total bacterial number (TBN) of caecal microbiota of rats fed the experimental GFDs. Values are expressed as means ± standard deviation; O, group fed a GF diet with the recommended calcium content; R, group fed a GF diet with restricted calcium content; OI, group fed a GF diet with the recommended calcium content and inulin; RI, group fed a GF diet with restricted calcium content and inulin. Significant differences between groups O, R, OI and RI are indicated with superscripts only when C × I interactions were statistically significant ($p < 0.05$).

3.5. Effect of a Gluten-Free Diet Enriched with Inulin on the Qualitative Profile of Caecal Microbiota

A qualitative analysis (PCR-DGGE) of bacterial groups influenced by the experimental GFDs was performed based on the results of a quantitative analysis of caecal microbiota. Based on the sequenced DGGE bands, gel regions were assigned to a specific bacterial family (*Lachnospiraceae*, *Peptostreptococcaceae*, *Erysipelotrichaceae* and *Selenomonadaceae*) or genus (*Bifidobacterium*, *Olsenella*) (Figure S1). A comparison of eubacterial DGGE profiles obtained for all animal groups revealed no changes that could be attributed to a particular diet (Figure S1). However, a paired comparison of DGGE profiles supported the determination of changes resulting from restricted intake of dietary Ca or inulin intake (Figure 2). Restricted Ca intake influenced the structure of the predominant caecal microbiota in 50% of the examined animals (Figure 2a). The DGGE profiles of microbiota of four animals from groups O and R were characterised by nearly 70% similarity, whereas the microbiota in the remaining four samples from groups O and R was grouped into clusters with minimum 80% overall similarity. Cluster A1 formed by group R microbiota harboured *Olsenella* and *B. animalis* (members of the phylum Actinobacteria) and *Faecalibacterium rodentium* (*Firmicutes*), but were devoid of selected *Lachnospiraceae*- and *Peptostreptococcaceae*-specific bands which were dominant in the DGGE profiles of cluster A2 bacteria detected in four O group animals. Dietary inulin stimulated bifidobacteria, especially *B. animalis* species in group fed a GFD with the recommended amount of Ca

(Figure 2b). Whereas, the addition of inulin to a calcium-deficient GFD (Figure 2c) or different Ca levels in inulin-supplemented GFDs (Figure 2d) had no effect on the banding patterns of the predominant bacteria. However, it should be stresses that in 75% of animals fed GFD with inulin, *B. animalis* was present in caecal microbiota (Figure 2d).

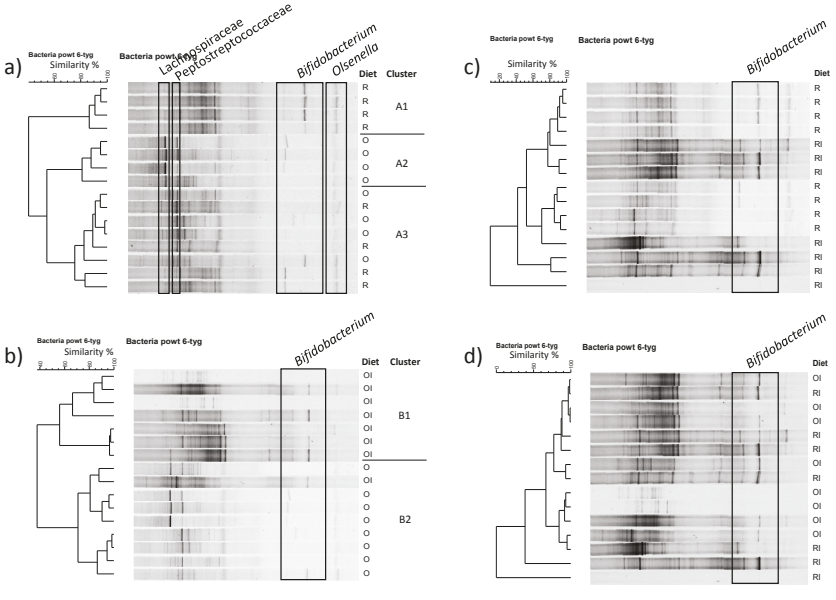


Figure 2. DGGE banding patterns of caecal bacteria obtained with universal primers. Frames depict gel positions assigned to a specific bacterial taxon based on band sequencing (see Supplementary Information, Figure S1). O and R—diets with recommended and restricted calcium intake, respectively. OI and RI—diets O and R supplemented with inulin. Paired comparisons, O vs. R (**a**), O vs. OI (**b**), R vs. RI (**c**) and OI vs. RI (**d**), were performed based on profile similarities calculated using the Pearson's product-moment correlation coefficient. Dendrograms were constructed using the UPGMA algorithm.

Similar comparisons were performed for the DGGE profiles of Bacteroides, and they revealed that inulin changed the profiles of these bacteria in groups fed GFDs with both recommended (Figure 3b) and restricted Ca levels (Figure 3c). The observed changes included the inhibition of *Bact. stercoris* (weak or absent DGGE band), a decreased representation of *Bact. uniformis* (lower number of species-specific bands; Figure 3b,c) and stimulation of *Bact. eggerthii* in group RI (Figure 3c). The limited intake of dietary Ca had no influence on the *Bacteroides* profile, and the majority of the analysed profiles revealed at least 80% similarity (Figure 3a). In the presence of inulin, different levels of dietary Ca had no effect on DGGE patterns, and most profiles had at least 80% similarity. A comparative analysis of all DGGE profiles of *Bacteroides* (Figure S2), *Lactobacillus* (Figure S3) and *C. leptum* group bacteria (Figure S4) and a paired comparison of the two latter bacterial groups (data not shown) did not reveal any effects that could be attributed to any of the tested factors.

A composite data set analysis was performed for the DGGE profiles of eubacteria, *Bacteroides*, *C. leptum* group and *Lactobacillus*. Multidimensional scaling of average profile similarities in the composite data set analysis revealed that caecal microbiota in the group O fed a GFD with the recommended amounts of Ca was most uniform and that supplementation of diet O with inulin (OI) scattered the samples along the y axis. A calcium-deficient GFD differentially impacted microbiota and divided the group into two subgroups (Figure 4), whereas the RI diet resulted in the most scattered distribution of samples along the x axis.

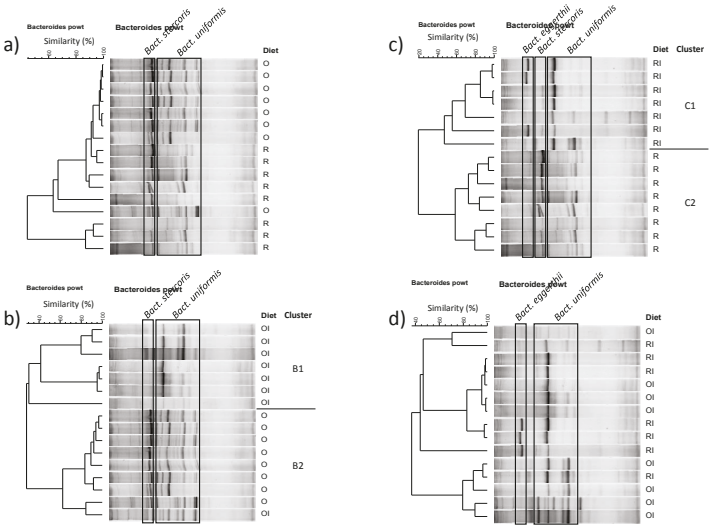


Figure 3. DGGE banding patterns of caecal *Bacteroides* population. Frames depict gel positions assigned to a specific bacterial taxon based on band sequencing (see Supplementary Information, Figure S1). O and R–diets with recommended and restricted calcium intake, respectively. OI and RI—diets O and R supplemented with inulin. Paired comparisons, O vs. R (**a**), O vs. OI (**b**), R vs. RI (**c**) and OI vs. RI (**d**), were performed based on profile similarities calculated using the Pearson's product-moment correlation coefficient. Dendrograms were constructed using the UPGMA algorithm.

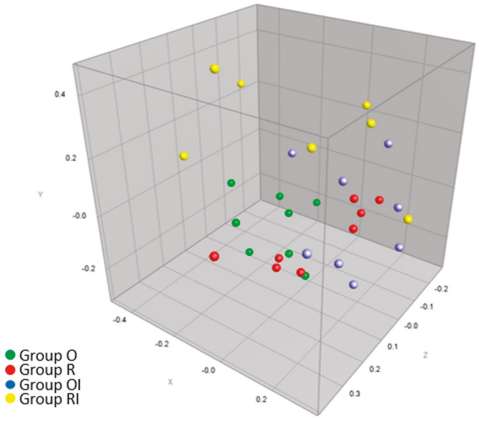


Figure 4. A three-dimensional MDS plot presenting the average similarities between the caecal microbiota of rats fed diets with the recommended (O, green dots) and decreased calcium levels (R, red dots) and supplemented with inulin (OI (blue dots) and RI (yellow dots), respectively). A multidimensional scaling analysis was preformed based on the similarity matrix generated during the composite data analysis of DGGE profiles of eubacteria, *Bacteroides*, *Lactobacillus* and *C. leptum* group bacteria.

3.6. Effect of a Gluten-Free Diet Enriched with Inulin on Intestinal Absorption and Retention of Calcium, Magnesium and Phosphorus

The Ca supply x inulin interaction significantly influenced ($p < 0.001$) daily Ca intake (Table 5). As expected, in groups fed GFDs with reduced dietary Ca levels (R and RI), daily Ca intake was

approximately 60% lower than in groups O and OI fed diets with the recommended Ca content. Two-way ANOVA revealed that faecal Ca excretion was influenced by both dietary Ca content and the presence of inulin in the experimental diets. Calcium excretion with faeces, expressed in mg/day, was significantly ($p < 0.001$) lower when dietary Ca levels were low, and it was also significantly ($p = 0.0232$) reduced by inulin intake. Consequently, relative Ca absorption (%) was significantly ($p < 0.05$) higher in groups fed a GFDs with inulin. According to statistical analysis, daily urinary Ca excretion (mg/day) was also affected by both dietary factors. Insufficient dietary Ca intake significantly decreased ($p < 0.001$) Ca excretion with urine, whereas inulin intake increased Ca urinary excretion, in particular in the group fed a GF diet with the recommended Ca level. Relative Ca retention (%) was significantly ($p < 0.001$) higher in groups fed calcium-deficient GFDs, regardless of the presence or absence of inulin. Daily magnesium (Mg) intake from GFDs was not affected by the analysed experimental variables, whereas Mg excretion with faeces and Mg absorption were significantly affected (Table 5). Faecal Mg excretion decreased significantly in response to low levels of dietary Ca as well as inulin intake ($p < 0.001$ and $p = 0.0173$, respectively), which increased relative absorption. Inulin increased urinary Mg excretion, but it did not influence the relative retention of Mg (%) which was calcium-dependant. The phosphorus balance was associated exclusively with the amount of Ca provided by the experimental GFDs, which significantly reduced faecal excretion and increased absorption in groups fed calcium-deficient diets (R and RI).

Table 5. Intestinal absorption and retention of calcium, magnesium and phosphorus in rats fed experimental GF diets *.

	Diets				Ca Effect	Inulin Effect	Ca × I
	O	R	OI	RI			
Calcium							
Intake (mg/day)	86.21 ± 7.87 [a]	35.64 ± 4.24 [b]	80.46 ± 5.37 [a]	33.95 ± 2.44 [b]	<0.001	0.0024	0.0220
Faecal excretion (mg/day)	22.68 ± 4.08	2.01 ± 0.43	20.05 ± 4.43	0.84 ± 0.32	<0.001	0.0232	0.2026
Absorption (%) †	73.28 ± 3.13	94.36 ± 0.80	74.60 ± 4.86	97.51 ± 0.92	<0.001	0.0414	0.3879
Urinary excretion (mg/day)	6.92 ± 1.98	0.54 ± 0.15	9.44 ± 2.98	0.52 ± 0.18	<0.001	0.0179	0.0673
Retention (%) ‡	65.63 ± 2.23	93.05 ± 1.84	62.62 ± 6.18	94.18 ± 3.80	<0.001	0.5916	0.0753
Magnesium							
Intake (mg/day)	6.35 ± 0.56	6.30 ± 0.75	6.46 ± 0.25	6.21 ± 0.43	0.1695	0.9289	0.2754
Faecal excretion (mg/day)	1.72 ± 0.37	0.42 ± 0.07	1.48 ± 0.19	0.32 ± 0.06	<0.001	0.0173	0.3777
Absorption (%) †	72.94 ± 5.06	93.28 ± 0.99	76.54 ± 1.50	95.21 ± 1.26	<0.001	0.0078	0.3920
Urinary excretion (mg/day)	3.03 ± 0.24	3.11 ± 0.41	3.25 ± 0.28	3.27 ± 0.47	0.4112	0.0321	0.2432
Retention (%) ‡	28.54 ± 8.57	43.84 ± 3.30	28.51 ± 4.04	43.68 ± 5.07	<0.001	0.4611	0.7504
Phosphorus							
Intake (mg/day)	35.26 ± 3.10	34.74 ± 4.13	35.44 ± 2.42	32.79 ± 2.35	0.1515	0.4407	0.3269
Faecal excretion (mg/day)	13.19 ± 1.54	4.68 ± 0.73	12.62 ± 1.83	4.57 ± 0.24	<0.001	0.0517	0.9248
Absorption (%) †	62.66 ± 2.79	86.42 ± 2.25	63.23 ± 1.05	88.90 ± 3.48	<0.001	0.0994	0.2963
Urinary excretion (mg/day)	0.37 ± 0.07	9.37 ± 1.39	0.37 ± 0.15	8.91 ± 0.74	<0.001	0.2644	0.2606
Retention (%) ‡	61.60 ± 2.79	60.21 ± 3.01	63.62 ± 4.13	62.30 ± 8.00	0.0927	0.0314	0.7850

* Values are expressed as means ± standard deviation. O, group fed a GF diet with the recommended calcium content; R, group fed a GF diet with restricted calcium content; OI, group fed a GF diet with the recommended calcium content and inulin; RI, group fed a GF diet with restricted calcium content and inulin. † Absorption: [(Intake—Faecal excretion)/Intake] × 100. ‡ Retention: [(Intake—Faecal excretion-Urinary excretion)/Intake] × 100. [a,b,c] Mean values in rows with different superscript letters are significantly different ($p < 0.05$). The differences between groups O, R, OI and RI are indicated with superscripts only when Ca × I interactions were statistically significant ($p < 0.05$).

3.7. Effect of a Gluten-Free Diet Enriched with Inulin on Intestinal Absorption and Retention of Calcium, Magnesium and Phosphorus

Plasma Ca concentration was characterised by non-significant variations (2.337 to 2.545 mM/L), but an increasing trend was noted in groups fed inulin-supplemented GF diets (p = 0.0601), in particular when dietary Ca supply was restricted (Table 6). Phosphorus concentration was significantly (p = 0.0026) affected by the Ca × I interaction. Insufficient dietary Ca intake and inulin supplementation led to a significant ($p < 0.001$ and p = 0.0063) increase in plasma P concentration. Consequently, the Ca to phosphorus ratio decreased significantly ($p < 0.001$) in groups fed calcium-deficient GFDs.

Table 6. Plasma calcium and phosphorus concentration in rats fed experimental GFDs *.

	Diets				Ca Effect	Inulin Effect	Ca × I
	O	R	OI	RI			
Calcium (mM/L)	2.522 ± 0.084	2.337 ± 0.059	2.545 ± 0.204	2.537 ± 0.238	0.1083	0.0662	0.1393
Phosphorus (mM/L)	1.861 ± 0.247 ^{b}	2.202 ± 0.166 ^{a}	1.835 ± 0.211 ^{b}	2.670 ± 0.215 ^{a}	<0.001	0.0063	0.0026
Ca:P	1.4	1.1	1.4	1.0	<0.001	0.3116	0.1177

* Values are expressed as means ± standard deviation. O, group fed a GF diet with the recommended calcium content; R, group fed a GF diet with restricted calcium content; OI, group fed a GF diet with the recommended calcium content and inulin; RI, group fed a GF diet with restricted calcium content and inulin. ^{a,b,c} Mean values in rows with different superscript letters are significantly different ($p < 0.05$). The differences between groups O, R, OI and RI are indicated with superscripts only when Ca × I interactions were statistically significant ($p < 0.05$).

4. Discussion

In the present study, all experimental GFDs had similar energy value, thus no effect on rats body weight gain was observed. Inulin in GFDs increased the weight of the small intestine and its acidification that suggested that inulin could be metabolised to a certain extent by aerotolerant small intestinal bacteria. Recently, van den Bogert et al. [36,37] demonstrated that small-intestinal streptococci *S. mitis*, *S. bovis* and *S. salivarius* differed considerably in their carbohydrate metabolism, whereas Veillonella species utilised lactic acid produced by carbohydrate-fermenting streptococci, which contributed to the synthesis of acetic and propionic acids, and led to environment acidification.

Similarly to other prebiotics, inulin affects mainly caecal parameters by influencing resident microbiota, in particular anaerobic species [38]. In the present study, the pH of a large intestinal digesta decreased whereas the weight of caecal and colonic tissues as well as their digesta increased considerably in animals fed GFDs with inulin. The observed inulin-mediated increase in the weight of tissue of the large intestinal segments could be attributed to fructans' ability to stimulate bacterial growth and proliferation, however in the present study, the DM content of the digesta did not increase. This indicates that apart from bacterial counts, bacterial activity considerably influenced intestinal parameters. In view of the above, the increase in the weight of caecal and colonic tissues could be partially attributed to inulin fermentation products, mainly SCFAs. Butyric acid and, to some extent, also propionic acid, are the main energy substrates for colonocytes [39]. Butyrate stimulates the physiological proliferation of enterocytes, induce histological changes in the gut epithelium and modifications of the mucosal architecture [40]. Whereas, the noted increase in the weight of large intestinal contents could have simply resulted from the inulin-mediated bulking effect and a higher content of water in the large intestinal digesta [41]. Nevertheless, a fructan-mediated increase in the weight of caecal digesta could have adverse consequences and could cause discomfort to the host [42]. On the other hand, a considerable effect on intestinal parameters could also be exerted by the level of dietary Ca intake. Recent research has demonstrated that Ca plays a number of important roles in eukaryotes as well as in prokaryotic cells [43]. Ca^{2+} ions affect the growth, division and differentiation of prokaryotic cells, and in the absence of Ca^{2+}, *E. coli* cells ceased to divide and proliferate, and they were eventually lysed and died [44].

The caecum is a site of intensive carbohydrate fermentation as well as proteolytic activity which is largely mediated by microbiota, where ammonia is the main metabolite. In our study, an

inulin-mediated decrease in a caecal ammonia concentration was noticed that could result from luminal acidification and, consequently, inhibited protein degradation in an acidic environment. Proteases are more active at neutral or slightly alkaline pH than in acidic pH [45]. In general, a reduction in caecal ammonia concentration is a favourable change since ammonia is the most toxic form of nitrogen and a metabolic disruptor [46]. The results of this study suggest that inulin and/or its fermentation products could facilitate to the utilisation and/or elimination of ammonia.

Changes in the concentrations of SCFAs, bacterial enzyme activities and DGGE profiles of eubacteria and *Bacteroides* affirmed an intensive fermentation of inulin by caecal microbiota. The degree of polymerisation (DP) and the solubility of fructooligosaccharides are vital criteria for SCFAs formation, which is why oligosaccharides with a low DP produced high levels of butyric acid, whereas oligosaccharides with a high DP, such as inulin, produced high levels of propionic acid [47]. In this study, the high propionate to total SCFAs ratio appears to be typical for the fermentation of long-chain inulin. On the other hand, a high concentration of propionate in rats fed inulin could be linked with an abundance of *Bacteroidetes* and several *Firmicutes* species which utilise succinate as a substrate for propionate synthesis through decarboxylation of methylmalonyl-CoA to propionyl-CoA [48]. The addition of inulin to the experimental GFD increased butyrate concentration, but only in the group fed a GFD with the recommended Ca content, which suggests that such diets could create a favourable environment for *Firmicutes*. *Firmicutes* species, including *Faecalibacterium*, *Eubacterium* and *Roseburia*, are the main butyrate-producers in the colon which are able to convert butyryl-CoA to butyrate in a single-step enzymatic reaction, the butyryl-CoA: acetate CoA-transferase pathway [49]. Surprisingly, the low concentration of butyrate in group RI could be linked with changes in the utilisation of β-hydroxybutyryl-CoA. Prokaryotic cells maintain tight control of their cytosolic Ca^{2+} by means of non-proteinaceous polyhydroxybutyrate-polyphosphate (PHB-PP) complexes or Ca^{2+} channels. In the latter case, Ca ions are extruded by Ca^{2+}-translocating ATPases or electrochemical potential-driven Ca^{2+} transporters [50]. β-hydroxybutyryl-CoA is one of key elements of butyrate synthesis in both butyryl-CoA:acetate CoA-transferase and butyrate kinase pathways [51]. However, it is also a precursor for PHB synthesis that relies on poly-3-hydroxybutyrate (PHB) synthase [52]. It could be hypothesized that prebiotic fermentation increases acetyl-CoA synthesis and, consequently, acetoacetyl-CoA and β-hydroxybutyryl-CoA. This leads to a metabolic switch from butyrate production to the synthesis of PHB which acts as a universal regulator of internal ion concentrations by selectively transporting ions across membranes [53]. It is likely that a calcium-deficient GFD without inulin is not a sufficient source of carbohydrates for the production of acetyl-CoA in amounts that could be metabolically economic for the butyrate→PHB switch. Therefore, bacteria cope with low Ca levels by activating a proteinaceous system of Ca transport [50]. On the other hand, it cannot be ruled out that nearly all available butyrate was used up by the enlarged intestinal tissue and that butyrate levels in the intestinal lumen were depleted. This observation is supported by the lowest values of Ca excretion with faeces and highest Ca absorption. Further research is needed to verify the above hypotheses.

SCFAs deliver health benefits [54]. The possible connection between selected inflammatory processes and a reduction in the populations of butyrate- and propionate-producing bacteria has turned the researchers' attention to the metabolism of propionate and butyrate. Machiels et al. [55] reported a decrease of in the counts of butyrate-producing species in ulcerative colitis. Based on above, it could be expected that the acidification of the large intestine resulting from SCFAs production could promote the growth and proliferation of beneficial microbiota and reduce the number of potentially pathogenic species. Our speculations were also supported by the results of a quantitative analysis of branched SCFAs which revealed that the addition of inulin to a GFDs suppressed putrefaction processes when the intake of dietary Ca was low.

In animals fed a GFD with the required Ca content, inulin exerted a similar influence on microbiota (stimulation of *B. animalis*) to that reported in other studies [56]. An analysis of eubacterial DGGE patterns indicates that members of the phylum Actinobacteria (*Olsenella* and *Bifidobacterium*) seem to be less susceptible to low Ca levels than *Firmicutes*, in particular the family *Lachnospiraceae*. Moreover,

the MDS analysis based on DGGE profiles revealed that limited intake of dietary Ca was associated with two microbiological phenotypes, which points to individual variations in response to low Ca levels. Moreover, the combined effect of dietary factors (restricted dietary Ca intake and inulin intake) led to the most scattered distribution of samples. Therefore, it could be assumed that inulin together with low Ca levels trigger substantial changes in microbial structure due to individual differences in microbial composition. The observed in the present study changes in the structure and metabolic activity of caecal microbiota seem to indicate that the combination of low Ca intake and inulin has an undesirable effect on intestinal bacteria. The presence of *Bacteroides eggerthii*, which was recently proposed as a colitis-promoting species [57], could be of particular interest in further studies on maintaining a properly balanced commensal microbiota.

The inulin-mediated modification of gut microbiota was also manifested by changes in the activity of bacterial enzymes in the caecum. The experimental GFDs with inulin increased the concentration of α- and β-glucosidase and β-galactosidase, whereas the activity of β-glucuronidase was suppressed in groups fed GFDs with inulin. Similar results were reported in other studies of fructooligosaccharides [42,58] which demonstrated that moderate FOS content significantly reduced the caecal activity of β-glucuronidase and increased the activity of β-galactosidase. Bile flow modulates the activity of β-glucuronidase in the large intestines of rats [59], therefore, the inulin-induced decrease in β-glucuronidase activity could be attributed to intensified intestinal peristalsis. In general, a reduction in β-glucuronidase activity is beneficial because this enzyme exerts toxic, carcinogenic, and mutagenic effects in the gastrointestinal tract [60]. Thus, the addition of inulin to the experimental GF diets seems to selectively modulate the composition of microbiota, and it potentially eliminates harmful bacteria that enhance the activity of β-glucuronidase in the caecum.

Fermentable carbohydrates, including inulin, were found to increase Ca and Mg absorption in the large intestine [21,22], but this effect was dependent on the dose, structure of fermentable carbohydrates as well as the duration of the experiment. In the present study, we hypothesised that the addition of inulin to a GFD would increase Ca absorption in the large intestine, in particular in animals with low dietary Ca intake. Inulin intake stimulated Ca and Mg absorption in the group fed a calcium-deficient GFD however, the increase in Ca and Mg absorption was not accompanied by changes in their retention. Coudry et al. [23] found that dietary inulin had a more beneficial effect on Ca absorption in shorter-term (17 days) than in long-term experiments (up to 40 days), and that inulin-mediated changes were more profound when dietary Ca was in low supply. In contrast, in another study, a 6-week prebiotic intervention (oligofructose + acacia gum) had no effect on Ca absorption or Ca retention in aged ovariectomized rats [61]. In the present study performed on growing rats, which are characterised by a higher demand for Ca and higher Ca absorption capacity, the increase in Ca and Mg absorption could be attributed to prolonged inulin fermentation in the caecum, which was manifested by changes in large intestinal parameters, mainly an increase in SCFA synthesis and acidification of caecal and colonic contents. This indicates that acidic caecal pH promotes the solubilisation of Ca and Mg. The observed increase in Ca absorption in animals fed GFD with inulin is a promising result that could generate favourable surplus Ca for bone, however further in vivo studies are required to assess the impact of a dietary application of inulin on calcium metabolism and bone mineralisation.

5. Conclusions

Our results indicated that the effect of inulin on intestinal microbiota characteristics and activity, and mineral utilization in growing rats depended on the dietary Ca intake in GFDs. Generally, dietary inulin stimulated the SCFAs formation, increased the luminal acidification and decreased caecal ammonia concentration. However, unsufficient Ca intake in GFD influenced negatively the structure of the predominant caecal microbiota, while a dietary inulin stimulated bifidobacteria, in particular *B. animalis* species if recommended amount of Ca was provided in rats diet. Most benefits to mineral utilization from inulin consumption were seen in rats fed GFD of restricted Ca amount where it

increased the relative Ca absorption. Obtained results allow to conclude that the administration of inulin to a GFDs could be a promising dietary strategy for beneficial modulation of intestinal ecosystem and by that for the improvement the Ca absorption.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/7/702/s1, Figure S1: DGGE profiles of rats' caecal bacteria obtained with universal primers. Rats were fed diet with optimal (O) or restricted (R) calcium supply supplemented with inulin (diet OI and RI, respectively). Arrows indicate band taken for identification which results are shown in a table below. Figure S2. DGGE profiles of rats' caecal *Bacteroides* population. Rats were fed diet with optimal (O) or restricted (R) calcium supply supplemented with inulin (diet OI and RI, respectively). Arrows indicate band taken for identification which results are shown in a table below. Figure S3. DGGE profiles of rats' caecal *Lactobacillus* population. Rats were fed diet with optimal (O) or restricted (R) calcium supply supplemented with inulin (diet OI and RI, respectively). Arrows indicate band taken for identification which results are shown in a table below. Figure S4. DGGE profiles of rats' caecal *C. leptup* group (clostridial cluster IV). Rats were fed diet with optimal (O) or restricted (R) calcium supply supplemented with inulin (diet OI and RI, respectively). Table S1: Nutritional composition of experimental gluten-free diets, Table S2: Primers and amplification conditions used for qualitative (PCR-DGGE) analysis of caecal microbiota, Table S3: Denaturing gradient applied for separation of PCR products in DGGE technique; Table S4: Primers used for real-time PCR analysis.

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Lactobacillus reuteri I5007 Modulates Intestinal Host Defense Peptide Expression in the Model of IPEC-J2 Cells and Neonatal Piglets

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Abstract: Modulation of the synthesis of endogenous host defense peptides (HDPs) by probiotics represents a novel antimicrobial approach for disease control and prevention, particularly against antibiotic-resistant infections in human and animals. However, the extent of HDP modulation by probiotics is species dependent and strain specific. In the present study, The porcine small intestinal epithelial cell line (IPEC-J2) cells and neonatal piglets were used as in-vitro and in-vivo models to test whether *Lactobacillus reuteri* I5007 could modulate intestinal HDP expression. Gene expressions of HDPs, toll-like receptors, and fatty acid receptors were determined, as well as colonic short chain fatty acid concentrations and microbiota. Exposure to 10^8 colony forming units (CFU)/mL of *L. reuteri* I5007 for 6 h significantly increased the expression of porcine β-Defensin2 (PBD2), pBD3, pBD114, pBD129, and protegrins (PG) 1-5 in IPEC-J2 cells. Similarly, *L. reuteri* I5007 administration significantly increased the expression of jejunal pBD2 as well as colonic pBD2, pBD3, pBD114, and pBD129 in neonatal piglets ($p < 0.05$). This was probably associated with the increase in colonic butyric acid concentration and up-regulating expression of Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) and G Protein-Coupled Receptor 41 (GPR41) ($p < 0.05$), but not with stimulation of Pattern-Recognition Receptors. Additionally, supplementation with *L. reuteri* I5007 in the piglets did not affect the colonic microbiota structure. Our findings suggested that *L. reuteri* I5007 could modulate intestinal HDP expression and improve the gut health of neonatal piglets, probably through the increase in colonic butyric acid concentration and the up-regulation of the downstream molecules of butyric acid, PPAR-γ and GPR41, but not through modifying gut microbiota structure.

Keywords: *Lactobacillus reuteri*; intestinal epithelial cells; neonatal piglets; host defense peptide; gut microbiota

1. Introduction

On a global basis, it is estimated that 5.9 million children under the age of five years died in 2015, most of which are caused by infectious diseases associated with bacteria that are resistant to antibiotics [1]. As key components of the innate immune system, host defense peptides (HDPs) play

critical roles in fighting against infections for their ability of possessing antimicrobials and a low propensity for the development of bacterial resistance in younger children with immature neonatal immune systems [2,3]. HDPs have been commonly studied for their antimicrobial properties and have been shown to kill bacteria, viruses, fungi, protozoa, and even cancer cells [3]. Due to their potential therapeutic activities, HDPs are attractive candidates as alternatives for antibiotics [4]. Swine and humans share high similarity in physiologic and anatomic characteristics, which makes the former the ideal model for human health and disease [5,6]. In vertebrate animals, HDPs are generally grouped into two major families; defensins and cathelicidins [7,8].

As an important first line of defense, HDPs are produced mainly by intestinal epithelial cells and phagocytes in the gastrointestinal tract. In addition to infection or inflammation, HDPs can also be induced by dietary compounds, including saccharides, essential amino acids, butyrate, vitamin D3, and zinc [4,9–12]. Moreover, probiotic lactobacilli could stimulate HDP expression in human cells and piglets without provoking inflammatory responses like pathogenic strains [13,14]. However, different lactobacilli strains show a varying magnitude of HDP-inducing activity [13].

Lactobacillus reuteri is considered to be an indigenous species in the gastrointestinal tract of humans and animals [15]. Numerous studies have demonstrated that *L. reuteri* has excellent probiotic properties and has been widely used as a probiotic in humans and animals [16]. *L. reuteri* I5007, initially known as *L. fermentum* I5007, was isolated from the colonic mucosa of healthy weaning piglets [17]. Compelling evidence shows that *L. reuteri* I5007 has several important probiotic properties including: (1) resistance to gastric acid and bile [18]; (2) strong adhesion [17,19]; (3) competitive exclusion against pathogens [19]; (4) alleviation of weaning stress in piglets [20]; (5) improvement of piglet performance [21,22]; (6) and positive regulation of redox status and immune function in piglets [23,24]. Notably, oral administration of *L. reuteri* I5007 increased the concentration of butyrate and branched chain fatty acids in the colonic digesta of suckling piglets [22,24]. It has been shown that butyrate, produced by butyrate-producing bacterial strains, has strong capacity to induce HDP expression in vitro. However, whether *L. reuteri* I5007 could modulate intestinal HDP expression through modifying gut microbiota and its metabolite butyrate in neonatal piglets is still unknown.

The aim of the current study was to investigate the effects of *L. reuteri* I5007 on the gut microbiota and HDP expression. We initially studied the in vitro effect of *L. reuteri* I5007 by inducing HDP expression in a porcine intestinal epithelial cell line. We subsequently determined the effects of *L. reuteri* I5007 supplementation on the colonic bacterial community and HDP expression in formula-fed neonatal piglets.

2. Materials and Methods

2.1. Ethics Statement

The procedures used in this experiment were approved by the China Agricultural University Institutional Animal Care and Use Committee (CAU20144-2, Beijing, China).

2.2. Bacterial Strain, Growth and Storage Conditions

L. reuteri I5007 was grown in De Man Rogosa Sharpe media under anaerobic conditions at 37 °C for 20 h. For cell culture assays, after incubation, bacterial cells were obtained by centrifugation (8000× *g* for 10 min at 4 °C). Then the bacterial cells were washed with phosphate-buffered saline (PBS, a balanced salt solution used for a variety of cell culture applications), reconstituted in DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, 1:1 mixture of DMEM and Ham's F-12) medium supplemented with 10% fetal bovine serum (FBS) and adjusted to the required cell concentration. After centrifugation, the culture supernatant of *L. reuteri* I5007 was passed through a 0.2-μm-pore-size filter (Corning Inc., Corning, NY, USA), and it was preserved for subsequent treatment with a 10% (*v*/*v*) concentration. For heat killed bacteria, heat inactivation was carried out in a water bath at 65 °C for 1 h. The bacterial cells were centrifugated, and the pellet was washed with

PBS and adjusted to a density of 1×10^8 colony forming units (CFU)/mL with DMEM/F12 medium supplemented with 10% FBS. The freeze-dried powder, containing 5×10^{10} CFU/g, was produced according to Liu et al. [22].

2.3. Cell Culture and Treatment

The porcine small intestinal epithelial cell line (IPEC-J2) was kindly provided by Dr. Wu at Texas A & M University (College Station, TX, USA). IPEC-J2 cells were cultured in DMEM/F12 medium supplemented with 10% FBS at 37 °C in a 5% CO_2 and 95% air atmosphere with 90% humidity.

For stimulation experiments, undifferentiated cells were seeded at a density of 1×10^6 cells per well in 6-well plates (Costar, Corning Inc., Corning, NY, USA). After overnight growth (cells were grown to ~80% confluence in the culture wells), the cells were treated in duplicate with *L. reuteri* I5007. To prevent any influence of antibiotics on the immune response, the medium did not contain antibiotics. The FBS showed no effect on expression.

For dose-dependent *L. reuteri* I5007 stimulation experiments, IPEC-J2 cells were incubated with a control or 10^5, 10^6, 10^7, 10^8, or 10^9 CFU/mL *L. reuteri* I5007 for 6 h. For time-dependent *L. reuteri* I5007 stimulation experiments, IPEC-J2 cells were incubated with 10^8 CFU/mL *L. reuteri* I5007 for 3, 6, or 12 h.

IPEC-J2 cells were also treated for 6 h with 10^8 CFU/mL *L. reuteri* I5007 exposed to different processing conditions. The processing conditions included a solvent control without *L. reuteri* I5007 (Control, DMEM/F12 medium supplemented with 10% FBS), 10^8 CFU/mL live *L. reuteri* I5007 (Live I5007), 10^8 CFU/mL heat-killed *L. reuteri* I5007 (Dead I5007, incubated in a water bath at 65 °C for 1 h), adhered *L. reuteri* I5007 (Adhered I5007, treated with 10^8 CFU/mL *L. reuteri* I5007 for 1 h, rinsed three times in PBS with fresh medium added, followed by continued incubation for 5 h), and 200 μL of *L. reuteri* I5007-free culture supernatant of *L. reuteri* I5007 (Supernatant, diluted 1:10 in basal medium). In addition, a Transwell Insert System (Costar, Corning Inc., Corning, NY, USA) was used to avoid direct contact between the IPEC-J2 cells and *L. reuteri* I5007 (Separate I5007). Herein, *L. reuteri* I5007 cells in an upper chamber and IPEC-J2 cells in a lower chamber were separated by a 0.2-μm-pore-size filter membrane support (Corning Inc., Corning, NY, USA), thereby minimizing any direct contact between the *L. reuteri* I5007 cells and IPEC-J2.

2.4. Animals and Treatments

The in vivo experiment was conducted in the Metabolism Laboratory of the Ministry of Agriculture Feed Industry Centre (Beijing, China). Twenty-two, full-term, crossbred (Duroc × Large White × Landrace) male piglets, obtained from six litters, were used in this study. The piglets were delivered vaginally and allowed colostrum for 48 h after birth. The piglets were individually housed in stainless steel cages (1.4 m × 0.45 m × 0.6 m) in a temperature (32 ± 1 °C) and relative humidity (65–70%) controlled room programmed to deliver a light:dark cycle of 16:8 h.

On the third day after parturition, the piglets were trained to suckle from bottles filled with milk replacer (Jiaduonai H001, DaChan Tianyao, Tianjin, China, Table 1), which was dissolved in warm previously boiled water (45 °C, *w*/*v* 1:9). The fresh liquid milk replacer was fed to piglets individually from a feeder five times daily (6:00, 10:00, 14:00, 18:00, and 22:00 h) for 20 days. After feeding, the remaining milk was measured and the feeders were cleaned before adding new fresh milk replacer. The formula did not contain any antibiotics or other medicine.

On day 4, the neonatal piglets were allocated to one of two treatments balanced for litter of origin and body weight (initial body weight of 1.81 ± 0.31 kg) with 11 piglets assigned to each treatment ($n = 11$). The treatments were comprised of a control treatment (the piglets were given a placebo of 4 mL of 0.1% peptone) and a *L. reuteri* I5007 treatment, which involved oral administration of 1.0×10^{10} CFU *L. reuteri* I5007 dissolved in 4 mL of 0.1% peptone water daily for 20 days.

The health status for each piglet was recorded, and the occurrence of diarrhea was assessed two times a day (monitoring time: 10 a.m. and 4 p.m.) according to the method of Marquardt et al. [25] and Ou et al. [26]. Scores were 0 = normal, solid feces; 1 = slight diarrhea, soft and loose feces; 2 = moderate

diarrhea, semi-liquid feces; or 3 = severe diarrhea, liquid and unformed feces. The occurrence of diarrhea was defined as maintaining a score of two or three for one day. The incidence of diarrhea (%) was calculated as ((number of piglets with diarrhea × number of days of diarrhea)/(total number of experiment piglets × number of days of the whole experiment)) × 100%.

Table 1. Composition and nutrient levels of the experimental diets (%, as-fed basis) [1].

Items	Content
Crude protein	24.78
Gross energy (MJ/kg)	20.46
Lactose	35.10
Calcium	0.92
Total phosphorus	0.73
The analyzed contents of amino acids in diets	
Asparate	2.58
Threoline	1.72
Serine	1.35
Glutamate	4.52
Proline	1.54
Glycine	0.55
Alanine	1.30
Valine	1.48
Isoleucine	1.42
Leucine	2.54
Tyrosine	0.81
Phenylalanine	0.92
Histidine	0.52
Lysine	2.03
Methonine	0.65
Argine	0.76

[1] Values are the means of a chemical analysis conducted in duplicates.

On days 4, 14, and 24, the piglets were weighed. On day 24, all piglets were euthanized with Zoletil 50® (Virbac, Carros, France), and all the intestinal tissues from the jejunum, ileum, and proximal distal colon were collected, frozen in liquid nitrogen, and then stored at –80 °C until total RNA was extracted. The colonic digesta were gently squeezed into sterile Eppendorf tubes, frozen in liquid nitrogen, and subsequently stored at −80 °C until processing.

2.5. Analysis of Porcine Gene Expression by Real Time PCR

The cells and tissues (about 0.04 mg) were lysed directly in TRIzol (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer's instructions. RNA concentrations were measured using a NanoDrop Spectrophotometer (P330, Implen, Germany). The purity was determined by the ratio of A260:A280 and A260:A230 by NanoDrop, and then the quality was checked with 1% Agarose Gel Electrophoresis following the procedures outlined by Aranda et al. [27].

The first-strand cDNA was synthesized by reverse transcription of 1 μg of total RNA using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's protocol and stored at –80 °C. The primers used are listed in Supplemental Table S1. Porcine β-Defensin (PBD) 1, pBD2, pBD3, pBD114, pBD129, Protegrins (PG) 1-5, Epididymis Protein 2 Splicing Variant C (PEP2C), toll-like Receptors (TLR) 2, TLR4, TLR6, TLR9, Nucleotide-Binding Oligomerization Domain (NOD) 1, Mucin 1 (MUC1), Peroxisome Proliferator Activated Receptor-γ (PPAR-γ) ,G Protein-Coupled Receptor (GPR) 41, and GPR43 were determined [4,28–31].

Real-time PCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Singapore) using SYBR Green PCR Master Mix (Takara, Dalian, China). All reactions were run in triplicate. Relative gene expression was calculated according to the $\Delta\Delta C_t$ method ((C_t gene of

interest − C_t internal control) treatment − (C_t gene of interest − C_t internal control) control) using porcine β-actin as the reference gene.

2.6. Colonic Short Chain Fatty Acid Concentrations

The concentrations of SCFA (short-chain fatty acid) were determined with a Dionex ICS-3000 Ion Chromatography System (Dionex Corporation, Sunnyvale, CA, USA) following the procedures of Qiu and Jin [32] with modification. Samples of colonic digesta (0.5 g) were weighed, diluted in a ratio of 1:5 with ultrapure water, homogenated with 8 mL ultrapure water, and then centrifuged at 10,000× *g* for 20 min at 4 °C. The supernatant was kept in a 2 mL screw-capped vial. The concentrations of formic, acetic, propionic, butyric, and lactic acid were measured with the Dionex ICS-3000 Ion Chromatography System (Dionex, Sunnyvale, CA, USA).

2.7. Fecal Microbiota Analysis

Microbial genomic DNA was extracted and purified from colon digesta samples using a QIAmp DNA stool mini kit (Qiagen, GmbH Hilden, Germany) modified to contain a bead-beating step. Successful DNA isolation was confirmed by agarose gel electrophoresis. PCR primers flanking the V3-V4 hyper variable region of bacterial 16S rDNA were designed. The barcoded fusion forward primer was 341F(5′-CCTAYGGGRBGCASCAG-3′), and the reverse primer was 806R(5′-GGACTACNNGGGTATCTAAT-3′). The optimized conditions for amplification were as follows: one pre-denaturation cycle at 95 °C for 5 min, 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The resulting amplicons were gel purified, quantified, pooled, and sequenced on the Illumina HiSeq 2500 platform. Microbiota sequences were processed through QIIME 1.8 (QIIME Team). After quality filtering, the sequences were denoised using denoise_wrapper.py. The denoised sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity against the GreenGenes OTU database (gg_13_8_otus). The chimeric OTUs were removed using UCHIME v4.2. Representative sequences for each OTU were picked and aligned using QIIME 1.8. Taxon-dependent analysis was conducted using the Ribosomal Database Project (RDP) classifier. The OTUs were counted for each sample to express the richness of bacterial species with an identity cutoff of 97%. Alpha and beta diversity calculations and taxonomic community assessments were performed using QIIME 1.8 scripts.

2.8. Statistical Analysis

Statistical analyses were performed using SPSS 17.0 Software (SPSS Inc., Chicago, IL, USA). All pairwise comparisons for the in vivo and in vitro data were examined using an unpaired Student's two-tailed *t*-test. Chi square was used to test differences in diarrhea incidence between the two groups. The level of significance was set at $p < 0.05$. The results were expressed as mean ± standard error of the mean (SEM). Principle component analysis (PCA) plots were used to visualize differences in bacterial community composition among samples. The PCA plots were produced based on a euclidean metric. Linear discriminant analysis effect size (LEfSe) analysis was used to identify the OTUs or taxa, which were responsible for the differences between the groups. An effect size threshold of two was used for the biomarkers discussed in this study. The metastats program from R-script was used to identify statistically different phylotypes among groups. Only taxa with average abundances greater than 10^{-3}, $p < 0.05$ and low *Q* values (low risk of false discovery) were considered significant.

3. Results

3.1. Effects of L. reuteri I5007 on Host Defense Peptide Expression in IPEC-J2 cells

The mRNA expressions of porcine HDP, including pBD1, pBD2, pBD3, pBD114, pBD129, PG1-5, and pEP2C, were determined in IPEC-J2 cells to study the effects of *L. reuteri* I5007 on the modulation of HDP. First the dose-dependence of HDP gene expression following treatment of IPEC-J2 cells

with *L. reuteri* I5007 was examined. Our results indicate that a 6 h treatment with *L. reuteri* I5007 markedly increased the mRNA expression of pBD2, pBD3, and pBD114 in a dose-dependent manner in IPEC-J2 cells, peaking at 10^8 CFU/mL (Figure 1A). The mRNA expression levels of pBD129 and PG1-5 were also dose-dependently induced by *L. reuteri* I5007 in IPEC-J2 cells, with the maximal response occurring at 10^9 CFU/mL (Figure 1A). However, the magnitude of induction varied obviously among the five genes, with PG1-5 showing an approximately 17-fold increase, whereas pBD3, pEP2C, and PG1-5 showed only 5-, 5-, and 7-fold induction at the peak response, respectively (Figure 1A).

An obvious time-dependent induction of pBD2, pBD3, pBD114, pBD129, and PG1-5 was also observed in IPEC-J2 cells after 10^8 CFU/mL *L. reuteri* I5007 treatment (Figure 1B). The maximum HDP mRNA was expressed after 6 h of incubation and decreased at 12 h of incubation. It is noteworthy that pBD1 and pEP2C were largely unaltered in the IPEC-J2 cells following *L. reuteri* I5007 treatment.

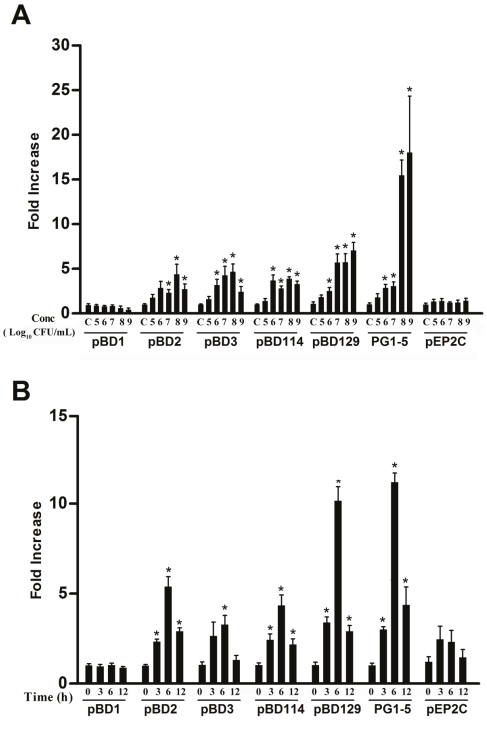

Figure 1. *L. reuteri* I5007-induced expression of pBD1, pBD2, pBD3, pBD114, pBD129, PG1-5, and pEP2C in porcine IPEC-J2 cells. Cells were incubated in duplicate with the indicated concentrations of (**A**) *L. reuteri* I5007 for 6 h or (**B**) 10^8 CFU/mL for 3, 6, or 12 h. Gene expression was analyzed by real-time PCR. The relative fold changes over the unstimulated control were calculated with the $\Delta\Delta C_t$ method using the β-actin gene for normalization. Data are mean ± standard error obtained in three independent experiments. "C" in X-axis, control group. * $p < 0.05$ by unpaired Student's *t*-test. pBD, porcine β-Defensin; PG, protegrins; pEP2C, Epididymis Protein 2 Splicing Variant C; IPEC-J2, The porcine small intestinal epithelial cell line; CFU, colony forming units.

3.2. Effects of Different Processing Conditions on L. reuteri I5007 Induced Host Defense Peptide Expression in IPEC-J2 Cells

To further examine whether *L. reuteri* I5007 induced HDP mRNA expression is altered by different processing conditions, IPEC-J2 cells were treated for 6 h with *L. reuteri* I5007 produced under different

processing conditions (Figure 2). We studied whether a heat-killed strain had the ability to stimulate the expression of HDP. The results indicated that heat-killed *L. reuteri* I5007 was much less effective than the live strain and only stimulated pBD3 expression.

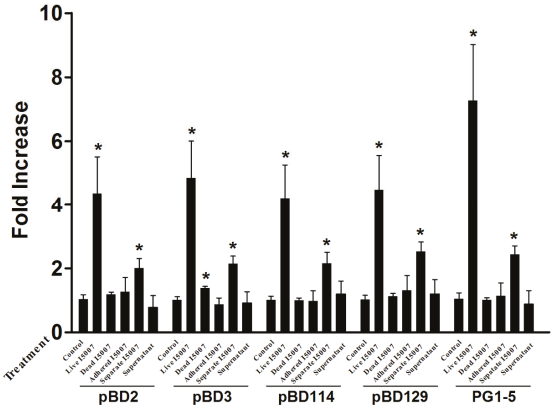

Figure 2. Regulation of pBD2, pBD3, pBD114, pBD129, and PG1-5 expression by *L. reuteri* I5007 subjected to different processing conditions. Porcine IPEC-J2 cells were incubated with 10^8 CFU/mL *L. reuteri* I5007 (Live I5007), heat-killed *L. reuteri* I5007 (Dead I5007), adhered *L. reuteri* I5007 (Adhered I5007), *L. reuteri* I5007 without direct contact with IPEC-J2 (Separate I5007), culture supernatant of *L. reuteri* I5007 (Supernatant), and the solvent control (Control). Gene expression was analyzed by real-time PCR. The relative fold changes over the unstimulated control were calculated with the $\Delta\Delta C_t$ method using the β-actin gene for normalization. Data are mean ± standard error obtained in three independent experiments. * $p < 0.05$ by unpaired Student's *t*-test.

According to a previous study conducted in our lab, *L. reuteri* I5007 has strong adhesion ability to monolayer cells when co-cultured with cells for 1 h [14]. IPEC-J2 cells were treated with *L. reuteri* I5007 for 1 h, non-adherent bacteria were washed away, and incubation continued for a further 5 h. The results revealed that the adherent bacteria were insufficient for *L. reuteri* I5007-induced HDP expression.

To further examine whether cell-to-cell contact is required for *L. reuteri* I5007 to stimulate HDP expression in IPEC-J2 cells, a Transwell Insert System, in which the bacteria and host cells are partitioned by a 0.22-μm membrane, was used to prevent all direct cell-to-cell contact between *L. reuteri* I5007 and IPEC-J2 cells. Under these conditions, *L. reuteri* I5007 enhanced the mRNA expression of pBD2, pBD3, pBD114, pBD129, and PG1-5 (Figure 2). These findings indicate that direct cell-to-cell contact is not required for *L. reuteri* I507 to stimulate HDP expression, and that some metabolite produced by *L. reuteri* I5007 may be able to cross the membrane and induce HDP expression.

To further elucidate whether the culture supernatant was involved in *L. reuteri* I5007 induced HDP expression, a culture supernatant of *L. reuteri* I5007 was also added to IPEC-J2 cells. Compared with the control group, cells treated with *L. reuteri* I5007 culture supernatant demonstrated no significant change in the mRNA expression of HDP. These findings indicate that there are no compounds in the culture supernatant without *L. reuteri* I5007 that stimulate HDP production in IPEC-J2 cells.

3.3. Effects of L. reuteri I5007 on Neonatal Piglet Performance

In this study, pigs in the two treatments (*L. reuteri* I5007 group and control group) started at the same age (day 4) and had similar body weights ($p = 0.99$) (Table 2). At the end of the experimental period (day 24), the piglets treated with *L. reuteri* I5007 had 15.07% higher ($p < 0.05$) average daily gain than control piglets. Moreover, the diarrhea incidence and diarrhea scores were lower in piglets

administrated with *L. reuteri* I5007 compared with the control, although not significant (3.64 vs. 5.91%, 0.12 vs. 0.20).

Table 2. Effects of *L. reuteri* I5007 on neonatal piglet performance and diarrhea incidence [1]

Items	Control	*L. reuteri* I5007	SEM	*p* Value
Body weight at day 4 (kg)	1.81	1.81	0.07	0.99
Body weight at day 14 (kg)	2.45	2.50	0.07	0.72
Body weight at day 24 (kg)	3.72	4.00	0.10	0.16
Average daily gain (g)				
4–14 days	64	69	3.91	0.52
14–24 days	127 [b]	151 [a]	4.86	0.01
4–24 days	96 [b]	110 [a]	3.31	0.03
Average feed intake (g/day)				
4–14 days	53	58	3.43	0.08
14–24 days	140	152	5.25	0.82
4–24 days	97	105	3.24	0.18
Diarrhea score [2]	0.20	0.12		
Diarrhea incidence [3] (%)	5.91	3.64		0.32

[a,b] Means within a row with different superscripts are significantly different ($p < 0.05$). [1] SEM, standard error of the mean, n =11 for each treatment. [2] Diarrhea scores were 0 = normal, solid feces; 1 = slight diarrhea, soft and loose feces; 2 = definitely unformed, moderately fluid feces; or 3 = very watery and frothy diarrhea; [3] The occurrence of diarrhea was defined as maintaining a score of 2 or 3 for one day. The incidence of diarrhea (%) was calculated as ((number of piglets with diarrhea × number of days of diarrhea)/(total number of experiment piglets × number of days of the whole experiment)) × 100%.

3.4. Effects of L. reuteri I5007 on Host Defense Peptide Expression in Neonatal Piglets

Twenty-two male neonatal piglets were orally administrated with 0.1% peptone solution or 1×10^{10} CFU of *L. reuteri* I5007 daily for 20 days. The levels of mRNA expression of pBD1, pBD2, pBD3, pBD114, pBD129, PG1-5, and pEP2C in the jejunum, ileum, and colon were measured and are presented in Figure 3. Compared with the control group, no significant difference was observed in the ileal HDP expression in the *L. reuteri* I5007 group. Only pBD2 expression in the *L. reuteri* I5007 group was observed to be significantly higher than that of the control group in the jejunum. However, pBD2, pBD3, pBD114, and pBD129 mRNA expression were significantly up-regulated in the colon of piglets administrated with *L. reuteri* I5007 compared with the control piglets.

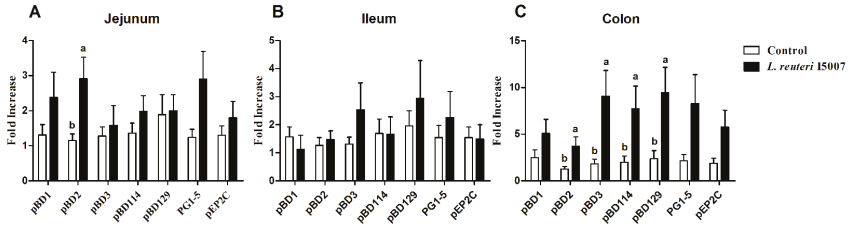

Figure 3. Regulation of pBD1, pBD2, pBD3, pBD114, pBD129, PG1-5, and pEP2C expression by *L. reuteri* I5007 in the (**A**) jejunum, (**B**) ileum, and (**C**) colon of neonatal piglets. Twenty-two male neonatal piglets were orally administrated with 0.1% peptone solution (control) or 1×10^{10} CFU of *L. reuteri* I5007 daily for 20 days. Gene expression was analyzed by real-time PCR. The relative fold changes over the control were calculated with the $\Delta\Delta C_t$ method using the β-actin gene for normalization. White bars or black bars represent control or *L. reuteri* I5007 treatments, respectively. Values are presented as mean ± standard error of the mean, n = 11 piglets per treatment. Bars with different letters differ, $p < 0.05$ by unpaired Student's *t*-test.

3.5. Effects of L. reuteri I5007 on Short Chain Fatty Acid Concentrations in Colonic Digesta

The concentrations of SCFA in colonic digesta are presented in Table 3. The concentration of butyric acid was higher in piglets treated with *L. reuteri* I5007 compared with the control group ($p < 0.05$). The concentration of formic acid tended to be higher in *L. reuteri* I5007 treated piglets ($p = 0.08$), while the acetic, propionic, and lactic acid concentrations did not differ between the two treatments.

Table 3. Effects of *L. reuteri* I5007 on short chain fatty acid concentrations (mmol/kg, wet weight) in colonic digesta obtained from neonatal piglets [1].

Fatty acid	Control	*L. reuteri* I5007	SEM	*p* Value
Formic acid	0.03	0.03	0.01	0.66
Acetic acid	32.49	37.74	2.03	0.20
Propionic acid	12.99	15.90	0.83	0.08
Butyric acid	7.10 [b]	9.51 [a]	0.58	0.04
Lactic acid	3.67	3.66	0.35	1.00

[a,b] Means within a row with different superscripts are significantly different ($p < 0.05$). [1] SEM, standard error of the mean, n =11 for each treatment. Means within a row with different superscripts are significantly different ($p < 0.05$).

3.6. Effects of L. reuteri I5007 on PPAR-γ, GPR41 and GPR43 in Colonic Tissue

The relative transcription levels of PPAR-γ, GPR41, and GPR43 in the colonic tissue were analyzed using real-time PCR (Figure 4). The relative abundances of mRNA for PPAR-γ and GPR41 were significantly increased in the colonic tissue of piglets treated with *L. reuteri* I5007 compared with the control treatment ($p < 0.05$).

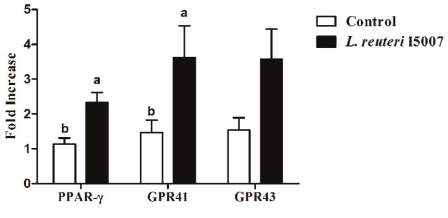

Figure 4. Effects of *L. reuteri* I5007 on PPAR-γ, GPR41, and GPR43 expression in the colonic tissue of neonatal piglets. Gene expression was analyzed by real-time PCR. The relative fold changes over the control were calculated with the $\Delta\Delta C_t$ method using the β-actin gene for normalization. Data are mean ± standard error ($n = 11$). Bars with different letters differ, $p < 0.05$ by unpaired Student's *t*-test.

3.7. Effects of L. reuteri I5007 on Bacterial Community Structure in Colonic Digesta

A total of 810,187 high quality sequences were obtained from all fecal samples, with an average of 36,826 sequences per sample. These sequences were assigned to 680 operational taxonomic units (OTUs). The Shannon diversity indices reached stable values, suggesting that the present study captured the dominant phylotypes. The fecal samples of all of the pigs were dominated by four phyla; *Bacteroidetes*, *Firmicutes*, *Spirochaetes*, and *Proteobacteria*, with *Firmicutes* and *Bacteroidetes* accounting for >95% of the bacteria (Figure 5A). The abundance of *Firmicutes* (76.69 ± 3.16 vs. 69.41 ± 3.46; $p = 0.13$, $Q = 0.87$) and *Bacteroidetes* (18.26 ± 3.04 vs. 25.81 ± 3.63; $p = 0.13$, $Q = 0.87$) was not significantly different between groups (Supplemental Table S2). An analysis of colonic microbiota composition by principal component analysis revealed that *L. reuteri* I5007 administration did not affect the overall composition of the fecal microbiota (Supplemental Figure S1). No significant differences were observed in relative abundance of bacterial taxa or operational taxonomic units between placebo-treated and *L. reuteri*–treated piglets except that three bacterial taxa were identified by LEfSe analysis (linear

discriminant analysis, LDA score >2) (Figure 5B). These significant differences were further confirmed by metastats analysis, in which genera *Sharpea* were significantly increased in *L. reuteri* I5007 group ($p = 0.03$ and 0.02, respectively) (Supplemental Table S2). To determine whether the difference in colonic butyrate concentration was caused by the alteration of genera *Sharpea*, the correlation between the butyrate level and *Sharpea* was analyzed. However, no significant correlation was found between the butyrate level and genera *Sharpea* ($r = 0.05$, $p = 0.82$, Spearman).

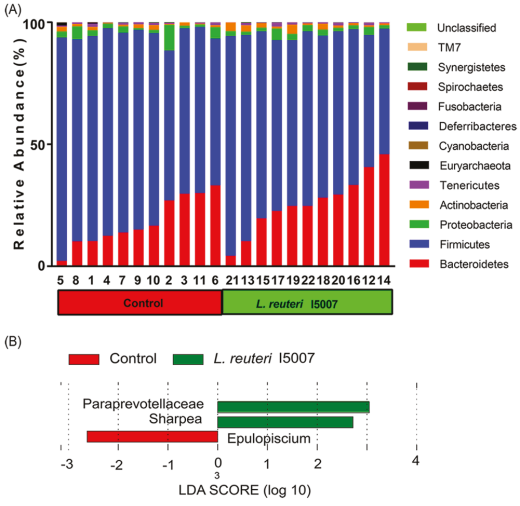

Figure 5. Effects of *L. reuteri* I5007 on bacterial community structure in colonic digesta. (**A**) Relative abundance levels of the bacterial phyla present in control group and *L. reuteri* I5007 group; (**B**) Histogram of the LDA scores computed for taxa differentially abundant between control and *L. reuteri* I5007 treated piglets ($n = 11$).

3.8. Effects of L. reuteri I5007 on Pattern-Recognition Receptor (PRRs) Expression in Intestinal Tissue

An analysis of PRRs by real-time PCR revealed that the expressions of TLR2, TLR4, TLR6, TLR9, and NOD1 were not changed with *L. reuteri* I5007 treatment in the jejunum, ileum, or colon ($p > 0.05$). The relative abundance of mRNA for MUC1 was significantly increased in the colon with *L. reuteri* I5007 treatment compared with the control treatment ($p < 0.05$), while no significant differences were observed in the jejunum and ileum (Supplemental Figure S2).

4. Discussion

In the present study, we observed that the probiotic *L. reuteri* I5007 induced the expression of HDP in a porcine small intestinal epithelial cell line (IPEC-J2). In addition, we observed that oral administration of *L. reuteri* I5007 stimulated colonic HDP expression in neonatal piglets. Finally, we found that *L. reuteri* I5007 increased concentrations of butyric acid in neonatal piglets but did not affect the colonic bacterial community structure.

Probiotics have been shown to induce β-Defensin (hBD-2) in human cells, and different probiotic strains show different HDP-inducing activity [13]. In pigs, *L. salivarius* can induce pBD2 production in the digestive tract [14]. *L. reuteri* is one of dominant species in the gastrointestinal tracts of humans and animals and is currently used as a probiotic in pigs [16]. Oral administration of *L. reuteri* modulates ileum microbial composition, intestinal development, and immune status in pigs [22,24]. However, there are no reports of the effects of *L. reuteri* on the stimulation of HDP gene expression in IPEC-J2 cells and pigs.

Previous studies have indicated that pBD1, pBD2, pBD3, pBD114, pBD129, PG1-5, and pEP2C are expressed in IPEC-J2 cells [4]. In this study, we found that *L. reuteri* I5007 administration increased pBD2, pBD3, pBD114, pBD129, and PG1-5 gene expression in these same cells. The time-dependent experiments showed a similar pattern, as previously described by Wehkamp et al. [33] and Schlee et al. [13], with the maximum level of HDP being induced after 6 h of incubation. The dose-dependent experiment showed that *L. reuteri* I5007 induced HDP production when the concentration of *L. reuteri* I5007 reached 10^6 CFU/mL.

Previously, Schlee et al. [13] used heat-killed bacteria, but we decided to research the effects of live strains. Since *L. reuteri* I5007 is used as a probiotic, the results for live strains may be helpful for future in vivo studies. Wehkamp et al. [33] also found a living form of *E. coli* Nissle 1917, which showed a strong induction of hBD-2 after incubation with Caco-2 cells for 4.5 h. A previous study demonstrated that heat-killed bacteria induced hBD-2 [13], but we found that only pBD3 expression was significantly increased with heat killed *L. reuteri* in the present study. Compared with the live strain, the capacity of the heat-killed *L. reuteri* I5007 to induce HDP was visibly diminished.

The suspension without bacteria did not induce HDP, which is consistent with the findings described by Wehkamp et al. [33] for *E. coli* Nissle 1917. In order to determine whether *L. reuteri* I5007 induced HDP by cell-to-cell contact, a Transwell Insert System was used. Our results indicate that *L. reuteri* I5007 without contact with IPEC-J2 cells also induced HDP, which suggests that a metabolite produced by *L. reuteri* I507 may be playing a role.

Neonatal piglets have an immature immune system, are susceptible to infections, and often suffer from diarrhea and growth retardation if infected [4]. Our previous studies showed that the optimum dosage (about 10^{10} CFU/day of *L. reuteri* I5007) could improve performance and reduce diarrhea incidence in neonatal piglets [22]. In this study, we obtained a similar result. We also found that the administration of *L. reuteri* I5007 induced pBD2 in the jejunum and pBD2, pBD3, pBD114, and pBD129 in the colon. Similar results have been obtained for *L. salivarius* induced expression of pBD2 in the pig jejunum [14]. The spatial heterogeneity patterns of this induction effect were probably due to the production of butyric acid, mainly triggered in the hindgut after *L. reuteri* I5007 administration [34]. It has been reported that most of the porcine HDP (e.g., pBD1, pBD2, pBD114, pBD129, PG1-5, pEP2C) show various activity against Gram-negative and Gram-positive bacteria, including *Salmonella typhimurium*, *Escherichia coli*, and *Clostridium perfringen*, the blooms of which are involved in the occurrence of diarrhea [35,36]. In addition to its antimicrobial properties, pBD3 could also regulate the expression of IL-8 and intestinal tight junction protein and exhibits a strong immunoregulatory ability [37]. In addition, β-defensins (hBD2) have been observed to have the capacity to recruit leukocytes. These actions can directly modify the inflammatory response [38]. The induction of the expression of HDP genes allows the immature intestinal epithelial surfaces of neonatal piglets to cope with these continuously complex microbial challenges [38]. Furthermore, previous studies showed that the supplementation of synthetic HDP could improve nutrient digestibility, intestinal morphology, and growth performance in weanling pigs [39] and broiler chickens [40]. These results suggest that the induction of HDP gene expression by *L. reuteri* I5007 may be responsible for the body weight increase and decrease in diarrhea incidence, which was mostly caused by the bloom of pathogens that can be eliminated by the HDPs [35,41,42].

Short chain fatty acids are the major metabolites of microbial digestion in the colon and have been considered as contributing an important role in normal colonic morphology and function [34]. Recent published data have proven that HDP could be induced by SCFA, especially by butyrate [4]. Our previous work and that of others indicated that administration of probiotics increased butyrate levels in the colonic digesta and fecal samples [22,24]. In this experiment, after the administration of *L. reuteri* I5007, the quantity of butyric acid in the colonic digesta was increased, which is consistent with the finding of Liu et al. [22] and may be the mechanism through which *L. reuteri* I5007 induces the expression of HDP.

Exposure to SCFA, such as butyrate, triggers profound changes in epithelial gene expression in vitro [43,44], which are mediated at least in part through the SCFA sensor PPAR-γ [45]. GPR41 (Free Fatty Acid Receptor 3, FFA3) and GPR43 (FFA2) are related G Protein-Coupled Receptors that are activated by short chain carboxylic acids [46,47]. In order to further confirm the changes of SCFA, we determined the changes of short-chain fatty acid receptors in the colon. PPAR-γ and GPR41 were observed to be higher in mRNA expression after *L. reuteri* I5007 administration compared with piglets in the control treatment.

Changes in the structure of gut microbiota alter the gut-microbial metabolism and eventually influence intestinal mucosal immunity and host metabolism [48,49]. Previous studies have reported increases of lactic acid bacteria after the intake of individual lactobacilli strains [50–53]. The administration of *Lactobacillus rhamnosus* GG increases the fecal butyrate level through expanding butyrate-producing bacterial strains [54]. To examine whether the significant difference in butyrate concentration of colonic digesta was induced by the change of microbiota structure after *L. reuteri* I5007 administration, the colonic microbial community was determined. Unexpectedly, the results of the next generation high throughput sequencing showed no significant changes in the colonic microbiota composition or stability, which was consistent with our previous finding using polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) profiling [22]. Similar observations have been reported in a recent review [55], suggesting that probiotics do not significantly modify the gut microbiota composition of healthy subjects. Additionally, microbiomes were compared at different taxonomic levels, and no differences were detected except the specific increase of genera *Sharpea*, which is a member of *Clostridium XVII* and exhibits a close phylogenetic association with *Lactobacillus catenaformis* and *Lactobacillus vitulinus* [56]. However, no significant correlation was found between the butyrate level and genera *Sharpea*, which indicated that the increase of butyrate levels was not due to the alteration of genera *Sharpea*. It has recently been reported that the introduction of probiotics significantly changes the microbiome's transcriptional profile but has no significant impact on the structure, leading us to speculate that *L. reuteri* I5007 may increase the butyrate concentration through modulating the microbial metabolic activities. Taken together, these results suggest that *L. reuteri* I5007 might increase butyrate levels through modulating the microbiota at the transcript level, rather than modifying the bacterial community structure in the colonic digesta [57,58]. PRRs could recognize conserved molecular motifs present on a wide range of different microbes, which have been termed Microbe-Associated Molecular Patterns (MAMPs). TLRs and NOD proteins are two classes of PRRs involved in innate immune detection [59]. Agreeing with the result of colonic bacterial community analysis, the mRNA levels for PRRs were unaffected by *L. reuteri* I5007 treatment, which indicated that *L. reuteri* I5007 induction of HDP expression was not induced through regulating the structure of the colonic bacterial community or expression of PRRs [57,58,60].

5. Conclusions

In conclusion, our study indicates that *L. reuteri* I5007 could regulate the expression of pBD2, pBD3, pBD114, pBD129, and PG1-5 in IPEC-J2 cells and stimulate the mRNA expression of colonic pBD2, pBD3, pBD114, and pBD129 in the neonatal piglets, which is probably mediated by increased butyric acid production and the up-regulation of the downstream molecules of butyric acid, PPAR-γ and GPR41, but not by modulation of the colonic microbial community. These findings suggest that the probiotic *L. reuteri* I5007 enhances HDP expression, which strengthens the mucosal antimicrobial barrier of neonatal piglets and is one explanation for the growth promoting effect of *L. reuteri* I5007. The identification of *L. reuteri* I5007 with favorable odors will expedite its application as a non-antibiotic, immune boosting additive for infectious disease prevention and control in pigs and other animal species; perhaps human beings as well.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/6/559/s1. Figure S1: Effects of *L. reuteri* I5007 on bacterial community structure in colonic digesta, Figure S2: Effects of *L. reuteri* I5007 on TLR2, TLR4, TLR6, TLR9, NOD1, and Muc1 expression in the (A) jejunum, (B) ileum,

and (C) colon of neonatal piglets, Table S1: Primer sequences used in the study, Table S2: Effect of *L. reuteri* I5007 on the relative abundance (%) of bacterial groups at the phylum, family, and genus level (above 1% abundance in at least one sample) detected in colonic digesta microbiota of piglets (n = 11).

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Author Contributions: H.L., C.H., and S.Q. conceived and designed the experiments; H.L., C.H., G.W., H.J., and H.Y. performed the experiments; H.L., X.Z., and C.H. analyzed the data and wrote the paper; and H.J. contributed reagents/materials/analysis tools.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

HDP, host defense peptide; pBD, porcine β-defensin; PPAR-γ, peroxisome proliferator-activated receptor-γ; GPR, G protein-coupled receptor; SCFAs, short chain fatty acids; LDA, linear discriminant analysis; CFU, colony forming units; PRRs, pattern-recognition receptors; pEP2C, porcine epididymis protein 2 splicing variant C; PG, cysteine-rich protegrins; TLR, toll-like receptor; NOD, nucleotide-binding oligomerization domain; MUC, Mucin.

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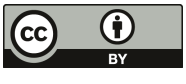

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Consumption of Dairy Yogurt Containing *Lactobacillus paracasei* ssp. *paracasei*, *Bifidobacterium animalis* ssp. *lactis* and Heat-Treated *Lactobacillus plantarum* Improves Immune Function Including Natural Killer Cell Activity

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Abstract: The aim of this study was to investigate the impact of consuming dairy yogurt containing *Lactobacillus paracasei* ssp. *paracasei* (*L. paracasei*), *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) and heat-treated *Lactobacillus plantarum* (*L. plantarum*) on immune function. A randomized, open-label, placebo-controlled study was conducted on 200 nondiabetic subjects. Over a twelve-week period, the test group consumed dairy yogurt containing probiotics each day, whereas the placebo group consumed milk. Natural killer (NK) cell activity, interleukin (IL)-12 and immunoglobulin (Ig) G1 levels were significantly increased in the test group at twelve weeks compared to baseline. Additionally, the test group had significantly greater increases in serum NK cell activity and interferon (IFN)-γ and IgG1 than placebo group. Daily consumption of dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum* could be an effective option to improve immune function by enhancing NK cell function and IFN-γ concentration (ClinicalTrials.gov: NCT03051425).

Keywords: immune function; IFN-γ; NK cell activity; probiotics

1. Introduction

The population of individuals above the age of 60 is steadily increasing in Korea. Elderly individuals suffer from more frequent and more severe infections than younger individuals for reasons including epidemiological factors, immunosenescence and malnutrition as well as various age-associated physiological alterations [1]. Human immune function also undergoes adverse changes with aging, including immune senescence, which potentially increases the risk of certain infections and cancers [2,3].

Consumption of yogurt could improve immune function based on its composition of probiotics, zinc, vitamin B6, and protein, which are associated with immune enhancement [4,5]. Among these components, probiotics are regarded as the most important in terms of stimulating the immune system [6]. Many studies have demonstrated that intake of some probiotic strains can affect the immune response with different manifestations [7–10]. For example, Rizzardini et al. [10] showed

that supplementation with *Bifidobacterium animalis* ssp. *lactis* (BB-12®) and *Lactobacillus paracasei* ssp. *paracasei* (L. casei 431®) could be an effective means to improve immune function by augmenting the systemic immune response to challenge using a vaccination model in healthy subjects. Makino et al. [9] showed that consumption of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 augmented natural killer (NK)-cell activity and reduced the risk of infection in elderly individuals. Additionally, Kawashima et al. [8] observed that *Lactobacillus plantarum* (*L. plantarum*) strain YU has a beneficial effect in activating helper T lymphocyte (Th)-1 immune responses and preventing viral infection.

Interestingly, some studies of heat-treated or heat-killed *Lactobacillus* have also shown a relationship with enhanced immunity. Tobita et al. [11] reported that heat-treated *Lactobacillus crispatus* KT strains could modulate the type 1/type 2 Th cell balance, reducing allergic symptoms in mice. Murosaki et al. [12] revealed that heat-killed *L. plantarum* L-137 might augment immunity in response to increased plasma levels of interleukin (IL)-12, previously known as natural killer cell stimulatory factor, which were obtained in mice treated with this strain. Hirose et al. [13] also found that heat-killed *L. plantarum* L-137 positively influenced acquired immune responses in healthy adults. Moreover, Lee et al. [14] reported that dead nano-sized *L. plantarum* reduced the expression of inflammatory markers in mouse colonic tissues.

Therefore, the aim of this study was to investigate the impact of consuming dairy yogurt containing *Lactobacillus paracasei* ssp. *paracasei* (*L. paracasei*), *Bifidobacterium animalis* ssp. *lactis* (*B. lactis*) and heat-treated *L. plantarum* on NK cell activity and circulating levels of cytokines and immunoglobulin (Ig) in elderly individuals (≥60 years).

2. Materials and Methods

2.1. Study Subjects

The study included 200 nondiabetic (fasting serum glucose concentration <126 mg/dL) subjects over 60 years in age with white blood cell levels between $4 \times 10^3/\mu L$ and $10 \times 10^3/\mu L$. Participants were recruited from the Goyang-si Heendol Community Welfare Center (Goyang, Korea) via poster advertisements between March 2016 and December 2016. Volunteers who agreed to participate and provided written informed consent were screened to measure white blood cell and fasting serum glucose levels. After the screening, those who met the inclusion criteria were enrolled. The following exclusion criteria were applied: regular consumption (more than 5 times per week) of any probiotic products or taking medicine related to inflammation within one month before screening, history/presence of diabetes, allergy to milk protein, epilepsy, liver disease, kidney disease, immune disease, cancer, or medication/alcohol abuse. Before participation, the purpose of the study was carefully explained to all subjects and written informed consent was obtained. The study protocol was approved by the Institutional Review Board of Yonsei University (1040917-201603-BR-151-06) and was conducted in accordance with the Helsinki Declaration.

2.2. Study Design and Intervention

A randomized, open-label, placebo-controlled study was conducted on 200 nondiabetic subjects. Over a twelve-week period, the test group (n = 100) consumed one bottle (120 mL) of dairy yogurt containing *L. paracasei* (L. casei 431®) at 12.0×10^8 cfu/day, *B. lactis* (BB-12®) at 12.0×10^8 cfu/day and 0.0175% heat-treated *L. plantarum* (nF1) once per day. The placebo group (n = 100) consumed the same volume of milk once per day (NCT03051425, http://www.clinicaltrials.gov). The two probiotic strains (L. casei 431® and BB-12®) and heat-treated *L. plantarum* (nF1) were provided by Chr.Hansen A/S (Hθrsholm, Denmark) and Biogenics Korea Co., Ltd (Seoul, Korea), respectively. The final products, including dairy yogurt and milk, were provided by Purmil Co., Ltd. (Seoul, Korea). Computer-generated block randomization was used (placebo:dairy yogurt = 1:1). The study was divided into two periods: the pre-ingestion period, in which nondiabetic subjects did not ingest test or

placebo products for two weeks (from screening to week 0), and the ingestion period, in which subjects ingested dairy yogurt or milk during the twelve-week study (from week 0 to week 12).

2.3. Anthropometric Parameters and Blood Pressure

Body weight (in lightweight clothes and without shoes) (UM0703581; Tanita, Tokyo, Japan) and height (GL-150; G-tech International, Uijeongbu, Korea) were measured in the morning, and body mass index (BMI) was calculated in units of kilograms per square meter (kg/m^2). Anthropometric parameters were assessed at weeks 0 and 12. During each testing session, systolic and diastolic blood pressure (BP) were assessed in the supine position after a resting period (20 min). BP was measured twice on the left arm using an automatic BP monitor (FT-200S; Jawon Medical, Gyeongsan, Korea); the two measurements were then averaged.

2.4. Serum Glucose and Lipid Profiles

Serum fasting glucose levels were measured via the hexokinase method; fasting triglyceride and total and low density lipoprotein (LDL) cholesterol levels were measured via enzymatic assays; and high density lipoprotein (HDL) cholesterol levels were measured via selective inhibition. All measurements were taken using a Hitachi 7600 autoanalyzer (Hitachi Ltd., Tokyo, Japan).

2.5. Serum Albumin, White Blood Cell, High-Sensitivity C-Reactive Protein and Immunoglobulin G Levels

Serum albumin concentrations were analyzed via the BCG method using an ALB kit (Roche, Basel, Switzerland) with a Hitachi 7600 autoanalyzer (Hitachi Ltd., Tokyo, Japan). White blood cell levels were determined using a HORIBA ABX diagnostic analyzer (HORIBA ABX SAS, Parc Euromedicine, Montpellier, France). Serum high-sensitivity C-reactive protein (hs-CRP) levels were measured via the turbidity method by a latex agglutination immunoassay using a Hitachi 7600 autoanalyzer. The concentrations of serum immunoglobulin G1 and G3 were analyzed via immunoturbidmetric assay using a COBAS Integra 800 (Roche Diagnostics, Rotkreuz, Switzerland).

2.6. Cytokine Assays

The serum level of tumor necrosis factor (TNF)-α was measured using a Bio-Plex™ Reagent Kit on a Bio-Plex™ system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Interferon (IFN)-γ in the serum was analyzed using an IFN gamma High-Sensitivity Human ELISA Kit (Covalab, Villeurbanne, France) according to the manufacturer's instructions. IL-12 in the serum was measured using a High-Sensitivity Human IL-12 (P70) ELISA kit (Bosterbio, Pleasanton, CA, USA). The absorbance of the reaction mixtures was read at 450 nm using a Victor™ $\times$ 5 Multilabel HTS Reader (PerkinElmer, Waltham, MA, USA).

2.7. Isolation of PBMCs

Whole blood was mixed with the same volume of RPMI 1640 (Gibco, Invitrogen Co., Waltham, MA, USA), gently overlaid on Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), and then centrifuged at 1800 rpm for 20 min at 15 °C. After separation, the peripheral blood mononuclear cell (PBMC) layer was isolated, washed twice, and resuspended in RPMI 1640. PBMCs were cultured with streptomycin for NK cell cytotoxicity assays.

2.8. Cytotoxic Activity of NK Cells

The cytolytic activity of NK cells was determined using a CytoTox96® Non-radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA). To assay NK cell cytotoxic activity, PBMCs isolated from each subject were incubated with K562 cells. Briefly, PBMCs (effector cells, E) were seeded with 2×10^4 K562 cells (target cells, T) per well at ratios of 10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1. The plates were treated at different E:T ratios (10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1) and were incubated at

37 °C with 5% CO_2 overnight, according to the manufacturer's instructions. Finally, NK cell activity was measured using a Victor^{TM} × 5 Multilabel Plate Reader (PerkinElmer, Waltham, MA, USA) at 490 nm and was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100 \quad (1)$$

2.9. Daily Energy Intake and Physical Activity Measurements

Information about the subjects' usual diets was obtained using both a 24-h recall method and a semi-quantitative food frequency questionnaire. We used the former to carry out our analyses and the latter to check if the data collected by the 24-h recall method were representative of their usual dietary patterns. All subjects were given written and verbal instructions by a registered dietitian on how to complete a three-day (two weekdays and one weekend day) dietary record every six weeks. Dietary energy values and nutrient contents from these 3-day food records were calculated using the Computer Aided Nutritional analysis program (CAN-pro 3.0, Korean Nutrition Society, Seoul, Korea). A standardized 3-day physical activity record was also completed at home on the same days in which the dietary record was completed. Total energy expenditure (kcal/day) was calculated from each subject's activity patterns, including their basal metabolic rate, physical activity over 24 h, and specific dynamic action of the food consumed. Basal metabolic rates for each subject were calculated using the Harris-Benedict equation.

2.10. Statistical Analysis

Statistical analysis was performed using SPSS, version 23.0 (IBM/SPSS, Chicago, IL, USA). Skewed variables were logarithmically transformed. Independent *t*-tests were used to compare parameters between the placebo and test groups. Paired *t*-tests were used to compare parameters between the baseline measurements and those collected at the 12-week follow-up. Pearson's correlation coefficient was used to examine the relationships between variables. The results are expressed as the mean ± standard error. For descriptive purposes, mean values are presented using untransformed values. A two-tailed *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects on Clinical Characteristics Following Twelve Weeks of Consuming Dairy Yogurt Containing L. paracasei, B. lactis and Heat-Treated L. plantarum

This study initially enrolled 200 subjects: 48 subjects (21 placebo and 27 test subjects) were later omitted, with 23 subjects discontinuing the study for personal reasons and 4 participants requiring antibiotics. Ten participants who had poor compliance (less than 80%) and 4 subjects who experienced weight change greater than 5% from baseline were excluded from the final analysis. Seven subjects were also excluded because their white blood cell level at baseline did not meet the study criteria. Ultimately, 152 subjects (79 placebo and 73 test subjects) were included in the final analysis. No adverse events were reported from the participants. Table 1 shows the clinical characteristics at baseline and at twelve weeks for the placebo and test groups. At baseline, there were no significant differences between the two groups in age, gender distribution, smoking and drinking, BMI, systolic and diastolic BP, serum glucose, lipid profiles, albumin, leukocyte counts, or hs-CRP (Table 1). After 12 weeks of treatment, significant increases were found in serum triglyceride in the test group (yogurt group) and in hs-CRP in the placebo group (milk group). However, there were no significant changes (differences from baseline) in triglyceride or hs-CRP between the placebo and test groups (Table 1). The estimated total calorie intake, physical activity, percent protein intake, percent fat intake, and percent carbohydrate intake did not significantly differ between the two groups at baseline, week six, or week twelve (data not shown).

Table 1. Effects on clinical and biochemical characteristics following 12 weeks of consuming dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum*.

	Total Subjects (n = 152)				p^a	p^b	p^c
	Placebo (n = 79)		**Test (n = 73)**				
	Baseline	**Follow-up**	**Baseline**	**Follow-up**			
Age (year)	65.7 ± 0.56		65.7 ± 0.50		0.988		
Male/Female n, (%)	24 (30.4)/55 (69.6)		21 (28.8)/52 (71.2)		0.828		
Current smoker n, (%)	4 (5.1)		7 (9.6)		0.282		
Current drinker n, (%)	32 (40.5)		25 (34.2)		0.426		
BMI (kg/m^2)	23.7 ± 0.31	23.7 ± 0.33	23.6 ± 0.25	23.7 ± 0.26	0.856	0.895	
Change	−0.03 ± 0.06		0.09 ± 0.06				0.131
Systolic BP (mmHg)	125.7 ± 1.73	123.4 ± 1.72	124.3 ± 1.94	122.9 ± 1.94	0.601	0.832	
Change	−2.23 ± 1.50		−1.42 ± 1.61				0.713
Diastolic BP (mmHg)	77.1 ± 0.93	76.9 ± 1.22	76.8 ± 1.19	75.4 ± 1.42	0.822	0.408	
Change	−0.20 ± 0.99		−1.41 ± 1.00				0.394
Glucose (mg/dL) $^\natural$	88.3 ± 1.19	89.0 ± 1.27	87.8 ± 0.93	88.9 ± 1.35	0.863	0.955	
Change	0.73 ± 0.85		1.10 ± 1.01				0.783
Triglyceride (mg/dL) $^\natural$	122.6 ± 6.31	126.3 ± 6.25	123.0 ± 8.41	138.0 ± 7.39 **	0.816	0.230	
Change	3.66 ± 5.49		15.0 ± 8.11				0.243
Total cholesterol (mg/dL) $^\natural$	206.7 ± 4.38	204.8 ± 4.35	209.2 ± 4.10	208.3 ± 4.07	0.585	0.488	
Change	−1.86 ± 3.00		−0.93 ± 2.32				0.807
HDL-cholesterol (mg/dL) $^\natural$	54.8 ± 1.54	54.0 ± 1.63	54.8 ± 1.64	54.7 ± 1.71	0.920	0.810	
Change	−0.75 ± 1.15		−0.11 ± 0.88				0.662
LDL-cholesterol (mg/dL) $^\natural$	127.4 ± 4.39	125.6 ± 4.09	129.8 ± 4.02	126.0 ± 3.64	0.529	0.747	
Change	−1.85 ± 2.71		−3.82 ± 2.49				0.595
Serum albumin (mg/dL) $^\natural$	4.55 ± 0.02	4.55 ± 0.03	4.55 ± 0.02	4.52 ± 0.03	0.939	0.497	
Change	−0.01 ± 0.02		−0.03 ± 0.02				0.361
White blood cells ($\times 10^3/\mu$L) $^\natural$	5.33 ± 0.12	5.29 ± 0.14	5.61 ± 0.13	5.71 ± 0.18	0.089	0.070	
Change	−0.03 ± 0.12		0.10 ± 0.13				0.424
hs-CRP (mg/L) $^\natural$	0.80 ± 0.07	2.01 ± 0.71 *	1.24 ± 0.26	1.77 ± 0.50	0.449	0.781	
Change	1.21 ± 0.72		0.53 ± 0.55				0.460
TNF-α (pg/mL) $^\natural$	22.5 ± 4.93	22.9 ± 5.58	23.1 ± 4.46	21.4 ± 3.13	0.798	0.754	
Change	0.39 ± 2.23		−1.77 ± 2.62				0.529
Immunoglobulin G3 (mg/dL) $^\natural$	265.6 ± 18.1	266.2 ± 18.6	256.8 ± 20.4	246.0 ± 18.5	0.565	0.506	
Change	0.66 ± 14.6		−10.8 ± 11.9				0.548

Mean ± SE. $^\natural$ tested by logarithmic transformation, p^a-values derived from independent t-tests at baseline. p^b-values derived from independent t-tests at follow-up. p^c-values derived from independent t-tests at changed value. * $p < 0.05$ and ** $p < 0.01$ derived from paired t-tests.

3.2. Effects on Serum Cytokine and Immunoglobulin Concentrations Following Twelve Weeks of Consuming Dairy Yogurt Containing L. paracasei, B. lactis and Heat-Treated L. plantarum

No significant differences were found in serum concentrations of TNF-α, IgG3 (Table 1), IL-12, IFN-γ, or IgG1 (Figure 1) at baseline between the placebo and test groups. After twelve weeks of treatment, significant increases were found in IL-12 and IgG1 in the test group. In comparing differences from baseline between the placebo and test groups, the test group exhibited greater increases in serum IFN-γ ($p = 0.041$) and IgG1 ($p = 0.022$) concentrations (Figure 1).

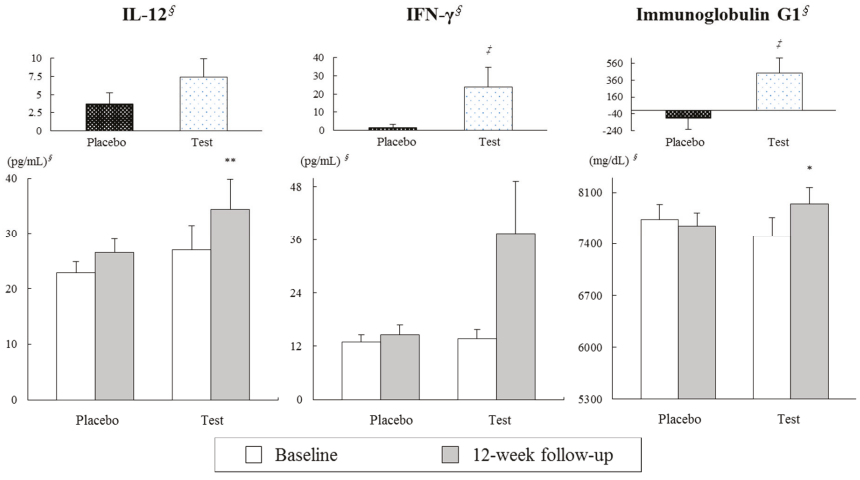

Figure 1. Effects on serum interleukin (IL)-12, interferon (IFN)-γ, and immunoglobulin G1 concentrations following 12 weeks of consuming dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum*. Mean ± SE. § tested by logarithmic transformation. * $p < 0.05$ and ** $p < 0.01$ derived from paired *t*-tests within each group. ‡ $p < 0.05$ derived from independent *t*-tests at changed value and adjusted for baseline values, sex, and changes in diastolic blood pressure (BP).

3.3. Effects on NK Cell Activity Following Twelve Weeks of Consuming Dairy Yogurt Containing L. paracasei, B. lactis and Heat-Treated L. plantarum

NK cell activity (%) was measured based on E:T ratios of 10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1. As shown in Table 2, no significant differences were found in NK cell activity measured at baseline between the placebo and test groups under any condition. Compared to baseline, NK cell activity significantly increased for all E:T ratios in the test group at twelve weeks, whereas the placebo group showed no significant changes. When we compared the changes between the placebo and test groups, the test group had greater increases in serum NK cell activity at ratios of E:T = 10:1 ($p < 0.001$), E:T = 5:1 ($p < 0.001$), E:T = 2.5:1 ($p < 0.001$), E:T = 1.25:1 ($p = 0.003$) and E:T = 0.625:1 ($p = 0.002$) after adjusting for baseline values, sex, and changes in diastolic BP (Table 2).

Table 2. Effects on natural killer (NK) cell activity following 12 weeks of consuming dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum*.

	Total Subjects (n = 152)				p^a	p^b	p^c	p^d
	Placebo (n = 79)		Test (n = 73)					
	Baseline	Follow-up	Baseline	Follow-up				
NK cell activity 10:1 (%) §	23.9 ± 1.72	26.1 ± 1.79	22.0 ± 1.77	35.1 ± 2.16 ***	0.395	<0.001		
Change	2.26 ± 1.82		13.2 ± 2.00				<0.001	<0.001
NK cell activity 5:1 (%) §	17.3 ± 1.34	18.0 ± 1.26	14.6 ± 1.34	25.2 ± 1.87 ***	0.349	0.002		
Change	0.69 ± 1.55		10.6 ± 1.78				<0.001	<0.001
NK cell activity 2.5:1 (%) §	12.4 ± 0.94	13.1 ± 1.09	12.0 ± 1.19	20.4 ± 1.54 ***	0.157	<0.001		
Change	0.73 ± 1.22		8.33 ± 1.55				<0.001	<0.001
NK cell activity 1.25:1 (%) §	10.5 ± 1.01	11.6 ± 1.15	9.78 ± 1.14	16.9 ± 1.61 ***	0.190	0.001		
Change	1.11 ± 1.32		7.08 ± 1.60				0.004	0.004
NK cell activity 0.625:1 (%) §	10.2 ± 1.15	9.57 ± 0.95	8.48 ± 1.20	14.7 ± 1.75 ***	0.059	0.019		
Change	−0.65 ± 1.36		6.23 ± 1.66				0.002	0.002

Mean ± SE. § tested by logarithmic transformation, p^a-values derived from independent *t*-tests at baseline. p^b-values derived from independent *t*-tests at follow-up. p^c-values derived from independent *t*-tests at changed value. p^d-values adjusted for baseline values, sex, and changes in diastolic BP for changed value. *** $p < 0.001$ derived from paired *t*-tests.

4. Discussion

The main findings of the present study indicated that daily supplementation of one bottle (120 mL) of dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum* led to beneficial immunostimulatory effects in healthy elderly subjects. In comparison with the placebo group, the test group showed significantly greater increases in serum NK cell activity at E:T ratios of 10:1, 5:1, 2.5:1, 1.2.5:1 and 0.625:1 after adjusting for baseline values, sex, and changes in diastolic BP. This finding is important because NK cells play an important role in the innate immune response. Indeed, Kawashima et al. [8] showed that the *L. plantarum* strain YU enhances NK cell activity in spleen cells, and Dallal et al. [7] recently reported that oral administration of *L. casei* significantly increased NK cytotoxicity in spleen cell cultures from mice bearing invasive ductal carcinoma. Takeda et al. [15] demonstrated that habitual intake of a fermented milk drink containing the *L. casei* strain Shirota increased NK cell activity in middle-aged volunteers. Moreover, an enhancement in NK cell activity in mouse spleen cells was also found after oral administration of *L. bulgaricus* OLL1073R-1 or yogurt fermented with this strain [16]. Therefore, the present result showing enhanced NK cell activity in the test group probably resulted from consumption of yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum*, which could enhance the immune response, particularly in immunocompromised populations such as the elderly.

NK cells provide a substantial defense against viral infection [17], and low NK cell activity was shown to be associated with the development of infections in healthy elderly subjects [18]. In a murine experiment, NK cells were shown to be a major source of IFN-γ, a potent immune-stimulatory cytokine [19] also known for its antiviral, immunoregulatory, and anti-tumor properties [20]. IFN-γ also has an effect on NK cell regulation [21]; therefore, the increase in serum IFN-γ concentration accompanying the increase in NK cell activity measured in the test group in this study could have contributed to the immune-enhancing action of supplementation with dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum*.

IFN-γ is produced not only by NK cells but also T and B cells [22]. Th cells are divided into two functional subclasses, Th1 and Th2, based on the cytokines they produce and their effects on cell-mediated and humoral immunity [23]. Th1 cells produce IL-2, IFN-γ, TNF-α, and IL-12 and enhance cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and up-regulate humoral immunity. Increased IL-12 production exerts a protective effect, which may be related to increased cellular immunity and phagocytic function [24]. IL-12 is also important for the induction of Th1 immunity [25] and directly activates CD56+ NK cell-mediated cytotoxicity [26]. In the present study, IL-12 levels significantly increased in the test group after twelve weeks of daily consumption of dairy yogurt compared with before supplementation.

The result that IgG1 levels significantly increased after the yogurt supplementation suggests that Th cell activity was promoted. Notably, in addition to being preferentially correlated with Th cells, IgG1 is also associated with optimal activation of complement [27].

The current study had several limitations. First, dietary intake was based on self-reports obtained from weighed food, which could have led to errors. However, measurement errors from self-reported dietary intake and lifestyle variables have been shown to be relatively small [28]. Second, we specifically focused on Korean nondiabetic subjects greater than 60 years in age. Typically, Korean adults consume low amounts of dairy products (60–69 years, 70.1 ± 5.4 g/day) according to the Korea Health Statistics 2013: Korean National Health and Nutrition Examination Survey (KNHANES VI-1). Therefore, our data cannot be generalized to other ethnic groups, other age groups, or severely obese subjects. Finally, we used milk as placebo product. Therefore, we could not conclude the results of this study were derived from probiotics or differences between milk and yogurt. Despite these limitations, this study showed that after consumption of dairy yogurt containing probiotics for 12 weeks, significant increases were found in NK cell activity and serum levels of IL-12 and IgG1. The test group also exhibited greater increases in NK cell activity and serum IFN-γ and IgG1 concentrations than the controls. Importantly, this is the first clinical study to investigate the effect of heat-treated *L. plantarum* (nF1)

on improving immune function. The results demonstrated that consumption of yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum* could enhance the immune response, particularly in immunocompromised populations such as the elderly.

5. Conclusions

In this study, we examined the impact of consuming dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum* over a twelve-week period on immune function. Consumption of yogurt containing probiotics increased NK cell activity, IL-12 and IgG1 in the test group, and increases in NK cell activity and IFN-γ and IgG1 in the test group were significantly greater than placebo group. The results suggest that daily consumption of dairy yogurt containing *L. paracasei*, *B. lactis* and heat-treated *L. plantarum* could improve immune function by enhancing NK cell activity.

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Mismatch between Probiotic Benefits in Trials versus Food Products

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Abstract: Probiotic food products contain a variety of different bacterial strains and may offer different health effects. The objective was to document the prevalence and dosage of probiotic strains in the Canadian food supply and to review the literature investigating these strains in order to understand what health benefits these products may offer. The Food Label Information Program was used to identify probiotic-containing products in the food supply. PubMed, Web of Science, and Embase were searched for randomized controlled trials that tested the health effects of these strains in humans. There were six probiotic strains/strain combinations identified in the food supply. Thirty-one studies investigated these strains and found that they are associated with decreased diarrhea and constipation, improved digestive symptoms, glycemic control, antioxidant status, blood lipids, oral health, and infant breastfeeding outcomes, as well as enhanced immunity and support for *Helicobacter pylori* eradication. There were a limited number of studies investigating these strains. Many studies were funded by the food industry and tested dosages that were up to twenty-five times the dosage found in most food products. Probiotic food products could have health benefits not currently reported on their labels. However, many dosages are too low to provide the benefits demonstrated in clinical trials. Further research is needed to enable more effective use of these functional foods.

Keywords: probiotics; yogurt; functional foods; microbiome; dairy products; food supply; packaged foods; Canada; public health; preventive medicine

1. Introduction

Probiotics are "live microorganisms that when administered in adequate amounts confer a health benefit on the host" [1,2]. The benefits of consuming bacteria have been known since ancient times, when fermented milk was commonly prescribed to treat an upset stomach [3]. Today, the term "probiotic" has been defined and qualified by the World Health Organization, which put also forward guidelines to support their use. Accordingly, different probiotics have been shown to prevent or treat a wide range of health issues, including respiratory tract infections, infectious diarrhea, atopic eczema associated with cow's milk allergy, infant colic, necrotizing enterocolitis, pouchitis, bacterial vaginosis, *Clostridioides* (formerly *Clostridium*) *difficile*-associated diarrhea, and urinary tract infections [4–6].

Probiotic food products are one of the fastest growing product markets globally [7]. Currently, commercial probiotic food products contain a variety of different probiotic species and strains. Certain health benefits are common to most or all probiotic species. These effects are considered "core benefits"

and include the regulation of intestinal transit, normalization of perturbed microbiota, turnover of enterocytes, competitive exclusion of pathogens, colonization resistance, and short-chain fatty acid production [2]. Meanwhile, some probiotic effects are found only among specific species of probiotics. Examples include vitamin synthesis, gut-barrier reinforcement, bile salt metabolism, enzymatic activity, and neutralization of carcinogens [2]. Lastly, certain benefits may only be found among specific strains of bacteria; this includes neurological effects, immunological effects, endocrinological effects, and the production of bioactives [2].

Therefore, probiotic food products currently in the marketplace may have the potential to offer a variety of different health benefits, depending on the specific species and strains of bacteria they contain. However, depending on in which country the products are being sold, consumers have varying degrees of information about the health benefit a probiotic product has been designed to provide.

The WHO has recommended that, where scientific evidence exists, strain specific probiotic health claims should be allowed to enable the linkage of a product to a specific health effect [8]. However, in the European Union, there are no approved probiotic health claims [9]. In fact, even the word "probiotic" is considered a health claim and is not permissible on food packages. In the US, products containing probiotics can state that they "support" the body or "maintain" general well-being (for instance, some products state; "help support your immune system" or "helps naturally regulate the digestive tract") [10]. Meanwhile, in Canada, products contain a general health claim (such as "promotes a healthy gut flora") but could provide more specific benefits depending on the species and strain(s) they contain [8,11,12].

To date, the majority of systematic reviews investigating probiotics have focused on the effects of different strains on a single health outcome or the effects of a single strain on different health outcomes. Furthermore, there have been no reviews focused exclusively on probiotics delivered in food formats.

This study had two objectives; first, to document the prevalence and dosage of probiotic species/strains in the Canadian food supply and, second, to review the literature investigating these species/strains in order to understand what health benefits consumers could potentially receive from the probiotic products in the marketplace.

2. Materials and Methods

2.1. Investigation of Probiotic Strains in the Food Supply

Data was derived from the Food Label Information Program (FLIP), a database of Canadian food package label information derived from major outlets of the three largest grocery chains in Canada (Loblaws, Metro, and Sobeys) and one major western retailer (Safeway) [13]. This database represents 75.4% of the grocery retail market share in Canada [14] and provides a detailed assessment of the nutrition information found on Canadian packaged food labels. Grocery store shelves were systematically scanned, and data for every food product with a Nutrition Facts table (NFt), including all available national and private label brands, were collected. Data for food products sold at multiple retailers were collected only once. When multiple sizes of a product were available, only one size was collected. However, all flavors and varieties of a product were collected. Information collected for each product included the Universal Product Code, company, brand, price, Nutrition Facts table information (serving size, calories etc.), ingredients, container size, nutrient content claims, disease risk reduction claims, function claims, front of pack symbols, children's marketing, and other claims (e.g., organic, natural, and gluten-free), in addition to the date and location of sampling. The FLIP database is updated every three years. Presently, two collections have been completed (in 2010 and 2013) and have been described in greater detail elsewhere [13,15]. The packages were visually inspected, and ingredient lists of the 15,341 unique products collected in 2013 were searched to identify probiotic-containing products. Fermented foods were not considered to be probiotic products unless they were labeled as being probiotic. The species, strain(s), and dosage found in the 92 probiotic-containing products were recorded and tabulated. In July 2016, Loblaws, Metro, and Sobeys were revisited to identify if

any probiotic strain and dosage information had changed and to investigate if new probiotic products had entered the marketplace. Four new probiotic products were identified and included in this study. Companies that listed species names without strain information were contacted via e-mail to inquire whether strain data could be disclosed. One company provided strain information via e-mail.

2.2. Review of Randomized Controlled Trials Testing the Probiotic Strains Found in the Canadian Food Supply

A systematic search of the peer-reviewed literature investigating each strain/strain combination found in the food supply was conducted in 2016 in accordance with the preferred reporting items for systematic reviews and meta-analysis protocols (PRISMA) checklist (with the exception of items related to meta-analyses) [16]. The full detailed protocol for this is available at PROSPERO registry CRD42106042660 [17].

2.2.1. Eligibility Criteria

Study Design, Treatment, and Participants

Double-blind randomized-controlled trials that tested the effects of probiotic strains in the food supply were considered. The probiotic strains were required to be administered in a food format similar to the formats found in the food supply.

Studies that administered probiotics in supplement form, that tested synbiotics, or investigated the safety, tolerance, persistence, or viability of probiotics were not included.

Studies on humans of all ages were considered, with the exception of infants under six-months. Individuals with a chronic disease (like diabetes), infections (such as *Helicobacter pylori*), or conditions (like constipation or Irritable Bowel Syndrome) were included.

Outcome Measures

This was not a traditional systematic review. This was an exploratory review and descriptive synthesis that aimed to understand what health effects these food products may offer. Therefore, any and all health-related outcome measures in humans were recorded. This ranged from serum lipid and glycemic levels, to incidence/duration of infections and illness, to markers of inflammation. Effects detected in-vitro were not included. Effects on cellular immunomodulation (e.g., increased number of lymphocytes) were not included.

2.2.2. Literature Search

PubMed, Web of Science, and Embase were searched by two independent reviewers (Beatriz Franco-Arellano and Sarah Murphy) from the earliest record to July 2016. The following keywords were searched in the title/abstract: (multiple iterations of each strain name) and (yogurt OR yoghurt OR milk OR fermented milk OR dairy) with (randomized controlled trial) in any field. When the strain was not found in a dairy product, the dairy keywords were omitted. The search was limited to full-manuscripts in English. Only randomized controlled trials in humans were searched.

Study Selection

After the removal of duplicates, two independent reviewers (Beatriz Franco-Arellano and Sarah Murphy) screened the title and abstracts of retrieved studies against the a priori selection criteria. The selection criteria included any double-blind randomized controlled trial reported in a peer-reviewed journal that included strain/strain combinations found in the food supply, a control group, a quantified dose of the probiotic, a quantified measure of the food treatment, and oral administration of the probiotic via a food format. The study could test any clinical health endpoint on any human population (healthy or sick, including pregnant and breastfeeding mothers). Full-text screening was completed independently by two of the authors of this paper (Mary Scourboutakos and Sarah Murphy), with consensus required for inclusion or exclusion.

Data Extraction

The following information was extracted from each manuscript; information related to the article (complete citation plus author, country, and year of publication), the probiotic species and strain(s) tested, strain dosage, food format, population characteristics (e.g., adults, children, male, female, both), health status of the population (e.g., healthy, population with constipation, diabetic population), sample size, study duration, primary outcome measure, secondary outcome measure(s), significant outcomes, and source of funding. Data was independently extracted by one author (Mary Scourboutakos) and verified by a different author (Sarah Murphy). When articles reported insufficient information, attempts were made to contact their authors via-e-mail to retrieve further information.

Assessment of Methodological Quality

The study quality was independently assessed by one author (Mary Scourboutakos) using Health Canada's quality appraisal tool for intervention studies [18] and independently checked by another author (Sarah Murphy). This tool is used to evaluate the quality of studies that provide evidence to support health claim submissions. The risk of bias was assessed using the Cochrane risk-of-bias tool (Table A1) [19].

Data Synthesis

All studies were grouped according to the strain/strain combination they investigated, and health outcomes were recorded accordingly.

3. Results

The probiotic strains found in the Canadian food supply and a summary of their health effects are shown in Table 1. The initial search of the probiotic strains found in the food supply and their health benefits yielded 188 papers, with 95 remaining after the removal of duplicates (Figure 1). After reviewing the titles and abstracts, 59 remained for full-text review, 29 of which were eligible for inclusion (Table 2). All studies were deemed to be of a 'high quality' according to Health Canada's quality appraisal tool for intervention studies. The majority of studies were judged to have an overall low risk of bias (Table A1).

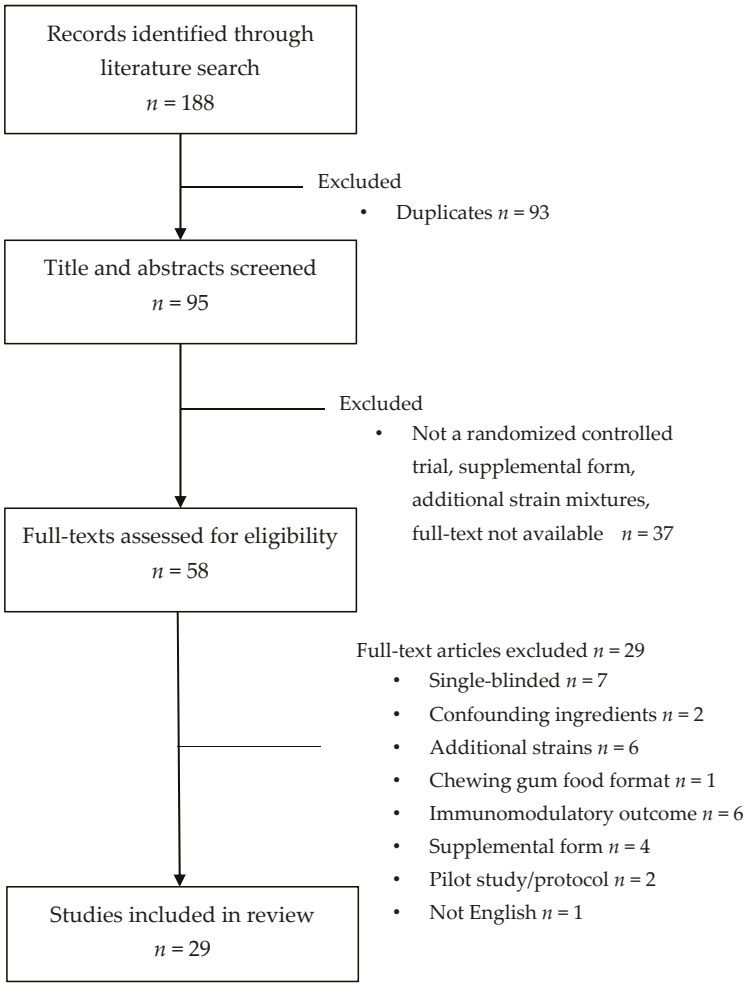

Figure 1. Identification of eligible studies.

Table 1. Strains in probiotic food products and reported health effects associated with these strains.

Strain(s)	Manufacturer and Product Brand	Food Type	Probiotic Dosage in Food (CFU */Serving)	Dosage Tested in Studies (CFU */Day)	Duration of Study	Health Effects Investigated in Healthy Populations										
						Acute Diarrhea	Antibiotic-Associated Diarrhea	Constipation	Digestive Symptoms	Glycemic Control	*Helicobacter pylori* Eradication	Immunity	Infant Breastfeeding Outcomes	Inflammation	Serum Lipids/Blood Pressure	Oral Health
Bifidobacterium lactis BB12 + *Lactobacillus acidophilus* LA-5	Yoplait's *Yoptimal*, Luceme's *Organics* †	Yogurt	>1 × 10^9	2 × 10^6–3 × 10^9	7 days–6 weeks		X [20] $			O [21]	O [20] $				O [22,23]	x [24,25]
Bifidobacterium lactis BB12	Iogo's *Probio* **, Yoplait's *Minigo*	Yogurt	>1 × 10^9	1 × 10^{10}–3.5 × 10^{10}	10 days—3 months							o [26] $ [27]		O [28] $		X [29]
Lactobacillus casei DN 114-001	Danone's *DanActive*	Drinkable yogurt	1 × 10^{10}	1 × 10^{10}–3 × 10^{10}	2 weeks –6 months	x [30] $, [31] o [32] $	X [33] $				x [34] $	X [35,36] $ x [26,30] $	x [37] $			
Bifidobacterium lactis DN-173 010	Danone's *Activia*	Yogurt	>1 × 10^9	8 × 10^9–2.5 × 10^{10}	2–4 weeks			o [38] $	X [39,40] $ x [38] $							O [41] $
Lactobacillus acidophilus NCFM + *Bifidobacterium lactis* Bi-07	Astro's *BioBest*	Yogurt	1 × 10^9	1 × 10^{10}	6 months	o [42] $						x [42] $				
Lactobacillus acidophilus NCFM	President's Choice's *ProAdvantage* †	Yogurt	1 × 10^9	1 × 10^{10}	6 months	o [42] $						x [42] $				

X = beneficial effects observed in healthy adults; x = beneficial effects observed in healthy children, O = studies that have investigated this outcome and have found no significant effect in adults, o = studies that have investigated this outcome and found no significant effect in children, $ = indicates that the research was funded by the company that uses that particular strain in their products. A blank square indicates that no research investigating the effects of that strain/strain combination was identified during the systematic review of all literature published up to 21 July 2016, as described in the methods. All effects reported in this table were found in healthy populations that were not diagnosed with a chronic disease or condition. Definition of health effects: Constipation = improved stool frequency, consistency, or condition; Acute diarrhea = decreased incidence or severity of acute diarrhea; Antibiotic-associated diarrhea = decreased incidence of antibiotic-associated or *Clostridium difficile*-associated diarrhea; Digestive symptoms = decreased abdominal pain/discomfort, bloating, flatulence, or overall GI well-being; Glycemic control = improved fasting glucose, insulin, HbA1c (marker of long-term glycemic control), or HOMA-IR (measure of insulin sensitivity); *Helicobacter pylori* eradication = enhanced eradication of *Helicobacter pylori* infections; Immunity = decreased incidence and/or duration of common infectious diseases, including fever, cough, common respiratory infections (rhinitis, sore throat), common gastrointestinal infections (gastroenteritis, vomiting), asthma, or days missed from school; Infant breastfeeding outcomes = infants (2–6 months old) of mothers who consume this strain while breastfeeding had decreased incidence of gastrointestinal episodes and lower medication-use rates; Inflammation = decreased levels of inflammatory markers (ex. C-reactive protein); Lipids = decreased serum total cholesterol, low density lipoprotein (LDL), triglyceride levels, or increased high density lipoprotein (HDL); Oral health = decreased levels of cavity causing bacteria. * CFU = colony forming units. ** Iogo's *Probio* reported two strains on its label in 2013 (*Bifidobacterium lactis* BB12 + *Lactobacillus acidophilus* LA-5) and only one strain on its label in 2016. † These products were available in 2013 but may no longer be available in the Canadian market. Note: All cited references were deemed to be of high quality according to Health Canada's quality appraisal tool for intervention studies [18].

Table 2. Results of the review of randomized controlled trials investigating the health effects of probiotic strains found in the Canadian food supply [1].

Strain	Study, Country (Year)	Population (*n*)	Probiotic Dosage (CFU per Day)	Study Duration	Outcome Measures (Primary and Secondary)	Statistically Significant Effects (Relative to Placebo Group)	Funding Source
B. lactis BB12 + *L. acidophilus* LA-5	Ivey et al. [21] Australia (2014)	Overweight adults *n* = 156	3×10^9	6 weeks	**Primary:** Glycemic control (fasting blood glucose, insulin, HbA1c, and HOMA-IR)	Increased HOMA-IR (worsened insulin sensitivity)	Sir Charles Gairdner Hospital
	Sadrzadeh-Yeganeh et al. [23] Iran (2010)	Females *n* = 90	3.9×10^7	6 weeks	**Primary:** Serum total cholesterol, HDL, LDL, and triglycerides	No observed effects	Tehran University Grant
	Ivey et al. [22] Australia (2015)	Overweight adults *n* = 156	3×10^9	6 weeks	**Primary:** Blood pressure, total cholesterol, HDL, LDL, and triglycerides	No observed effects	Sir Charles Gairdner Hospital
	deVrese et al. [20] Germany (2011)	H pylori infected adults *n* = 88	5×10^6	5 weeks	**Primary:** *Helicobacter pylori* activity; **Secondary:** Frequency, intensity and duration of abdominal pain; stool frequency/consistency; duration of diarrhea episodes; IBS symptoms; orofecal transit time	Decreased duration of antibiotic-associated diarrhea episodes	Chr. Hansen GmbH J. & Co., KG, NOM AG [$]
	Ashwin et al. [24] India (2015)	Children *n* = 60	2×10^6	7 days	**Primary:** Salivary levels of streptococcus mutans (a cavity causing bacteria)	Reduced salivary *mutans streptococci*	Funded by study author
	Singh et al. [25] India (2011)	Children *n* = 40	5.4×10^7	10 days	**Primary:** Salivary levels of salivary *mutans streptococci* and *lactobacilli* (cavity causing bacteria)	Reduced salivary *mutans streptococci*	Not disclosed
	Ejtahed et al. [43] Iran (2011)	Type II Diabetics *n* = 64	$>1 \times 10^9$	6 weeks	**Primary:** Fasting blood glucose, HbA1c, insulin and antioxidant molecules (superoxide dismutase, glutathion peroxidase, catalase activity, malondialdehyde concentration, and total antioxidative status)	Decreased fasting blood glucose and HbA1c; increased activity of superoxide dismutase, glutathoine peroxidase, and total antioxidative status	Iran Dairy Industry [$]
	Mohamadshahi et al. [44] Iran (2014)	Type II Diabetics *n* = 44	$>1 \times 10^9$	8 weeks	**Primary:** Serum triglycerides, LDL, HDL, triglycerides, LDL:HDL	Decreased LDL:HDL, increased HDL	Nutrition Disease Research Center
	Ejtahed et al. [45] Iran (2012)	Type II Diabetics *n* = 60	6×10^8	6 weeks	**Primary:** total cholesterol, triglycerides, HDL, LDL, total cholesterol:HDL, LDL:HDL	Decreased total cholesterol, LDL, LDL:HDL and total cholesterol:HDL	Grant from Tabriz University
	Nabavi et al. [46] Iran (2014)	Non-alcoholic fatty liver disease patients *n* = 72	$>1 \times 10^9$	8 weeks	**Primary:** Blood levels of liver enzymes (alanine aminotransferase and aspartate aminotransferase); fasting blood glucose; total cholesterol, triglycerides, LDL, HDL.	Decreased blood levels of liver enzymes, total cholesterol, triglycerides, and LDL	Nutrition Research Center, Tabriz University
	Tonucci et al. [47] Brazil (2015)	Type II Diabetics *n* = 45	2×10^9	6 weeks	**Primary:** Glycemic control (fasting blood glucose, insulin, HOMA-IR, fructosamine, HbA1c); lipid profile (total cholesterol, LDL, VLDL, triglycerides, total cholesterol:HDL); total antioxidant status and cytokine concentrations (Il-6, Il-10, TNF-α, adiponectin, and resistin); fecal short-chain fatty acids	Decreased fructosamine, LDL, and total cholesterol; significant change in HbA1c	Brazilian Agri-Research; Foundation to Support the State of Miras Gerais
B. lactis BB12	Caglar et al. [29] Turkey (2008)	Healthy young adults *n* = 24	5×10^8	10 days	**Primary:** Salivary levels of *mutans streptococci* and *lactobacilli* (cavity causing bacteria)	Decreased salivary *mutans streptococci*	Funded by researchers
	Merenstein et al. [48] USA (2010)	Children *n* = 182	1×10^{10}	90 days	**Primary:** Missed days of school due to illness; **Secondary:** Diarrhea, stool consistency, respiratory infection, missed parental work, doctor visits, illnesses, and overall parental satisfaction	No observed effects	The Gerber Foundation [$]
	Merenstein et al. [27] USA (2011)	Healthy children *n* = 172	1×10^{10}	90 days	**Primary:** Missed days of school due to illness; **Secondary:** Diarrhea, stool consistency, respiratory infection, missed parental work, doctor visits, illnesses	No observed effects	USDA
	Kekkonen et al. [28] Finland (2008)	Healthy adults *n* = 62	3.5×10^{10}	3 weeks	**Primary:** Blood levels of inflammatory markers including C-reactive protein and cytokines (TNF-α, IL-6, IFN-γ, IL-10)	No observed effects	Research Council Finland and Valio [$]
L. acidophilus NCFM + *B. lactis* Bi-07	Leyer et al. [42] China (2009)	Healthy children *n* = 326	1×10^{10}	6 months	**Primary:** Frequency and duration of fever, cough, rhinorrhea, vomiting, diarrhea, physicians' visits and antibiotic prescriptions; **Secondary:** School absences	Decreased incidence of fever, cough, rhinorrhea, antibiotic use, and days missed from school. Reduced symptom duration.	Danisco [$]

Table 2. *Cont.*

Strain	Study, Country (Year)	Population (*n*)	Probiotic Dosage (CFU per Day)	Study Duration	Outcome Measures (Primary and Secondary)	Statistically Significant Effects (Relative to Placebo Group)	Funding Source
B. lactis DN-173 010	Pinto et al. [41] Brazil (2013)	Healthy adults $n = 26$	not reported	2 weeks	**Primary:** Salivary levels of cavity-associated microorganisms (*mutans streptococci*, *lactobacilli* and total microorganisms) in saliva	No observed effects	Not Disclosed
	Tabbers et al. [38] Netherlands and Poland (2011)	Constipated children $n = 159$	$>8 \times 10^9$	3 weeks	**Primary:** Stool frequency; **Secondary:** Stool consistency, frequency of faecal incontinence, pain during defecation, abdominal pain, flatulence	Decreased flatulence	Danone [$]
	Guyonnet et al. [39] Germany (2009)	Healthy adult women $n = 192$	2.5×10^{10}	4 weeks	**Primary:** Overall GI well-being (intestinal transit, stool frequency and consistency, abdominal pain/discomfort, bloating, flatulence, stomach rumbling); **Secondary:** Frequency of digestive symptoms including abdominal pain/discomfort, bloating, flatulence, stomach rumbling; stool frequency and consistency; health-related quality of life	Improved overall GI well-being; decreased frequency of flatulence, stomach rumbling, improved stool consistency, and health-related quality of life.	Danone [$]
	Agrawal et al. [40] United Kingdom (2008)	Adult females with IBS $n = 34$	2.5×10^{10}	4 weeks	**Primary:** Abdominal distension and bloating; **Secondary:** Orocaecal and colonic transit times; incidence and severity of IBS symptoms (abdominal pain/discomfort, bloating, flatulence); overall IBS symptom severity; time and consistency of bowel movements; feelings of incomplete evacuation at time of stool passage	Decreased maximal abdominal distension, orocaecal and colonic transit times, overall IBS symptom severity, and abdominal pain/discomfort.	Danone [$]
L. casei DN 114-001	Guillemard et al. [35] Germany (2010)	Healthy adult shift workers $n = 1000$	$>2 \times 10^{10}$	3 months	**Primary:** Cumulative number of common infectious diseases (CID) (e.g., sore throat, sinusitus, nasal discharge, ear ache, influenza, pneumonia, cough, GI infection, diarrhea, nausea vomiting.) **Secondary:** Occurrence of having at least one CID: time to first CID, severity, duration, cumulated duration; occurrence and duration of fever, sick days, medication use	Decreased occurrence and time to first CID; decreased duration of fever; decreased cumulative number of CIDs (post-hoc analysis)	Danone [$]
	Merenstein et al. [26] USA (2010)	Healthy children $n = 638$	$>2 \times 10^{10}$	3 months	**Primary:** Change in behaviour due to illness (e.g., missed school, missed sports activity); incidence of common infectious diseases (CIDs) **Secondary:** Absences from daycare or school, missed parental work, days with diarrhea, vomiting, stomach pain, constipation, runny nose, cough, decreasing appetite, fever, rash, medication use	Decreased incidence of CID	Danone [$]
	Guillemard et al. [36] France (2009)	Elderly adults $n = 1072$	$>2 \times 10^{10}$	3 months	**Primary:** Cumulative number of all common infectious diseases (CID) **Secondary:** The occurrence of CID (defined as the number of subjects experiencing at least one CID), duration of CID (cumulative and per episode), time to first CID, severity of CID, fever associated with CID, occurrence or duration of medication use	Decreased duration of CID episodes and cumulative duration of CID	Danone [$]
	Sykora et al. [34] Czech Republic (2005)	Children w/H Pylori $n = 86$	1×10^{10}	14 days	**Primary:** Eradication rate of *Helicobacter pylori* infection	Increased *Helicobacter pylori* eradication rates	Ministry of Health and Danone [$]
	Ortiz-Andrellucchi et al. [37] Spain (2008)	Breastfeeding infants $n = 104$	3×10^{10}	6 weeks	**Primary:** Immunomodulatory molecules in breast milk (not included in this review) **Secondary:** Infant growth and weight; incidence of gastrointestinal episodes, respiratory symptoms, medication use, allergies and dermatitis	Reduced incidence of gastrointestinal episodes and lower rate of medication use in infants	Danone [$]
	Agarwal et al. [31] India (2002)	Children $n = 150$	$2–3 \times 10^{10}$	9 months	**Primary:** Duration of acute diarrhea	Decreased duration of acute diarrhea	Not Disclosed
	Hickson et al. [33] United Kingdom (2007)	Elderly in-patients $n = 137$	2×10^{10}	2 weeks	**Primary:** Incidence of antibiotic-associated diarrhea and *Clostridium difficile* associated diarrhea	Decreased incidence of antibiotic- and *Clostridium*-associated diarrhea	Danone [$]
	Giovannini et al. [30] Italy (2007)	Children with asthma/rhinitis $n = 187$	1×10^{10}	12 months	**Primary:** Episodes and duration of asthma and rhinitis (runny/stuff nose) **Secondary:** Episodes and duration of abdominal symtoms, diarrhea and fever	Decreased asthma and rhinitis episodes, decreased duration of diarrhea in children with rhinitis	Danone [$]
	Giralt et al. [49] Spain (2008)	Gynecological cancer patients $n = 85$	2.8×10^{10}	6 months	**Primary:** Frequency and severity of radiation induced diarrhea **Secondary:** Time to the development of diarrhea, stool consistency	Improved stool consistency	Danone [$]

[1] All probiotic strains in the Canadian food supply were recorded and a systematic review of their health effects was conducted. All literature published up to 21 July 2016 was included, as described in the methods. All studies included in the review were deemed to be of a 'high quality' according to Health Canada's quality appraisal tool for intervention studies and thus are considered eligible to substantiate a health claim [18]. [$] Indicates that funding was provided by the food industry HbA1c = hemoglobin A1c, a long-term measure of glycemic control; HOMA-IR = a measure of insulin sensitivity; LDL = low-density lipoprotein; HDL = high-density lipoprotein; VLDL = very low-density lipoprotein; IBS = irritable bowel syndrome; CID = common infectious diseases.

Danone's *DanActive* contained one proprietary strain (*Lactobacillus casei* DN 114-001). This was one of the most well studied strains in the food supply with eleven studies, all funded by Danone, investigating its effects [35]. Three studies showed decreased incidence [26,35] and duration [36] of common infectious diseases (ranging from upper respiratory tract infections to sore throats and influenza) in adults, children, and seniors. Of these, one study showed decreased duration of acute diarrhea in children [31]. One study of hospitalized elderly adults showed decreased incidence of *Clostridium difficile* and antibiotic-associated diarrhea [33]. Other effects associated with this strain included decreased asthma and rhinitis episodes [30] and increased *Helicobacter pylori* eradication rates in children [34]. One study tested the effect of this strain when consumed by breastfeeding mothers and showed that their infants had a reduced incidence of gastrointestinal episodes and a lower rate of medication use [37]. The probiotic dosage administered in these studies was up to three times the dosage found in one serving of this product.

Danone's *Activia* contained a different proprietary strain, *Bifidobacterium lactis* DN-173 010. This strain was associated with improved overall GI well-being, including decreased flatulence [38], decreased stomach rumbling, and improved stool consistency [39]. In one study of women with irritable bowel syndrome (IBS), this strain was shown to decrease overall IBS symptom severity and to decrease maximal abdominal bloating [40].

President's Choice's *ProAdvantage* contained *Lactobacillus acidophilus* NCFM. One study tested this strain in children and found decreased incidence of fever, cough, rhinorrhea, antibiotic use, symptom duration, and days missed from school [42]. However, the dosage tested in the study (10 billion colony forming units (cfu) per day) was ten times the dosage found in the product (1 billion cfu per day). Astro's *BioBest* contained *Lactobacillus acidophilus* NCFM in combination with *Bifidobacterium lactis* Bi-07. This combination was tested in the same study reported above and was found to have the same effects and dosage discrepancy.

Bifidobacterium lactis BB-12 was found in two brands; Iogo's *Probio* and Yoplait's *Minigo* (a product intended for children). This strain was investigated in four studies. In one study, testing a dosage that was half of what is found in these products, this strain was associated with decreased levels of a cavity causing bacteria (*mutans streptococci*) in saliva [29]. Two studies tested the effect of this strain (at a dosage that was ten-times the dosage found in the product) on children's risk of illness and absences from school [27,48]. No effects were seen. One study tested a dosage that was thirty-five times the dosage found in the products containing this strain and showed no effect on inflammatory markers (C-reactive protein and cytokines) [28].

Bifidobacterium lactis BB-12 in combination with *Lactobacillus acidophilus* LA-5 was found in two brands (Yoplait's *Yoptimal* and Lucerne's *Organics*). Eleven studies investigated this strain combination. Three studies tested dosages that were substantially smaller than the dosage found in commercial products. Two of those studies showed reduced salivary levels of cavity causing bacteria (*Streptococcus mutans*) [24,25], while one showed decreased duration of antibiotic-associated diarrhea in patients infected with *Helicobacter pylori* [20]. Two studies investigated the impact of these strains on blood lipids and found no effects despite the fact that one study tested a dosage that was lower than found in commercial products and the other tested a dosage that was higher [22,23]. One study investigated the effect of these strains on glycemic control [21]. It tested a dosage that was three-times the dosage found in commercial products and found decreased insulin sensitivity. There were four studies that tested the effect of these strains on type-two diabetics and used dosages that were similar to those found in commercial products (Table 3). These studies showed improved glycemic control [43,47], improved blood lipid levels [44,45,47], and enhanced antioxidant status [43] in diabetics.

Nine brands labeled species names without identifying the strain (Table 4). Therefore, strain-specific health benefits could not be inferred for these products.

Table 3. Strains in probiotic food products and reported health effects in populations with a diagnosed non-communicable disease/condition.

Population	Strain/Strain Combination	Manufacturer and Product Brand	Probiotic Dosage in Food (CFU */Serving)	Dosage Tested in Studies (CFU */Day)	Health Effects Investigated in Populations with a Disease/Condition						
					Antioxidant Status	Digestive Symptoms	Glycemic Control	Inflammation	Liver Damage	Radiation Induced Diarrhea	Serum Lipids
Type II Diabetics	*Bifidobacterium lactis* BB12 + *Lactobacillus acidophilus* LA-5	Yoplait's *Yoptimal*, Lucerne's *Organics* †	$>1 \times 10^9$	6×10^8–$>1 \times 10^9$	X [43] $ O [47]		X [43] $, [47]	O [47]			X [44,45,47]
Patients with Non-Alcoholic Fatty Liver Disease				$>1 \times 10^9$					X [46]		X [46]
Females with Irritable Bowel Syndrome	*Bifidobacterium lactis* DN-173 010	Danone's *Activia*	$>1 \times 10^9$	2.5×10^9		X [40] $					
Gynecological Cancer patients undergoing radiation therapy	*Lactobacillus casei* DN 114-001	Danone's *DanActive*	1×10^{10}	2.8×10^{10}						X [49] $	

X = beneficial effects observed; O = studies have investigated this outcome and have found no significant effects; $ = indicates that the research was funded by the dairy industry. A blank square indicates that no research investigating the effects of that strain/strain combination was identified during the systematic review of all literature published up to 21 July 2016, as described in the methods. Effects reported in this table were observed in populations that were diagnosed with a disease or condition Definition of health effects: Antioxidant status = activity of superoxide dismutase, glutathoine peroxidase, and total antioxidant status; Digestive symptoms: decreased abdominal distension/pain/discomfort, decreased fecal transit time, reduced IBS symptom severity; Glycemic control = decreased fasting blood glucose, insulin, and/or HbA1c (long-term measure of blood glucose control); Inflammation = Increased levels of anti-inflammatory markers (cytokines: IL-6, IL-10, TNF-α, adiponectin, and resistin) Liver damage = decreased serum levels of liver enzymes (alanine aminotransferase and aspartate aminotransferase) [a marker of decreased liver damage]; Radiation Induced Diarrhea = incidence and severity; Serum Lipids = decreased serum total cholesterol, LDL, or triglyceride levels; increased HDL; improved lipid ratios. * CFU = colony forming units. † This product may no longer be available in the marketplace Note: All cited references were deemed to be of high quality according to Health Canada's quality appraisal tool for intervention studies [18].

Table 4. Additional probiotic products with undetermined health effects.

A: Products whose specific health effects are undetermined because there is no research on the strain/strain combination in their specific food format			
Bacterial strains identified in the food database *	Manufacturer and product brand	Food type	Probiotic dosage (CFU) per serving
Lactobacillus acidohpilus Bi-07	Irresistibles' *Life Smart*	Frozen fruit and yogurt blend	Not indicated
Lactobacillus acidophilus LA-5	Breuggens *Yog Active Cereal*	Cereal with yogurt flakes	1×10^9
Bacillus coagulans GBI-30 6086	ShaSha Co's *Spelt Ginger Snaps*	Cookies	Not indicated
Lactobacillus acidophilus ATCC 4356T *Lactobacillus helveticus* ATCC 10797 *Lactobacillus helveticus* ATCC 12046 *Lactobacillus helveticus* ATCC 15009T *Lactobacillus kefir* ATCC 35411T *Lactobacillus kefir* ATCC 8007 *Lactobacillus brevis* ATCC 14869T *Lactobacillus brevis* ATCC 13648 *Lactobacillus kefirgranum* LMG 15132T *Lactobacillus parakefir* LMG 15133T *Lactobacillus kefiranofaciens* ATCC 43761T *Leuconostoc mesenteroides* ATCC 8293T *Leuconostoc mesenteroides* LMG 14531 *Leuconostoc mesenteroides* LMG 6909T *Leuconostoc pseudomesenteroides* ATCC 12291T *Lactococcus lactis* subsp. *lactis* LMG 6890T *Lactococcus lactis* LMG 7931 *Lactococcus lactis* subsp. *cremoris* LMG 6897	† Liberte's *Kefir* (effervescent)	Fermented milk	4.5×10^{10}

Table 4. *Cont.*

B: Products whose specific health effects are undetermined because they only indicate the species and not the specific strain of the bacteria they contain			
Bifidobacterium	Lucerne's *Eating Right*	Yogurt	Not indicated
Lactobacillus casei	Liberte's *BioOrganic*	Yogurt	$>1 \times 10^9$
Bifidobacterium lactis + *Lactobacillus acidophilus* + *Lactobacillus casei*	Liberte's *Classic*	Yogurt	$>1 \times 10^9$
	Riviera's *Petit Pot Yogurt* **		1×10^9
Bifidobacterium lactis + *Lactobacillus acidophilus*	Liberte's *Goat Yogurt*	Yogurt	$>1 \times 10^9$
	Skotidakis' *Greek Yogurt* **	Yogurt	not indicated
Lactobacillus casei *Lactobacillus acidophilus* *Bifidobacterium lactis* *Lactobacillus rhamnosus* *Lactococcus lactis* subsp. *cremoris* *Lactococcus lactis* subsp. *lactis biovar diacetylactis* *Lactobacillus delbrueckii* subsp. *lactis* *Lactobacillus delbruecki* subsp. *bulgaricus* *lauconostoc mesenteroides* subsp. *cremoris*	Liberte's *Kefir* (non-effervescent)	Fermented milk	$>1 \times 10^9$
Bifidobacterium infantis *Bifidobacterium lactis* *Lactobacillus acidophillus* *Lactobacillus fermentum* *Lactobacillus lactis* *Lactobacillus paracasei* *Lactobacillus rhamonus* *Lactococcus lactis* subsp. *Cremoris* *Lactococcus lactis* subsp. *Lactis* *Lactococcus lactis* subsp. *lactis biovar diacetylactis* *Lactobacillus delbrueckii* subsp. *bulgaricus Leuconostoc mesenteroides* *Leuconostoc pseudomensenteroides*	Iogo's *Probio Kefir*	Fermented milk	2×10^9
Bifidobacterium bifidum + *Bifidobacterium longum* subsp *longum* + *Bifidobacterium animals* subsp. *lactis*	President's Choice's *Kefir* **	Fermented milk	2×10^9
Lactobacillus acidophilus + *Bifidobacterium lactis*	President's Choice's *Greek Probiotic* **	Yogurt	1×10^9

* Probiotic containing foods were identified in the Food Label Information Program (FLIP), a database of Canadian food package label information. FLIP data was collected in 2013 from major outlets of the four largest grocery retail chains in Canada. Probiotic species/strain information was obtained from the ingredients list and package of each probiotic product. Data was re-verified in 2016 to ensure that the species and dosage information had not changed. ** These products were not included in the 2013 database but were identified when grocery chains were revisited in 2016. † The strains associated with Liberte's effervescent Kefir are not listed on the product label. This data was obtained via an inquiry with the company. All companies that listed species names without strains were contacted to inquire whether strain data could be disclosed.

Most products contained one or two different strains. Kefir (here a fermented milk with added probiotics) products had the largest strain and species diversity, as well as the highest dosage (45 billion colony forming units per serving). However, not all kefir products contained this dosage and diversity.

4. Discussion

Probiotic food products in the Canadian marketplace contained bacterial strains that were associated with a wide variety of health benefits ranging from enhanced immunity to improved glycemic control in diabetics, suggesting that probiotic products could potentially offer health benefits that are not advertised on their labels. However, many of the current probiotic dosages in products were lower than the dosages tested in randomized controlled trials.

4.1. Dosage

In order to obtain many of the health benefits reported in the randomized controlled trials that were reviewed in this study, consumers would need to eat anywhere from two to twenty-five servings of these products each day. The WHO has recommended that "the suggested serving size (on the product label) must deliver the effective dose of probiotics related to the health claim" [8]. Currently most products contain one billion colony forming units (CFU) of probiotics because that is the minimum required in order to provide core benefits and thus be eligible to display the probiotic health claim "promotes a healthy gut flora" in Canada [12]. Therefore, if strain-specific health claims were implemented (in addition to the existing general probiotic health claim), companies would have greater incentive to provide the higher dosages needed to convey some of the health benefits reported in this review.

4.2. Strain Diversity

Most products contained one or two strains. However, research has shown that, in some cases, strain mixtures can be more effective than single strains [50–54], as "different strains (that are) targeted toward different ailments can be blended into one preparation", enabling cultures to complement each other's health effects and produce synergistic benefits [55]. For instance, *Bifidobacterium lactis* BB-12 (found in the food supply) has been shown to have greater gut-adherence when accompanied by *Lactobacillus rhamnosus* GG (one of the most well-studied probiotic strains [4], which is mainly available in supplement form) [56]. Furthermore, evidence from Leyer et al.'s investigation of *Lactobacillus acidophilus* NCFM alone and in combination with *Bifidobacterium lactis* Bi-07 showed that the combination of strains resulted in a lower risk of fever, coughing, and rhinorrhea when compared to the single strain [42]. It is understandable that our results found that fewer products contained strain mixtures, as, presently, food companies have no incentive to utilize strain synergies. Not to mention that single strain probiotics are more easily patentable than multi-strain probiotics [52]. Therefore, current health claims that are based on a single strain encourage the addition of single strains and could therefore be partially responsible for promoting a potentially suboptimal pharmaceutical-like approach to probiotic foods. That being said, it should be noted that not all strain mixtures are beneficial, as strains can antagonize one another. Therefore, research is needed to verify if mixtures are synergistic or antagonistic [50]. It has been previously noted that there is a lack of research on multi-strain probiotics because such research is more difficult to conduct and thus more expensive [57].

4.3. Strengths and Limitations

A strength of this study is the use of FLIP to derive information on all marketed products, which is why we chose to focus on the Canadian market as a model. Obviously, different markets will have different probiotic-containing products, which may come with benefits that overlap or differ from those discussed here. It is important that future research focuses on these other markets to the benefit of both the consumers and the industry. Furthermore, many probiotic benefits could vary depending

on an individual's lifestyle and baseline microbiome. Therefore, it is expected that the health effects noted in this review may not benefit all consumers equivalently.

Limitations include the fact that studies in this review tested various strains, dosages, and health outcomes. Therefore, at this point in time, there is no consensus on what strain, dose, or product is best. For example, while our review showed that one strain (*Bifidobacterium lactis* DN-173 010) was associated with decreased digestive symptoms, this was the only strain for which this outcome was assessed. Hence, we cannot conclude that other strains/products would not also have these benefits. Therefore, these results show what is known according to the limited amount of literature that currently exists. Additionally, since much of the current research was funded by the companies that sell probiotic products and therefore dictated which strains were studied, there is a need for further research on a broader range of species/strains that is supported by alternate funding bodies.

Despite the WHO's recommendation that genus, species, and strain should be designated on a product's label [8], nearly half of the brands in this study did not disclose strain information. Thus, the potential health benefits for these products could not be deduced.

Previous research has shown that industry funded nutrition-related research may bias conclusions in favor of the sponsors' products [58]. Most of the studies included in this review were funded by the companies making the products [26,30,32–37,40,59] or were published in journals that are funded by the food industry (Table 1) [45,46]. Many of these studies investigated a large number of outcome measures but did not make statistical adjustments to control for testing multiple hypotheses. Furthermore, in many of these studies, the primary outcome measure was not significant, and, instead, significance was detected in secondary outcomes or through post-hoc analyses. Therefore, while these studies were deemed to be of high quality and were published in peer-reviewed journals, elements of their analysis suggest that their results should be interpreted with caution.

5. Conclusions

Probiotic food products sold in Canada could offer a variety of health benefits depending on the strain(s) and dosage they contain. That being said, the probiotic dosages contained in most food products are currently too low to provide the benefits shown in clinical trials. Therefore, with higher dosages, or with trails substantiating the current dosage, there is potential for the strains that are already in food products to provide more benefits to the consumer.

Currently there is only a small volume of literature investigating the health benefits of the probiotic strains used in the Canadian food supply. Thus, additional clinical trials, particularly ones that are not sponsored by the food industry, are needed. Hopefully this work will encourage funding and regulatory agencies to fund more research investigating probiotics. A larger number of well conducted studies and clear evidence-based labeling regulations will ultimately help the consumers to make informed choices and derive substantiated benefits form the products they choose to consume.

Overall, considering the wide range of diseases and health conditions for which probiotics have been shown to have benefits, further research to promote the optimal design of probiotic food products is needed to enable more effective use of these functional foods.

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Author Contributions: Scourboutakos and L'Abbe had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Scourboutakos and Comelli conceived and designed the study; Scourboutakos, Norsen, Franco-Arellano, Murphy, and L'Abbé acquired the data; Scourboutakos, Comelli, and L'Abbé analyzed and interpreted the data; Scourboutakos wrote the manuscript; Scourboutakos, Norsen, Franco-Arellano, Murphy, and L'Abbé reviewed the manuscript for important intellectual content; L'Abbé and Comelli obtained funding; L'Abbé and Comelli supervised the study.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Appraisal of the risk of bias of the included studies using the Cochrane risk-of-bias tool.

Strain Studied	Study	Sequence Generation	Allocation Concealment	Blinding	Incomplete Outcome Data	Selective Reporting	Overall
Bifidobacterium lactis BB12	Caglar et al. [29]	L	L	L	L	L	L
	Merenstein et al. [48]	L	L	L	U	L	L
	Merentstein et al. [27]	L	L	L	U	L	L
	Kekkonen et al. [28]	U	U	L	L	L	U
Bifidobacterium lactis DN-173 010	Pinto et al. [41]	L	L	L	L	L	L
	Tabbers et al. [38]	L	L	L	L	L	L
	Guyonnet et al. [39]	U	U	L	L	L	U
	Agrawal et al. [40]	U	U	L	L	L	U
Lactobacillus acidophilus NCFM + *Bifidobacterium lactis* Bi-07	Leyer et al. [42]	L	U	L	L	L	L
Bifidobacterium lactis BB12 + *Lactobacillus acidophilus* LA-5	Ivey et al. [21]	L	L	L	L	L	L
	Ivey et al. [22]	L	L	L	L	L	L
	Sadrzadeh-Yeganeh et al. [23]	U	U	L	U	L	U
	de Vrese et al. [20]	U	U	L	L	L	U
	Ashwin et al. [24]	U	U	L	U	L	U
	Singh et al. [25]	L	L	L	U	L	L
	Ejtahed et al. [45]	L	L	L	L	H	L
	Mohamadshahi et al. [44]	L	L	L	U	L	L
	Ejtahed et al. [43]	L	L	L	L	H	L
	Nabavi et al. [46]	L	L	L	L	U	L
	Tonucci et al. [47]	L	U	L	L	L	L
Lactobacillus casei DN 114-001	Guillemard et al. [36]	L	U	L	L	L	L
	Merenstein et al. [26]	L	L	L	L	U	L
	Guillemard et al. [35]	L	U	L	L	L	L
	Sykora et al. [34]	L	H	L	L	L	L
	Ortiz-Andrellucchi et al. [37]	U	L	L	L	U	U
	Agarwal et al. [31]	L	U	L	L	L	L
	Hickson et al. [33]	L	L	L	U	L	L
	Giovannini et al. [30]	L	H	L	L	L	L
	Giralt et al. [49]	L	L	L	L	L	L

Note: H = high risk of bias, L = low risk of bias, and U = unclear risk of bias.

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Bacteriological and Immunological Profiling of Meconium and Fecal Samples from Preterm Infants: A Two-Year Follow-Up Study

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Abstract: An abnormal colonization pattern of the preterm gut may affect immune maturation and exert a long-term influence on the intestinal bacterial composition and host health. However, follow-up studies assessing the evolution of the fecal microbiota of infants that were born preterm are very scarce. In this work, the bacterial compositions of fecal samples, obtained from sixteen 2-year-old infants were evaluated using a phylogenetic microarray; subsequently, the results were compared with those obtained in a previous study from samples of meconium and feces collected from the same infants while they stayed in the neonatal intensive care unit (NICU). In parallel, the concentration of a wide range of cytokines, chemokines, growth factors and immunoglobulins were determined in meconium and fecal samples. Globally, a higher bacterial diversity and a lower interindividual variability were observed in 2-year-olds' feces, when compared to the samples obtained during their first days of life. Hospital-associated fecal bacteria, that were dominant during the NICU stay, seemed to be replaced, two years later, by genera, which are usually predominant in the healthy adult microbiome. The immune profile of the meconium and fecal samples differed, depending on the sampling time, showing different immune maturation statuses of the gut.

Keywords: prematurity; infant gut microbiota; DNA microarray; immune maturation

1. Introduction

The microbial composition of the gastrointestinal tract in humans undergoes remarkable changes in our life span [1–3]. The most dynamic period of changes in human intestinal microbiota is reported after birth, when the scarce bacteria present in the intrauterine environment make space for a complex microbial community. The conventional belief is that the development of the intestinal microbiota in term infants involves early colonization by facultative anaerobes, which generate a reducing environment, favoring the growth of strictly anaerobic bacteria [4–7]. From an initial low diversity and

low complexity, the intestinal bacterial community of the infant will gradually develop and mature, reaching an enduring adult state after 2–3 years of age [6,8,9].

However, the establishment and development of the intestinal microbiota differ between preterm and healthy term infants [10,11]. In the former, several factors, including mother/infant antibiotic therapy, Caesarean section, early separation from parents, delayed enteral feeding, invasive medical procedures or a long stay in the neonatal intensive care unit (NICU), are assumed to exert strong influences on early colonization of the infant gut [5,12,13]. Previous studies monitoring the bacterial communities, using culture-dependent and independent techniques, in term and preterm infants, have detected a reduced number of bacterial species in the fecal microbiota of preterm infants, compared with term infants [14–16]. In healthy, full-term, vaginally-delivered newborns, gut microbial colonization is initiated with facultative anaerobic microorganisms, which decrease the intestinal redox potential, helping the subsequent establishment of strict anaerobic microorganisms, such as *Bifidobacterium*, *Bacteroides* or *Clostridium* [8,17]. In preterm infants, the establishment of obligate anaerobes, especially bifidobacteria, are delayed, compared with full-term infants and facultative anaerobes, such as enterobacteria, enterococci and staphylococci, seem to persist for several weeks at high levels in the preterm infant's fecal microbiota [14,15,18–20]. The abnormal gut colonization in preterm infants during their first weeks of life [14,17,21] may affect the maturation of the gut barrier as well as its nutritional and immunological functions at that time and later [22,23].

There is circumstantial evidence that initial microbial gut colonization and the resulting immune and metabolic programming could have a long-lasting influence on the risk for future diseases [6,24]. However, little is known about the possible influence of gut microbiota on the human immune system and how early bacterial colonization affects immune maturation [25,26], particularly among preterm infants [16]. Several studies have assessed immune compounds in saliva, umbilical cord blood or peripheral blood of infants [25,27–31], but few have described the presence of cytokines, chemokines, growth factors or immunoglobulins in fecal samples of preterm babies [32–35].

In this context, the objectives of this study were, firstly, to study if the abnormal initial colonization of preterm babies previously studied [36] may affect their fecal bacterial composition when they are 2 years old, by using a phylogenetic microarray [37] and, secondly, to characterize and compare the immune profiles of the meconium and infant feces, obtained from such infants in the first weeks after birth and, also, at the age of 2.

2. Materials and Methods

2.1. Patients and Sampling

This prospective study included sixteen 2-year-old infants, who were born prematurely at the Hospital Universitario 12 de Octubre, Madrid (Spain) (Table 1).

Table 1. Demographic data for the infant cohort.

Infant	Gestational Age (week)	Delivery Mode	Gender	Birth Weight (g)
2	30	Caesarean section	Male	1550
4	27	Caesarean section	Female	1080
5	30	Caesarean section	Male	2030
6	30	Vaginal	Male	1760
7	24	Caesarean section	Female	600
9	27	Vaginal	Male	1540
10	26	Caesarean section	Female	790
13	32	Vaginal	Female	1310
15	30	Vaginal	Female	1350
18	24	Vaginal	Male	740
21	28	Caesarean section	Male	1100
22	31	Vaginal	Female	1430
28	27	Vaginal	Female	1040
29	29	Caesarean section	Male	680
30	30	Vaginal	Female	1370
31	28	Caesarean section	Female	1150

Written informed parental consent was obtained for each infant before inclusion in the study, which was approved by the Ethical Committee on Clinical Research of the Hospital Clínico San Carlos of Madrid (10/017-E). This study was conducted in accordance with the Declaration of Helsinki. Characteristics to be eligible for enrolment have been described previously [32]. Relevant clinical data recorded for each infant, such as length of antibiotic therapy, parenteral nutrition, nasogastric feeding, mechanical ventilation, hospital stay and type of feeding, are described in Table 2. All infants were fed with human milk (donor milk and/or their own mother's milk) and, occasionally, with preterm formula.

The medical staff from the Department of Neonatology of the hospital had collected first spontaneously evacuated meconium and fecal samples during the infants' stays at the NICU [36]. Later, when the infants reached 2 years of age, parents were contacted to provide an additional fecal sample, if their infants had not taken antibiotics within the previous 2 months. All the samples were stored at −20 °C until analysis and processed as described previously [36].

Table 2. Clinical characteristics of the preterm infants recruited in this study.

Infant	Hospital Stay (days)	Antibiotic Therapy (days)	Mechanical Ventilation (days)	Parenteral Nutrition (days)	Nasogastric Feeding Tube (days)	Meconium Expulsion (h)
2	42	3	0	5	38	59
4	60	4	0.5	3	48	24
5	27	3	2	0	26	11
6	27	0	0	0	26	7
7	113	5	9	8	107	116
9	68	4	2	5	58	96
10	84	7	0.5	6	70	0
13	28	3	0	0	21	12
15	44	3	1	7	38	11
18	116	27	35	13	112	144
21	73	7	10	14	62	48
22	37	3	0	4	35	3
28	68	7	0	7	62	12
29	70	15	8	9	60	144
30	41	4	0	3	40	
31	52	3	0	6	47	9
Mean (95% CI)	58.64 (41.35; 75.93)			4.85 (2.66; 7.05)	52.40 (36.03; 68.82)	46.4 (17.22;75.58)
Median (IQR)		3.5 (3.0–7.0)	0.25 (0.00–1.75)			

CI: Confidence interval; IQR: Inter-quartile range.

2.2. Human Intestinal Tract Chip (HITChip) Analysis

DNA extraction from fecal samples was performed following the protocol described in Moles et al. (2013) [36]. All the steps for the HITChip microarray analysis, including polymerase chain reaction (PCR) amplification of 16S rRNA genes, RNA production and labeling, hybridization and data extraction, were performed as described previously [33]. Then, the PCR products were purified, using the High Pure PCR Product Purification kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. In vitro transcription of the T7 promoter-carrying 16S rRNA genes was performed using the Riboprobe System (Promega, Madison, WI, USA) while amino-allyl-modified nucleotides were coupled with CyDye using the Post-Labeling Reactive Dye (Amersham Biosciences, Little Chalfont, UK).

Data were extracted from the microarray images, using Agilent Feature Extraction software, version 9.1 (http://www.agilent.com), subsequently normalized, and further analyzed using a set of R-based scripts (http://r-project.org) in combination with a custom-designed relational database that runs under the MySQL database management system. Hierarchical clustering of probe profiles was carried out using the Pearson's distance and Ward's minimum variance method. Normalized hybridization signals for 23 Level 1 (phylum-like) groups and 131 Level 2 (genus-like) groups, as defined previously [37], are available as Supplementary Materials in Tables S1 and S2, respectively.

Data, available on the HITChip database, on fecal samples from 2–4-year-old healthy children and healthy adults were used for comparison purposes. Demographic data and other information from the 2–4-year-old healthy children has been published previously [38]. The bacterial compositions of fecal samples from healthy adult subjects were selected from the CO-MIC cohort, a study of the intestinal microbiome among Irritable Bowel Syndrome patients and healthy individuals. None of the individuals selected received antibiotics.

2.3. Immunological Analysis

The concentrations of 18 cytokines, chemokines, and growth factors, including interleukins (IL) 1_{β}, 2, 4, 5, 6, 7, 8, 10, 12(p70), 13, and 17, interferon-gamma (IFN-γ), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), growth regulated oncogene-alpha (Gro-α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1_{β} (MIP-1_{β}) and tumor necrosis factor-alpha (TNF-α), were determined in meconium and fecal samples by using the Human Cytokine group I and II assay kits (Bio-Rad Laboratories Inc., Hercules, CA, USA) in a Bioplex 200 system instrument (Bio-Rad). Briefly, 0.1 g of fecal samples was suspended in 1 mL of PBS. After homogenization, the samples were centrifuged (14,000$\times$ *g*, 15 min, 4 °C) and the supernatants ($\geq$200 µL) were collected. Determinations in meconium and fecal samples were carried out in duplicate.

The concentrations of immunoglobulin (Ig) G1, IgG2, IgG3, IgG4, IgM and IgA in the samples were determined using the Bio-Plex Pro Human Isotyping Assay kit (Bio-Rad) using the Bioplex 200 system. For this purpose, the samples were conditioned, as described above, for cytokine analysis. All analyses were carried out in duplicate, following the manufacturer's protocol.

Calibration curves for each analyte were constructed using triplicate values for each known concentration and the Bio-Plex Manager 6.0 software (Bio-Rad, Hercules, CA, USA).

2.4. Statistical Analysis

Quantitative data were expressed as the mean and 95% confidence interval (CI) of the mean or, when they were not normally distributed, as the median and interquartile range (IQR). The richness and diversity of the meconium and fecal microbiota were determined by calculating the Shannon–Weaver diversity index, which takes into account the number and evenness of the bacterial species. Friedman's non-parametric repeated measures comparisons were applied to determine differences between the hybridization signal intensities of genus-like bacterial groups across time. Chi-square independency tests were used to evaluate differences in the detection frequencies of immune compounds. One-way ANOVA or Kruskal–Wallis tests were used to compare differences in the concentrations of immune compounds at different sampling times. The immune compounds were further analyzed, applying exploratory multivariate analyses, such as the principal component analysis (PCA), multiple discriminant analysis (MDA) and cluster analysis (CA). Differences were considered significant at $p < 0.05$. Statgraphics Centurion XVI version 16.1.15 (Statpoint Technologies Inc., Warrenton, VA, USA) and R 2.13.2 (R Foundation for Statistical Computing, https://www.r-project.org, Vienna, Austria) software were used to carry out the analyses cited above.

For comprehensive multivariate statistical analyses, Canoco software for Windows 5.0 (Wageningen, The Netherlands) was used [34]. A redundancy analysis was performed to assess correlations between the microbial groups detected by the HITChip and the sample characteristics. The log-transformed hybridization signals of 130 genus-level phylogenetic groups targeted by the HITChip were used as biological variables. Gestational age, gender, birth weight, Z score, vaginal vs. cesarean section, age, antibiotics (mother and/or infant), time of first passage of meconium, type of nutrition, time of enteral and parenteral nutrition, sepsis and hospital stay were included as explanatory variables. The Monte Carlo Permutation testing (MCPT) was used to assess the significance of the variation in large data sets.

To evaluate the significance of the difference between datasets not-normally distributed, p values were calculated by Wilcoxon rank sum tests.

3. Results

3.1. Characteristics of the Infants

The 16 infants that participated in this study had, at birth, a mean gestational age of 28 weeks (ranging from 24 to 32 weeks) and a mean birth weight of 1220 g (ranging from 600 to 2030 g) (Table 1). Half of the infants (n = 8) were born by Cesarean section. All of them, except one, received antibacterial prophylaxis at least for the first 3 days of life, and nine needed mechanical ventilation (Table 2). Infants were fed either with their own mother's milk, donor milk and/or preterm formula by nasogastric feeding tube for, at least 21 days after delivery. The time required for spontaneous delivery of the first meconium oscillated between the first minutes to day 6 after birth. In addition to the meconium samples, fecal samples were available from the same infants, obtained after 3 weeks (±21 days) and 2 years (±730 days) after birth.

3.2. HITChip Analysis

The microarray datasets of the 16 fecal samples, collected two years after birth and those previously obtained from meconium and feces, collected in the third week of life [36] were analyzed and hierarchically clustered, based on the signal intensity of the 3699 distinct HITChip oligonucleotide probes (Figure 1, Table S1). The obtained unsupervised microbiota profiles clustered into three different groups, according to the sampling times, except for those of the meconium samples of infants 4, 6 and 22 (Figure 1).

The relative contribution of the major phyla (Table S2) was assessed as the percentage of the phylum taxa among the total microbiome detected in each fecal sample (Figure 2). Globally, approximately two thirds of the DNA phylotypes retrieved from the meconium samples were ascribed to the *Firmicutes* phylum (63.4%). The phylum *Proteobacteria* dominated in the 21-day fecal samples (about 60.0%) but became a minor species in the 2-year samples (2.5%). Interestingly, *Firmicutes* was again the most abundant phylum (78.5%) in the later samples, followed by *Actinobacteria* (9.9%) and *Bacteroidetes* (7.7%).

Within the *Firmicutes*, those belonging to the class *Bacilli* were the most abundant, both in meconium and 21-day feces (18.8%). However, the diversity of *Firmicutes* was notably higher in 2-year fecal samples, where *Clostridium* cluster XIVa (35.2%) and *Clostridium* cluster IV (27.1%) became the predominant classes (Figure 3).

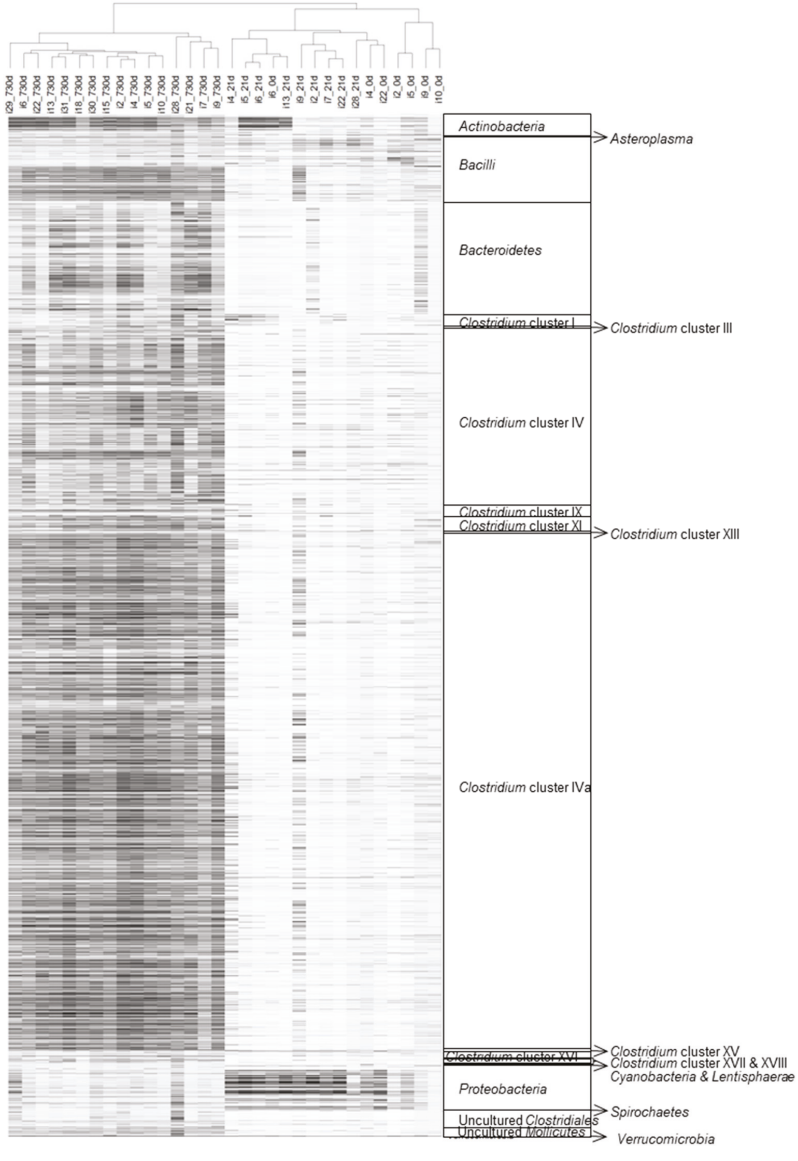

Figure 1. Hierarchical clustering of the intestinal microbiota. Unsupervised clustering was performed for HITChip oligoprofiles, obtained from fecal samples of the preterm infants at meconium (0 days), third week (21 days) and 2-years (720 days). Each line represents a different probe and the darkness of the lines represents the probe abundance in the sample. The highest phylogenetic levels represented are shown on the right side of the figure. Pearson's correlation and Ward's clustering methods were used.

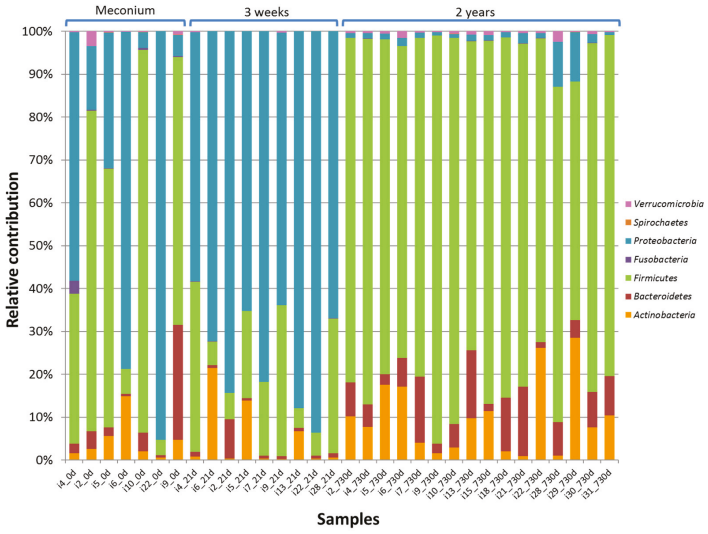

Figure 2. Development of the intestinal microbiota composition at the phylum level. The relative contribution is shown for the phyla detected in meconium, third week and 2-year fecal samples of the preterm infants.

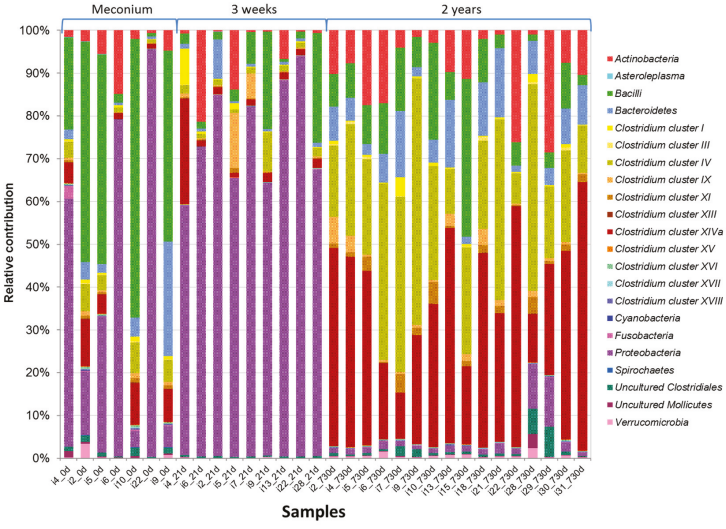

Figure 3. Development of the intestinal microbiota composition at the phylum/order level. The relative contribution is shown for the phylum/order-like phylogroups of the microbiota of meconium, third week and 2-year fecal samples of the preterm, as assessed using the HITChip microarray. Only phylum/order-like phylogroups that contributed at least 0.1% to a given profile are shown.

On a lower taxonomic level, the comparison of the 130 hybridization signals, corresponding to the genus-like bacterial groups (Level 2, Table S3), obtained from the 21-day and 730-day fecal samples, showed that 91 phylogenetic groups differed significantly between both types of samples.

Among them, 60 phylogenetic groups contributed >0.1% to the microbial profile of each sample (Table 3). The presence of 10 genus-like groups (Level 1) significantly decreased in the 2-year fecal samples, compared to the 21-day ones. It should be highlighted that most of the genera that were significantly reduced (Table 3, positive fold change ↑) are typically associated with hospital environments, including bacteria related to *Enterobacter aerogenes*, *Enterococus* spp. *E. coli*, *Granulicatella* spp., *K. pneumoniae*, and *Proteus*, *Serratia* and *Yersinia* spp. In contrast, there was a very high increase (≥40-fold) from the 21-day to 2-year microbiota, in the abundance of bacteria related to the known carbohydrate degraders, *Bacteroides vulgatus*, *Lactococcus* spp., *Ruminococcus bromii* and *Ruminococcus obeum* as well as the butyrate producers, *Anaerostipes caccae*, *Coprococcus eutactus* and *Eubacterium hallii* (Table 3, negative fold change ↓).

Table 3. Relative counts of genus-like bacterial groups. The genus-like phylogenetic groups (Level 2; [39]), detected in fecal samples, collected at 21 days and 2 years after birth, from preterm infants, are shown [a].

Phylum/Order	Genus-Like Phylogenetic Group [¥]	21 Days Mean (95% CI)	730 Days Mean (95% CI)	*p*-Value *	Fold Change #
Actinobacteria	*Collinsella*	0.04 (0.03; 0.04)	0.25 (0.18; 0.32)	0.009	↑6.77
Bacteroidetes	*Allistipes et rel.*	0.09 (0.08; 0.11)	0.38 (0.17; 0.59)	0.009	↑4.06
	Bacteroides ovatus et rel.	0.06 (0.05; 0.07)	0.38 (0.05; 0.71)	0.009	↑6.69
	Bacteroides plebeius et rel.	0.04 (0.03; 0.04)	0.12 (0.08; 0.16)	0.009	↑3.23
	Bacteroides splachnicus et rel.	0.09(0.08; 0.11)	0.26 (0.11; 0.41)	0.016	↑2.73
	Bacteroides stercoris et rel.	0.04 (0.03; 0.04)	0.11 (0.05; 0.16)	0.009	↑2.97
	Bacteroides vulgatus et rel.	0.08 (0.05; 0.09)	3.12 (1.54; 4.70)	0.009	↑39.50
	Parabacteroides distasonis et rel.	0.06 (0.05; 0.07)	0.32 (0.16; 0.48)	0.009	↑5.20
	Prevotella melaninogenica et rel.	0.11 (0.09; 0.13)	0.88 (−0.16; 1.92)	0.033	↑8.20
	Prevotella oralis et rel.	0.04 (0.03; 0.04)	0.24 (−0.02; 0.50)	0.016	↑6.55
	Prevotella tannerae et rel.	0.02 (0.02; 0.03)	0.10 (0.07; 0.13)	0.009	↑4.20
	Tannerella et rel.	0.05 (0.04; 0.06)	0.13 (0.11; 0.16)	0.009	↑2.63
Bacilli	*Enterococcus*	3.49 (−1.05; 8.03)	0.10 (0.06; 0.13)	0.009	↓36.18
	Granulicatella	1.00 (0.19; 1.80)	0.02 (0.01; 0.02)	0.009	↓61.31
	Lactococcus	0.02 (0.01; 0.02)	0.73 (−0.02; 1.48)	0.056	↑41.05
	Staphylococcus	0.19 (−0.05; 0.43)	0.02 (nd)	0.033	↓10.86
	Streptococcus intermedius et rel.	0.04 (0.02; 0.05)	0.15 (0.11; 0.20)	0.033	↑3.96
Clostridium cluster III	*Clostridium stercorarium et rel.*	0.03 (0.03; 0.04)	0.28 (0.15; 0.41)	0.009	↑9.26
Clostridium cluster IV	*Clostridium cellulosi et rel.*	0.13 (0.11; 16)	3.49 (1.20; 5.79)	0.009	↑26.48
	Clostridium leptum et rel.	0.18 (0.09; 0.28)	2.16 (0.74; 3.57)	0.009	↑11.81
	Clostridium orbiscindens et rel.	1.03 (−0.68; 2.73)	5.59 (3.90; 7.29)	0.042	↑5.44
	Faecalibacterium prausnitzii et rel.	0.17 (0.14; 0.20)	5.02 (1.81; 8.22)	0.009	↑29.35
	Oscillospira guillermondii et rel.	0.15 (0.13; 0.18)	3.10 (−0.13; 6.32)	0.009	↑20.04
	Papillibacter cinnamivorans et rel.	0.06 (0.05; 0.07)	0.50 (0.34; 0.66)	0.009	↑8.56
	Ruminococcus bromii et rel.	0.02 (0.02; 0.03)	1.31 (0.20; 2.43)	0.009	↑52.65
	Ruminococcus callidus et rel.	0.08 (0.07; 0.09)	0.86 (−0.06; 1.78)	0.009	↑10.57
	Sporobacter termitidis et rel.	0.16 (0.14; 0.19)	1.89 (0.96; 2.81)	0.009	↑11.56
	Subdoligranulum variable at rel.	0.11 (0.09; 0.13)	2.98 (1.48; 4.48)	0.009	↑27.51
Clostridium cluster IX	*Dialister*	0.05 (0.02; 0.07)	0.75 (−0.03; 1.54)	0.009	↑16.10
Clostridium cluster XI	*Anaerovorax odorimutans et rel.*	0.05 (0.04; 0.06)	0.41 (0.27; 0.55)	0.009	↑7.79

[a] Relative counts (log-transformed hybridization signals) are expressed as the mean and 95% confidence interval. nd, no data. [¥] The genus-like phylogenetic groups shown, contributed at least 0.1% to the microbial profile of a given sample. * *t*-tests were used to evaluate differences in the hybridization signal intensities of genus-like bacterial groups across time. # Fold changes were calculated as log-transformed hybridization signals at 2 years over those at 3 weeks.

Table 3. *Cont.*

Phylum/Order	Genus-Like Phylogenetic Group ¥	21 Days Mean (95% CI)	730 Days Mean (95% CI)	*p*-Value *	Fold Change #
Clostridium cluster XIVa	*Anaerostipes caccae et rel.*	0.09 (0.05; 0.12)	3.94 (2.10; 5.77)	0.009	↑45.14
	Bryantella formatexigens et rel.	0.14 (0.08; 0.21)	0.69 (0.50; 0.89)	0.009	↑4.82
	Butyrivibrio crossotus et rel.	0.16 (0.09; 0.23)	1.41 (1.14; 1.68)	0.009	↑8.89
	Clostridium colinum et rel.	0.05 (0.04; 0.06)	0.14 (0.06; 0.23)	0.009	↑2.77
	Clostridium sphenoides et rel.	0.12 (0.09; 0.14)	0.85 (0.56; 1.14)	0.009	↑7.38
	Clostridium symbiosum et rel.	0.22 (0.13; 0.32)	1.81 (1.38; 2.24)	0.009	↑8.09
	Coprococcus eutactus et rel.	0.08 (0.06; 0.09)	4.96 (2.87; 7.04)	0.009	↑64.93
	Dorea formicigenerans et rel.	0.16 (0.08; 0.23)	2.63 (1.86; 3.40)	0.009	↑16.72
	Eubacterium hallii et rel.	0.04 (0.02; 0.06)	2.23 (1.40; 3.05)	0.009	↑52.41
	Eubacterium rectale et rel.	0.06 (0.04; 0.08)	0.42 (0.26; 0.58)	0.009	↑7.09
	Eubacterium ventriosum et rel.	0.05 (0.02; 0.07)	0.28 (0.13; 0.42)	0.009	↑6.00
	Lachnobacillus bovis et rel.	0.07 (0.05; 0.07)	0.31 (0.26; 0.36)	0.009	↑4.43
	Lachnospira pectinoschiza et rel.	0.10 (0.07; 0.13)	0.40 (0.28; 0.53)	0.009	↑4.08
	Roseburia intestinalis et rel.	0.03 (0.01; 0.05)	0.24 (0.15; 0.34)	0.009	↑7.86
	Ruminococcus lactaris et rel.	0.03 (0.02; 0.03)	0.42 (−0.18; 1.02)	0.009	↑16.30
	Ruminococcus obeum et rel.	0.26 (0.21; 0.31)	11.42 (8.18; 14.66)	0.009	↑43.96
Uncultured *Clostridiales*	Uncultured *Clostridiales I*	0.17 (0.14; 0.20)	1.11 (0.12; 2.10)	0.009	↑6.48
	Uncultured *Clostridiales II*	0.18 (0.16; 0.21)	0.36 (0.21; 0.51)	0.009	↑1.97
Uncultured *Mollicutes*	Uncultured *Mollicutes*	0.08 (0.07; 0.10)	0.32 (−0.07; 0.70)	0.022	↑3.94
Proteobacteria	*Burkholderia*	0.01 (nd)	0.10 (−0.04; 0.24)	0.016	↑10.98
	Enterobacter aerogenes et rel.	14.82 (8.07; 21.57)	0.21 (0.10; 0.32)	0.009	↓69.90
	Escherichia coli et rel.	37.06 (24.37; 49.76)	0.82 (−0.25; 1.88)	0.009	↓45.41
	Klebisiella pneumoniae et rel.	15.75 (10.18; 21.32)	0.11 (0.05; 0.18)	0.009	↓138.34
	Oxalobacter formigenes et rel.	0.02 (0.02; 0.03)	0.17 (0.02; 0.32)	0.009	↑7.35
	Proteus et rel.	0.29 (0.17; 0.42)	0.09 (0.05; 0.12)	0.009	↓3.46
	Serratia	5.18 (1.88; 8.49)	0.15 (−0.04; 0.33)	0.009	↓35.00
	Sutterella wadsworthia et rel.	0.08 (0.07; 0.10)	0.23 (0.07; 0.39)	0.009	↑2.79
	Vibrio	0.11 (0.08; 0.15)	0.04 (0.03; 0.04)	0.009	↓2.94
	Yersinia et rel.	1.16 (0.63; 1.68)	0.04 (0.03; 0.04)	0.009	↓31.05
Verrucomicrobia	*Akkermansia*	0.06 (0.00; 0.13)	0.62 (0.34; 0.91)	0.016	↑9.64

[a] Relative counts (log-transformed hybridization signals) are expressed as the mean and 95% confidence interval. nd, no data. ¥ The genus-like phylogenetic groups shown, contributed at least 0.1% to the microbial profile of a given sample. * *t*-tests were used to evaluate differences in the hybridization signal intensities of genus-like bacterial groups across time. # Fold changes were calculated as log-transformed hybridization signals at 2 years over those at 3 weeks.

The comparison of genus-like bacterial groups (level 2) obtained from the 730-day fecal samples of preterm infants and 2–4-year-old term infants showed differences in 65 genus-like bacterial groups (Supplementary Materials Table S4) belonging to five different phyla: *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria*. Almost all the *Firmicutes*, the majority butyrate-producing bacteria were more abundant in 2-year-old children born preterm, than those born at term. In contrast, lactic acid bacteria accounted for a higher amount of the hybridization signal in children born at term represented by *Lactobacillus plantarum et rel*. The genus, *Bifidobacterium*, accounted for less than 0.1% of the total hybridization signal in 2-year-old children, born preterm, while in 2–4-year-old children, born at term, levels were high [38].

The Shannon–Weaver diversity indices, obtained from meconium, 21-day and 2-year fecal samples were compared with data available on the HITChip database for fecal samples from 2–4-year-old healthy infants and, also, from healthy adults (Figure 4). The microbial diversity increased with age, both in preterm and healthy individuals. Diversity indices differed significantly among all the groups, except for (a) the meconium and 21-day samples, and (b) the 2-year samples obtained in this study and the data from 2–4-year-old healthy infants existing in the HITChip database (paired *t*-test; $p = 0.511$ and $p = 0.957$ respectively).

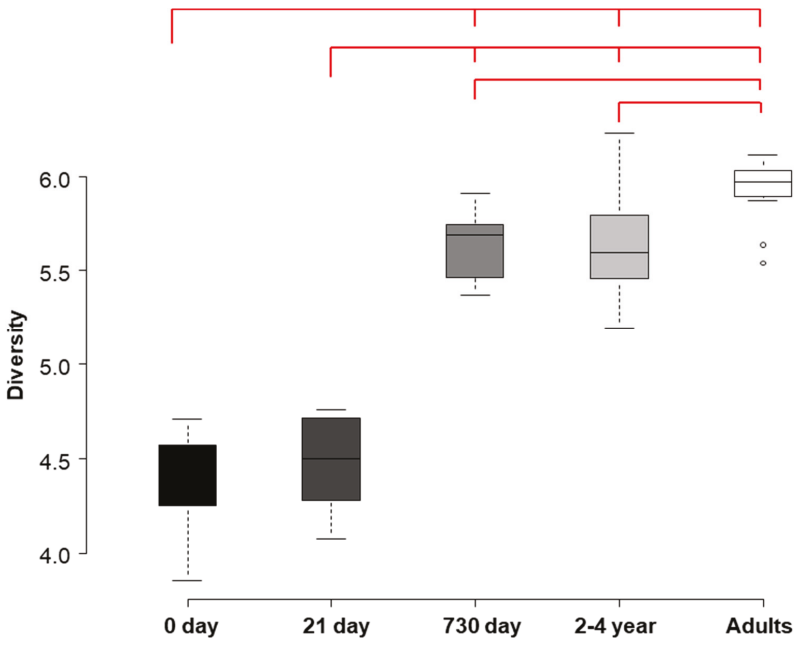

Figure 4. Development of the intestinal microbiota diversity. Shannon–Weaver diversity indices are shown for the intestinal microbiota in the meconium, third week and 2-year fecal samples of preterm infants. Boxes at the right and middle represent the Shannon–Weaver diversity index obtained in meconium, 21-day and 2-year feces. Boxes on the right represent Shannon–Weaver diversity indexes previously obtained with the Human Intestinal Tract Chip (HITChip) in feces, from 2 and 4-year-old healthy infants and from healthy adults. The red bars on the top represent pairs of indices that were found to be statistically different ($p < 0.001$).

The relationships between the observed differences in the bacterial profiles detected in meconium, 21-day and 2-year feces, and several demographic and clinical variables (Tables 1 and 2), were explored with a multivariate cluster analysis. A redundancy analysis revealed that, among all variables explored, only the age of the infant had a significant effect on the bacterial community composition at the different sampling times ($p = 0.006$, Monte Carlo Permutation Procedure) (Figure 5). More specifically, the observed distribution could explain 41.4% of the total variation in the dataset (Figure 5). Bacterial groups that were positively associated with increased age included bacteria belonging to butyrate-producing species, such as *Butyrivibrio crossotus*, *Eubacterium rectale* and *Eubacterium hallii*. In contrast, the groups that were negatively associated with age included potentially pathogenic Gram-negative bacteria, typically related to hospital environments, such as *Escherichia*, *Klebsiella*, *Serratia* and *Yersinia*. The uniform length of all vectors depicted in Figure 5 indicates that the strength of the correlation was similar for all bacterial species. The length of the hospital stay was also found to be associated (although not significantly) with 21-day fecal samples and with specific bacterial groups (most notably the *Proteobacteria*). None of the other variables significantly influenced the sample separation.

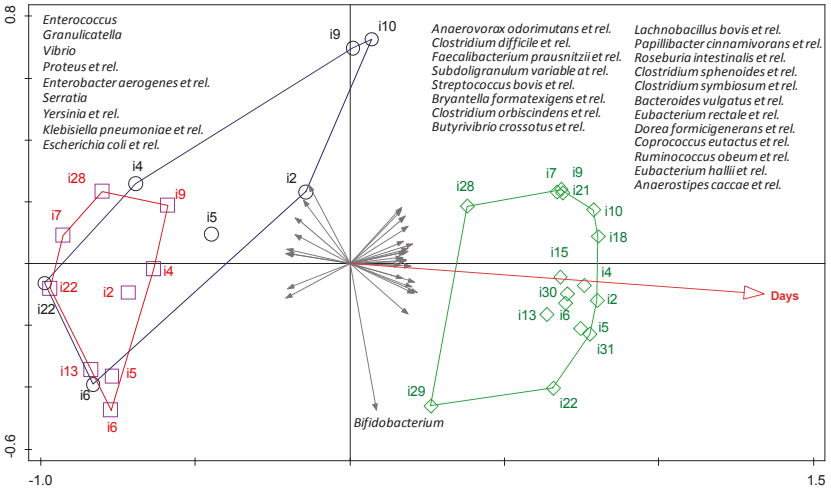

Figure 5. Correlation analysis for the intestinal microbiota. The results of a redundancy analysis are shown for the bacterial composition of meconium (blue circles), third week (red squares) and 2-year (green rhombus) fecal samples of the preterm infants. Arabic numbers indicate the different infants. Gray arrows indicate the bacterial groups associated with the different samples. The plotted first and second ordination axes explained 41.4% of the variability in the data set. Age was the only variable that was significantly related to the sample distribution ($p = 0.006$, Monte Carlo Permutation Testing (MCPT) with forward selection).

3.3. Immunological Analysis

The concentration of a broad range of immune compounds, including cytokines, chemokines, growth factors and immunoglobulins was measured in nine meconium and 9, 15 and 16 fecal samples from the first week, third week and second year of life, respectively. Initially, an exploratory screening was performed, in order to detect outliers; this analysis revealed that the 2-year fecal sample from infant 2 was very different from the rest of the sample set in the PCA analysis of immune compounds. The medical history from this infant reflected a high incidence of acute otitis media during the first two years of life and, in fact, a few days after the collection of this fecal sample, this infant was submitted to an emergency surgery. Therefore, this sample was excluded from the general immunological analysis.

Globally, the values obtained for all these immune factors showed high interindividual variability, in both detection frequencies and concentration. Meconium samples showed a lower presence of immune-related compounds, compared to fecal samples, except for MIP-1β and GM-CSF (Table 4). Most cytokines related to either innate or acquired immunity were detected in less than 50% of the samples, except for IL-1β, in the first and third weeks of life; IL-4 (for which detection frequency increased over time); and IL-17 in the third week and after two years from birth. Among the chemokines, MCP-1 (2-year samples) and MIP-1β (meconium, first and third week fecal samples) were also detected in more than 50% of the samples, while the hematopoietic factors, G-CSF and GM-CSF, were detected in higher numbers (Table 4). All the detection frequencies (except those for IL-6 and IL-13) changed significantly ($p < 0.018$) depending on the sampling time (Table 4). However, only GM-CSF showed a statistically significant difference ($p = 0.002$), with higher levels in meconium and 2-year samples, when the evolution over time of the concentrations of all the immune compounds was considered (Table 4). Some immune compounds (IL-1β, IL-2 and MIP-1β) showed a decreasing tendency, while others (IL-6, IL-12(p70), IFN-γ, TNF-α, MCP-1 and IL-5) tended to increase over time.

The concentrations of IgG1, IgG2, IgG3, IgG4, IgM and IgA in meconium and fecal samples, taken at 7, 21 and 730 days after birth, are shown in Table 5. With the exception of IgA, a high degree of variability and a low frequency of detection were observed for immunoglobulins. Similarly to cytokines, the detection frequencies of all the immunoglobulins changed significantly, depending on the sampling time but no statistically significant differences were found in relation to their concentrations. IgA was the most abundant immunoglobulin in all the tested samples with a significant change ($p = 0.004$) over time. The median IgA concentrations in the first week fecal samples (26.62 mg/g) were approximately 26 times higher than those detected in the meconium ones (1.02 mg/g); the concentration then decreased in the later time samples.

Immune profiles of fecal samples, collected at 21 and 720 days after delivery of preterm infants, were compared with immune profiles of fecal samples collected at 90 and 300 days after delivery of healthy infants born at term. Despite the difference between time of collection of the samples, interesting divergences were observed. Both concentration and frequency of detection of cytokines were higher in fecal samples of preterm infants than in infants born at term. On the other hand, the frequency and the concentration of immunoglobulins were higher in fecal samples of infants born at term than preterm, except for IgA.

When a multiple discriminant analysis (MDA) was applied to all the variables, taking sampling time as the discriminant factor, two derivative functions with an eigenvalue >1 were obtained. The predictive power of the MDA was 85.42% and the expression of the first derivative function (Function 1) had a canonical correlation factor of 0.9018 that was statistically significant ($p = 0.002$). The standardized coefficients for this function were the following: IL-1b (−0.96), IL-2 (−3.38), IL-4 (1.22), IL-5 (−0.56), IL-6 (−0.48), IL-7 (−1.72), IL-8 (−2.81), IL-10 (−4.56), IL-12(p70) (4.50), IL-13 (−1.10), IL-17 (−5.61), G-CSF (4.38), GM-CSF (−0.67), IFN-γ (4.49), MCP-1 (0.81), MIP-1β (8.88), TNF-α (1,60), GRO-α (−4.78), IgG_1 (2.13), IgG_2 (−6.37), IgG_3 (−2.71), IgG_4 (−0.50), IgM (0.36), IgA (0.30). In fact, the MDA representation allowed a differential classification of fecal samples, according to infant age, when considering their profiles of immune compounds (Figure 6). Furthermore, the centroid of each group of samples (meconium, first week, third week and 2-year feces) was located in a different quadrant of the coordinates delimitated by the zero values of the axes (Figure 6).

Table 4. Immunological analysis of intestinal samples. The presence and concentrations are shown for cytokines and other immune compounds in the meconium and fecal samples collected in this study.

	Meconium (n = 9)		Feces (7 days) (n = 9)		Feces (21 days) (n = 15)		Feces (2 years) (n = 15)		p-Value ‡	p-Value ǂ
	n (%) [1]	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)		
Innate immunity										
IL-1β	2 (22.22)	1.24 (0.68–1.80)	7 (77.78)	0.13 (0.05–0.30)	13 (86.67)	0.05 (0.03–0.38)	0	-	0.000	0.520
IL-6	1 (11.11)	0.03	2 (22.22)	0.02 (0.02–0.03)	3 (20.00)	0.05 (0.05–0.71)	4 (26.67)	0.3 (0.15–0.47)	0.094	0.255
IL-12p70	1 (11.11)	0.06	2 (22.22)	0.11 (0.08–0.15)	4 (26.67)	0.07 (0.05–0.12)	4 (26.67)	0.84 (0.28–1.38)	0.018	0.601
IFN-γ *	0	-	0	-	1 (6.67)	3.27	3 (20.00)	9.27 (−7.44; 25.98)	0.000	0.521
TNF-α	1 (11.11)	0.14	2 (22.22)	0.15 (0.13–0.17)	5 (33.33)	0.18 (0.10–0.25)	3 (20.00)	0.27 (0.17–0.43)	0.002	0.934
Acquired immunity										
IL-2	1 (11.11)	0.92	1 (11.11)	0.09	2 (13.33)	0.04 (0.03–0.05)	4 (26.67)	0.04 (0.02–0.15)	0.003	0.373
IL-4	4 (44.44)	0.01 (0.00–0.01)	6 (66.67)	0.01 (0.00–0.01)	11 (73.33)	0.01 (0.00–0.01)	15 (100.00)	0.01 (0.00–0.01)	0.000	0.292
IL-10	0	-	1 (11.11)	0.03	1 (6.67)	0.04	4 (26.67)	0.03 (0.02–0.23)	0.000	0.675
IL-13 *	1 (11.11)	0.03	1 (11.11)	0.05	2 (13.33)	0.08 (−0.10; 0.26)	1 (6.67)	0.05	0.568	0.571
IL-17	4 (44.44)	3.09 (2.32–3.31)	2 (22.22)	0.08 (0.05–0.12)	9 (60.00)	0.07 (0.03–0.19)	12 (80.00)	0.08 (0.04–0.06)	0.000	0.122
Chemokines										
IL-8 *	4 (44.44)	0.07 (−0.08; 0.21)	1 (11.11)	0.05	3 (20.00)	0.09 (−0.02; 0.20)	0	-	0.000	0.865
MCP-1	3 (33.33)	0.03 (0.02–0.03)	1 (11.11)	0.03	4 (26.67)	0.05 (0.03–0.06)	9 (60.00)	0.19 (0.06–0.88)	0.000	0.080
MIP-1β	6 (66.67)	0.62 (0.05–1.78)	6 (66.67)	0.05 (0.04–0.16)	8 (53.33)	0.07 (0.02–0.10)	2 (13.33)	0.05 (0.04–0.07)	0.000	0.639
GRO-α	3 (33.33)	0.18 (0.16–8.28)	0 (0.00)	-	6 (40.00)	0.16 (0.11–0.19)	3 (20.00)	0.14 (0.13–0.22)	0.000	0.537
Hematopoyetic factors										
IL-5 *	0	-	0	-	1 (6.67)	0.03	5 (33.33)	0.25(0.03; 0.47)	0.000	0.322
IL-7	1 (11.11)	0.03	1 (11.11)	0.04	3 (20.00)	0.03 (0.02–0.05)	0	-	0.000	0.766
G-CSF	4 (44.44)	1.64 (0.34–4.29)	6 (66.67)	0.06 (0.04–0.17)	12 (80.00)	0.28 (0.15–0.55)	12 (80.00)	0.14 (0.11–0.46)	0.000	0.220
GM-CSF	9 (100.00)	0.59 [a] (0.28–55.03)	7 (77.78)	0.23 [ab] (0.12–0.40)	15 (100.00)	0.23 [ab] (0.18–0.26)	15 (100.00)	0.67 [ac] (0.62–0.87)	0.000	0.002

Concentrations (ng/g feces) are expressed as median and interquartile range (IQR). [1] n (%): number of samples in which the parameter was detected (relative frequency of detection). ‡ Chi-squared tests were used to evaluate differences in expression frequencies of the analyzed parameters. ǂ One-way ANOVA or Kruskal–Wallis tests were used to evaluate differences in concentration across time. Different superscript letters show which medians were different within groups. * These data sets were normally distributed and concentrations are expressed as mean and 95% CI.

Table 5. Detection and concentrations of immunoglobulins (Ig) in the meconium and fecal samples collected in this study.

	Meconium (n = 9)		Feces (7 days) (n = 9)		Feces (21 days) (n = 15)		Feces (2 years) (n = 15)		p-Value ‡	p-Value ǂ
	n (%) [1]	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)	n (%)	Median (IQR)		
IgG1	4 (44.44)	0.03 (0.02–0.06)	2 (22.22)	0.34 (0.18–0.50)	3 (20.00)	0.32 (0.17–0.76)	1 (6.67)	0.06	0.000	0.621
IgG2	6 (66.67)	1.54 (0.84–3.39)	7 (77.78)	0.99 (0.84–1.19)	3 (20.00)	0.35 (0.27–0.62)	2 (13.33)	0.34 (0.24–0.44)	0.000	0.062
IgG3 *	0	-	1 (11.11)	0.01	0	-	2 (13.33)	0.01 (−0.01; 0.03)	0.000	0.326
IgG4 *	2 (22.22)	0.00 (−0.01; 0.01)	3 (33.33)	0.01 (−0.00; 0.02)	1 (6.67)	0.00	1 (6.67)	0.01	0.000	0.510
IgM	1 (2.08)	0.55	6 (12.50)	2.27 (0.53–5.14)	11 (22.92)	0.54 (0.33–0.88)	1 (6.67)	0.08	0.000	0.368
IgA	5 (55.56)	1.02 [a] (0.48–67.45)	8 (88.89)	26.62 [ab] (10.42–58.88)	15 (100.00)	6.24 [ab] (3.69–26.13)	13 (86.67)	1.13 [ac] (0.81–3.18)	0.000	0.004

Concentrations (mg/g feces) arere expressed as median and interquartile range (IQR). [1] n (%): number of samples in which the parameter was detected (relative frequency of detection). ‡ Chi-squared tests were used to evaluate differences in expression frequencies of the analyzed parameters. ǂ One-way ANOVA or Kruskal–Wallis tests were used to evaluate differences in concentration across time. Different superscript letters show which medians were different within groups. * These data sets were normally distributed and concentrations are expressed as mean and 95% CI.

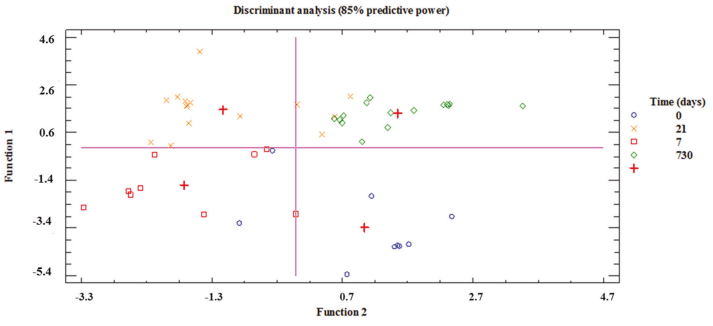

Figure 6. Multiple discriminant analysis (MDA) of the immune data. MDA was applied to the immunological data of meconium (blue circles), first week (red squares), third week (orange cross), and 2-year (green rhombus) feces, taking sampling time as the discriminant factor. The red cross represents the mathematical centroid for each sampling time group (meconium, first week, third week, and 2-year fecal samples). The first and second functions, which were plotted as the *x* and *y* axes, had a predictive power of 85% and internal axes (pink lines) match the zero values for both functions.

4. Discussion

In this study, the bacterial compositions of fecal samples obtained from 2-year-old infants that were born preterm were assessed and compared to those from meconium and third week of life fecal samples obtained from the same infants [36,40,41]. In addition, a wide range of cytokines, chemokines, growth factors and immunoglobulins were determined in all the meconium and fecal samples, in order to describe their immunological profiles, their changes over time and their potential relationships with bacterial colonization.

The results obtained from meconium and third week fecal samples showed a low diversity of bacterial species and high interindividual variability, while the opposite was observed in those taken from the same infants at the age of 2. Globally, the bacterial communities evolved towards an adult-like microbiota, which is the normal evolution of the microbiome of healthy term infants as they age [9,42]. Fecal samples taken 2 years after birth showed a distinctive bacterial composition when compared to that obtained from the same infants when they were 3-weeks-old. Those genera, related with a hospital environment—such as *E. coli*, *Klebsiella* or *Serratia*—and present in the third week of life seemed to be replaced, two years later, by genera belonging to *Clostridium* clusters, IV and XIVa. The predominance of such genera is a feature of the healthy adult gut microbiome, as a part of a complex microbiota, which is characterized by slow turnover, preference for low redox potential and high production of short chain fatty acids [43].

In this study, bacterial diversity increased with age, in agreement with previous works that have reported that the number of operational taxonomic units (OTUs) detected in fecal samples increases with age in different human populations [9]. The Shannon diversity index of the microbiota, present in 2-year-old infant stools was higher than that observed in the 21-day ones, similar to that calculated from the 2–4-year-old healthy infants from the HITChip database, and lower to that of healthy adults, as deduced using the same database. Remarkable changes occur in the gut colonization pattern throughout the first two or three years of life, but then, the microbiota stabilizes and starts to resemble that of adulthood [8,44]. The results of this study indicate that the diversity of the gut microbiota of 2-year-old infants, who were born preterm, has not yet reached the attributes of the adult microbiome.

Colonization of the infant gut by *Lactobacilli* and *Bifidobacteria* is often delayed or even absent in the case of antibiotic-treated infants [12,15,45], including preterm neonates [36]. The results obtained in this study indicate that this may be a long-lasting effect of prematurity, since, after two years from birth, the relative abundance of *Lactobacilli* was still low, compared to age-matched term infants (Supplementary Materials Table S4).

Previous studies, focused on the detection and quantification of cytokines, chemokines and immunoglobulins in blood samples, from term and preterm infants, have shown that there are differences in their immune profiles, depending on their gestational age [27,29,31]. However, as far as we know, this is the first study where a wide range of immune compounds has been assessed in the meconium and feces of preterm infants and followed up when they were 2 years old. Interestingly, each type of sample (meconium, 7-day, 21-day and 2-year feces) showed a different immune pattern and, in fact, the MDA analysis, performed with all the immune variables, exhibited a high predictive power, highlighting the differences in the immune profiles among the different sampling times.

In this study, the median IgA concentration increased notably from meconium to first week feces but in the third week of life, there was a progressive reduction, although not significant, reaching levels similar to those of healthy infants [46,47]. This probably results from the massive arrival of bacteria and other antigens to the gut after birth, since microbial gut colonization triggers the production of IgA by the gut-associated lymphoid tissue (GALT) [48]. This high IgA concentration in the first weeks of life became lower in the 2-year samples. In addition, lactating mammary glands are part of the secretory immune system, and IgA antibodies in breast milk reflect the antigenic stimulation of mucosal-associated lymphoid tissue [49]. Breast-milk antibodies are, thus, highly targeted against infectious agents and other exogenous antigens in the mother's environment, which are those likely to be encountered by the infant [32]. Therefore, breastfeeding represents an ingenious immunologic mother–infant integration [49–52]. This fact highlights the importance of the availability of own mother's or donor's milk to feed preterm neonates, a population particularly sensitive to infectious and inflammatory diseases. It should be noted that an abnormal gut microbial colonization predisposes the neonatal intestine to inflammation and to a cascade of pro-inflammatory and anti-inflammatory cytokine responses [53]. The ability of IgA to penetrate the gut mucosal surface, in conjunction with antigens and, as a consequence, to induce effector immune responses, plays a key role in the maintenance of intestinal microbiota and immune homeostasis [54].

The comparison of the immune profiles of fecal samples of preterm infants, collected at 720 days after delivery, with samples from infants born at term, collected at day 300, suggested a higher activity of B lymphocytes in the latter. This fact may be associated with a higher secretory maturity, whereas 2-year-old children born preterm have a higher activity of mediators of the immune system, which may be associated with a high activity of T lymphocytes.

Work is in progress to characterize the wide collection of bacterial isolates obtained from the biological samples analyzed in this study and, therefore, to elucidate at the strain or clone level if initial colonizers may persist later in life.

5. Conclusions

Hospital-associated fecal bacteria, dominant during NICU stay are replaced, two years later, by adult-like genera. In contrast to infants born at term, preterm infants have a low abundance of *lactobacilli* and *bifidobacteria* at two years of age. The immune profiles of the meconium and fecal samples differed, depending on the sampling time, showing different immune maturation statuses of the gut.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/12/1293/s1, Table S1: L1 HITChip composition, Table S2: L2 HITChip composition, Table S3: Oligoprofile HITChip composition, Table S4: Relative counts of significant different genus-like bacterial groups of preterm and at term 2-year-old children ($p < 0.05$).

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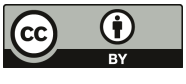

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Review

Immune-Mediated Mechanisms of Action of Probiotics and Synbiotics in Treating Pediatric Intestinal Diseases

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Abstract: The pediatric population is continually at risk of developing infectious and inflammatory diseases. The treatment for infections, particularly gastrointestinal conditions, focuses on oral or intravenous rehydration, nutritional support and, in certain case, antibiotics. Over the past decade, the probiotics and synbiotics administration for the prevention and treatment of different acute and chronic infectious diseases has dramatically increased. Probiotic microorganisms are primarily used as treatments because they can stimulate changes in the intestinal microbial ecosystem and improve the immunological status of the host. The beneficial impact of probiotics is mediated by different mechanisms. These mechanisms include the probiotics' capacity to increase the intestinal barrier function, to prevent bacterial transferation and to modulate inflammation through immune receptor cascade signaling, as well as their ability to regulate the expression of selected host intestinal genes. Nevertheless, with respect to pediatric intestinal diseases, information pertaining to these key mechanisms of action is scarce, particularly for immune-mediated mechanisms of action. In the present work, we review the biochemical and molecular mechanisms of action of probiotics and synbiotics that affect the immune system.

Keywords: probiotics; pediatric gastrointestinal infection; mechanism of action; intestinal microbiota; immune system

1. Introduction

Pediatric intestinal diseases comprise a variety of clinically important conditions, such as infectious diseases (acute diarrhea, antibiotic-associated diarrhea (AAD), *Clostridium difficile*-associated diarrhea, and *Helicobacter pylori* infection), and necrotizing enterocolitis (NEC), as well as some non-communicable chronic diseases, including inflammatory bowel diseases (IBD) (ulcerative colitis and Crohn disease) and cystic fibrosis.

Pediatric infectious diseases are the most important illnesses in children, especially in preschool centers [1]. Children who go to daycare centers are at a 2.2–3.5-fold greater risk of developing

gastrointestinal infections than children who stay at home [2,3]. In addition, studies have suggested that poor hygiene is related to the development of such infections [4,5].

Acute infectious diarrhea and AAD are the two primary manifestations of gastrointestinal pediatric infections. Acute diarrhea is frequently originated through viral infection, with rotavirus and Norwalk virus infections being common causes of gastroenteritis in children. In addition, important infectious bacteria are implicated in day care-associated gastrointestinal disorders, such as *Escherichia coli*, *Salmonella* sp., *Campylobacter jejuni*, *Clostridium difficile* and *Helicobacter pylori* [6–9].

In developing countries, more than 500,000 people die annually due to diarrhea associated with rotavirus gastroenteritis. In Europe, almost every child will have experienced an episode of rotavirus gastroenteritis, and one in 54 will need hospitalization [10].

AAD is known to disrupt the gastrointestinal microbiota that marks in a variety of medical symptoms. The AAD incidence in children in primary health services is approximately 10% [11,12]. *C. difficile* infections primarily occur in immunocompromised hosts and represent serious infection for which the primary treatment is antibiotic therapy. In fact, *C. difficile* infection is the leading cause related to antimicrobial therapy, accounting for nearly 15–25% of all AAD episodes [13].

Additional pediatric intestinal diseases that are frequently associated with intestinal dysbiosis include: NEC, a health condition that is principally appreciated in premature infants with bowel undergo necrosis [14]; ulcerative colitis, a chronic IBD of unknown etiology that is characterized by acute exacerbations of intestinal complications, followed by remissions; Crohn's disease, a systemic disorder in which the development of host genetic susceptibility represents an important etiological factor [15]; and cystic fibrosis, a fatal genetic disease without cure, affecting the digestive system and lungs with some typical complications, such as difficulty digesting fats and proteins, malnutrition and vitamin deficiencies resulting from an inability to absorb nutrients, chronic infections and aberrant inflammation [16].

The current treatment for pediatric infectious diseases focuses on oral or intravenous rehydration, nutritional support, and in some cases, antibiotics. The new therapeutic alternatives, for example antiemetics, antidiarrheal agents, and probiotics are often proposed. Oral rehydration therapy prevents only related dehydration [17], but does not affect the frequency of bowel movements, diarrheal duration, or intestinal barrier function [18,19]. Indeed, to better limit and heal intestinal damage, new treatment alternatives are required.

Alteration to the gut microbiome through the administration of beneficial microbes, typically referred to as probiotics, is an active area of investigation [15,20–22]. Probiotics are defined as "live microorganisms that confer a health benefit to the host when administered in adequate amounts, although dead bacteria and bacterial molecular components may also exhibit probiotic properties" [22]. Recent reviews and meta-analyses have suggested an effect of probiotics in the treatment and prevention of gastrointestinal and upper respiratory infections in children [23–30]. Probiotics appear to have a beneficial impact in treating acute infectious diarrhea and reducing AAD. However, the potential advantage of probiotics in the prevention of traveler's diarrhea and *C. difficile*-associated diarrhea, as well as the adverse effects in *H. pylori* eradication, NEC, IBD, and cystic fibrosis remain unclear, the principal reason being that the effects tend to be strain-specific [3,31–33].

A prebiotic is a non-viable food component that confers a health benefit to the host and is associated with the modulation of the intestinal microbiota. Using prebiotics and probiotics in combination is often described as synbiotics [34,35].

The administration of probiotics for the prevention and treatment of a variety of pediatric infectious diseases has received increasing attention worldwide. Many scientific reports from different societies, such as the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [24], the American Academy of Pediatrics [36], the World Gastroenterology Organization [37], and the Canadian Pediatric Society [38] have indicated the benefits of probiotics, supporting recommendations for the use of probiotics to treat acute gastroenteritis and for the reduction of AAD. Several important mechanisms that underlie the observed beneficial effects of probiotics

include secretion of antimicrobial substances, competitive adherence to the mucosa and epithelium, strengthening of the gut epithelial barrier, and modulation of the immune system [22,23,32]. Probiotic effects that are mediated through these mechanisms are an important issue that needs to be addressed. In addition, pro-inflammatory transcription factors, cytokines, and apoptosis-related enzymes can also be affected by probiotic strains. However, information on the mechanism of action of these effects, which are mediated by probiotics, is scarce, especially details related to the modulation of the immune system.

Therefore, the present review was conducted to investigate what is known from published research on the immune-mediated effects of probiotics and synbiotics in the prevention and treatment of pediatric intestinal diseases, with a special focus being placed on the mechanisms of action related to immune system modulation.

2. Materials and Methods

A comprehensive search of the relevant literature was performed using electronic databases, including MEDLINE (PubMed), EMBASE, and the Cochrane Library. We searched for scientific articles published between 2009 and 2017 in English in MEDLINE through PubMed. We used the MeSH terms "probiotics" and "synbiotics" combined with "infection" "pediatrics" and "gastrointestinal diseases". We evaluated the results that were obtained using the following equation search: (("infection"[MeSH Terms] OR "infection"[All Fields]) AND ("pediatrics"[MeSH Terms] OR "pediatrics"[All Fields] OR "pediatric"[All Fields])) AND (("probiotics"[MeSH Terms] OR "probiotics"[All Fields] OR "probiotic"[All Fields]) OR ("synbiotics"[MeSH Terms] OR "synbiotics"[All Fields] OR "synbiotic"[All Fields])). Our search yielded 211 articles, 31 of which were selected that specifically inform on probiotic mechanisms of action. Additionally, we searched the reference lists of the included articles for potential relevant literature.

3. Results and Discussion

3.1. Major Clinical Effects and Related Mechanisms of Action of Probiotics in Pediatric Intestinal Diseases

3.1.1. Gastrointestinal Infections

One thousand and sixty-two preschool children were enrolled in a double-blind, randomized, controlled study to test the effects of *L. casei rhamnosus*, *L. rhamnosus* T cell-1, multiple probiotics, and a placebo over 3 and 7 month periods [39]. Single strain probiotic supplementation significantly decreased the incidence of bacterial infections. Nevertheless, the multiple probiotic supplements did not show any effect. The only strain that decreased infectious disease at 3 months was *L. casei rhamnosus*. The authors hypothesized that the results were mediated through the action of lactobacilli species, as they can affect antigen-specific IgG1/IgG2 antibodies and cytokine responses and can also stimulate dendritic cells (DC) and produce a Th1 response [39].

L. rhamnosus GG strain is the most evaluated probiotic on pediatric population, alone or in combination with prebiotics or vitamins. A prospective, randomized, double-blind, placebo-controlled trial was performed to test the *L. rhamnosus* GG administration during 3 months in 281 children. The *L. rhamnosus* GG treatment was only successful only in the prevention of upper respiratory tract infections in children who attended primary health services [40]. This study showed no significant effect of the *L. rhamnosus* GG treatment with respect to the observed number of gastrointestinal infections and the number of diarrhea and vomiting episodes. In contrast, using a similar methodology in hospitalized children, Hojsak et al., 2010 observed that a *L. rhamnosus* GG treatment significantly reduced the gastrointestinal infections, vomiting, and diarrhea episodes [41]. In both studies, the authors only mentioned a few documented effects of probiotics related to antimicrobial properties and enhancement in mucosal barrier and their immunomodulatory action.

An immunological approach was performed in 124 children (82 infected with rotavirus and 42 with cryptosporidial diarrhea) that evaluated the *L. rhamnosus* GG effects on immune response and intestinal permeability. In the *L. rhamnosus* GG group less children had repeated diarrheal events and impaired intestinal function. Moreover, children that received *L. rhamnosus* GG have an increase in IgG levels post-intervention. *L. rhamnosus* GG administration exhibited a significant enhancement in intestinal permeability in children with cryptosporidial diarrhea [42]. The authors stated that the mechanism by which probiotics produce an immunomodulatory action is not completely understood and might be related to the modulation of immune responses (innate and adaptive), increasing serum IgG and secretory IgA to enteric pathogens, including *Salmonella typhi* and rotavirus [42].

Another study with *L. rhamnosus* GG was performed in 90 hospitalized children, and they received *L. rhamnosus* GG plus vitamin B, vitamin C, and zinc or placebo. The consumption of a combination of *L. rhamnosus* GG and micronutrients was effective in reducing the incidence of gastrointestinal infections and the length of hospitalization compared to placebo [43]. Furthermore, the duration and severity of symptoms were reduced [43]. No mechanism of action was reported to be associated with the observed effects.

The last *L. rhamnosus* GG study was a follow-up study that was conducted for 3 and 5 years in 109 and 96 children, respectively. Children received hydrolyzed protein formulas with *L. rhamnosus* GG, and the primary expected result (the decrease of the incidence of acute gastroenteritis mediated through the action of *L. rhamnosus* GG) was not observed at the analyzed time-points [44].

Studies on the administration of *L. rhamnosus* GG have shown contradictory results. In some studies, probiotic treatment decreased the frequency and gravity of gastrointestinal diseases, and in others, an effect was not observed. It is important to mention that the administration doses and time interventions were different in these studies, and future studies should be standardized to assess a potential successful result. Regarding the mechanism of action, the primary mechanism proposed for *L. rhamnosus* GG was the modulation of the innate and adaptive immune system, but this was mostly based on speculation.

The gastrointestinal effects and antibiotic sensitivity of *L. salivarius* CECT5713 were analyzed in 80 6-month-old children during 3 and 6 months of intervention. A probiotic treatment decreased the frequency of diarrhea and respiratory infections compared with placebo group [45]. Fecal concentration of butyric acid has augmented in *L. salivarius* CECT5713 group, this acid reduce the colonic pH and increase the peristaltic movements, promoting an advantageous environment for commensal bacteria. The authors observed a lower frequency of diarrhea in *L. salivarius* CECT5713 group, and they related this finding to in vitro assays, reporting on the production of antimicrobial compounds through *L. salivarius* CECT5713 action [45].

In 2012, 215 infants were enrolled to test the effects of *L. fermentum* CECT5716 plus galactooligosaccharides (GOS) during 6 months. *L. fermentum* administration was useful for the prevention of gastrointestinal infections in infants [46], increasing bifidobacteria and lactobacilli. The production of short chain fatty acids and IgA concentrations did not change during the study. The authors linked those changes in bifidobacteria and lactobacilli with the reductions in the number of gastrointestinal episodes observed in *L. fermentum* CECT5716 plus GOS group [46].

A follow-up study that lasted 3 years observed similar values of growth, frequency of infectious and non-infectious diseases in children that received the *L. fermentum* CECT5716 formula compared with placebo [47]. The proposed mechanism of action was the innate response activation through the *L. fermentum* CECT5716 plus GOS, although no changes in fecal IgA were detected [47]. The effects of *L. salivarius* CECT5713 were mediated through butyric acid production, and a *L. fermentum* CECT5716 plus GOS treatment appeared to produce changes in the intestinal microbiota.

Regular calcium content plus *L. casei* CRL431 or *L. reuteri* DSM17938 treatments were tested in 494 children. The frequency of diarrhea episodes was significantly lower in the *L. reuteri* group compared with the placebo group [48]. Similar results were shown in children supplemented with fermented milk with killed-*L. paracasei* CBA L74 or placebo for 3 months on a daily basis. The probiotic treatment

decreased the number of episodes of acute gastroenteritis compared to placebo [49]. These effects were mediated by an augment of immunity peptides. Moreover, to their antimicrobial character these peptides regulate the T cells activity, DC, macrophages, monocytes, and neutrophils, as well as the production of secretory IgA, in the killed-*L. paracasei* CBA L74 group [49].

The administration of *L. casei* DN-114 001 was effective in decreasing gastrointestinal infections in 638 3–6-year-old children in daycare centers/schools [50]. The authors recognized that this trial studied a precise probiotic strain, dose, and age group, and their findings cannot be generalized for other species or consequences without explaining a mechanism of action.

Conversely, several studies did not show clinical effects of probiotic strains over gastrointestinal infections. *L. reuteri* DSM 17,938 was administered in children, no differences were found between the probiotic and placebo groups [51]. In addition, healthy children over 4 months old were investigated in a study consisting of a 3-month product consumption period and a 1-month follow-up period with a fermented milk containing *L. casei* CNCM I-1518 [52]. These negative results were related to the administered doses (low doses), the strain specificity, and the intervention time of each study. Unfortunately, none of these studies evaluated the mechanism of action of the probiotics.

Members of the genus *Bifidobacterium* are another important group of probiotic species. *B. lactis* B94 plus inulin were tested in 79 children with diarrhea. Compared with 77 children in the placebo group, the synbiotic treatment reduced the length of diarrhea, and this reduction was most pronounced in the *Rotavirus* diarrhea cases [53]. The number of diarrheal stools on the third day was significantly smaller in the synbiotic group. Although the reduction in stool frequency on the third day was more pronounced, the number of diarrheal stools on the second day was also significantly smaller in the synbiotic group [53]. The authors stated that the presented effects were related to inulin administration. Although prebiotics are generally well tolerated, they can cause bloating, abdominal pain, and diarrhea when taken in excessive amounts. In this study, the patients were given 900 mg of inulin, and no symptoms of discomfort were observed [53]. Further studies in this field are still required to reveal the actual mechanism of action in a synbiotic treatment.

In contrast, with the previous study, the results of *B. animalis* subsp. *lactis* administration was tested in 727 children. The ingestion of probiotic strain failed to prevent gastrointestinal infections in children [54]. In another double-blind, placebo-controlled study with 109 children who were randomly divided into the receiving group being administered the same probiotic strain and the placebo group, the probiotic administration failed in decreasing the reported gastrointestinal symptoms or fever [55]. The authors hypothesized that xylitol, present in the administered tablets, might perform as a prebiotic ingredient to influence the gut colonization of probiotic strain.

Finally, a recent study was conducted in 290 infants that received a mixture of *B. animalis* subsp. *lactis* and *L. rhamnosus* GG for a 6-month intervention period. The outcomes revealed that the mixture administration did not decrease the absence of children from primary health services [3]. It is important to mention that the use of products containing other probiotics and prebiotics were not prohibited during the study [3]. A potential immunoprotective effect of breastfeeding, might thus have reduced the study power. Molecular mechanisms of action were not listed in the study.

Several probiotics strains were administered in the aforementioned studies, but it appears that the intervention time and method used to evaluate the primary outcome are the most important variables to obtain promising results. *L. rhamnosus* GG was the most assessed strain, showing contradictory results in the incidence and severity of gastrointestinal diseases. The proposed mechanism of action of this bacterium was the modulation of the innate and adaptive immune system. Other strains, such as, *L. salivarius* CECT5713, act through butyric acid production, and an *L. fermentum* CECT5716 plus GOS treatment generated a number of changes in the intestinal microbiota. In addition, some studies did not show clinical effects of the probiotic strains over gastrointestinal infections, even the strains belong to the *Lactobacillus* or *Bifidobacterium* genera. Table 1 shows the most relevant information regarding selected studies of children with probiotic approaches.

Table 1. Prevention and treatment of pediatric gastrointestinal infections with probiotics.

Reference	Participants	Probiotic Strain/Treatment	Time	Primary Outcome
Song-Lin et al., 2009 [39]	986 children	*L. casei rhamnosus*, *L. rhamnosus* T cell-1, and a mixture of strains	7 months	*L. casei rhamnosus* reduced respiratory infections, whereas multiple probiotic supplementation reduced the gastrointestinal disease. *L. rhamnosus* T cell-1 decreased the incidence of bacterial infection at 7 months
Hojsak et al., 2010 [40]	281 children	*L. rhamnosus* GG	3 months	Only prevention of upper respiratory tract infections
Hojsak et al., 2010 [41]	742 children	*L. rhamnosus* GG	1 week	*L. rhamnosus* GG treatment significantly reduced the risk for gastrointestinal infections, vomiting, and episodes of gastrointestinal infections
Kulandaipalayam et al., 2014 [42]	124 children	*L. rhamnosus* GG	1 month	*L. rhamnosus* GG decreased diarrheal episodes and restored normal intestinal permeability
Bruzzese et al., 2016 [43]	90 children	*L. rhamnosus* GG plus vitamins B, C and zinc	2 weeks	Treatment reduced incidence of gastrointestinal infections and length of hospitalization
Maldonado et al., 2010 [45]	80 children	*L. salivarius* CECT5713	6 months	*L. salivarius* CECT5713 decreased incidence of diarrhea and respiratory infections
Maldonado et al., 2012 [46]	215 children	*L. fermentum* CECT5716 plus GOS	6 months	Synbiotic administration prevented community-acquired gastrointestinal infections in infants
Maldonado et al., 2015 [47]	91 children	*L. fermentum* CECT5716 plus GOS	3 years follow-up	All variables measured were similar compared with placebo
Scalabrin et al., 2017 [44]	109 children	*L. rhamnosus* GG	5 years follow-up	A decrease in the incidence of acute gastroenteritis was not detected
Agustina et al., 2012 [48]	494 children	RCC plus *L. casei* CRL431, or RCC plus *L. reuteri* DSM17938	6 months	Incidence of all reported diarrhea and diarrhea incidence in children with a lower nutritional status were significantly lower in the *L. reuteri* group
Corsello et al., 2017 [49]	126 children	*L. paracasei* CBA L74	3 months	Probiotic treatment decreased the number of episodes of acute gastroenteritis
Merenstein et al., 2010 [50]	638 children	*L. casei* DN-114 001	3 months	*L. casei* DN-114 001 decreased gastrointestinal infections
Wanke et al., 2012 [51]	106 children	*L. reuteri* DSM 17938	1 week	No effects

Table 1. *Cont.*

Reference	Participants	Probiotic Strain/Treatment	Time	Primary Outcome
Prodeus et al., 2016 [52]	599 children	*L. casei* CNCM I-1518	3 months	No effects
Islek et al., 2014 [53]	156 children	*B. lactis* B94 plus inulin	1 week	Synbiotic treatment decreased the duration of diarrhea
Hoksak et al., 2015 [54]	727 children	*B. animalis* subsp. lactis BB-12	1 week	No effects
Taipale et al., 2016 [55]	67 children	*B. animalis* subsp. lactis BB-12	2 years follow-up	No effects
Laursen et al., 2017 [3]	290 children	*B. animalis* subsp. lactis and *L. rhamnosus* GG	6 months	No effects

Abbreviations: GOS, galactooligassacharides; mixture of strains, three bifidobacteria, seven lactobacilli, *S. thermophilus*, and *E. faecium*; RCC, regular calcium content.

3.1.2. Antibiotic-Associated Diarrhea (AAD)

The AAD happens when antibiotics disrupt the natural ecology in the mucosal tract, causing the increase of pathogens bacteria. The symptoms of AAD include abdominal pain and extensive bowel movements [56].

A recent study was performed in 97 children to test the effectiveness of *L. reuteri* DSM 17938 in the prevention of AAD. The probiotic administration did not change the frequency or rigorousness of AAD [57]. A weakness of this study was the lack of fecal analysis to confirm compliance and survival of the probiotic administration.

Twenty-three studies with 3938 participants were included in a systematic review of the treatment of AAD with probiotics. Analyzed trials included treatment with either *Bacillus* spp., *Bifidobacterium* spp., *Clostridium butyricum*, lactobacilli, *Lactococcus* spp., *L. cremoris*, *Saccharomyces* spp., or *Streptococcus* spp., alone or in combination. Two strains (*L. rhamnosus* or *S. boulardii*) may be the most recommended. In the case of immunocompromised or debilitated children, the use of probiotics needs a rigorous evaluation to assess patient safety [56].

Finally, the authors gave some evidence that proposes a defensive effect of probiotics in AAD. For that reason, further well-designed studies are desired to adjust the safety of probiotics as a treatment option for AAD. Currently, probiotic administration in the pediatric population with AAD is avoided. No specific mechanisms of action have been addressed in most of studies related to treatment of ADD with probiotics.

3.1.3. *Clostridium difficile*-Associated Diarrhea

Clostridium difficile-related diseases include severe diarrhea, colitis, and pseudomembranous colitis. The treatment is costly, and probiotics have been suggested as a cheap strategy to both prevent and treat *C. difficile*-associated diarrhea. In the last systematic review, a total of 31 studies with 4492 participants were discussed regarding this issue. The administration of probiotics was safe and effective for preventing *C. difficile*-associated diarrhea [58]. In contrast to AAD, probiotic administration seems to be safe, well tolerated, and indicated as coadjuvant therapy in *C. difficile*-associated diarrhea. In addition, immunocompromised or severely debilitated patients as a risk group always require a risk-benefit evaluation. Further clinical trials are needed to elucidate the mechanisms by which probiotics prevent *C. difficile*-associated diarrhea [58].

3.1.4. *Helicobacter pylori* Gastritis and Peptic Ulcer

Helicobacter pylori is accepted as a main etiological issue in the pathogenesis of gastritis and peptic ulcer disease. In pediatric population, the *H. pylori* eradication has a failure percentage of more than 30%, due to reduced compliance, antibiotic resistance, and the incidence of adverse events. A study was performed in sixty children with *H. pylori* who treated with *H. pylori* eradication treatment protocol (omeprazole + amoxycillin + furazolidon or other antibiotic), and randomly divided to receive either probiotic mixture or placebo. The probiotic treatment with *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *L. casei*, *S. thermophilus*, *B. infantis*, and *B. breve* increased the *H. pylori* eradication ratio. Moreover, it was the most effective treatment in lowering the frequency of nausea, vomiting and diarrhea [59]. The authors argued that the observed probiotic effects might have been mediated by *Lactobacillus* strains that interfered with the activity of *H. pylori* through preventing its adherence to epithelium and incapacitating its primary virulence factor, urease enzyme.

Developing countries have a higher prevalence of *H. pylori* infections. Therefore, new non-invasive therapies are preferred, among which the *H. pylori* stool antigen testing is included. Twenty-eight children with a positive stool test for *H. pylori* were randomized in a clinical trial, with individuals receiving *S. boulardii* or placebo for one month. The probiotic administration reduced the mean amount of antigen present during the study, but was not competent of causing the *H. pylori* abolition when used as a mono-therapy [60].

These results contrast with another study in which children who had biopsy-proven *H. pylori* infections were randomly divided to receive the *H. pylori* eradication treatment protocol plus *B. lactis* B94 and inulin for 14 days, and the standard therapy alone. The abolition ratio were similar in both groups. In addition, the synbiotic do not show advantage compared with standard therapy conducted alone [61]. The limitations of this study were that *H. pylori* culture and antibiotic susceptibility tests were not performed, *B. lactis* B94 colonization in the feces was not evaluated, and the authors stated that the sample size was relatively small. In addition, the authors only mention and speculate that the probiotic effects might be achieved by the production of short-chain fatty acids, autolysins, mucin, and bacteriocins and/or by the binding of some specific strains to the same glycolipid receptors as *H. pylori* [61].

Recently, Feng et al., 2017 described some important results from 29 trials involving 17 different probiotic treatments. When the standard therapy was accompanied with a probiotic strain, the eradication of *H. pylori* was successful. The most identified strain in the aforementioned effect was *L. casei* as mono-therapy, as a multi-strain therapy *B. infantis*, *B. longum*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. reuteri*, *L. rhamnosus*, *L. salivarius*, *L. sporogenes*, and *S. thermophilus* was the best in reducing the incidence of diarrhea. Indeed, probiotic ingestion is suggested to supplement *H. pylori* eradication treatment protocol in pediatric population, and the effectiveness of this therapy is related with the particular probiotic administration. Finally, the proposed mechanism of actions for *H. pylori* eradication include the following: increased competition with *H. pylori* to bind surface receptors of intestinal epithelial cells; inhibiting adhesion of *H. pylori* to mucosa; altering the inflammatory factors expression; strengthening of the intestinal mucosa barrier; and secretion of antimicrobial substances [62].

Taking into consideration the current literature, AAD is the only pathology for which probiotics should be avoided. Likewise, the mechanism of action and their clinical effects requires further investigation. Moreover, the administration of probiotics was a safe coadjuvant therapy in the case of *C. difficile*-associated diarrhea.

3.1.5. Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is a disturbing inflammatory disorder that primarily occurs in preterm neonates and causes high mortality rates (20–30%) [63]. In fact, NEC is the leading cause of death from gastrointestinal disease in premature newborns with low birth weight (LBW). Probiotics were recently reported to be beneficial to infants with NEC. Indeed, several meta-analyses indicated that probiotics reduce the risk of NEC and all causes of mortality, but not of sepsis, in preterm infants [14]. Such a treatment has also been shown to decrease mortality and days of hospitalization and to increase the effectiveness of exclusive enteral nutrition in the days following treatment [64,65].

The pathogenesis of NEC is still discussed, although besides prematurity and LBW, known risk factors include early formula feeding and an altered intestinal microbiota [66]. In 2010, Alfaleh et al., 2010 established that enteral supplementation with probiotics reduces the risk of severe NEC and mortality in preterm newborns [67]. Twenty-four trials showed that enteral probiotic supplementation significantly decreased the incidence of severe NEC and mortality. Probiotic preparations containing either *Lactobacillus* alone or in combination with *Bifidobacterium* were found to be positive.

Lactobacillus rhamnosus HN001 is a Gram-positive bacterium which is beneficial for inflammatory diseases treatment due to its probiotic actions [66]. Though the specific mechanisms of action of that probiotic are still unknown related to NEC, different studies, with strains such as *L. rhamnosus* HN001, have reported that the microbial DNA receptor Toll-like receptor-9 (TLR9) can be activated, which has been described as a potential therapeutic target. Thus, *L. rhamnosus* HN001 is capable of attenuating NEC in in vitro studies through via microbial DNA (Lr-DNA). Such protection requires activation of TLR9, which has no evidence of toxicity [68].

Lactobacillus reuteri is a probiotic bacterium which has also been studied with the aim to prevent NEC. This probiotic inhibits enteric infections and controls the immune system. *L. reuteri* produces

a potent antibacterial compound that inhibits the growth of microorganisms and modulates tumor factor necrosis alpha (TNF-α) synthesis from bacterial lipopolysaccharide (LPS)-activated monocytoid cells [69–71]. The human-derived *L. reuteri* strains DSM17938, ATCC PTA4659, ATCC PTA 5289, and ATCC PTA 6475 decreased the LPS induced-inflammation in small intestinal epithelial cells and in the ileum of neonatal rats [71]. Afterwards, Liu et al. (2012) reported that those *L. reuteri* strains reduced bowel inflammation by downregulating interleukin (IL)-6, TNF-α, TLR4, and nuclear factor κ-B (NF-κB) and upregulating IL-10 in newborn rats with NEC. In addition, *L. reuteri* led to a decrease in intestinal TLR4, TNF-α and IL1β in the experimental model, demonstrating a potential therapeutic profile of this probiotic in the prevention of NEC [72].

With respect to NEC, it has been described that a live probiotic from diet might remain effective at reducing the translocation of pathogens in a short-term animal model [73]. Copeland et al. (2009) studied whether a live probiotic diet, such as *L. lactis*, could have the same effects in a long-term neonatal rabbit model. The fortified probiotic diet produced a significant reduction in translocation of *Enterobacter* to the liver and the colonization in the stomach and lungs was also lower in rabbit pups. Moreover, colonization or translocation of the probiotic outside of the gastrointestinal tract was prevented by the diet and rabbits tested positive for *L. lactis* in the cecum, exhibiting the ability of this probiotic to survive the transit to the colon [66].

Immunity and subsequent resistance to enteric pathogens of gut predominate during initial infancy and do not appear during any other stage of life [74]. Thus, infections caused by enteric bacterial pathogens are one of the most important causes of severe infantile diarrhea. Likewise, it has been reported that probiotic treatments, such as *Lactobacillus acidophilus*, attenuate bacterial-mediated intestinal injury and inflammation, which enhanced the host defense against enteric bacterial infection [75]. Foye et al. (2012) showed that early inoculation of the probiotic *L. acidophilus* might improve host-protective immunity to enteric bacterial pathogens by means of the TGF-β (transforming growth factor b) response. Thus, the anti-inflammatory effects were triggered through decreasing Smad 7 expression, allowing TGF-β to activate IκB-α and lower NF-κB accumulation [76]. An in vivo study was carried out to assess the probiotic properties of *L. acidophilus*, a prebiotic, inulin or both (synbiotic) on pathogen-induced inflammatory reactions in neonatal mice. Mice were inoculated twice per week for 4 weeks with *L. acidophilus*, inulin, or the synbiotic and were challenged with the pathogenic bacterium *C. rodentium* at 5 weeks. They observed that *L. acidophilus* and/or prebiotic inulin consumption reduced *C. rodentium*-induced early morbidity and inflammation in mice. In addition, an in vitro study was carried out in mice. The intestinal epithelial cell line CMT-93 was treated with *C. rodentium* to determine changes in NF-κB and Smad (similarity to the *Drosophila* gene Mothers Against Decapentaplegic (Mad)) 7 levels. Thus, NF-κB was activated at 60 min post-*C. rodentium* infection, as indicated by IκB-α degradation in CMT93 cell line. These findings indicated that TNF-α production reveals that *C. rodentium* bacteria-induced NF-κB activation and Smad 7 response was associated with the pro-inflammatory cytokine production in intestinal epithelial cells. This study supports the fact that probiotics are capable of promoting host-protective immunity and of attenuating *C. rodentium*-induced bowel inflammation through mechanisms that affect NF-κB and Smad 7 expression [76].

Probiotics reduce the pro-inflammatory status by immunomodulation and by protecting tissues against microbial infection [77], and their mechanism of action consists of modifying the production of cytokines in diverse cell populations. *Lactobacillus rhamnosus* GG is a probiotic strain that is commonly integrated into fermented products. In rats, it has been demonstrated to decrease LPS-induced systemic inflammation [78]. Accordingly, it has been described that *L. rhamnosus* GG provokes IL-4, IL-10, and urocortin expression and inhibits LPS-induced TNF-α in trophoblast cells from human term placenta. Thus, these findings support the immunomodulatory effect of probiotics in human placenta [79,80]. Another study reported that the probiotic *L. rhamnosus* GG diminishes *Campylobacter jejuni* infection and butyrate transporter and receptor are expressed in differentiated Caco-2 cell monolayers. The butyrate protection against *C. jejuni* adhesion are correlated to the existence of HCAR2 and SLC5A8, which are a receptor and transporter of butyrate, respectively. Moreover, the *L. rhamnosus*

GG exerts the same effects [81]. Concerning inflammatory cytokine production, other described mechanisms include TGF-β/SMAD and NF-κB signaling pathway. The probiotic *L. acidophilus* was reported to decrease *Salmonella*-induced NF-κB activation in human intestinal Caco-2 cells. Moreover, TNF-α and IL-8 expression was significantly lowered and TGF-β1 and MIR21 levels were higher in *L. acidophilus*-treated cells compared with cells infected with only *Salmonella* [82]. In contrast, the levels of SMAD7, which it is a target of MIR21, were lower in cells treated with *L. acidophilus* or synbioticaly with inulin. Indeed, consistent with TGF-β1/MIR21 and SMAD7 expression, transcriptional activity of SMAD3/4 was significantly increased in cells treated with *L. acidophilus* or synbiotics. This suggests that TGF-β1/MIR21 expression might be useful as a marker to assess the anti-inflammatory effects of different *Lactobacillus* strains and that probiotics may be a new treatment approach for inflammation due to *Salmonella* infection [82].

Finally, Rojas et al., 2012 evaluated the use of prophylactic probiotics to prevent death and nosocomial infections in preterm newborns. Although they observed a 40% decrease in the incidence of NEC in the group treated with probiotics, they did not observe a primary outcome of the study. However, it may be clinically relevant and it is consistent with others studies that assessed NEC [83].

In summary, animal studies appear to be more widely used in the evaluation of potential mechanisms of actions for probiotics. The results from these studies show that probiotic treatment might improve inflammatory status by immunomodulation, treating and reducing NEC. Further analyses are required in human trials to ensure that no adverse effects occur from the treatment of probiotics. Table 2 shows the primary information relating to the mechanism of action in NEC.

3.1.6. Inflammatory Bowel Diseases

Ulcerative colitis and Crohn's disease are the primary manifestations of IBD. Recently, we published a review that focuses on the treatment of chronic diseases in in vitro, animal, and human studies after the probiotics treatment. The use of probiotic strains seems to be potentially well tolerated, effective, and safe in patients with IBD. Indeed, probiotics improved clinical symptoms in patients with mild to moderate active ulcerative colitis; the results in Crohn's disease are unclear. Some probiotics and their supernatants act by decreasing the pro- and inflammatory cytokines gene expression by the modulation of TLR, NF-κB, and mitogen-activated protein kinase (MAPK) pathways. Importantly, there is no recommendations of any probiotics strain for treatment of Crohn's disease in children. Additionally, probiotics definitely seem more favorable for ulcerative colitis, where some strains have previously confirmed to be effective [15].

3.1.7. Cystic Fibrosis

Intestinal inflammation is a common symptom in patients with cystic fibrosis, in whom bacterial overgrowth may also be present. Younger patients with cystic fibrosis might be good candidates for supplementation with probiotics, because their intestinal microbiota is often abnormal due to immense exposure to antibiotics, suggesting the disturbance of intestinal barrier function and the dysregulation of innate immune mediators [16]. A prospective randomized, double-blind, placebo-controlled trial was carried out in 61 children with cystic fibrosis to evaluate *Lactobacillus reuteri* ATCC55730 in altering the degree of respiratory exacerbations and of infections of upper respiratory and gastrointestinal tracts. Pulmonary exacerbations were significantly decreased in the probiotic group. Probiotic and control groups did not significantly change in the mean number and duration of hospitalizations as a consequence of pulmonary exacerbations, gastrointestinal infections, fecal calprotectin concentration, and tested cytokines (TNF-α and IL-8). The authors concluded that probiotic administration attenuates pulmonary exacerbations in cystic fibrosis patients with mild-to-moderate lung disease, and the mechanistic speculation of those results was that *Lactobacillus* strains effect immune responses beyond the intestinal tract [16].

Based on the complexity of cystic fibrosis, the probiotic treatment requires further and detailed investigation to ensure a safe therapy.

Table 2. General probiotic mechanisms of action in NEC.

Reference	Animal Species	Probiotic Strain/Treatment	Type of Study	Time	Primary Outcome
Good et al., 2014 [68]	Newborn mice/ premature piglets	*L. rhamnosus* HN001	In vivo and ex vivo	5 days	*L. rhamnosus* HN001 or its DNA could protect against the development of NEC in animals. This seems to require DNA receptor TLR9 activation
Liu et al., 2012 [72]	Newborn rats	*L. reuteri*	In vivo and ex vivo	3 days	*L. reuteri* strains reduced intestinal inflammation by down-regulating the IL-6, TNF-α, TLR4, and NF-kB and up-regulating the IL-10 in rats with NEC
Liu et al., 2010 [71]	Newborn rats	*L. reuteri*	In vivo and in vitro (IPEC-J2 intestinal cell line)	3 days	*L. reuteri* reduced the inflammation caused by LPS in intestinal epithelial cells and in the ileum
Copeland et al., 2009 [66]	Neonatal rabbit model	*L. lactis*, *E. cloacae*	In vivo	7 days	*E. cloacae* probiotic fortified diet was effective by reducing the colonization of pathogenic bacterium
Foye et al., 2012 [76]	Newborn mice	*L. acidophilus*	In vivo and in vitro (mouse intestinal epithelial cell line)	7 weeks	*L. acidophilus*, inulin, or synbiotic attenuate *C. rodentium*-induced intestinal inflammation through NF-κB and Smad 7 expression
Bloise et al., 2010 [80]		*L. rhamnosus GG*	In vitro (primary trophoblast cells from human placenta)	3 h	*L. rhamnosus* GG provokes IL-4, IL-10 and urocortin expression and inhibits LPS-induced TNF-α in trophoblast cells from human term placenta

Abbreviations: NEC, necrotizing enterocolitis; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor κ-B; TNF-α, tumor factor necrosis alpha; TLR, toll-like receptor.

3.1.8. Other Studies

Recently, our research group has reported different in vitro and in vivo studies related to probiotics. Thus, we examined the anti-inflammatory properties of probiotics in human DC generated from CD34+ progenitor cells (hematopoietic stem cells) collected from umbilical cord blood that showed surface antigens of dendritic Langerhans cells, resembling to the lamina propria DCs in the intestine [84–86]. We incubated these intestinal-like human DCs with *Lactobacillus paracasei* CNCM I-4034, *Bifidobacterium breve* CNCM I-4035, *L. rhamnosus* CNCM I-4036 or its cell-free supernatants (CFS), *Salmonella typhi* CECT 725, *E. coli* CECT 742, CECT 515, and CECT 729 or a mixture of these treatments for 4 h. These probiotic treatments provoked an upregulation of TLR-9, toll-interacting protein, and CASP8 gene expression. Probiotic supernatants diminished pro-inflammatory cytokines and chemokines in DCs that were challenged with *S. typhi* and restored TGF-β levels in the existence of *S. typhi*. In addition, supernatants enhanced innate immunity due to the activation of TLR signaling, especially TLR-9, TLR-2, and TLR-4 gene expression [84–86].

Other results from our research group using an experimental model of obesity in Zucker rats and in human healthy volunteers who received selected probiotic strains have documented a number of immunomodulatory effects. Administration of *B. breve* CNCM I-4035 produced a significant increase in fecal secretory IgA content. IL-4 and IL-10 were up-regulated, whereas IL-12 was lower in the serum of subjects after the treatment with any of the three strains. Serum TNF-α levels diminished in Zucker-Lepr$^{fa/fa}$ rats treated with *B. breve*, *L. rhamnosus*, or the mixture, whereas *L. paracasei* feeding showed a reduction of IL-6 levels in the serum of Zucker-Lepr$^{fa/fa}$ rats. Moreover, probiotic administration downregulated the gene and protein expression of *Adamdec1* and *Ednrb*, and that of *Ptgs1/Cox1* at the gene expression level. This result was partially mediated by a reduction in both macrophage and dendritic cell populations [21,22,77,87,88]. Additionally, we have studied the early administration of *L. fermentum* CECT5716, which is a probiotic strain added in infant formula, in children. We reported that this probiotic preparation was safe and it did not produce quantifiable differences in children compared with the control group, but no specific mechanism of action was addressed [47,89].

In line with our findings, Tsilingiri et al. (2012) have developed an in vitro model system that offers several physiological characteristics that can be representative of a mucosal microenvironment, containing the existence of an organized mucus layer and an apical to basolateral polarity. The authors evaluated the effects of *L. paracasei* supernatant against *S. typhimurium* in healthy and IBD tissue [90]. They concluded that probiotics could be more appropriately used in patients in remission and not for the period of the acute phase of the disease. Additionally, the use of supernatant might be an effective and safe alternative for the treatment of acute IBD. This effect was observed in the co-incubation treatment, mediated through the abrogation of TNF-α release without affecting IL-10 secretion [90]. Moreover, in a well-conducted in vitro study, Buccigrossi et al. (2014) have showed the effects of *S. boulardii* supernatant in cells infected with rotavirus. The aforementioned supernatant prevents oxidative stress produced by rotavirus infection, and inhibits chloride secretion in Caco-2 cells [91]. These findings suggest that probiotics and their supernatants can exhibit important molecular effects.

Finally, Figure 1 shows the general probiotic mechanism of action in pediatric intestinal diseases. Concerning gastrointestinal infections, the most important mechanism of action reported was the modulation of the immune system. With respect to NEC, the mechanism of actions are related to the TLR-signaling pathway and the butyric receptor.

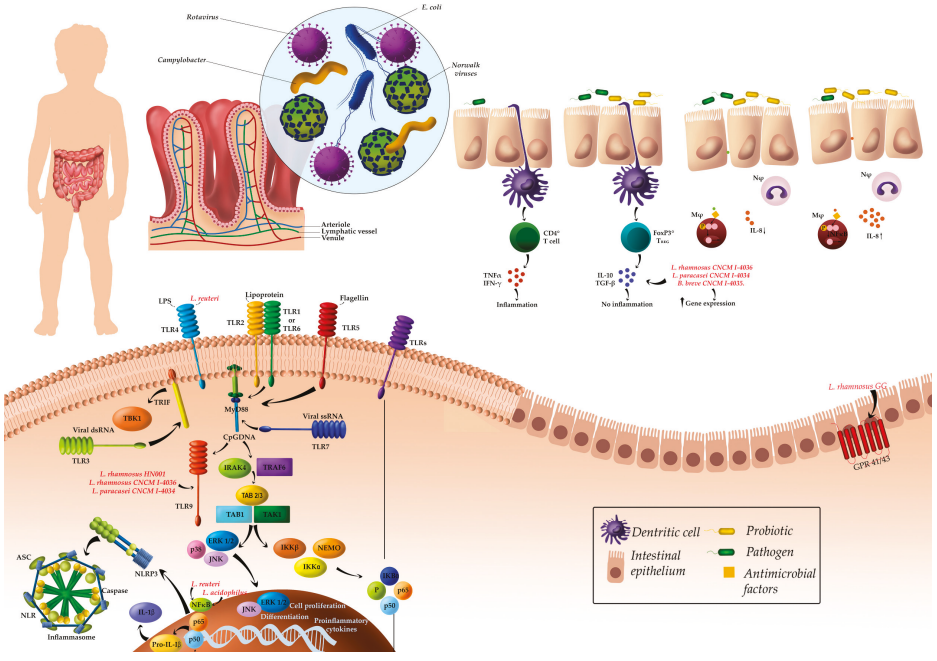

Figure 1. General probiotic mechanism of action in pediatric gastrointestinal infections. Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; ERK, extracellular regulated kinase; IKK, IκB kinase; IL, interleukin; IFN, interferon; IRAK4, IL-1 receptor-associated kinase 4; JNK, Jun N-terminal kinase; NF-κB, nuclear factor κ-B; NEMO, NF-κB essential modulator; TNF-α, tumor factor necrosis alpha; TLR, toll-like receptor, TAB1/2/3, TAK binding proteins; TAK1, ubiquitin-dependent kinase of MKK and IKK; TBK1, serine/threonine-protein kinase 1; TGF, transforming growth factor; TRAF6, Tumor necrosis factor receptor-associated factor 6.

4. Conclusions

Probiotics and synbiotics have been extensively studied in the evaluation of potential treatments for different gastrointestinal infections in children, such as acute gastroenteritis, AAD, *Clostridium difficile*-associated diarrhea, *Helicobacter pylori* gastritis, and peptic ulcer, as well as in other intestinal pathologies associated with gut dysbiosis. It appears that probiotics and synbiotics may be useful in improving such pathologies, except for AAD and Crohn's disease. In any case, major adverse effects of probiotics have since been reported. Although such effects are supported by numerous clinical studies, further research is required to corroborate the adequate doses and time of treatment. Some strains, such as *L. rhamnosus*, have been reported for the treatment of pediatric infections, despite the existence of other potential probiotics that should be studied. Immune-mediated mechanisms of action of probiotics include the modulation of both innate and adaptive immunity. However, most of the reported studies only made speculations and did not attempt to evaluate specific biomarkers of systemic or intestinal immunity. Only a few in vitro and animal studies have shown that modulation of the immune system can be mediated through the interaction of probiotics with intestinal TLR, which in turn affects inflammatory cascade signaling, expression of cytokines and some intestinal host genes involved in inflammation. Thus, there is a need for designing further studies using probiotics and synbiotics in pediatric intestinal diseases and addressing their potential mechanisms of action though appropriate biomarkers of immunity and inflammation to support and provide scientific reasons that are able to explain the clinical benefits of specific probiotic strains.

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nutrients

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Review

Probiotic, Prebiotic, and Brain Development

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Abstract: Recently, a number of studies have demonstrated the existence of a link between the emotional and cognitive centres of the brain and peripheral functions through the bi-directional interaction between the central nervous system and the enteric nervous system. Therefore, the use of bacteria as therapeutics has attracted much interest. Recent research has found that there are a variety of mechanisms by which bacteria can signal to the brain and influence several processes in relation to neurotransmission, neurogenesis, and behaviour. Data derived from both in vitro experiments and in vivo clinical trials have supported some of these new health implications. While recent molecular advancement has provided strong indications to support and justify the role of the gut microbiota on the gut–brain axis, it is still not clear whether manipulations through probiotics and prebiotics administration could be beneficial in the treatment of neurological problems. The understanding of the gut microbiota and its activities is essential for the generation of future personalized healthcare strategies. Here, we explore and summarize the potential beneficial effects of probiotics and prebiotics in the neurodevelopmental process and in the prevention and treatment of certain neurological human diseases, highlighting current and future perspectives in this topic.

Keywords: microbiota; prebiotics; probiotics; health; disease

1. Introduction

The micro-organisms that inhabit the human gastrointestinal tract (GI) have been implicated in the development and functioning of a number of basic physiological processes, such as digestion, immunity, and the maintenance of homeostasis. The GI microbiota may also play a role in multiple diseases, ranging from inflammation to obesity [1,2]. Recently, many studies have shown that gut microbiota play a very important role in the development and function of the central nervous system (CNS) through specific channels, such as metabolic, neuroendocrine, and immune pathways [3]. In particular, these researchers have found bi-directional communication between the brain and the gut microbiota, denominated the microbiota–gut–brain axis [4–6].

Although the molecular mechanisms by which the gut microbiota communicate with the brain are not yet clear, the link between both components is currently attributed to immune signals and the vagus nerve. Cellular components produced by gut microbiota, such as lipopolysaccharide (LPS), peptidoglycan, and flagellin, are recognized by pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), or RIG-1-like receptors (RLRs), on epithelial and immune cells, producing cytokines, hormones, and other molecular signals, which will act as neurotransmitters

within the CNS [7]. Several studies have found that, in the densely innervated gut, the vagus nerve is involved in the bi-directional communication of the microbiota–gut–brain axis [8,9], while others have shown vagus-independent effects [10,11]. Either way, a supplementing nutrition therapy with specific probiotic commensals and prebiotics can alter the excitability of enteric nervous system (ENS) sensory neurons [12–14]. Prebiotics-induced growth of probiotic members within the *Bifidobacterium* and *Lactobacillus* genera show multiple beneficial effects on host immunity and physiology [15]. Moreover, strong effects of *Bifidobacterium* and *Lactobacillus* spp. on the brain–gut axis have been reported [16].

This review summarizes current knowledge on the influence of the establishment of the gut microbiota in critical neurodevelopmental windows, and discusses recent findings on the interactions between the gut microbiota and the host's brain–gut axis communications. In addition, current research on the effects of the administration of probiotics and prebiotics in specific neurological disorders is reviewed. Finally, recommendations for future research on this topic are also discussed.

2. Establishment of Intestinal Microbiota during Early Neurodevelopmental Windows

Gut microbiota establish a beneficial cohabitation with the host that will prime for health later in life [17]. The assembly of the gut microbiota occurs during the first three years of life, starting from birth, where there is a rapid rate of colonization and expansion of gut bacteria dominated by *Actinobacteria* and *Proteobacteria* that shifts towards one dominated by *Firmicutes* and *Bacteroidetes*, increasing compositional diversity and stability while maturing into an adult-like state [18]. This process coincides in time with the intense synaptogenesis and pruning in the cerebral cortex during early life [18–20], ending in adolescence [21]. Therefore, perturbations of gut microbiota colonization and maturation by environmental factors may influence brain development. The dynamics of the microbial ecosystem's maturation during this critical period of CNS development is influenced by several environmental factors, such as mother-to-child bacterial transfer, mode of delivery, and type of feeding. The mother-to-child transfer of commensal bacteria in the uterus has been shown to influence an infant's immune system development [22,23]. Until recently, the idea that foetuses were sterile in the uterus and that the microbial colonization of the new-born started during and after birth had been widely accepted [24]. However, nowadays, this belief has been challenged by evidence of microbes in placenta and other tissues surrounding the foetus, such as umbilical cord blood after vaginal and caesarean birth [25–27]. Several studies have analysed the meconium of new-born babies and showed the presence of bacterial populations, including *Enterococcus*, *Lactococcus*, *Escherichia*, *Leuconostoc*, and *Streptococcus*, though at low levels, concluding that gut colonization occurs mainly after birth [28]. Based on these findings, prenatal probiotic intervention has been shown to modulate the expression of TLR-related genes in the placenta and foetal GI tract and to reduce atopic dermatitis [29,30]. Thus, prenatal and postnatal maternal oral probiotic therapy may represent an effective method of intervention to prevent pathologies such as allergy [31], atopic diseases [32], and neurodevelopmental disorders, reviewed below. Still, the origin of the microbiota colonizing the placenta is unknown and results have to be carefully interpreted, because, in samples with low microbial biomass, such as those from placenta, the risk of contamination is high when using high-throughput sequencing methods based on DNA amplification [29]. Further studies are needed to discern whether foetuses have contact with bacteria before birth or are colonized during and after parturition.

Regardless of mother-to-child transmission within the intrauterine environment, two different modes of maternal–infant transmission during delivery have been proposed: (a) horizontal, in which microbes are taken up from the environment for infants born by caesarean section; and (b) vertical, in which vaginal microbes are transferred during parturition to the infants [33]. Infants delivered by Caesarean section are more likely to suffer several diseases, such as asthma, obesity, or allergies, in adulthood [34]. Interestingly, a study carried out by Jasarevic et al. using a mouse model of early prenatal stress found that changes in the vaginal microbiome were associated with shifts in the abundance of *Lactobacillus* in the expression of maternal stress proteins related to vaginal

immunity, in offspring metabolic profiles related to energy balance, and in the amino acid profiles of the developing brain [35].

The third strong environmental factor that influences an infant's gut microbial development as well as neurodevelopment is the type of feeding. In recent years, several studies have reported that breastfeeding and particularly full breastfeeding has beneficial effects on child neuropsychological development [36]. Human milk is the optimal feeding source, since it provides all the nutrition factors that an infant needs for healthy development. Human milk is not sterile, and, during breastfeeding, bacteria from mother's skin and mammary gland via maternal dendritic cells and macrophages [37] are transferred to the baby [38]. Breast-fed infants tend to contain a more uniform population of gut microbes dominated by *Bifidobacterium* and *Lactobacillus* [39], whereas formula-fed infants exhibit higher proportions of *Bacteroides*, *Clostridium*, *Streptococcus*, *Enterobacteria*, and *Veillonella* spp. [21]. Bacteria belonging to the *Bifidobacterium* genus present in human milk are early colonizers that characterize the gut microbial composition of healthy breast-fed new-born's [40] with beneficial functions for the host, such as the acceleration of the maturation of the immune response, the limitation of excessive inflammation, the improvement of the intestinal permeability, and an increase of acetate production [41]. In mice, *B. infantis* produces antidepressant-like effects and normalizes peripheral pro-inflammatory cytokine and tryptophan concentrations, both of which have been implicated in depression [42–44]. Moreover, breastfeeding has an additional role in the establishment of an infant's gut microbiota, since it contains bioactive molecules that are increasingly recognized as drivers of microbiota development and overall gut health [45]. Among the nutrients present in human milk, oligosaccharides constitute the third-most abundant class of molecules in terms of concentration after lactose and lipids. Nowadays, more than 200 different structures have been identified as human milk oligosaccharides (HMOs) [35]. HMOs can act as prebiotics, stimulating the growth of specific bacterial groups such as *Staphylococci* [46] and *Bifidobacteria* [47].

These results suggest that postnatal neurodevelopment and gut microbiota establishment co-occur, suggesting the intriguing possibility of a bi-directional regulation of each other's maturation [48]. Further studies are needed in order to clarify whether those differences in bacterial acquisition during early life lead to neurodevelopmental differences in infants.

3. Gut Microbiota–Brain Axis

The brain and the gut reciprocally influence each other by constant communication (Figure 1). The brain–gut–microbiota axis includes the CNS, the endocrine-immune system, the hypothalamus–pituitary–adrenal (HPA) axis, the autonomic nervous system, the ENS, and the gut microbiota [49]. This bi-directional communication enables signalling from the brain to influence motor, sensory, and secretory modalities of the GI tract, and conversely, signalling from the gut to affect brain function, most notably the hypothalamus and amygdala that are implicated in stress [50–52].

Though communication between brain and gut was realized in the middle of the nineteenth century [53], gut microorganisms had not been considered important for the development and function of the CNS or for brain diseases until recently, expanding the term to microbiome–gut–brain axis [54]. In humans, evidence of microbiome–gut–brain axis interactions have been obtained from the association of shifts in gut microbiota composition with central nervous disorders (i.e., autism spectrum disorder (ASD) and anxiety and depressive behaviours) and functional gastrointestinal disorders [54]. Most of the data demonstrating the role of the microbiota in the gut–brain axis have been obtained from germ-free animals [55]. Mice fed with prebiotics showed diminished stressor-induced anxiety-like behavior [56]. In a mouse model of ASD, Buffington et al. showed that a maternal high-fat diet reduced the number of oxytocin immunoreactive neurons in the hypothalamus and induced dysbiosis that was restored by a commensal *Lactobacillus reuteri* strain [57]. In a mouse model of Parkinson's disease, Sampson et al. highlighted a negative interaction in the microbiome–gut–brain axis because the absence of gut bacteria decreased aggregated misfolded α-synuclein levels and reduced the severity of the animals' abnormal movements. The authors showed that short chain fatty acids

(SCFA), such as acetate, propionate, and butyrate, the end products of anaerobic fermentation of dietary fibre and starch, promoted a microglia-mediated immune response and increased α-synuclein aggregation, causing movement abnormalities [58]. Butyrate can cross the blood-brain barrier (BBB) and produce a dose-dependent increase in neuronal and glial nuclear histone H3 acetylation in mice due to its potential to inhibit histone deacetylation [59]. Another metabolite whose levels in the host are influenced by gut microbiota is tryptophan, the amino acid precursor of the neurotransmitter serotonin, and kynurenine, the main breakdown product of tryptophan catabolism [60]. Kynurenine intake during gestation and postnatal development, a time frame in which the maternal and offspring microbiota undergo major compositional and functional remodelling, produced neurochemical and cognitive deficits later in adulthood [61]. The prenatal inhibition of kynurenine synthesis modified hippocampal neuron morphology and changed neocortical and cerebellar protein expression that persisted into adulthood. In germ-free and in antibiotic-induced microbiota-depleted mice, despite increased circulating tryptophan levels, serotonin and kynurenin availabilities were decreased, suggesting that gut microbiota modulated kynurenin metabolism [62]. Distinct gut microbial species affect host physiology, producing diverse neuromolecules involved in mood regulation. *Lactobacillus* and *Bifidobacterium* spp. generate gamma-aminobutyric acid (GABA). *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* spp. synthesise serotonin while *Bacillus* spp. produces dopamine [63].

Gut microbiota also influence the regulation of BBB integrity. The BBB is an active interface between systemic circulation and the CNS that maintains brain homeostasis by preventing the entry of potentially toxic or harmful substances and regulates the transport of nutrients and the removal of metabolites [64]. Braniste et al. (2014) [65] showed that the transplantation of gut microbiota into germ-free mice normalized BBB permeability and upregulated the expression of tight junction proteins. Therefore, gut microbiota have a key role in regulating BBB permeability, suggesting that the maternal gut microbiome influences an offspring's BBB integrity. Together with the results discussed in the previous section, these findings open an intriguing question on the mechanism by which a mother's gut microbiota cooperate in regulating BBB integrity and ultimately brain function development.

Gut microbiota have direct effects on the immune system, which constitutes another route of communication between gut microbes and the brain. The signalling molecules of the immune system, cytokines and chemokines, access the brain from the periphery via the vagus nerve or directly via the circumventricular organs [66]. The administration of rifaximin (a non-systemic, broad-spectrum antibiotic) to stressed rats increased the abundance of *Lactobacillus* in the ileum and the expression of the tight junction protein occludin while decreasing the expression of pro-inflammatory interleukin 17, interleukin 6, and tumour necrosis factor α mRNA [67].

Since many of the above effects have been observed during early life, it is plausible that an environmentally induced dysbiosis of infants' microbiota (e.g., mode of birth, maternal transmission of a suboptimal microbiota, antibiotics) may generate altered patterns of microbial metabolites with detrimental effects in human CNS development. Further research is needed to unravel these mechanisms and develop probiotics or prebiotics therapies that shape gut microbial composition and metabolism to ultimately modulate CNS development.

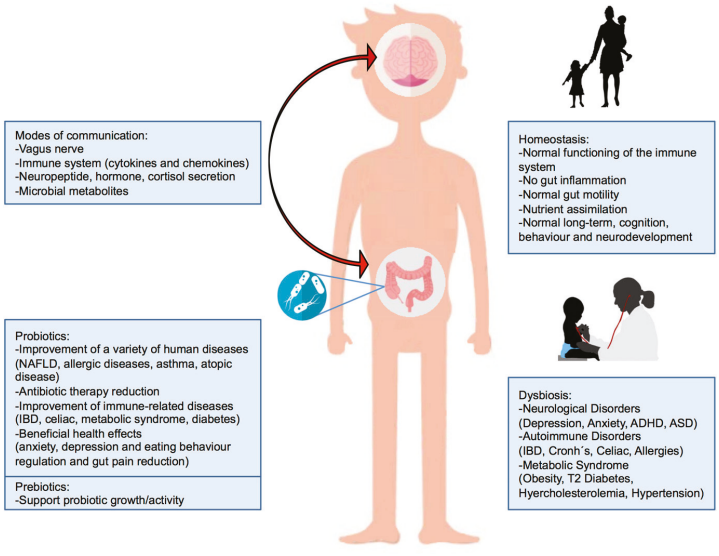

Figure 1. The gut microbiota–brain axis. The central part of the figure shows the bidirectional influence between the brain and gut microbiota. The left side of this figure shows modes of communication in the bidirectional crosstalk between gut microbiota and the brain and the possible influences of prebiotics and probiotics on human diseases. The right side of the figure shows the consequences of gut dysbiosis/homeostasis. Intestinal dysbiosis can adversely influence gut physiology, leading to inappropriate brain–gut axis signalling and associated consequences for CNS functions and disease states. Abbreviations: Non-Alcoholic Fatty Liver Disease (NAFLD), Inflammatory Bowel Disease (IBD), Attention deficit hyperactivity disorder (ADHD), Autism spectrum disorder (ASD).

4. Probiotics

In 2001, the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) proposed the following definition of probiotics: "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" [68], which was reaffirmed in 2014 [69]. Probiotics, comprised by strains of *Lactobacilli*, *Bifidobacteria* and *Saccharomycetes* have been suggested to play a role in fighting human diseases, such as non-alcoholic fatty liver disease (NAFLD), allergy diseases, and asthma. They also promote protection against atopic disease in the infant during pregnancy and breastfeeding [32,70,71]. In addition, probiotics also reduce the duration of antibiotic therapy, and reduce symptom severity in immune-related diseases, such as inflammatory bowel diseases (IBDs), celiac disease, metabolic syndrome and diabetes [72,73].

The search for probiotics that can affect cognitive functions, known as psychobiotics, has increased in recent years (Table 1). Psychobiotics are defined as live organisms that, when ingested in adequate amounts, produce beneficial health effects to patients suffering from psychiatric illness [74]. Depression is currently a major psychiatric disorder in developed countries, and is characterized by a low mood or loss of interest and anxiety affecting appetite and sleep. Messaoudi et al. [75,76] reported a double-blind, placebo-controlled, randomized study where a multispecies probiotic containing *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (PF) was administered to healthy women for 30 days. This treatment resulted in a decrease in the global scores of the hospital anxiety and depression scale (HADs) and the global severity index of the Hopkins symptoms checklist (HSCL-90) due to the decrease of the sub-scores of somatization, depression, and anger–hostility spheres. In a cohort of 124 healthy humans, Benton et al. reported that the consumption of *Lactobacillus casei*-containing yogurt improved the self-reported mood of those whose mood was

initially poor [77]. Similarly, Steenbergen et al. reported a significantly reduced overall cognitive reactivity to depression, in particular aggressive and ruminative thoughts, in forty healthy young adults that consumed either a probiotic supplement or placebo for 4 weeks [78]. Recently, Akkasheh et al. showed that the consumption of a probiotic supplement significantly decreased Beck Depression Inventory (BDI) scores, indicating overall improved symptoms, including mood, in 40 patients diagnosed with depression [79]. Conversely, Marcos et al. reported that probiotics decreased, respectively, levels of stress and anxiety assessed using the state-trait anxiety inventory (STAI) that remained unchanged in subjects under academic examination stress [80]. In a recent study carried out by Romijn et al. [81], administering a multispecies probiotic containing *L. helveticus* and *B. longum* in 79 participants that were not taking psychotropic medications at that moment and with at least moderate scores on self-report mood measures, found no evidence that the probiotic formulation was effective in treating low mood or in moderating the levels of inflammatory and other biomarkers. Improved cognitive function (neuropsychological and cognitive fatigue) was reported by Chung et al., which tested a *L. helveticus*-fermented milk in healthy 60–75 year olds, though no effects on stress or geriatric depression symptoms were observed [82].

Probiotics affect mood by their ability to modulate pain in the gut. A recent study reported that the administration of *Lactobacillus reuteri* DSM 17938 in the treatment of children with functional abdominal pain (FAP) and irritable bowel syndrome (IBS) is associated with a possible reduction of the intensity of pain [83]. In 35 patients suffering from chronic fatigue syndrome, Rao et al. showed that while the consumption of the probiotic improved anxiety scores, it had no effect on depressive symptoms [84]. Giannetti et al. also reported that a probiotic mixture of *B. infantis* M-63, *B. breve* M-16V, and *B longum* BB536 was associated with improvement in children with IBS, but not in children with functional dyspepsia (FD) [85]. In healthy women without gastrointestinal or psychiatric symptoms, the consumption of a fermented milk product containing *B. animalis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *L. lactis* subsp. *lactis* resulted in robust alterations in activity in the brain regions that control the central processing of emotions and sensations, as observed by functional magnetic resonance imaging [86].

Table 1. Studies evaluating probiotics supplementation on central nervous system (CNS) disorders.

Study (Reference)	Cohort Population	Probiotic Used	Key Findings
Messaoudi et al. (2011) [75,76]	55 healthy human volunteers plus 25 subjects with urinary free cortisol (UFC) levels less than 50 ng/mL (less stressed subjects), 10 subjects received the probiotic and 15 placebo.	*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (PF)	Beneficial effects on anxiety and depression related behaviors in healthy human volunteers and volunteers with lower levels of cortisol
Benton et al. (2007) [77]	124 healthy adults volunteers were randomly allocated to a group that consumed, on a daily basis, a probiotic-containing milk drink or a placebo	*Lactobacillus casei* Shirota	The consumption of a probiotic-containing yoghurt improved the mood of those whose mood was initially poor. However, there was not an increased frequency of defaecation.
Steenbergen et al. (2015) [78]	40 healthy young adults were randomly assigned to receive a 4-week intervention of either placebo or multispecies probiotics in a triple-blind intervention assessment design.	*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, and *Lactococcus lactis* (W19 and W58)	Participants who received multispecies probiotics showed a significantly reduced overall cognitive reactivity to sad mood, which was largely accounted for by reduced rumination and aggressive thoughts.
Akkasheh et al. (2016) [79]	40 patients with a diagnosis of major depressive disorder (MDD) whose age ranged between 20 and 55 years were randomized.	*Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*.	Patients who received probiotic supplements had significantly decreased Beck Depression Inventory total scores
Marcos et al. (2004) [80]	136 university students were randomized.	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* plus *Lactobacillus casei* DN-114001	There was no significant treatment effect on anxiety.
Romijn et al. (2017) [81]	79 participants not currently taking psychotropic medications with at least moderate scores on self-report mood measures. Participants were randomly allocated to receive a probiotic preparation or placebo.	*Lactobacillus helveticus* and *Bifidobacterium longum*	No significant difference was found between the probiotic and placebo groups on any psychological outcome measured.
Jadrešin et al. (2017) [83]	55 children with age between 4 and 18 years old, diagnosed as functional abdominal pain (FAP) or irritable bowel syndrome (IBS) were randomly allocated.	*Lactobacillus reuteri* DSM 17938	Administration of *L. reuteri* DSM 17938 was associated with a possible reduction of the intensity of pain and significantly more days without pain in children with FAP and IBS
Giannetti et al. (2016) [85]	48 children with IBS aged between 8 and 17.9 years and 25 with functional dyspepsia (FD) with age between 8 and 16.6 years were randomized.	*Bifidobacterium infantis* M-63, *breve* M-16V, and *longum* BB536	In children with IBS a mixture of *Bifidobacteria* is associated with improvement in abdominal pain (AP) and quality of life (QoL).
Kałuźna-Czaplińska et al. (2012) [87]	22 autistic children.	*Lactobacillus acidophilus*	The probiotic supplementation let to a significant decrease in D-arabinitol (DA) and the ratio of D-/L-arabinitol (DA/LA) and to a significant improvement in ability of concentration and carrying out orders
West et al. (2013) [88]	33 ASD children.	Delpro® (*Lactocillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbruecki*, *Bifidobacteria longum*, *Bifidobacteria bifidum*)	88% reported a decrease in total autism treatment evaluation checklist (ATEC) score, an improvement of ASD symptoms. Participants also had significant improvements in all ATEC domains (speech/language/communication, sociability, sensory/cognitive awareness, and health/physical/behavior)

Table 1. *Cont.*

Study (Reference)	Cohort Population	Probiotic Used	Key Findings
Tomova et al. (2015) [89]	10 children with autism, 9 siblings and 10 healthy children.	"Children Dophilus" containing three strains of *Lactobacillus* (60%), two strains of *Bifidobacterium* (25%), and one strain of *Streptococcus* (15%)	Probiotic diet supplementation normalized the *Bacteroidetes*/*Firmicutes* ratio, *Desulfovibrio* spp. and the amount of *Bifidobacterium* spp. in feces of autistic children. No significant difference was found to reduce symptom severity in patients with autism.
Santocchi et al. (2016) [90]	100 preschoolers with ASD on the basis of a symptom severity index specific to gastrointestinal (GI) disorders. Patients with and without GI disorders were blind randomized to regular diet with probiotics or with placebo	"Vivomixx®" (one strain of *Streptococcus thermophilus* DSM 24731, three strains of *Bifidobacterium* (*Bifidobacterium breve* DSM 24732, *B. longum* DSM 24736, *B. infantis* DSM 24737), and four strains of *Lactobacillus* (*Lactobacillus acidophilus* DSM 24735, *Lactobacillus plantarum* DSM 24730, *Lactobacillus paracasei* DSM 24733, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734))	Ongoing study
Dickerson et al. (2014) [91] and Tomasik et al. (2015) [92]	32 patients healthy and 33 patients with schizophrenia meeting DSM-IV criteria and with at least moderately severe psychotic symptoms	*Lactobacillus rhamnosus* strain GG and *Bifidobacterium animalis* subsp. *lactis* strain Bb12	No significant difference was found to reduce symptom severity in patients with schizophrenia. Probiotic regulate immune and intestinal epithelial cells through the IL-17 family of cytokines

Probiotics have been tested to normalize gut microbial composition and metabolism, enhance gut barrier, and relieve patients suffering from ASD. In 2012, Kaluzna-Czaplinska and Blaszczyk reported that the administration of *Lactobacillus acidophilus* in 22 ASD subjects decreased D-arabinitol concentration and the ratio of D-arabinitol to L-arabinitol in urine, and improved their ability to follow directions, as demonstrated through a comparison with data collected before the treatment [87]. Another study reported that a combination of *Lactobacillus acidophilus*, *L. casei*, *Lactobacillus delbrueckii*, *B. longum* and *Bifidobacterium bifidum*, formulated with the imunomodulator Del-Immune V (*Lactobacillus rhamnosus* V lysate), decreased the severity of ASD symptoms and improved GI symptoms in 33 children [88]. Moreover, a recent study of "Children Dophilus" (a combination of three species of *Lactobacillus*, two species of *Bifidobacterium* and one strain of *Streptococcus*) in 10 ASD children showed higher GI dysfunction in ASD children and siblings and a very strong association of the amount of *Desulfovibrio* spp. with the severity of autism. After the intervention, the *Bacteroidetes*/*Firmicutes* ratio, *Desulfovibrio* spp., and the amount of *Bifidobacterium* spp. were normalized in faeces of autistic children [89]. However, the effects of treatments with probiotics on children with ASD need to be evaluated through rigorous, controlled trials. In a recent clinical study currently in progress, Santocchi et al. are providing a multispecies probiotic (one strain of *S. thermophilus* DSM 24731, three strains of *Bifidobacterium* (*B. breve* DSM 24732, *B. longum* DSM 24736, and *B. infantis* DSM 24737), and four strains of *Lactobacillus* (*L. acidophilus* DSM 24735, *Lactobacillus plantarum* DSM 24730, *Lactobacillus paracasei* DSM 24733, and *L. delbrueckii* subsp. *bulgaricus* DSM 24734) to a group of 100 pre-schoolers with ASD. This study will try to provide new insights to clinical and neurophysiological patterns in response to a probiotic mixture in ASD patients [90].

Probiotics are also tested in the treatment of schizophrenia and bipolar disorder. One of the first trials of probiotic compounds in schizophrenia used a combined probiotic of *L. rhammosus* strain *GG* and *B. animals* subsp. *Lactis strain* Bb12. The results showed no significant difference in psychiatric symptom severity between probiotic and placebo supplementation [91]. However, other studies have found that probiotic supplementation significantly alters the levels of several serum proteins, including the von Willebrand factor and the brain-derived neurotrophic factor, and lowered the level of antibodies to the fungus *Candida albicans* [92,93].

Despite that the majority of the studies found positive results on symptoms in these neurological disorders, future studies are needed to identify potential probiotics for the effective modulation of these disorders as well as to define probiotics risk in therapeutic interventions. Gut microbial studies that use 16S rRNA gene sequencing to characterize bacteria must consider that highly similar bacteria (higher than 97% sequence identity) can have large differences in genomic sequences and profound differences in growth and metabolism. Hence, it is important to characterize probiotics to the strain level and apply next-generation sequencing techniques to analyse the functions encoded by their genome [94]. Therefore, the effects of one probiotic strain should not be generalized to others without confirmation in separate studies.

5. Prebiotics

Although the concept of a prebiotic was first defined in 1995 by Gibson, the current definition of a prebiotic is the one proposed by the International Scientific Association for Probiotics and Prebiotics (ISAPP): a substrate that is selectively utilized by host micro-organisms and confers a health benefit [95]. The group of substances recognized for their ability to influence gastrointestinal health comprise certain non-digestible oligosaccharides (NDOs), soluble fermentable fibres, and HMOs. NDOs are low molecular weight carbohydrates in nature that are intermediates between simple sugars and polysaccharides. The use of NDOs as prebiotics has rapidly increased because the enrichment of a diet with NDOs provides the opportunity to improve the gut microbial ecosystem, including bacterial populations, biochemical profiles, and physiological effects [96]. Fibre influences satiety by the following two mechanisms. One is by increasing the chewing time of fibre-rich foods, which promotes saliva and gastric acid production and increases gastric distension, triggering afferent vagal signals of

fullness contributing to this end. The other mechanism is by slowing gastric emptying and decreasing the rate of glucose absorption in the small intestine. Consequently, the insulin response may also be attenuated; this is sometimes correlated with satiation and satiety [97]. Various hormones (i.e., ghrelin, the polypeptide YY, and the glucagon-like peptide) have been related to satiety, and are sent to the brain, where they regulate food intake and overall energy balance [98].

Though prebiotic therapies potentially could be beneficial for children with a genetic pre-disposition to develop ASD or attention deficit hyperactivity disorder because of their selective enhancement of *Lactobacilli* and *Bifidobacteria* growth [99], a small number of studies has examined the effect of these prebiotics on disorders related to CNS (Table 2). Inductive evidence that prebiotics modulated emotional satisfaction was provided by Hume et al., who investigated the effect of oligofructose-enriched inulin/d administration versus a placebo (maltodextrin) in a randomized, double-blind, placebo-controlled trial with 42 children (who were aged 7–12 and were overweight and obese) [100]. Prebiotic supplementation improved subjective appetite ratings, reducing energy intake in older but not in younger children.

In a cohort of healthy male and female subjects (n = 45), Schmidt et al. tested the intake of fructo-oligosaccharides (FOS) and Bimuno®-galactooligosaccharides (B-GOS), and reported that only B-GOS reduced the waking-cortisol response [101]. Exaggerated waking cortisol is a biomarker of emotional disturbances, such as depression [102]. Besides this, the subjects also provided measures of vigilance, or attention to negative stimuli, which is also a behavioral marker of anxiety and depression [103]. B-GOS attenuated vigilance, suggesting a reduction in anxiety and depression [104]. Van den berg et al. found no evidence that the use of short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides in preterm infants at 24 months improves neurodevelopmental outcomes [105]. LeCouffe et al. studied the effect of an enteral supplementation of a prebiotic mixture (neutral and acidic oligosaccharides) in the neonatal period and found no effect on neurodevelopment [106], though lower *Bifidobacteria* counts are associated with serious neonatal infections and lower neurodevelopmental outcomes.

More studies are required to determine whether prebiotics exert a beneficial effect on neurodevelopmental disorders in infants, and to understand the mechanism of action, by stimulating certain bacterial taxa or bacterial activities within gut microbiota. Efficacy, safety, and dosing schedules should be established for each prebiotic product in long-term follow-up studies.

Table 2. Studies evaluating prebiotics and synbiotics supplementation on CNS disorders.

Study (Reference)	Cohort Population	Prebiotic Used	Key Findings
Prebiotics			
Hume et al. (2017) [100]	42 boys and girls, ages 7–12 years, with a body mass index (BMI) of ≥85th percentile	Oligofructose-enriched inulin/d	Prebiotic supplementation in children with overweight and obesity significantly increased feelings of fullness and reduced prospective food consumption in older but not in younger children
Schmidt et al. (2105) [101]	45 adults healthy volunteers	FOS and Bimuno®-galactooligosaccharides, B-GOS	B-GOS reduced waking-cortisol response and decreased attentional vigilance to negative versus positive information
van den Berg et al. (2016) [105]	77 preterm infants (gestational age <32 weeks and/or birth weight <1500 g), admitted to the level-III neonatal intensive care unit (NICU)	scGOS/lcFOS/pAOS	Neurodevelopmental outcomes were not different in the scGOS/lcFOS/pAOS and placebo group. Infections, lower bifidobacteria counts, and higher serum cytokine levels during the neonatal period were associated with lower neurodevelopmental outcomes at 24 months of age
LeCouffe et al. (2014) [106]	93 Infants, with a gestational age (GA) of less than 32 weeks and/or birth weight of less than 1500 g, participed in the study (prebiotic mixture group (n = 48) and placebo group (n = 45)	80% scGOS/lcFOS and 20% pAO	Short-term enteral supplementation of a prebiotic mixture in the neonatal period had no effect on neurodevelopmental outcome in preterm infants in the first year of life
Synbiotics			
Malaguarnera et al. (2007) [107]	60 cirrhotic patients (30 with synbiotics and 30 with placebo)	*Bifidobacterium longum* plus fructo-oligosaccharides	Patients with minimal hepatic encephalopathy (MHE) treated with *Bifidobacterium* + FOS, showed an improvement and a recovery of neuropsychological activities related to short-term memory, attention and computing ability, language, orientation ability, and cognitive activities
Firmansyah et al. (2011) [108]	393 healthy 12 month-old toddlers	The probiotic *Bifidobacterium longum* BL999 (ATCC: BAA 999) and *Lactobacillus rhamonosus*, LPR (CGMCC 1.3724), the prebiotics inulin (30%) and fructo-oligosaccharide (70%), and the LCPUFA, arachidonic acid (AA) and docosahexaenoic acid (DHA)	Changes in cognitive and adaptive behaviour scores between 12 and 16 months were higher but not significantly different in the synbiotics group compared with the control group

6. Synbiotics

The term synbiotic was primarily stated considering the benefits of a product that combines prebiotics and probiotics and in which the prebiotic compounds selectively favour the probiotic strains [109]. Several studies have shown positive synergistic effects for synbiotics on obesity, diabetes, non-alcoholic fatty liver disease, necrotizing enterocolitis in very low birth weight infants, and in the treatment of hepatic encephalopathy [110–114]. Despite these findings, few studies have tested the potential benefits of synbiotics on neurodevelopmental disorders (Table 2). Malaguarnera et al. reported that *B. longum* plus FOS improved cognitive function in the treatment of minimal hepatic encephalopathy (MHE) [107]. Firmansyah et al. provided milk containing synbiotics (BL999, LPR, and prebiotics) and LCPUFA to 393 healthy toddlers at 12 months-old for 12 months. The authors reported that the change in cognitive and adaptive behaviour scores between 12 and 16 months was higher but not significantly different in the synbiotics group compared with the control group [108]. Future work is needed to determine whether synbiotics may contribute to relieve neurological diseases and to explore the benefits of new potential synbiotics during critical time windows in an infant's CNS development and susceptibility to neurological disorders.

7. Future Perspectives

During the last decade, numerous in vivo and in vitro studies have explored the influence of probiotics and prebiotics in host physiology [115]. Their results showed that gut microbiota may modulate inflammation, adiposity, satiety, energy expenditure, and glucose metabolism. Most efforts have focused on studying the mechanisms by which certain probiotics regulate the colonization of and protect against pathogens through the activation of the mucosal immune system and competition for limited nutrients [116,117]. Alternate approaches such as recombinant probiotics expressing therapeutic biomolecules, faecal microbiota transplantation and phage therapy, need be explored for the manipulation of the gut ecosystem. A proof of concept was the experiment performed by Paton et al., where they created a recombinant probiotic by introducing glycosyltransferase genes from *Neisseria meningitidis* or *Campylobacter jejuni* in a harmless *Escherichia coli* strain (CWG308) to treat and prevent the diarrheal disease caused by enterotoxigenic *E. coli* strains [118]. The same group also developed a recombinant probiotic for the treatment and prevention of cholera [119]. A recent study showed that microbiota transfer therapy improves ASD symptoms in children, which persists for at least 8 weeks after the treatment ends [120]. And finally, phage therapy has become an interesting strategy to treat bacterial infections due to the rise of antibioticresistant microbial strains. The only approved phage therapy clinical trial in the human gut was carried out in 120 patients with diarrhoea caused by *E. coli*, who were infected by a coliphage mix. The treatment failed to solve diarrhoea, although no adverse effects of phage infection were observed [121]. Customized phage cocktails could be an alternative for future therapies. These phages would directly target pre-identified bacterial pathogens though the main drawback would be the high interindividual variation of the gut microbiome and legislative approval [122,123].

In conclusion, this review summarized the accumulating evidence on the modulation of gut microbial composition and metabolism as a potential strategy for neurological disorders and CNS development. Despite this wealth of information, the effect of probiotics and prebiotics is still largely unexplored, and numerous gaps and inconsistencies exist when the studies are compared. Differences in quantity of dose, type of strain, type of prebiotic, assessment of gut microbiota, duration of intervention, standardization of neurological measurements, variety and complexity of neurological symptoms, study design, and cohort size make it difficult to confirm evidence of efficacy. To this end, double-blind placebo in vivo studies that exploit the power of the latest robust high-throughput multi-omic technologies are required to identify the molecular mechanisms of the gut's microbial modulation of neurological disorders and CNS development and ultimately to design effective probiotic and prebiotic therapies.

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Review

A New Proposal for the Pathogenic Mechanism of Non-Coeliac/Non-Allergic Gluten/Wheat Sensitivity: Piecing Together the Puzzle of Recent Scientific Evidence

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Abstract: Non-coeliac/non-allergic gluten/wheat sensitivity (NCG/WS) is a gluten-related disorder, the pathogenesis of which remains unclear. Recently, the involvement of an increased intestinal permeability has been recognized in the onset of this clinical condition. However, mechanisms through which it takes place are still unclear. In this review, we attempt to uncover these mechanisms by providing, for the first time, an integrated vision of recent scientific literature, resulting in a new hypothesis about the pathogenic mechanisms involved in NCG/WS. According to this, the root cause of NCG/WS is a particular dysbiotic profile characterized by decreased butyrate-producing-*Firmicutes* and/or *Bifidobacteria*, leading to low levels of intestinal butyrate. Beyond a critical threshold of the latter, a chain reaction of events and vicious circles occurs, involving other protagonists such as microbial lipopolysaccharide (LPS), intestinal alkaline phosphatase (IAP) and wheat α-amylase trypsin inhibitors (ATIs). NCG/WS is likely to be a multi-factor-onset disorder, probably transient and preventable, related to quality and balance of the diet, and not to the presence of gluten in itself. If future studies confirm our proposal, this would have important implications both for the definition of the disease, as well as for the prevention and therapeutic-nutritional management of individuals with NCG/WS.

Keywords: non-coeliac gluten/wheat sensitivity; pathogenic mechanism; butyrate; amylase trypsin inhibitors; lipopolysaccharide; intestinal alkaline phosphatase; microbiota

1. Introduction

Non-coeliac/non-allergic gluten/wheat sensitivity (NCG/WS) is a clinical condition described for the first time in 1978 by Ellis and Linaker [1], and then in 1980 by Cooper et al. [2], who reported cases of patients presenting gluten-responsive clinical picture in absence of coeliac disease (CD). However, it was only in 2012 that NCG/WS has been considered to be within the gluten-related disorder (GRD) spectrum, together with CD and wheat allergy (WA) [3]. In 2012, a consensus on new nomenclature and classification of gluten-related disorders has been published [3], after the first of three International Expert Meetings on GRD, all leading to related publications [3–5] that outlined NCG/WS main clinical and diagnostic features. While CD is a chronic small intestinal, autoimmune enteropathy triggered by gluten and related prolamines in genetically predisposed individuals, and WA

is an adverse immunologic reaction to wheat proteins, NCG/WS is "a condition in which symptoms are triggered by gluten ingestion, in the absence of celiac-specific antibodies and of classical celiac villous atrophy, with variable Human Leukocyte Antigen (HLA) status and variable presence of first generation anti-gliadin antibodies (AGA)" [3,4]. HLA-DQ2 and -DQ8 are the genetic markers most strongly associated with CD, being positive in approximately 95% of coeliac patients. HLA haplotypes are found positive in about 50% of NCG/WS patients, only slightly higher than 30% of the general population [4].

The recent renewed interest of scientific community in NCG/WS is due to an increasing number of patients, not affected by CD or WA, referring intestinal and extra-intestinal symptoms after gluten/wheat ingestion; despite the initial skepticism on its very existence as a discrete entity, NCG/WS has been recognized as an independent disorder of clinical, social, and economic relevance. However, there are still questions about its separation from CD [3–9]. This immune-mediated disorder [10] affects individuals for whom CD and WA have been ruled out according to the respective current diagnostic criteria [5,11]. It is characterized by heterogeneous and not specific gastrointestinal (GI) symptoms, including abdominal pain, bloating, bowel habit abnormalities (diarrhea, alternating bowel and constipation), and extra-intestinal symptoms, including chronic tiredness, headache, 'foggy mind', joint and muscle pain, limb numbness, eczema or skin rash, depression, anemia, of variable severity, occurring within hours or a few days after the ingestion of gluten-containing foods; symptoms improve or rapidly disappear with the exclusion of the latter and recur following their reintroduction [3–5,11].

In the last few years, several studies have suggested that both innate and adaptive immunity are involved [9,10,12], but there still remains an absence of confirmed and validated specific biomarkers. Therefore, according to the Salerno Experts, a diagnosis of NCG/WS should be made after a positive double-blind placebo-controlled gluten challenge with crossover (DBPCC) [5,11]. However, this procedure has been shown to be an "imperfect gold standard" [13,14], and in daily clinical practice, the diagnosis remains based on the evaluation of symptoms, the exclusion of CD and WA and improvement in symptoms after elimination of gluten/wheat from the diet (the latter of which is often influenced by placebo effects) [9,15]. Diagnostic difficulty, the lack of correct and scientific diagnostic work-up by some clinicians, and the considerable attention have given by the media to this "young" disorder, have contributed to the spread of the "self-diagnostic" phenomenon and the devotion of many people to gluten-free diets (GDF), which are often self-administered [4,9,12,16,17]. In fact, as for CD and WA, a GFD is the only possible treatment for NCG/WS, to date, but the severity and duration of this diet are not yet well defined, because of the uncertainty about the pathogenesis and triggers, the lack of specific biomarkers, and the strong inter-individual differences among patients [11]. All these factors also hamper the performance of accurate research because of the resulting heterogenous criteria for the selection of patients for the studies, and the following poorly comparable data [12]. Although risk factors for this disorder have not yet been identified, NCG/WS seems to be more common in females and in young/middle age adults [12]. Because of the "self-diagnostic" phenomenon and the absence of biomarkers, the overall prevalence of NCG/WS remains vague and possibly ranging from 0.6% to 6% [4,9]; the prevalence in children is still unknown [12].

There are increasing doubts among the scientific community regarding whether gluten is the trigger of NCG/WS; these doubts are supported by several studies [13,18–24]. It has been suggested that other molecules could determine the onset of NCG/WS [25]; possible candidates are fermentable oligo-, di-, monosaccharides and polyols (FODMAPs) [18], wheat amylase trypsin inhibitors (ATIs) [19–21], wheat germ agglutinin (WGA) [26,27] and exorphins [28].

Regarding the pathogenic mechanism of NCG/WS, a recent study conducted by Uhde et al. [29] clarified some aspects of this condition [10]. This study also suggested that some biomarkers (fatty acid-binding protein 2 or FABP2, soluble CD14 or sCD14, lipopolysaccharide (LPS)-binding protein or LBP, endotoxin-core antibodies or EndoCAb IgM, anti-flagellin IgM and IgG) could be useful as possible diagnostic tools, although they are yet to be confirmed and validated. Uhde demonstrated the

presence of enterocyte injury and translocation of microbial components from the intestinal lumen to the blood circulation, resulting in activation of the systemic innate and adaptive immune response [29]. This study confirmed the already verified [30,31] existence of an increased intestinal permeability in individuals with NCG/WS, and provided evidence for a possible pathogenic role of the intestinal microbiota [12]. One of the main questions that remains to be answered is what induces the increased intestinal permeability, allowing microbial and food-borne antigens to cross into the lamina propria, and how does this occur [12].

Starting from the best of our current knowledge, in the present paper we connect some recent scientific evidence, which were never been expressly linked together till now, and propose a new hypothesis on the pathogenic mechanism of NCG/WS, by reconstructing the possible "chain reaction" involved in the onset of this disease. Finally, we provide some starting points for further research that, if confirmed by future studies, could imply important changes both to the substantial definition of the disease and to the therapeutic-nutritional management of individuals with NCG/WS.

2. Scientific Background

2.1. Diagnostic Difficulties

Because a clear pathogenic mechanism and specific, confirmed and validated biomarkers are yet to be identified, the evaluation of symptoms remains fundamental for diagnosis of NCG/WS [9,10,12]. Unfortunately, the symptoms are also shared by CD [3,14], irritable bowel syndrome (IBS) [4,5,14] and non-immunoglobulin E (IgE)-mediated food allergies [4,14,32–34], and, as for CD, symptoms must be considered a poor indicator and predictor of the disease [29,35,36].

The current diagnostic criteria for NCG/WS [5,11] are insufficient for a certain identification of sensitive individuals, both for clinical practice [9,13] and, in our opinion, for research. They are based on the exclusion of CD and WA, and on the clinical responsiveness of individuals to a GFD and gluten rechallenge [5,11]. However, CD and WA cannot always be adequately excluded [14]. According to a recent systematic review [8], up to 20% of NCG/WS patients in literature were eventually reclassified as coeliac patients, after re-evaluation after gluten challenge, or advanced diagnostic investigations, such as characterization of γδ intraepithelial lymphocytes (IELs), immunohistological detection of anti-tTG2 IgA, duodenal aspirate or biopsy culture, and HLA-DQ2-gliadin tetramer test [9,13]. This subgroup of NCG/WS patients was characterized by lymphocytic enteritis (LE, representing Marsh 1 lesion level), negative serology for CD (anti-endomysial-EmA- and anti-tissue transglutaminase 2-tTG2-IgA), and positive genetics for CD (HLA-DQ2/DQ8 haplotype) [9,13]. Furthermore, according to the current diagnostic criteria, non-IgE-mediated WA can fall within the NCG/WS spectrum [4,32–34]. Non-IgE-mediated food allergies have different systemic and GI symptoms, similar to NCG/WS in terms of quality and time of onset. Without any biomarker, aside from an increased number of eosinophils in normal intestinal mucosa, they can be diagnosed by a positive response to an elimination diet followed by a DBPCC [14,32,34].

With regard to the DBPCC proposed to confirm the diagnosis of NCG/WS [5], besides being cumbersome, time consuming and costly, it is subject to important precebo, placebo, and nocebo effects and presents with a series of parameters that still need standardization and validation [9,13,14]; moreover, Molina-Infante and Carroccio show that more than 80% of recruited patients undergoing a DBPCC cannot reach a formal diagnosis of NCG/WS, 40% have a nocebo response and only 16% show gluten-specific symptoms, reaching 30% when the challenge is performed with wheat [13]. These findings highlight why DBPCC remains an "imperfect gold standard" for NCG/WS, and raise doubts about the role of gluten in the actual triggering of the disease, as suggested by many other studies [13,18–24].

The shared intestinal manifestations also make it hard to distinguish NCG/WS from IBS [14], a chronic functional GI disorder diagnosed exclusively on the basis of non-specific clinical

characteristics [37–39]. Many researchers suggest that NCG/WS may be a subgroup of IBS, rather than an independent clinical entity. This hypothesis is supported by the fact that the clinical picture in NCG/WS is almost always dominated by some GI symptoms, among those previously mentioned, most of which are also present in IBS, and that FODMAPs, rather than gluten, are responsible for them [6,18,25,39–43]. FODMAPs are present in grains and related products (especially those gluten-related), legumes, fruit, vegetables, milk and honey [9,44,45], and could in fact induce distension of the intestine because they are osmotically active molecules and fermentative substrates [25,40,46]. However, FODMAPs are known to inhibit rather than cause inflammation, by inducing beneficial changes in the intestinal microbiota and generation of short-chain fatty acids (SCFAs) [42,46–48]. It is unlikely that FODMAPs are the sole responsible for symptoms reported by NCG/WS subjects [42,49]: many individuals in clinical remission with a GFD do continue to ingest FODMAPs from legumes [18], containing quantities of these carbohydrates that are comparable to those of gluten-containing grains [44,45]. In our opinion, rather than triggers, FODMAPs should be considered as possible additional elements of disturbance that, in this specific case, could exacerbate symptoms associated with the gut lumen which is already compromised, due to other causes. Moreover, it is possible that individuals with NCG/WS could also have, at the same time, a lack of one or more enzymes for the digestion of FODMAPs or other nutrients; this would also explain cases of people defining themselves NCG/WS because of an association between the appearance of typical manifestations and the ingestion of gluten-containing foods, but then continuing to report persistent symptoms despite adhesion to a GFD [41]. In this regard, Balakireva [50] claims that both conditions could coexist independently, without necessarily sharing a common pathophysiological basis.

2.2. ATIs as a Trigger of NCG/WS?

Recently, the scientific community has focused on the possible role of ATIs in GRDs [19–21,51–53]. Wheat α-amylase trypsin inhibitors belong to the family of water soluble albumins [21,53], which, together with globulins, represent 10–20% of total wheat proteins [19]; in the endosperm of plant seeds, they support the natural defense against parasites and insects and may regulate starch metabolism during seed development and germination. They are a family of compact protease-resistant proteins with strong disulfide bonds and high secondary structural homology, that copurify specifically with ω-gliadins. ATIs can be grouped into three subfamilies of approximately 50–60, 24–30 and 12–15 kDa [19,21].

Studies on immune stimulating activity of ATIs have been conducted in human and murine macrophages, monocytes and dendritic cells (DCs), in cultures of coeliac intestinal biopsies as well as in vivo in mice [19,21]. These studies have demonstrated that ATIs (in particular CM3 and 0.19 types, of about 15 KDa) are strong activators of dendritic cells (DCs), macrophages and monocytes [19,21]. On these cells they engage the TLR4-MD2-CD14 complex, thereby activating both the classical (nuclear factor kappa B or NF-kB) and the non-classical (interferon responsive factor 3 or IRF-3) pathway. It results in an up-regulation of maturation markers and the early release of innate proinflammatory cytokines, IL-1β, IL-6, TNFα, and then later, IL-8 and MCP-1 [19,21]. Contrary to wild type mice, studies have shown that TLR4- or TLR4-signalling-deficient mice do not show intestinal and systemic innate immune activation after oral challenge with ATIs [20,21]. Furthermore, the in vitro and in vivo experiments performed by Junker [21] found that gliadins or gliadin peptides, such as p31–43 or p31–49, are not innate immunity stimulators. Zevallos [19] reported that in mice fed with an ATI/gluten-free diet for four weeks, a single gavage of about 12 mg/mouse of commercial gluten (containing 0.2 mg ATIs) increased parameters of innate inflammation along the whole intestine; this did not happen in mice challenged with the same dose of gluten that was 70% de-enriched of ATIs by prior extraction. This appears to indicate that pure dietary gluten itself has no relevant immune stimulating activity in normal mice, in contrast to what has been suggested in ex vivo settings [19].

Intestinal myeloid cells probably sense ATIs through DCs body extensions into the gut lumen while probing it for the presence of antigens and/or through an active transport of intact ATIs across

the intestinal epithelial layer, as occurs with gliadin peptides [20,54,55]. A direct interaction between ATIs and TLRs may also occur on the surface of enterocyte membrane, leading to the development of intestinal inflammation [56].

Schuppan [20] suggests that ATIs could have an adaptive adjuvant effect on pre-existing intestinal inflammation, in addition to promoting an innate immune response. A healthy adult person with a daily consumption of 150–250 g of wheat flour, therefore exposed to about 0.5–1 g of ATIs, would have a modest or moderate innate intestinal immune activation without development of symptoms, thanks to immune tolerance mechanisms [20,21]. According to Schuppan [20], individuals with NCG/WS could be those with pre-existing or chronic inflammatory diseases in whom the sensing/uptake of ATIs is increased, probably as a consequence of the disruption of intestinal homeostasis and barrier integrity. Thus, even modest activation of innate immunity could exacerbate inflammatory conditions already present, by indirectly promoting an adaptive response through the strengthening of pre-existing antigenic exposition of antigen presenting cells (APC) to T cells. According to Schuppan, such adaptive responses would occur in the gut, as well as in nearby or more remote lymph nodes or lymphatic organs, resulting in the typical NCG/WS extra-intestinal inflammation [20]. Zevallos [19] observed such adjuvant effect of nutritional ATIs in mice with pre-existing dextran sodium sulfate (DSS)-induced small intestinal or colonic inflammation. However, as suggested by Zevallos [19], further preclinical and clinical studies in human are warranted to assess the effect of an ATIs-free diet on intestinal and extra-intestinal inflammatory diseases.

In light of these findings, the recently proposed "FODMAPs hypothesis" concerning NCG/WS would be hardly sustainable, as discussed by Zevallos [19]. As previously mentioned, FODMAPs cannot induce the inflammatory responses occurring in NCG/WS patients, but rather could contribute to "mechanically" worsen their symptoms. In general, scientific evidence seems to better support the role of ATIs as a trigger of NCG/WS, which seems likely to be an immune-mediated disorder [10].

2.3. Microbial Lipopolysaccharide, Intestinal Alkaline Phosphatase and Intestinal Permeability

TLR4 activation by ATIs resembles that induced by LPS, the strongest TLR4 agonist. In fact, TLR4 activation by ATIs is CD14-dependent and engages MyD88-mediated downstream signaling (leading to activation of NF-kB and transcriptional upregulation of proinflammatory cytokines and chemokines such as IL-8, TNFα and MCP-1), or TRIF-mediated signaling (resulting in secretion of type I interferons and RANTES) [21].

LPS is the major cell wall component of Gram negative bacteria, consisting of a hydrophobic portion (lipid A) and a hydrophilic portion (an oligosaccharidic core plus an antigenic polysaccharide called O-antigen); it is released from bacterial cell wall by shedding or bacterial lysis [57]. As opposed to ATIs, ingested and luminal LPS is usually completely inactivated by gastric acids and intestinal alkaline phosphatase (IAP) [19–21].

The latter is an important brush-border enzyme involved in preventing intestinal inflammation and preserving the gut microbiota homeostasis. Mainly produced by the proximal small intestine epithelial cells, it is secreted both luminally and basolaterally and inactivates, by dephosphorylation, the microbial components normally present at high concentrations in the gut lumen [58–61]. In particular, IAP may play a pivotal role in the maintenance of intestinal barrier integrity by detoxification of LPS [62]. IAP is downregulated in settings where gut barrier dysfunctions are critical in the development of diseases, such as inflammatory bowel disease (IBD): low levels of IAP have been found in inflamed colonic biopsies of patients with Crohn's disease and ulcerative colitis (UC) [63,64]. Expression levels and activity of duodenal IAP were found to be particularly low, and related to the disrupted intestinal barrier integrity, in severe cases of CD in young patients [62,65]. IAP knock-out (KO) mice show higher LPS influx to the systemic circulation [66]. Exogenous IAP supplementation prevents the development of colitis in both human and mice [67,68], and prevents LPS-induced barrier dysfunctions in vitro [58].

In this regard, in healthy individuals with intact intestinal barriers, plasma concentrations of LPS range from undetectable levels up to 0.2 ng/mL; a variety of physiological factors can result in permeability alterations that lead to plasma LPS levels ranging about 1–2 ng/mL, while patients with intestinal permeability disorders such as necrotizing enterocolitis (NEC) and IBD can reach levels between 2 and 10 ng/mL [69–78]. Guo et al. [57] found that relatively low but physiologically and clinically relevant levels of LPS in the interstitial fluid of enterocytes can lead to a reversible, time-dependent increase in paracellular permeability in vitro (filter-grown Caco-2 monolayers) and in an in vivo (mouse intestinal perfusion) intestinal epithelial model system, without inducing cell death [57]. This occurs through a TLR4-MD2-CD14-mediated intracellular mechanism, engaged by LPS linked to LBP, and involves a TLR4-dependent up-regulation of CD14 membrane expression [57]. A variety of physiological factors such as prolonged physical exertion, high-fat diet, physiological stresses, or intestinal permeability disorders can readily achieve these concentrations of LPS in the interstitial fluid of enterocytes [57]. The consequent paracellular permeability variation occurs within four or five days [57], and can be dynamically regulated by altering both the expression levels and localization of tight junction proteins (TJPs) [58]. Inflammatory pathways, such as LPS-induced NF-kB activation and the consequent production of cytokines, result in disrupted levels and localization of TJPs [79,80]. This, in turn, can increase the passage of intestinal contents to the gut mucosa and to the systemic circulation [57,58]. As circulating LPS is an important determinant of the inflammatory response and multi organ failure, it could therefore play an important role in further deterioration and prolongation of intestinal TJ barrier defects in intestinal permeability disorders and inflammatory gut diseases [57].

Regarding intestinal permeability, the early belief that it was reduced in NCG/WS [81] has been definitively rejected [9]. Several studies have shown that an increased one is present even in non-coeliac patients, in particular in NCG/WS [30,31], IBS [31,82], and generic "non-coeliac" patients with persistent dyspeptic complaints [83]. Barbaro [31] proposed that zonulin could play a role in the pathophysiology of NCG/WS because of increased zonulin serum levels and a correlation with symptoms found in NCG/WS patients, suggesting disassembly of TJs. Hollon [30] analyzed intestinal permability of duodenal biopsy explants in four study groups (coeliacs with active disease (ACD), coeliacs in remission, non-coeliac gluten sensitives and non-coeliac dyspeptic controls) through the measurement of transepithelial electrical resistance (TEER). This study found an increase in permeability in all groups after pepsin-trypsin digested gliadin (PT-gliadin) exposure, compared to the media alone, with a greater increase in individuals with ACD and with NCG/WS. Finally, the findings by Uhde [29] about damage to enterocytes and translocation of microbial components from the lumen to the intestinal mucosa and blood circulation, further support the presence of increased gut permeability in NCG/WS. Uhde [29] also found an association between improvement in symptoms after GFD and normalization of biomarkers levels, although the magnitude of the latter did not correlate significantly with that of the symptoms scores. The damage to enterocytes was deduced by serological levels of FABP2 [29], a cytosolic protein specific to intestinal epithelial cells, rapidly released into systemic circulation after cellular damage, reflecting changes in the rate of enterocyte turnover [84–87]. In individuals with NCG/WS, elevated levels of circulating FABP2 are comparable to those of individuals with CD, and correlate with systemic innate and adaptive immune responses to microbial antigens. These are respectively evidenced by the significant increase in serological levels of soluble CD14 (sCD14) and LBP, and of antibodies towards LPS (EndoCAb IgM) and flagellin (anti-flagellin IgM and IgG). All these serum levels were significantly increased in NCG/WS patients, compared both to healthy and CD cohorts, except anti-flagellin IgM and IgG which were significantly increased only when compared to healthy controls [29]. It has been reported that LBP and sCD14 bind to circulating LPS and, depending on their relative concentrations, transfer LPS either to the TLR4-MD2-CD14 complex on myeloid cells, resulting in inflammatory stimulation, or to high density lipoproteins (HDL), lowering bioactivity of LPS [88,89]. Plasma lipoproteins, and in particular HDL, play an important role in neutralization of circulating LPS by transporting it to the liver for

metabolization and excretion in the bile. In humans, low HDL plasma levels were found in septic patients, while raised ones are associated with a reduction in LPS-induced inflammation [90]. Low HDL levels could favour the transfer of LPS to the TLR4-MD2-CD14 complex, favouring inflammatory processes; by failing or decreasing the passage of LPS to HDL, peripheral inflammatory events would have a "green light". In this regard, it would be interesting to assess HDL levels in individuals with NCG/WS to demonstrate a correlation between presence and intensity of symptoms and their improvement after dyslipidemia correction.

We must highlight that in the mentioned studies on intestinal permeability in NCG/WS patients, issues about different inclusion/exclusion criteria recur, as in most of the studies about this condition, due to the lack of specific confirmed biomarkers.

3. New Hypothesis on the Pathogenic Mechanism of NCG/WS

The intestinal epithelial surface is in constant contact with the enteroma; in diseases such as IBD, NEC and HIV infections there is a correlation between compromised epithelial integrity and immune responses consequent to translocation of microbial components from the intestinal lumen to the blood circulation [88,91]. Human microbiota, mainly composed of species belonging to *Firmicutes* and *Bacteroidetes* phyla, is suggested to play immunological, structural and metabolic functions such as, for example, the preservation of GI barrier integrity through the production of SCFAs [88].

Among these, butyrate is the major source of energy for the colonic mucosa, in turn promoting epithelial cell differentiation and injury repair; it also seems to play an important protective role in colorectal carcinogenesis. Butyrate stimulates the secretion of mucin and epithelial antimicrobial peptides, the synthesis of TJPs, and prevents microbial translocation [92–95]. Recently Yan and Ajuwon [92] found that butyrate significantly and dose-dependently protects intestinal barrier integrity from LPS-induced impairment. This effect was indicated by the restoration of paracellular permeability, measured by TEER and paracellular uptake of fluorescein isothiocyanate-dextran (FITC-dextran), and was carried out through the selective stimulation of TJPs and downregulation of TLR-4 expression.

Butyrate-producing bacteria are a functional group within the human gut microbial population [96]. Most of these bacteria belong to *Firmicutes* phylum, in particular Clostridial Clusters IV and XIVa (saccharolytic, strictly anaerobic Gram positive bacteria). Numerically, two of the most important groups appear to be *Faecalibacterium prausnitzii* (Clostridial cluster IV), and *Eubacterium rectale*/*Roseburia* spp. (Clostridial cluster XIVa) with a detection rate in faeces of healthy adults of about 2–15% compared to total bacteria [96,97]. Human studies are often limited to faecal samples analysis that, however, do not provide information about microbiota in the mucus layer, where butyrate-producing *Firmicutes* (b-p*F*) mainly reside, according to a validated in vitro gut model (M-SHIME) [98]. In fact, Clostridial cluster XIVa and IV species constitute respectively 59% and 19% of the mucin-adhered *Firmicutes* microbiota (94% of the mucin layer total community), with major representatives in *Roseburia intestinalis* and *Eubacterium rectale*. In contrast, *Proteobacteria* and *Bacteroidetes* prefer the lumenal milieu [98].

Bifidobacteria (Gram positive, anaerobic, saccharolytic bacteria belonging to the phylum *Actinobacteria*) contribute to the maintainance of adequate levels of intestinal butyrate by providing acetate and lactate to b-p*F*, in turn converting them into butyrate (cross-feeding interaction) [96,97,99].

Similarly, even mucins may indirectly serve as a growth substrate for b-p*F*, possibly via cross-feeding with mucin-degrading microbes, such as *A. muciniphila*, which provide partial breakdown products, acetate and lactate [98,100].

Low levels of b-p*F* are found in patients with IBD and their inflamed tissues compared to healthy individuals [96,101]. Low levels of b-p*F* and *Bifidobacteria* are also found in IBS and are associated with increased IBS symptoms [102,103]. Furthermore, the absence of butyrate in colonic tissue is associated with mucosal atrophy and coloncyte apoptosis [104,105].

In light of the above findings and considerations, a particular dysbiotic profile characterized by low levels of b-p*F* and *Bifidobacteria* could not provide an adequate butyrate level for enterocytes,

resulting in reduced or absent butyrate trophic and protective effects. This would "starve" enterocytes, would negatively influence TJPs expression and localization, and would also predispose GI epithelial cells to possible cell damage or premature death. In fact, Uhde observed high FAPB2 serum levels in NCG/WS patients [29]. Moreover, as butyrate stimulates mucine secretion [95], a decrease or lack of butyrate could result in mucus layer alterations. Mucus is produced by goblet cells and forms a protective physical barrier covering enterocytes, thus preventing microrganisms and noxious substances from reaching epithelial surface [106]; so mucus layer impairment could further enhance the direct contact between enterocytes and microbial and food-borne antigens, and would create unfavorable conditions for b-p*F*, thereby creating a vicious circle. For example, recent in vivo and in vitro studies have revealed that IBD, in which the mucus layer becomes thinner and more discontinuous, is associated with low levels of mucosal butyrate producers, such as *Roseburia* and *Faecalibacterium*, indicating that a damaged mucus layer may lower the ecological fitness of specific butyrate producers [98].

Such a situation could allow sufficient LPS quantities to arrive in the interstitial fluid, resulting in a further increase in permeability, as explained by Guo [57]. In our opinion, this could constitute the pre-existing condition allowing the onset of NCG/WS. As a consequence, simultaneous paracellular translocation of microbial components and food-borne antigens, such as intact ATIs, could occur. Active ATIs would directly, and in greater quantities, stimulate the maturation of DCs, monocytes and macrophages of the GI tract, leading to activation of the innate immune response in the lamina propria [19–21]. Here, the stimulation of the TLR4-MD2-CD14 complex by ATIs would be additional to that of translocated LPS, amplifying the proinflammatory effect. Further, direct stimulation of the TLR4-MD2-CD14 complex on the surface of epithelial cells may occur to a greater extent because of the impaired mucus layer. Such a mechanism may also be amplified because TLR4 and CD14 expression and membrane co-localization are increased after LPS exposure in vitro and in vivo [57]. This would explain the typical local and rapid-onset intestinal symptoms of NCG/WS after the ingestion of gluten/ATI-containing foods. Uhde found that these translocated molecules could also arrive in the bloodstream and activate both innate and adaptive systemic immunity, as shown by the respective detection of serum LBP and sCD14, and EndoCAb and anti-flagellin antibodies. Translocated circulating antigens could bind to TLRs on other cells to trigger inflammatory responses in other parts of the body, explaining the extra-intestinal manifestations of NCG/WS [29].

Luminal LPS is usually completely inactivated by IAP: the latter, although exclusively secreted by brush border epithelial cells of the proximal small intestine, preserves its activity along the entire GI tract [107,108]. Several in vitro and in vivo studies in mice and rats have shown that IAP prevents adhesion of both pathogenic and commensal bacteria to the intestinal epithelial cells [109], prevents their translocation [107,110,111], inactivates possibly translocated pathogen-associated molecular patterns (PAMPs), and inhibits PAMP-induced NF-kB-mediated inflammatory responses [60,108]. IAP also stimulates gene expression of TJPs, such as ZO-1, ZO-2 and occludin, and their correct cellular localization, thus playing a direct role in intestinal barrier functionality [58]. However, we must highlight that butyrate is an inducer of IAP expression, and increases its activity [93,107,112,113]. Therefore, it is likely that a dysbiosis characterized by low levels of b-p*F* and/or *Bifidobacteria* could indirectly cause a decrease in IAP levels and activity, resulting in an insufficient detoxification of luminal microbial components, including LPS. The latter would have a greater green light for translocation across the previously described compromised NCG/WS GI barrier. In support of this hypothesis, also Goldberg [107] asserts that IAP silencing could result in impairment of the host's ability to protect itself from luminal LPS exposure. Moreover, butyrate-induced IAP gene expression is inhibited by cytokines such as IL-1β and TNFα, and according to Malo [114], "cytokine-mediated IAP gene silencing may have important implications for gut epithelial function in the setting of intestinal inflammatory conditions". Interestingly, IL-1β and TNFα are two of the cytokines produced after ATI stimulation [19], so that the GI inflammation, triggered by translocated antigens, could contribute to further silencing IAP, resulting in another vicious cycle.

In Junker's in vivo experiments in mice to characterize the inflammatory activity of ATIs, orally ingested LPS was not found to cause increased transcription of cytokines, and thus, inflammation [21]. Junker, and later Schuppan and Zevallos, properly described this finding to be attributed to LPS inactivation by gastric acids and IAP activity [19–21]. In a paper on NCG/WS, Schuppan [20] defined ATIs as "the only relevant luminal TLR4 activator in the gastrointestinal tract". In light of what discussed above, it could be that not only ATIs, but also luminal LPS from the resident microbiota, are involved in immune response activation in NCG/WS, thus accumulating their stimulating effects.

In NCG/WS individuals AGA are detected in a variable trend: AGA IgG are found in about half of patients [81,115], finding that created great discussion about their possible utility as a diagnostic tool for this disease [49,115–118], while AGA IgA are rarely detected [29,115]. Interestingly, in the study by Uhde [29] IgM responses to gliadin and microbial components (LPS and flagellin) were also investigated and found enhanced, in clear contrast with CD and healthy cohorts. Uhde suggests that acute microbial translocation from the gut would be expected to enhance the secretion of IgM antibodies in the periphery via a TLR9-dependent activation of B cells, independent of direct contact with respective antigens or T-cell involvement. IgM B cells would be further stimulated by direct interaction with specific antigens, such as translocated gliadins and microbial components, thus contributing to the observed IgM antibody responses [29]. However, according to our hypothesis, the triggers of NCG/WS are ATIs and microbial components, LPS in particular; we think that the host immune response would be mainly addressed against these ones, that should be further investigated in this regard. Even if our hypothesis is confirmed by future studies, further research will be necessary to understand pathophysiological and immunological events, and most of aspects of NCG/WS, such as variability in antibody reactivity among patients.

Contrary to CD patients, individuals with NCG/WS do not present villous atrophy or mucosal architecture abnormalities at duodenal level. For this reason, regarding NCG/WS, several authors have recently suggested possible elective damage sites aside from the duodenum [11,29,119]. Di Liberto et al. [119] suggested that in sensitive patients the immunological response could be greater in the colon rather than in the duodenal mucosa, because NCG/WS is generally characterized by GI symptoms also present in IBS, which is very often characterized by colonic mucosal inflammation [40,120,121]. In fact, Di Liberto observed innate lymphoid cell infiltrates (ILC1) in the rectal mucosa of individuals with NCG/WS, where a greater expression of proinflammatory cytokines such as IFNγ was also detected [119]. Uhde suggested the jejunum as an alternative damage location because it is the primary expression site of FABP2 [29]. On the basis of our proposal, we believe that the whole intestine could be involved in NCG/WS, maybe with an increasing trend in the colon. This hypothesis is based on several factors: first, the stimulating activity of ATIs progressively increases along the intestinal tract and is more marked in the colon rather than in the small intestine [19]; second, the colon contains the most "dense" and metabolically active microbiota within the adult GI tract, however the Clostridial cluster XIVa spp. is also present in the small intestine [97]. Regardless, it could be that epithelial changes associated with NCG/WS do not lead to overt remodelling of the mucosa, and therefore, they could require confocal laser endomicroscopy (CLE) for visualization [10,29].

In summary, the "culture medium" for the rooting of NCG/WS appears to be a pre-existing dysbiosis characterized by low levels of b-p*F* and/or *Bifidobacteria*, leading to a decrease in butyrate. Beyond a critical threshold of the latter, the gut would no longer be capable of dealing with different inflammatory stimuli, these being, an exogenous one from the diet (ATIs) and an endogenous one from the resident microbiota (LPS and other microbial components); as a consequence, it would begin a chain reaction as illustrated in Figure 1. In line with this, although no consensus has been agreed upon, Bennet [122] declared that in IBS patients there is a temporary decreased stability of the gut microbiota, leading to a dysbiosis generically defined as a weak tendency for a reduction in the beneficial bacteria of the gut countered with an increase in pathogenic species. Furthermore, Bennet highlighted the beneficial effects of butyrate and suggested that, although inconsistent, reduced levels of

butyrate-producing *Eubacterium*, *Faecalibacterium* and *Roseburia* spp. could potentially be an ancillary cause of IBS symptoms in some patients [122]. Chassard [103] suggests that low levels of butyrate found in a subgroup of IBS patients could be due to the lowering of some butyrate-producing bacteria and may reduce the potential health benefit of this metabolite, including anti-inflammatory effects and the colonic defence barrier. All of this could be important in our perspective, considering that, to date, NCG/WS patients may be still diagnosed as suffering from IBS. Finally, with regard to NCG/WS, Volta [9] suggests that microbial dysbiosis driven by aberrant changes in the normal composition of the gut microbiota may contribute to intestinal barrier defects and inflammatory responses.

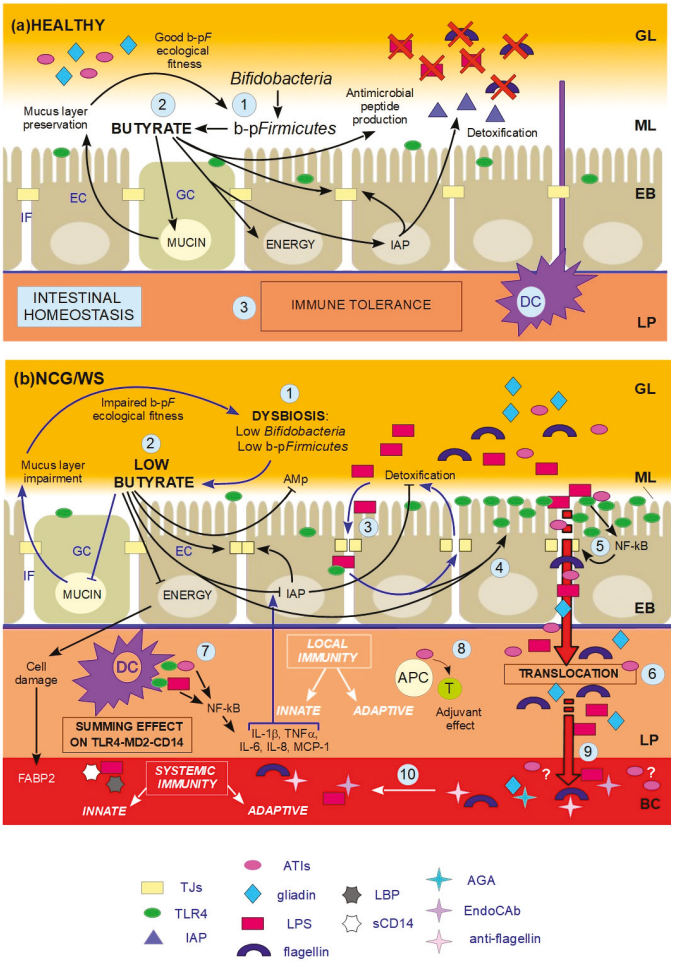

Figure 1. Schematic drawing that shows what happens in a healthy gut versus a non-coeliac gluten/wheat sensitivity gut according to our hypothesis. **NCG/WS** = non-coeliac gluten/wheat sensitivity; **EC** = enterocyte; **GC** = goblet cell; **IF** = interstitial fluid; **GL** = gut lumen; **ML** = mucus layer; **EB** = epithelial barrier; **LP** = lamina propria; **BC** = blood circulation; numbers in light blue balloons indicate the various steps in the chain reaction; → indicates stimulation; ⊣ indicates inhibition. (**a**) HEALTHY: 1. Butyrate-producing *Firmicutes* (b-p*F*) provide adequate levels of butyrate

in the ML and *Bifidobacteria* support the production of butyrate thanks to cross-feeding interactions with b-p*F*; 2. Butyrate in the ML, close to ECs, plays different trophic and protective functions: it stimulates GCs in the production of mucins, resulting in the preservation of the ML, and thus in a good b-p*F* ecological fitness. Butyrate constitutes the major energy supply for ECs; it favours the preservation of tight junctions (TJs) integrity by stimulating the expression and membrane co-localization of tight junction proteins (TJPs). Butyrate stimulates the production of antimicrobial peptides (AMp), and the expression and activity of intestinal alkaline phosphatase (IAP), thereby favouring the detoxification of microbial components; 3. All these functions together prevent that the content of the GL directly contacts and/or translocates across the EB, and, together with dendritic cells (DCs) which probe the GL for the presence of antigens, allow gut homeostasis and immune tolerance. (**b**) NCG/WS: (1) A dysbiosis characterized by low levels of b-p*F* and/or *Bifidobacteria* results in not sufficient levels of butyrate in the ML; (2) As a consequence, a chain reaction of events and vicious circles occur: the production of mucins is no longer stimulated, resulting in impairment of the ML. The consequent lowering of b-p*F* ecological fitness further promotes low levels of butyrate. ECs, without adequate energy source, run into inefficiency and cell damage, resulting in high serum levels of fatty acids binding protein 2 (FABP2). Moreover, TJs integrity is compromised, and the production of AMp is decreased. Low levels of butyrate also cause a decrease in the expression levels and activity of IAP; as a consequence, TJs integrity is further impaired, and the detoxification of microbial components is not sufficient; (3) The failed detoxification enables microbial lipopolysaccharide (LPS) to penetrate in the IF, where it increases paracellular permeability, with a consequent vicious cycle; (4) Furthermore, both LPS in the IF and low levels of butyrate upregulate toll-like receptors 4 (TLR4); (5) Because of the compromised ML, the lumenal content can reach EC surface. LPS and wheat amylase trypsin inhibitors (ATIs) can stimulate overexpressed TLR4, resulting in the production of NF-kB, and then later, inflammatory cytokines, which further damage TJs integrity; (6) Food-borne antigens and microbial components can cross the leaky EB; (7) In the LP, both translocated LPS and ATIs stimulate, at the same time, the TLR4-MD2-CD14 complex on myeloid cells, such as DCs, resulting in a local innate immune response with the production of inflammatory cyokines and chemokines. Among the latter, IL-1β and TNFα further inhibit the activity of IAP, thus maintaining this condition; (8) Moreover, ATIs have an adjuvant effect on possible pre-existing antigenic exposition of antigen-presenting cells (APC) to T-cells (T), triggering an adaptive immune response; (9) Microbial and food-borne antigens translocate in the BC (10), and trigger a systemic innate and adaptive immune response, respectively resulting in high serum levels of lipopolysaccharide-binding protein (LBP) and soluble CD14 (sCD14), and EndoCAb, anti-flagellin and anti-gliadin (AGA) antibodies.

In terms of what induces the dysbiosis, this could be an "erroneous" diet and lifestyle, and/or an epigenetic predisposition. In fact, the first bacterial colonization in utero can modulate immunological and metabolic "fetal programming", with potential long-term consequences on the risk of developing GI diseases, such as CD and IBD, in addition to allergies, autoimmune and metabolic diseases in the adult life [123,124]. Further, breastfeeding and adult diet are known to strongly influence microbiota. For example, given the scarceness of fiber and the excess of animal fats and proteins, "Western-like diets" are associated with an increase in the secretion of bile salts, resulting in the selection of bile-resistant and sulfate-reducer bacteria with proteolytic and putrefactive action. This is in contrast with a healthy saccharolytic microbiota, promoted, for example, by the "Mediterranean diet", which allows the preservation of adequate leves of SCFAs and butyrate in particular. Moreover, the richness of Western diets, particularly with regard to trans and satured fats, could result in a greater translocation of LPS and greater inflammation [94,123–129].

4. Implications of the New Hypothesis

Based on the previously described literature and evaluations, it could be considered that NCG/WS is a potentially transient and preventable condition, strongly related to diet quality and balance, rather than to the presence or absence of gluten-containing foods. Without the suggested dysbiotic conditions, the extent of gliadin exposure effects would remain limited to those described in healthy

subjects by Hollon [30]. This hypothesis could also be supported by the current lack, to the best of our knowledge, of both a specific and proven genetic background [12,14], and clear and defined evidence of hypersensitivity to gliadin [23,115] in NCG/WS patients.

Regardless, a GFD is currently the cornerstone of treatment for NCG/WS [11]. Undertaking a GFD, if self-administered, means exposure to a series of nutritional risks, and the possibility of obesity and related comorbidities, or in the least excessive weight gain. Moreover, a GFD is usually not economical and is very difficult to follow because of cross-contaminations and/or the presence of small amounts of gluten in food and drugs. In general a GFD appears to be unbalanced and inadequate in terms of both macro- and micronutrients, so it is fundamental that people start a GFD only if, and for the time, strictly necessary [11,50,130–135]. Also according to the current NASPGHAN guidelines on GRDs [11], given the uncertainty about the pathogenesis and triggers of NCG/WS, it is not clear if a GFD is actually the optimal treatment for this disease, although it could lead to improved symptoms in self-reported or diagnosed sensitives; it is also not clear how strict and how prolonged this diet should be, and how its efficacy should be monitored independently from the clinical response, given the strong inter-individual differences and the lack of clear guidelines for a standardized follow-up [8,11].

The validation of a co-causal role of specific alterations of the intestinal microbiota in this disorder would be fundamental to the management of NCG/WS patients at a nutritional level. As such, sensitive individuals would no longer need to follow a prolonged, if not permanent, restrictive diet based on the exclusion of gluten. All the more so that this restriction has been shown to affect microbiota richness and composition by reducing beneficial bacteria such as *F. prausnitzii* and *B. longum* [43,136]. On the contrary, by directing the dietetic choice of these patients towards a targeted "prebiotic" type, we could aim to restore a state of eubiosis thanks to the food's ability to shape the microbiota [126,137,138].

According to what is hypothesized here, targeted probiotic intake could be another fundamental aspect of the treatment of individuals with NCG/WS. In line with this, we might speculate if the weak correlation between magnitude of change in analyzed biomarkers and magnitude of change in symptom score after GFD in the study by Uhde [29] could be explained simply by the absence of an associated probiotic therapy.

One of the chief contributions of our microbiota is its partecipation in food digestion, mainly through a saccharolytic or a proteolytic catabolic pathway [125,139]. With the first one, saccharolytic bacteria, such as *Bifidobacteria* and *Lactobacilli*, hydrolize complex polysaccharides in monomeric sugars, then converted in SCFAs (mainly acetate, propionate, and butyrate). The latter seem to to have a positive role in regulating some physiological processes [94], well summarized in a review by Macfarlane G.T. and Macfarlane S. [139]. For example, SCFAs are known to affect lipid, cholesterol and glucose metabolism in various tissues, and to control the release of satiety hormones; recently, they have also been suggested to play a critical role in the regulation of the gut-microbiota-brain cross talk [94]. Furthermore, SCFAs have been shown to promote intestinal barrier integrity, affect epithelial cell transport and metabolism, epithelial cell growth and differentiation, and elicit direct transcriptional responses in immune cells [125,139]. Both carbohydrate and protein fermentation result in SCFAs production, although in quantitative terms, protein is a minor contributor [139]. Furthermore, besides SCFAs, the proteolytic pathway yields a variety of end-products including co-metabolites such as CO_2, H_2, H_2S, ammonia, amines, thiols, phenols, and indoles, many of which are potentially toxic and are believed to promote the onset of "Western diseases", such as colon cancer and chronic systemic disorders [125,139]. Saccharolytic bacteria contribute to protect the host from toxic products associated with putrefaction, by requiring them for incorporation into cellular proteins [139]. For these reasons, although the composition of microbiota is a highly personalized peculiarity influenced by several factors such as diet, drugs and lifestyle, it seems that a healthy and balanced microbiota should be mainly saccharolytic with a prevalence of *Bifidobacteria* and *Lactobacilli* [125]. Among SCFAs, butyrate seems to be the most interesting

one, thanks to its many important physiological properties [139], some of which have been previously mentioned. Among butyrate-producing bacteria that colonize the human gut, *F. prausnitzii*, *Roseburia* spp., and *E. hallii* belong to the so called "*emerging probiotics*" [100,140]. Of particular interest, *F. prausnitzii* has well characterized immunomodulatory properties in vitro and in vivo [100]: it is able to abolish the NF-kB pathway in intestinal epithelial cells, and prevents colitis in animal models [141]. Currently, *emerging probiotics* culturing and functional characterization difficulties do not allow well-designed pre-clinical and intervention studies yet [140]. For this reason, we have to wait for developing new probiotic products aimed to directly restore butyrate-producing bacteria levels in NCG/WS subjects.

Fortunately, the contribution of cross-feeding interactions between *Bifidobacteria* and butyrate-producing *Firmicutes* is remarkable in the stimulation of butyrate production [99] and thus we believe it could be a strategy in the "attack phase" of NCG/WS treatment. For this purpose, *Bifidobacteria* such as *B. bifidum*, *B. longum* and *B. adolescentis*, the colonic dominant species of the genus, could be useful [97,99]: besides their role in butyrate production, they promote other important anti-inflammatory effects [142–147]. For example, some strains of *B. bifidum* have been shown to promote strong anti-inflammatory effects through the inhibition of LPS-induced NF-kB-activation in a strain and dose-dependent manner [142]; some strains of *B. longum* ssp. *longum* and *B. longum* ssp. *infantis*, as well as *B. adolescentis* and *B. animalis* have been shown to induce significant levels of IL-10 in different human cell cultures and in human colonic lamina propria [144]; IL-10 is a regulatory cytokine with an important role in the maintenance of intestinal homeostasis [148,149], and it is absent in gut explants from NCG/WS patients, according to Hollon [30]. *B. longum* ssp. *longum* CCM 7952, is able to increase expression of zonulin-1 and occludin in the intestinal epithelium, preserving TJs and epithelial barrier function [144], thus potentially contributing to a reduction in inflammatory stimuli in the intestinal mucosa of patients with NCG/WS. Finally, *Bifidobacteria*, alone or together with particular *Lactobacilli* and *Streptococci* strains in specific multispecies probiotic mixtures, have been shown to be effective in the prevention and/or treatment of DSS-induced colitis [144,146,150], IBS symptoms [151] and GI inflammatory diseases, such as UC in mice and humans [143,146,147,152,153]. In particular, *Bifidobacterium bifidum* BGN 4 seems to reduce the production of pro-inflammatory cytokines, thanks to the chiro-inositol present in its cell membrane [154]. According to a recent study [155], the administration of a specific probiotic blend containing *Bifidobacterium bifidum* BGN 4, *Lactobacillus salivarius* and *Lactobacillus acidophilus* (Acronelle®, Bromatech srl, Milan, Italy) decreases inflammation in patients with IBD, in combination with mesalazine treatment.

Other microbes besides *Bifidobacteria* have demonstrated important anti-inflammatory activities that could be useful in the treatment of NCG/WS: *L. rhamnosus* GG, found in the mucus layer environment [98], has been shown to attenuate intestinal barrier dysfunctions and proinflammatory signals [156], and in particular, to restore in vitro gliadin-induced epithelial barrier disruption and related TEER in the presence of polyamines [157]; *Akkermansia muciniphila*, a Gram-negative strictly anaerobic mucin-degrader, contributes to the production of SCFAs and the maintenance of a healthy protective GI barrier by continuously renovating the mucosae cover [100]. The use of probiotic therapy could be strengthened by the association with prebiotics such as resistant starch, inulin, xylans and fructo-oligosaccharides (FOS) which would, directly and indirectly, stimulate butyrate production [94,96–99,126].

Obviously, if our hypothesis is validated in future studies, further research would be necessary to define the best formulation of treatment, including correct dosages and posology.

5. NCG/WS Is a Cancer Risk Factor?

Whether CD is associated with an increased risk of cancer malignancy is still under debate. However, some literature seems to support this hypothesis. A European multi-centre study reported more than a three-fold increased risk of non-Hodgkin's Lymphoma (NHL) in patients with

clinically diagnosed CD [158]. Also an increased incidence and/or mortality for NHL have been reported in patients with CD [159]. An increased risk of GI cancers, duodenal ones in particular, was also documented [160]. On the contrary, other studies suggested that CD may be associated with a reduced risk of some cancers, including breast and lung ones [161,162], although the rationale for this relationship remains unknown.

Overall, it has been observed that the correlation between all types of cancer and CD remained significant for more than one year after diagnosis.

As regards possible correlations between malignancies and NCG/WS, to date data are missing [12]. However, Elfestrom et al. [163] in a study including 28.882 CD patients, 12.860 subjects with small intestinal inflammation (Marsh 1–2) and 3.705 patients with latent CD, found that all the three groups had an increased risk for GI cancers in the first year after diagnosis, but not thereafter. Moreover, an Irish population-based retrospective cohort study [164] reported that malignancy and mortality from malignant neoplasms were increased in patients with positive AGA and negative EmA tests. However, only on the basis of those serological results, the authors could not classify patients as having CD or NCG/WS according to recognised diagnostic criteria, so they could not provide reliable information about a real correlation between NCG/WS and cancer risk.

In conclusion, more investigations are necessary in this field to determine if NCG/WS may be correlated with cancer malignancy.

6. Starting Points for Future Research

The data and hypotheses described herein could contribute to the clarification of some controversial aspects of this "young" clinical condition, and could provide some novel avenues for future research. First, it would be interesting to verify the existence of an actual association between the particular dysbiotic profile proposed and NCG/WS. Second, it is of interest to reassess, at the colonic level, the expression of TJPs and TLRs. In this regard, according to a study by Sheth [165] on the effects of LPS on cholangiocytes TJs, LPS disrupts barrier function and increases paracellular permeability in a time- and dose-dependent manner, and also induces a redistribution of TJPs from intercellular junction sites. This study also reported that the LPS-induced disruption of TJs is mediated by TLR4 and LBP [165]. This could raise the question whether, in individuals with NCG/WS, the localization mechanisms of TJPs could be altered, rather than their expression levels. Third, we could speculate that individuals with NCG/WS may have an "overactive" TLR4, attributable to "gain-of-function" mutations. This could also contribute to explain the LPS-induced increase in gut permeability, according to the mechanisms proposed by Guo [57] and Sheth [165], and even more so, may help explain the extent of the immune response caused by the hypothetical combined stimulating effect of ATIs and LPS on the TLR4-MD2-CD14 complex.

Finally, future verification that critical intestinal butyrate levels are pivotal for the onset of NCG/WS could also provide the premise for studies regarding possible associated long-term major complications, such as intestinal lymphoma or gastrointestinal malignancies, as observed in CD. In the current literature there are no such reports, due to a lack of longitudinal data and prospective studies on the natural history of NCG/WS [12]. However, as previously mentioned, butyrate has been suggested to play an important protective role in colorectal carcinogenesis, and has been shown to reduce size and number of tumors in rat models of bowel cancer. Moreover, in vitro, it influences morphology and motility, inhibits proliferation and induces apoptosis in a variety of cancer cells [93,139].

The starting points for future research offered by this paper are described in Table 1.

Table 1. Starting points for research. The listed starting points are suggested for testing our hypothesis on the pathogenic mechanism of non-coeliac gluten/wheat sensitivity (NCG/WS). b-p*F* = butyrate-producing Firmicutes, IAP = intestinal alkaline phosphatase, TJPs = tight junctions proteins, TLR4 = toll-like receptor 4, ATIs = amylase trypsin inhibitors, LPS = lypopolysaccharide, HDL=high density lipoproteins.

Suggested Starting Points For Testing Our Hypothesis On NCG/WS
1. Association between NCG/WS and dysbiosis, in particular focusing on b-p*F* and *Bifidobacteria* levels
2. Association between NCG/WS and an impaired mucus barrier
3. Roles of butyrate and IAP
4. Presence of TJPs co-localization defects and role in the alteration of gut permeability
5. Expression of TJPs and TLR4 at the colonic level
6. Presence of simultaneous stimulation of the TLR4-MD2-CD14 complex by ATIs and LPS
7. Existence of anti-ATIs antibodies
8. Mutations of TLR4 coding gene and related functional studies
9. Association between NCG/WS and HDL levels

7. Conclusions

This review investigates and discusses, for the first time, novel plausible connections among recent scientific evidence, which have never been linked together in an integrated vision. We also propose a new theory on the pathogenic mechanism of NCG/WS, schematized in Figure 1.

NCG/WS may be considered a multi-factor-onset disorder, potentially transient and preventable, to date without a specific genetic pattern. It may have, instead, an epigenetic component, strongly related to quality and balance of the diet, and consequently, to the microbiota. If the hypotheses posed here are confirmed, NCG/WS could be still defined as a gluten-related disease because of the substantial coexistence of gluten and the stimulating activity of ATIs, for which a GFD is essentially ATIs-free [19].

More precisely, NCG/WS could be considered to be an ATIs/low butyrate-producing *Firmicutes*/low *Bifidobacteria*-dysbiosis-induced disorder, which would more appropriately be referred to as "dysbiosis-induced ATIs sensitivity" (DIAS).

In future, once excluded CD and WA in all their forms, the diagnosis of NCG/WS could ideally be determined also thanks to the aid of the immunological and enterocyte damage biomarkers suggested by Uhde, and the analysis of gut microbiota. According to our hypothesis, host immune system may be mainly addressed towards two different inflammatory stimuli, an exogenous one from the diet (ATIs) and an endogenous one from the resident microbiota, LPS in particular. In our opinion, further studies should be focused into this field, including possible existence of anti-ATIs antibodies. However, first of all, more appropriate diagnostic criteria for NCG/WS and standardized inclusion/exclusion criteria are warranted to perform more reliable studies on it. In our opinion, more appropriate diagnostic criteria are warranted for GRDs in general, because even a certain exclusion of CD still does not seem to be guaranteed, as well as exclusion of non-IgE mediated WA, with a consequent possible contamination of NCG/WS sample, thus creating confounding and possibly biased results. In this regard, it is important to underline that HLA haplotypes should not to be considered as suggestive of NCG/WS: according to the systematic review by Molina-Infante [8], not all studies on this disorder could adequately exclude CD and confirm diagnosis of NCG/WS, as well as not all studies performed, clearly defined and fully described genetic tests, and related the latter to histology [14]. Positive HLA-DQ2/DQ8 haplotypes are necessary, but not sufficient, to develop CD, and their absence excludes the latter, but so far, no genetic markers have been identified for NCG/WS [9], and according to Bardella [14], "other haplotypes, not CD-related, should be investigated".

According to our hypothesis, the treatment for NCG/WS would be completely different, and no longer necessarily based only on a restrictive and prolonged GFD, but on a targeted "prebiotic" type of nutrition together with specific probiotic therapy, all to be formulated in the future. In particular, we suggest that such a treatment should be specifically addressed to the direct or indirect restoration

of adequate levels of butyrate-producing *Firmicutes*, and consequently of intestinal butyrate. In fact, in our opinion, the decrease of bp-*F* is at the basis of increased intestinal permeability of NCG/WS, via insufficient butyrate levels, according to the supposed chain reaction. However, we do not know at the moment what should be exactly referred to as "adequate levels" of b-p*F* or intestinal butyrate, also considering that a state of eubiosis results from a balance among all species of our microbiota. Because of culturing and functional characterization difficulties, new strategies are warranted to study the functional group of bp-*F*, later allowing pre-clinical and interventation studies necessary to confirm their usefulness in the treatment of this condition. Furthermore, clinical studies on human are necessary also to define if patients would benefit from an ATI-reduced or -free diet, as well as from IAP and/or butyrate supplementation.

We are aware that the objective which we aim at with this paper could seem ambitious, but we think that it is our duty as part of scientific community, to continuously pose questions and formulate related hypothetical plausible theories, even if not yet supported by experimental evidence: such theories could be at the basis of developing new research projects, which could lead to a step forward in the comprehension of critical aspects in a field, even if negative results are obtained. With this paper, in a very humbly way, we would like to make our idea/intuition available to scientific community, to stimulate it in investigating at various levels about aspects not yet taken into account, and that could have a role in the onset of NCG/WS. In our hypothesis we have critically put together recent scientific evidence in NCG/WS, highlighting diagnostic difficulties and absence of standardized procedures. We are aware that our proposed integrated vision opens up more questions than it closes, by taking into account many aspects which were never been expressly linked together till now in regard to NCG/WS, such as ATIs, IAP, LPS, butyrate and microbiota.

Coordinated studies in different areas of research will be necessary to confirm or reject our hypotheses, and to develop a full understanding of the pathogenesis of NCG/WS, that still seems to retain many intriguing secrets to uncover.

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Review

Probiotic Supplementation in Preterm Infants Does Not Affect the Risk of Bronchopulmonary Dysplasia: A Meta-Analysis of Randomized Controlled Trials

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Abstract: Probiotic supplementation reduces the risk of necrotizing enterocolitis (NEC) and late-onset sepsis (LOS) in preterm infants, but it remains to be determined whether this reduction translates into a reduction of other complications. We conducted a systematic review and meta-analysis to evaluate the possible role of probiotics in altering the risk of bronchopulmonary dysplasia (BPD). Fifteen randomized controlled trials (4782 infants; probiotics: 2406) were included. None of the included studies assessed BPD as the primary outcome. Meta-analysis confirmed a significant reduction of NEC (risk ratio (RR) 0.52, 95% confidence interval (CI) 0.33 to 0.81, $p = 0.004$; random effects model), and an almost significant reduction of LOS (RR 0.82, 95% CI 0.65 to 1.03, $p = 0.084$). In contrast, meta-analysis could not demonstrate a significant effect of probiotics on BPD, defined either as oxygen dependency at 28 days of life (RR 1.01, 95% CI 0.91 to 1.11, $p = 0.900$, 6 studies) or at 36 weeks of postmenstrual age (RR 1.07, 95% CI 0.96 to 1.20, $p = 0.203$, 12 studies). Meta-regression did not show any significant association between the RR for NEC or LOS and the RR for BPD. In conclusion, our results suggest that NEC and LOS prevention by probiotics does not affect the risk of developing BPD in preterm infants.

Keywords: probiotics; bronchopulmonary dysplasia; sepsis; necrotizing enterocolitis

1. Introduction

Bronchopulmonary dysplasia (BPD), a chronic lung disease of prematurity, is considered one of the major complications of premature birth [1–4]. The incidence of BPD is inversely proportional to gestational age, with rates reaching up to 60–90% in extremely preterm infants (22–25 weeks gestation). Infants suffering from BPD are at increased risk of death and long-term pulmonary and neurodevelopmental morbidities [5–7].

The pathogenesis of BPD is initiated by the arrest in alveolar and lung vascular development, due to premature birth, and sustained by inflammatory events that play a paramount role in the progression of BPD [3,4,8,9]. The initiation of the inflammatory response can already occur in utero, in the setting of chorioamnionitis [3,4,10,11]. Nevertheless, postnatal stimuli, such as the ex-utero higher oxygen partial pressures, the need for oxygen administration or mechanical ventilation, and the occurrence of postnatal infections (including late onset sepsis (LOS) and necrotizing enterocolitis

(NEC)), perpetuate inflammation and lead to the establishment of BPD [12–14]. A dysregulation of the immune system, toward a sustained status of inflammation which is characteristic of very preterm infants, completes the multifactorial pathophysiological picture [15].

Several treatments, most of which focused on anti-inflammatory or homeostasis-restoring properties, have been attempted in order to prevent or treat BPD [16]. However, meta-analyses could confirm a reduction of BPD only for vitamin A and dexamethasone [16,17]. Moreover, vitamin A showed only a modest effect [17], while the use of dexamethasone is limited in preterm infants by its well-known long- and short-term side effects [18]. Adequate timing, dose, and formulation of steroid therapy is still under investigation in preterm infants at risk for BPD. Lately, regenerative medicine has received a great deal of attention as a promising therapeutic option for complications of prematurity, including BPD [19,20]. However, the knowledge of stem cell function is still incomplete, and further studies are needed to elucidate the impact of several manufacturing aspects that may determine the success or failure of this therapy [19,20]. In summary, despite the continuous advances in neonatal care, BPD remains a significant burden for the premature population, lacking a safe, effective and easily available treatment.

Probiotics are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host [21,22]. Probiotic supplementation in preterm infants is one of the most studied interventions in neonatal medicine [23–30]. Many randomized controlled trials (RCTs) involving the use of probiotics have been performed in the last years. Several meta-analyses combined these RCTs and demonstrated that probiotic supplementation reduces mortality, NEC, and LOS, as well as the time to achieve full enteral feeding in preterm infants [23–31]. Although until now no study has been performed to analyze the effect of probiotics on BPD as primary outcome, a number of RCTs included BPD as a secondary outcome. There are several hypothetical mechanisms by which probiotics may exert a protective effect against BPD: (1) by reducing postnatal inflammatory processes such as NEC and LOS; (2) by modulating the immune function [32,33]; (3) by improving the nutritional status and growth of the infants [30,31,34]; and (4) through the antioxidant properties of probiotics [35]. Therefore, in the present systematic review we aimed to collect and analyze the current evidence on the effects of probiotic supplementation on the risk of developing BPD in preterm infants.

2. Materials and Methods

A protocol was developed prospectively that detailed the specific objectives, criteria for study selection, the approach to assessing study quality, clinical outcomes, and statistical methodology. The study is reported according to the PRISMA checklist [36].

2.1. Data Sources and Search Strategies

A comprehensive literature search was undertaken using PubMed, EMBASE and CENTRAL (the Cochrane Central Register of Controlled Trials, The Cochrane Library) from their inception to 1 July 2017. Combinations of the following terms (including MeSH terms) were used to search for relevant publications: (probiotic(s) OR lactobacillus OR saccharomyces OR bifidobacterium OR streptococcus) AND ("preterm infant" OR "premature infant" OR "extremely low birth weight infant" OR "very low birth weight infant"). Language was not restricted. Additional strategies to identify studies included manual review of reference lists of key articles that fulfilled our eligibility criteria, use of the "related articles" feature in PubMed, use of the "cited by" tool in Web of Science and Google Scholar, and manual review of reference lists of meta-analyses on probiotics in preterm infants [23–25,27,28,30,34,37–44]. The search method used to identify all relevant articles was discussed and developed by two authors (EV-M and EV) and the final search string was approved by all authors.

2.2. Eligibility Criteria and Study Selection

The initial search was performed by two reviewers (EV-M and EV), who eliminated clearly irrelevant articles based on the title and abstract as defined by the pre-set selection criteria. The final

selection of articles was made by mutual consideration of both authors. Studies were included if they were RCTs involving the use of probiotics in preterm infants (gestational age, GA < 37 weeks) and reported results on BPD. BPD was defined as dependence on supplementary oxygen either at 28 days of life (BPD28) or at a postmenstrual age (PMA) of 36 weeks (BPD36) [2]. However, the use of another BPD definition was not an exclusion criterion.

Studies were reviewed to ensure that study populations did not overlap by checking subject sources and studying time-frame. Where two or more studies reported on the same population, the most recent study was preferentially used (provided it reported data on BPD) to avoid duplicate data.

2.3. Data Extraction and Assessment of Risk of Bias

Two groups of investigators (EV-M/EV and MP/GC) extracted the data independently by using a data collection form designed for this review. Data extracted included: gestational age (GA) and birth weight (BW) of participants, patient inclusion criteria, study design (age at the first day of intervention, duration of intervention, dosage, and type of probiotic), and outcomes of interest (BPD, LOS, NEC, and mortality).

Two reviewers (EV-M and EV) independently assessed risk of bias in each trial by using the Cochrane "Risk of Bias Assessment Tool" [45]. For each domain (allocation sequence, allocation concealment, blinding of participants and outcome assessors, incomplete outcome data, selective outcome reporting, and other potential sources of bias) the risk of bias was assessed as low, high, or unclear. Potential discrepancies during the data extraction process and assessment of risk of bias were resolved by discussion and consensus among all reviewers.

2.4. Statistical Analysis

Studies were combined and analyzed using comprehensive meta-analysis V3.0 software (Biostat Inc., Englewood, NJ, USA). We used a random-effects model to account for anticipated heterogeneity, resulting from the differences in methodology between studies. However, analysis using a fixed-effect model was also carried out to ensure that the model used for the meta-analysis would not affect the results. Effect size was expressed as Mantel–Haenszel risk ratio (RR) and 95% confidence interval (CI). Statistical heterogeneity was assessed with the Cochran's Q statistic and by the I^2 statistic, which is derived from Q and describes the proportion of total variation that is due to heterogeneity beyond chance [45]. An I^2 value of 0% indicates no observed between-study heterogeneity, and large values show increasing between-study heterogeneity. The risk of publication bias was assessed by visual inspection of the funnel plot and using an Egger test. To identify any study that may have exerted a disproportionate influence on the summary effect, we calculated the summary effect excluding studies one at a time. To explore differences between studies that might be expected to influence the effect size, we performed subgroup sensitivity analysis and univariate random-effects meta-regression (method of moments) [46,47]. A potential pitfall with meta-regression analysis is that with few trials and many possible covariates, false positive findings and data dredging can happen [47]. We chose to prespecify NEC, LOS, and mortality as covariates to analyze with meta-regression to protect against this issue. A probability value of less than 0.05 (0.10 for heterogeneity) was considered statistically significant.

3. Results

There was no substantial disagreement between reviewers on articles for inclusion, data extraction, and risk of bias assessment. Based on the titles and abstracts of 1456 citations, we identified 63 potentially relevant studies, of which 15 met the inclusion criteria [48–62] (Figure 1). The main characteristics of the studies are shown in Table 1. The 15 studies included 4782 infants of which 2406 infants received probiotics. Twelve studies [48–51,53–58,60,62] included very preterm (GA < 32 weeks) and/or very low BW (VLBW) infants (<1500 g). One study [48] included extremely low BW preterm infants (<1000 g). Two studies included larger preterm infants; one [52] included infants with GA < 34 weeks and the other [59] included infants with GA < 37 weeks. The included

studies randomized infants to different preparations, times of initiation, and duration of therapy (Table 1). Details of the risk of bias analysis are depicted in Appendix A, Table A1. None of the included studies reported serious adverse events potentially associated with the use of probiotics.

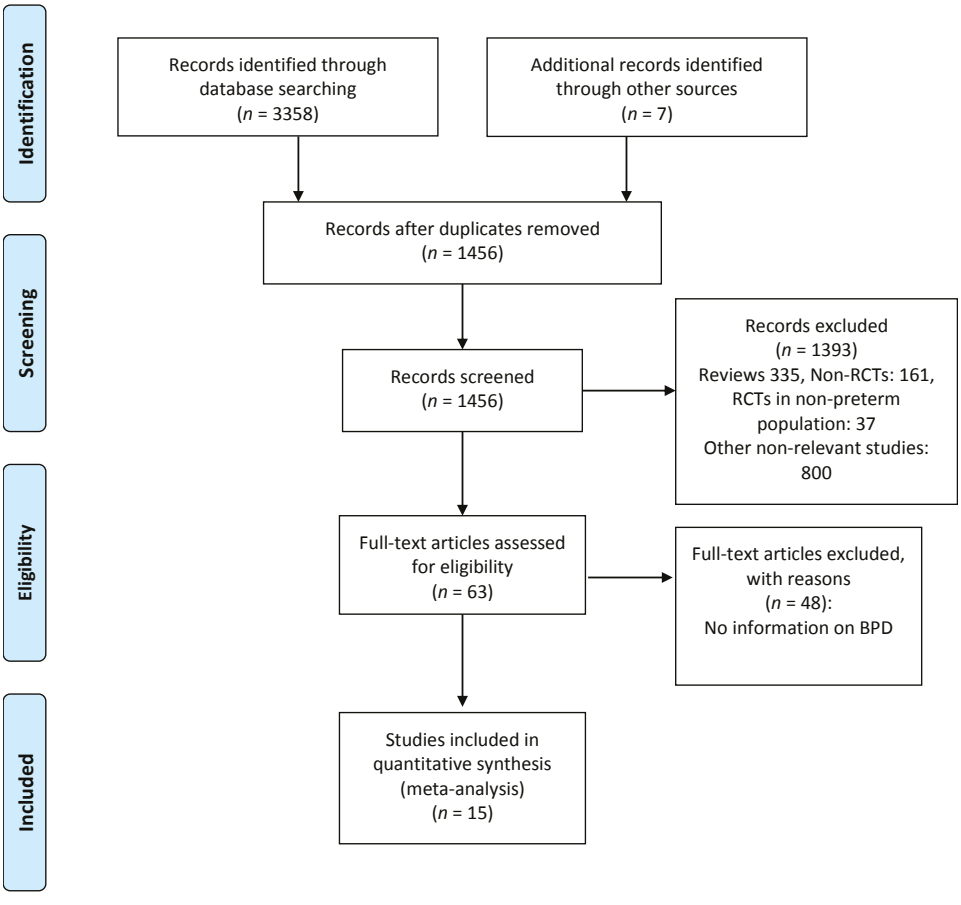

Figure 1. Flow diagram of literature search process. RCTs: randomized controlled trials; BPD: bronchopulmonary dysplasia.

Table 1. Characteristics of the included studies.

Study	Participants	Sample Size, GA (Weeks), BW (g)		Intervention	Duration of Intervention	Primary Outcome	BPD Definition
		Probiotics	Control				
Akar 2017 [49]	GA ≤ 32 weeks or BW ≤ 1500 g	*n* = 124 GA: 28.9 (2.1) BW: 1138 (257)	*n* = 125 GA: 28.6 (2.5) BW: 1142 (267)	*Lactobacillus reuteri* vs. no probiotics	From first feed until discharge	Neurodevelopmental outcome	BPD36
Al Hosni 2012 [48]	BW 501–1000 g	*n* = 50 GA: 25.7 (1.4) BW: 778 (138)	*n* = 51 GA: 25.7 (1.4) BW: 779 (126)	*Lactobacillus rhamnosus* + *Bifidobacterium infantis* vs. no probiotics	Once daily from the time of initiation of enteral feeds, until discharge or 34 weeks PMA	% infants <10th centile at 34 weeks PMA	BPD36
Costeloe 2016 [62]	GA < 31 weeks	*n* = 650 GA (median): 28.0 (IQR: 26.1–29.4) BW: 1039 (312)	*n* = 660 GA (median): 28.0 (IQR: 26.1–29.6) BW: 1043 (317)	*Bifidobacterium breve* BBG-001 vs. placebo	Commenced within 48 hours of birth, until 36 weeks PMA or discharge	NEC ≥ stage 2, LOS, death	BPD36, Severe BPD
Demirel 2013 [50]	GA ≤ 32 weeks and BW ≤ 1500 g	*n* = 135 GA: 29.4 (2.3) BW: 1164 (261)	*n* = 136 GA: 29.2 (2.5) BW: 1131 (284)	Saccharomyces *boulardii* vs. no probiotics	Once daily from the time of initiation of enteral feeds, until discharge	NEC ≥ stage 2 or death	BPD28
Dilli 2015 [51]	GA < 32 weeks and BW < 1500 g	*n* = 100 GA: 28.8 (1.9) BW: 1236 (212)	*n* = 100 GA: 28.2 (2.2) BW: 1147 (271)	*Bifidobacterium lactis* vs. placebo	From day 8 of life, once daily until discharge or a maximum of 8 weeks	NEC ≥ stage 2	BPD28, BPD36
Fujii 2006 [52]	GA < 34 weeks	*n* = 11 GA: 31.3 (3.2) BW: 1378 (365)	*n* = 8 GA: 31.2 (2.0) BW: 1496 (245)	*B. breve* M-16V vs. placebo	From several hours after birth until discharge	Serum cytokine levels and expression of Transforming growth factor beta signaling Smad molecules	BPD28
Jacobs 2013 [53]	GA < 32 weeks and BW < 1500 g	*n* = 548 GA: 27.9 (2.0) BW: 1063 (259)	*n* = 551 GA: 27.8 (2.0) BW: 1048 (260)	*B. infantis* + Saccharomyces *thermophilus* + *B. lactis* vs. placebo	From enteral feed ≥ 6 mL/day until discharge or term corrected age.	LOS	BPD28, BPD36
Lin 2008 [54]	GA <34 weeks and BW < 1500 g	*n* = 217 BW: 1029 (246)	*n* = 217 BW: 1077 (214)	*Lactobacillus acidophilus* + *Bifidobacterium* bifidum vs. no probiotics	From first feeding, for 6 weeks.	Death or NEC ≥ Stage 2	BPD36
Manzoni 2009 [55]	BW < 1500 g	*n* = 151 GA: 29.8 (2.8) BW: 1138 (253)	*n* = 168 GA: 29.5 (3.2) BW: 1109 (269)	*L. rhamnosus* GG + lactoferrin vs. placebo	From day 3 of life, for 6 weeks or until discharge	LOS	BPD36
Saengtawesin 2014 [56]	GA ≤ 34 weeks and BW ≤ 1500 g	*n* = 31 GA: 31.0 (1.8) BW: 1250 (179)	*n* = 29 GA: 30.6 (1.8) BW: 1208 (199)	*L. acidophilus* + *B. bifidum* vs. no probiotics	From first enteral feed until 6 weeks of age or discharge	NEC ≥ Stage 2	BPD28

Table 1. *Cont.*

Study	Participants	Sample Size, GA (Weeks), BW (g)		Intervention	Duration of Intervention	Primary Outcome	BPD Definition
		Probiotics	Control				
Sari 2012 [57]	GA <33 or BW < 1500 g	*n* = 86 GA: 29.7 (2.5) BW: 1241 (264)	*n* = 88 GA: 29.8 (2.3) BW: 1278 (273)	*Lactobacillus sporogenes* vs. no probiotics	From first enteral feed until discharge	Growth and neurodevelopment at 18–22 months	BPD36
Serce 2013 [58]	GA ≤ 32 weeks and BW ≤ 1500 g	*n* = 104 GA: 28.8 (2.2) BW: 1126 (232)	*n* = 104 GA: 28.7 (2.1) BW: 1162 (216)	*S. boulardii* vs. placebo	From first enteral feed until discharge	NEC ≥ Stage 2 or death or LOS	BPD36
Stratiki 2007 [59]	GA 27–37 weeks	*n* = 41 GA (median): 31 (range: 27–37) BW (median): 1500 (range: 900–1780)	*n* = 36 GA (median): 30.5 (range: 26–37) BW (median): 1500 (range: 700–1900)	*B. lactis* vs. no probiotics	From day 2 to discharge	Intestinal permeability by the sugar absorption test	Undefined
Totsu 2014 [60]	BW < 1500 g	*n* = 153 GA: 28.6 (2.9) BW: 1016 (289)	*n* = 130 GA: 28.5 (3.3) BW: 998 (281)	*B. bifidum* vs. placebo	Commenced within 48 h of birth and continued until discharge	Postnatal day when enteral feed exceeding 100 mL/kg/day	BPD28, BPD36
Underwood 2009 (CUL) [61] [1]	GA < 35 weeks and BW 750–2000 g	*n* = 30 GA: 29.5 (2.6) BW: 1394 (356)	*n* = 29 GA: 29.3 (2.6) BW: 1393 (363)	*L. rhamnosus* GG + inulin vs. placebo	From first feed until 28 days or discharge	Weight gain	BPD36
Underwood 2009 (PBP) [61] [1]	GA < 35 weeks and BW 750–2000 g	*n* = 31 GA: 30.2 (2.4) BW: 1461 (372)	*n* = 29 GA: 29.3 (2.6) BW: 1393 (363)	*L. acidophilus* + *Bifidobacterium longum* + *B. bifidum* + *B. infantis* + inulin vs. placebo	From first feed until 28 days or discharge	Weight gain	BPD36

[1] Culturelle® (CUL) and ProBioPlus DDS® (PBP) were the names assigned by the authors to the probiotic preparations. BPD: bronchopulmonary dysplasia; BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; Severe BPD: defined as any baby at 36 weeks PMA still receiving mechanical ventilator support or in at least 30% oxygen or more than 0.1 L/min of low flow oxygen. BW: birth weight; GA: gestational age; IQR: interquartile range; NEC: necrotizing enterocolitis; PMA: postmenstrual age; LOS: late-onset sepsis. Data for GA and BW given in mean (standard deviation), unless noted otherwise.

BPD was not the primary outcome in any of the included studies. Six studies [53,55,58,60–62] clearly defined BPD as BPD28 and/or BPD36, whereas nine studies did not [48–52,54,56,57,59]. A clarification on BPD definition was kindly provided by the authors of eight studies [48–52,54,56,57]. After these clarifications, data on BPD28 were available from six studies [50–53,56,60]. We decided to pool the study of Stratiki et al. [59] that did not specify a BPD definition, with studies reporting BPD28. Neither the individual studies nor the meta-analysis could detect a significant effect of probiotic supplementation on BPD28 (RR 1.01, 95% CI 0.91 to 1.11, $p = 0.900$, Figure 2). The use of a fixed effect model instead of a random effects model did not significantly affect the results of the meta-analysis (RR 1.00, 95% CI 0.91 to 1.10, $p = 0.999$). In sensitivity analyses, excluding one study at a time, the summary RR ranged from 0.99 (95% CI 0.89–1.10, $p = 0.900$), when the study of Totsu et al. [60] was excluded, to 1.04 (95% CI 0.86–1.25, $p = 0.703$), when the study of Jacobs et al. [53] was excluded (Appendix A, Table A2). The study of Fujii et al. [52] included larger infants than the other five studies (Table 1). However, when this study was excluded, overall results were not substantially affected (RR 1.01, 95% CI 0.91–1.11, $p = 0.983$). Exclusion of the study by Stratiki et al. [59], in which BPD was not clearly defined, did not significantly affect results (RR 1.01 95% CI 0.91–1.11, $p = 0.829$). Further sensitivity analysis and assessment of publication bias were not performed for BPD28 due to the low number of studies.

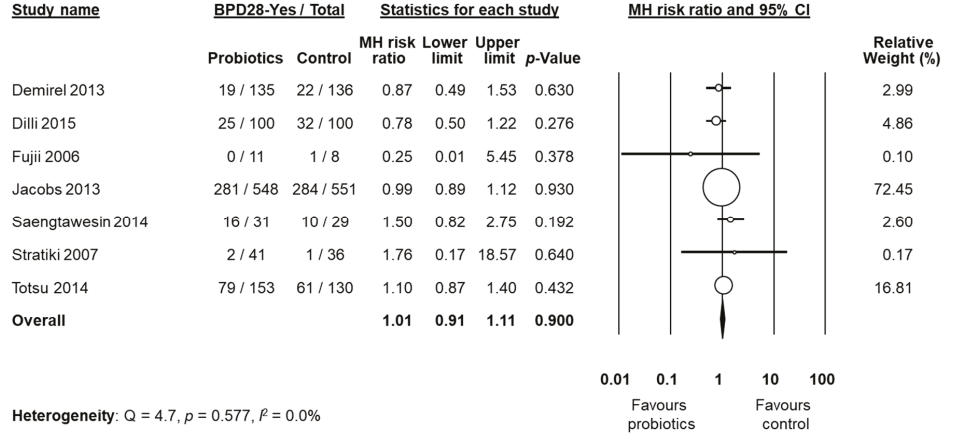

Figure 2. Random effects meta-analysis: Probiotic supplementation and risk of BPD28 (bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life). MH: Mantel–Haenszel; CI: confidence interval.

Data on BPD36 were available from 11 studies [48,49,51,53–55,57,58,60–62]. The study of Underwood et al. [61] randomized infants into three different groups: a placebo group and two treatment groups based on different probiotic preparations (Table 1). For the purposes of this analysis, the two treatment groups of the trial of Underwood et al. [61] were considered as two separate studies. The study of Lin et al. [54], showed a significant increase of the BPD36 risk in the infants receiving probiotics (RR 1.38, 95% CI 1.01 to 1.88, $p = 0.043$). In contrast, neither the other individual studies nor the meta-analysis could detect a significant effect of probiotic supplementation on BPD36 (RR 1.07, 95% CI 0.96 to 1.20, $p = 0.203$, Figure 3). Although some degree of asymmetry was observed by visual inspection of the funnel plot, Egger's test could not show any evidence of publication bias (Figure 4). The use of a fixed effect model instead of a random effects model did not significantly affect the results of the meta-analysis (RR 1.08, 95% CI 0.98 to 1.18, $p = 0.123$). In sensitivity analyses, excluding one study at a time, the summary RR ranged from 1.04 (95% CI 0.93–1.17, $p = 0.488$), when the study of

Lin et al. [54] was excluded, to 1.09 (95% CI 0.97–1.23, $p = 0.138$), when the study of Al Hosni et al. [48] was excluded (Appendix A Table A3).

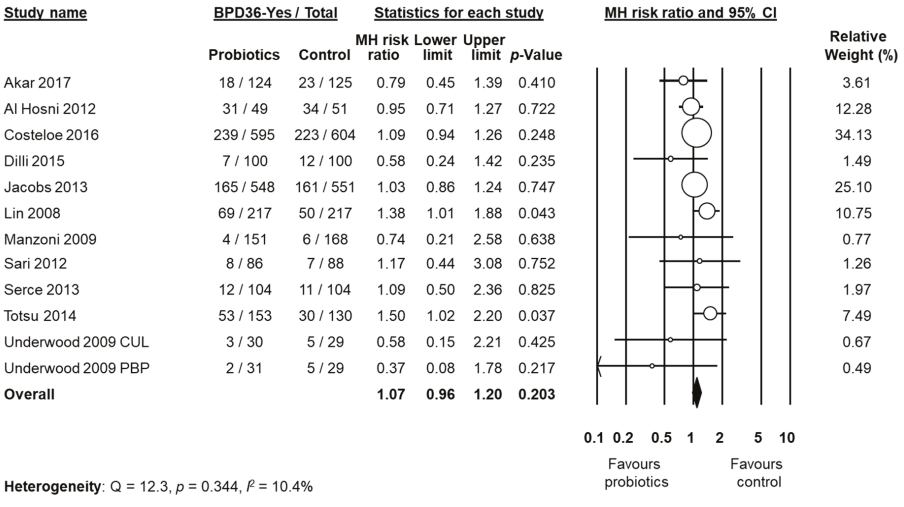

Figure 3. Random effects meta-analysis: Probiotic supplementation and risk of BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age). MH: Mantel–Haenszel; CI: confidence interval. CUL: Culturelle preparation; PBP: ProBioPlus DDS preparation.

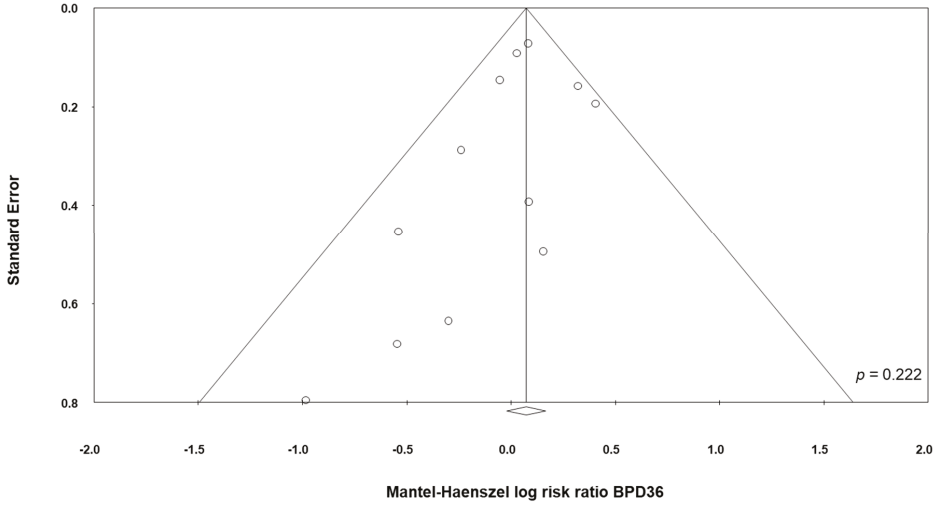

Figure 4. Funnel plot assessing publication bias for BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age).

One study [62] also included, besides BPD36, the category severe BPD (defined as any baby at 36 weeks PMA still receiving mechanical ventilator support or in at least 30% oxygen or more than 0.1 L/min of low flow oxygen) (Table 2). They report that the probiotics group did not have a significantly different risk of severe BPD compared to the control group (RR 1.21, 95% CI 0.90 to 1.62, $p = 0.200$).

Table 2. Subgroup analysis of probiotics and risk of BPD.

Subgroup	*k*	BPD Definition	Sample Size	MH RR	95% CI	*p*
Studies where *Lactobacillus* was part of the supplementation	6	BPD36	1335	1.01	0.80–1.29	0.904
Studies where *Bifidobacterium* was part of the supplementation	5 4	BPD28 BPD36	1601 2781	1.00 1.10	0.90–1.11 0.90–1.33	0.999 0.346
Single-strain supplementation	4 7	BPD28 BPD36	773 2372	0.97 1.08	0.79–1.18 0.88–1.32	0.763 0.480
Multiple-strain supplementation	2 5	BPD28 BPD36	1159 2012	1.01 1.06	0.90–1.13 0.87–1.29	0.829 0.574
Studies with infants mean BW < 250 g	5 9	BPD28 BPD36	1913 4091	1.01 1.08	0.91–1.11 0.96–1.22	0.893 0.195
Studies with low risk of bias on random sequence generation and allocation concealment	9	BPD36	3752	1.08	0.97–1.19	0.155
Studies with low risk of bias on incomplete outcome data	10	BPD36	3927	1.09	0.96–1.23	0.188
Studies with low risk of bias on selective reporting	9	BPD36	3493	1.06	0.94–1.18	0.344

BPD: bronchopulmonary dysplasia; BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; CI: confidence interval; *k*: number of studies included; MH RR: Mantel–Haenszel risk ratio.

For the outcome BPD36, we conducted additional sensitivity analysis by excluding studies that had uncertain/high risk of bias in the different domains. In addition, we carried out subgroup analyses of studies where *Bifidobacterium* was part of the supplementation, studies where *Lactobacillus* was part of the supplementation, studies where multiple-strain supplements were used, studies where single-strain supplements were used, and studies where infants had a mean BW < 1250 g. No subgroup analysis could demonstrate a significant effect of probiotics on BPD36 (Table 2).

All the included studies reported data on NEC (Table 3) and, when pooled, we observed that probiotics significantly reduced the risk of developing NEC (RR 0.52, 95% CI 0.33–0.81, $p = 0.004$, Table 4). This significant reduction of NEC was also observed when we pooled the studies that reported BPD28 (RR 0.40, 95% CI 0.18–0.88, $p = 0.022$), and when we pooled the studies that reported BPD36 (RR 0.48, 95% CI 0.29–0.81, $p = 0.006$, Table 4). We performed meta-regression analyses (methods of moments) to investigate the possible correlation between the effect size for NEC and the effect size for BPD. As shown in Figure 5, meta-regression could not detect a statistically significant correlation between the reduction in NEC produced by the probiotics and the effect size for BPD36.

All the included studies reported data on LOS (Table 3), and meta-analysis demonstrated a close to significant reduction of LOS in the probiotics group (RR 0.82, 95% CI 0.65–1.03, $p = 0.084$, Table 4). Similarly, the meta-analysis of studies that reported BPD28 found a close to significant effect of probiotics on LOS (RR 0.79, 95% CI 0.63–1.00, $p = 0.054$), and the meta-analysis of studies that reported BPD36 found a close to significant reduction in LOS (RR 0.80, 95% CI 0.62–1.04, $p = 0.090$, Table 4). We performed meta-regression analyses (methods of moments) to investigate the possible correlation between the effect size for LOS and the effect size for BPD36. As shown in Figure 6, meta-regression could not detect a statistically significant correlation between the reduction in LOS produced by the probiotics and the effect size for BPD36.

Table 3. NEC, LOS and mortality in the included studies.

Study	NEC (Affected/Total)		NEC Definition	LOS (Affected/Total)		LOS Definition	Mortality (Affected/Total)		Mortality Definition
	Probiotics	Control		Probiotics	Control		Probiotics	Control	
Akar 2017 [49]	1/124	6/125	NEC stage $\geq$ 2	8/124	19/125	Culture-proven sepsis	14/200	16/200	Death before 18–24 month follow-up
Al Hosni 2012 [48]	2/50	2/51	NEC stage $\geq$ 2	13/50	16/51	Culture-proven sepsis	3/50	4/51	Death before 34 weeks PMA
Costeloe 2016 [62]	61/650	66/660	NEC stage $\geq$ 2	73/650	77/660	Culture-proven sepsis > 72 h	54/650	56/660	Death during primary hospitalization
Demirel 2013 [50]	6/135	7/136	NEC stage $\geq$ 2	20/135	21/136	Culture-proven sepsis	5/135	5/136	Death after 7 days of life
Dilli 2015 [51]	2/100	18/100	NEC stage $\geq$ 2	8/100	13/100	Culture-proven sepsis > 72 h	3/100	12/100	Not defined
Fujii 2006 [52]	0/11	0/8	Not defined	1/11	1/8	Not defined	0/11	0/8	Death during primary hospitalization
Jacobs 2013 [53]	11/548	24/551	NEC stage $\geq$ 2	72/548	89/551	Culture-proven sepsis > 48 h	30/548	31/551	Death during primary hospitalization
Lin 2008 [54]	4/217	14/217	NEC stage $\geq$ 2	40/217	24/217	Culture-proven > 72h	2/217	9/217	Death during intervention (6 weeks)
Manzoni 2009 [55]	0/151	10/168	NEC stage $\geq$ 2	7/151	29/168	Culture-proven sepsis > 72 h	6/153	12/168	Death during primary hospitalization
Saengtawesin 2014 [56]	1/31	1/29	NEC stage $\geq$ 2	2/31	1/29	Not defined	0/31	0/29	Death during primary hospitalization
Sari 2012 [57]	3/86	7/88	NEC stage $\geq$ 2	24/86	19/88	Not defined	5/110	8/111	Death before 18 to 22 months of age
Serce 2013 [58]	7/104	7/104	NEC stage $\geq$ 2	19/104	25/104	Culture-proven sepsis	5/104	4/104	Death during primary hospitalization
Stratiki 2007 [59]	0/41	3/36	NEC stage $\geq$ 2	0/41	3/36	Culture-proven sepsis	0/41	0/36	Not defined
Totsu 2014 [60]	0/153	0/130	NEC stage $\geq$ 2	6/153	10/130	Culture-proven sepsis $\geq$ 1 week	2/153	0/130	Death during primary hospitalization
Underwood 2009 (CUL) [61] [1]	1/30	1/29	NEC stage $\geq$ 2	4/30	4/29	Culture-proven sepsis > 72 h	0/30	0/29	Death during primary hospitalization
Underwood 2009 (PBP) [61] [1]	1/31	1/29	NEC stage $\geq$ 2	2/31	4/29	Culture-proven sepsis > 72 h	0/31	0/29	Death during primary hospitalization

[1] Culturelle (CUL) and ProBioPlus DDS (PBP) were the names assigned by the authors to the probiotic preparations. LOS: late-onset sepsis; NEC: necrotizing enterocolitis.

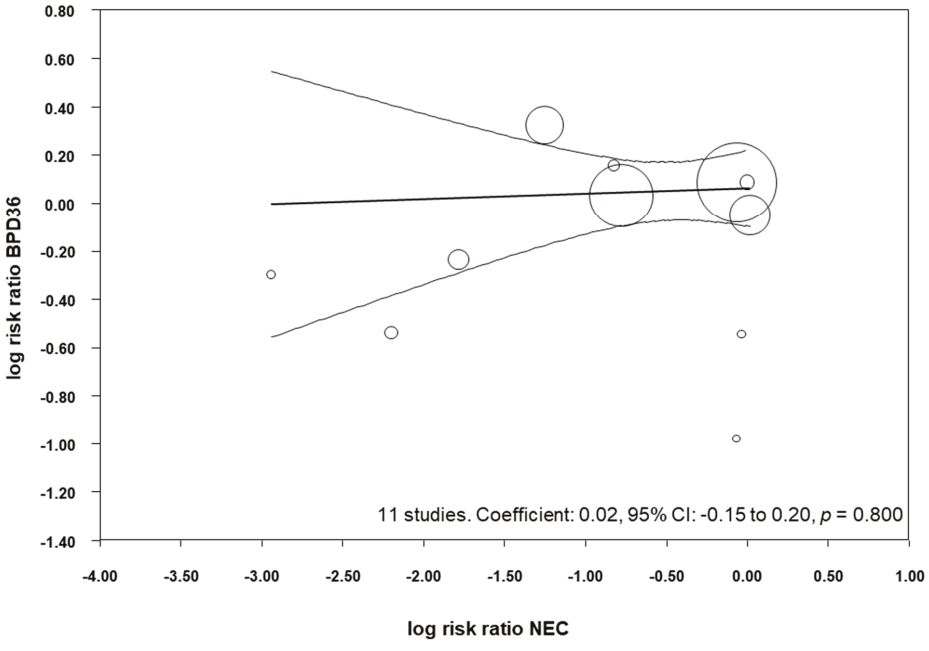

Figure 5. Meta-regression plot of probiotics and risk of BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age) and probiotics and risk of necrotizing enterocolitis (NEC), CI: confidence interval.

Table 4. Random effects meta-analysis of probiotics and LOS, NEC and mortality.

Meta-Analysis	*k*	BPD Definition	MH Risk Ratio	95% CI	Z	*p*	Heterogeneity		
							Q	*p*	I^2
Probiotics NEC	15	All	0.52	0.33 to 0.81	−2.88	0.004	22.0	0.055	40.9%
	7	BPD28	0.40	0.18 to 0.88	−2.29	0.022	6.3	0.175	37.0%
	12	BPD36	0.48	0.29 to 0.81	−2.73	0.006	20.4	0.025	51.1%
Probiotics LOS	15	All	0.82	0.65 to 1.03	−1.73	0.084	26.8	0.031	44.0%
	7	BPD28	0.79	0.63 to 1.00	−1.93	0.054	3.6	0.72	0.0%
	12	BPD36	0.80	0.62 to 1.04	−1.70	0.090	24.5	0.011	55.1%
Probiotics mortality	11	All	0.84	0.66 to 1.07	−1.38	0.169	10.4	0.410	3.4%
	4	BPD28	0.78	0.37 to 1.66	−0.65	0.518	5.2	0.155	42.8%
	10	BPD36	0.82	0.62 to 1.07	−1.45	0.146	10.3	0.328	12.5%

BPD: bronchopulmonary dysplasia; BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; CI: confidence interval; *k*: number of studies included; LOS: late onset-sepsis; MH: Mantel–Haenszel; NEC: necrotizing enterocolitis.

All the included studies reported data on mortality (Table 3), but meta-analysis could not demonstrate a significant reduction of mortality in the probiotics group (RR 0.80, 95% CI 0.60–1.06, $p = 0.114$, Table 4). Moreover, the meta-analysis of studies that reported BPD28 could not find a significant effect of probiotics on mortality (RR 0.78, 95% CI 0.37 to 1.66, $p = 0.518$), and neither could the meta-analysis of studies that reported BPD36 (RR 0.77, 95% CI 0.56 to 1.05, $p = 0.101$). We performed meta-regression analyses (methods of moments) to investigate the possible correlation between the effect size for mortality and the effect size for BPD36. This meta-regression could not detect a statistically significant correlation between the changes in mortality produced by the probiotics and the effect size for BPD36 (coefficient 0.04, 95% CI −0.13 to 0.21, $p = 0.638$).

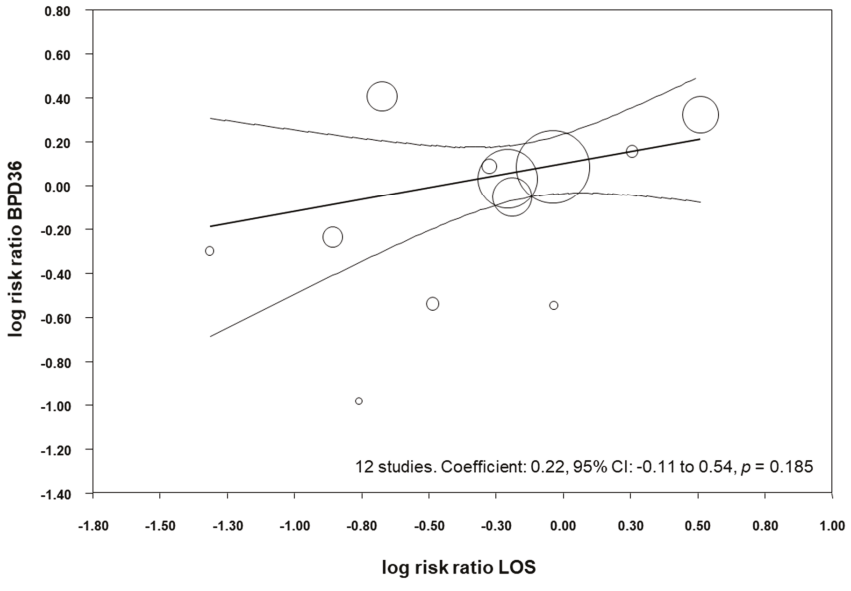

Figure 6. Meta-regression plot of probiotics and risk of BPD36 (bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age) and probiotics and risk of late-onset sepsis (LOS), CI: confidence interval.

4. Discussion

Inflammatory events, such as NEC and LOS, are not only life-threatening for (very) preterm infants but also may mediate major short- and long-term adverse outcomes [63,64]. Current evidence indicates that probiotic supplementation significantly reduces NEC and LOS in preterm infants, but our data suggest that this decrease is not accompanied by a concomitant reduction in BPD. The present meta-analysis could not demonstrate any significant effect of probiotic supplementation on the risk of developing of BPD. Similarly, in a recent meta-analysis we found that probiotics did not significantly affect the risk of retinopathy of prematurity (ROP) [44]. However, our results should be interpreted with caution since the included RCTs showed relevant methodological differences in terms of enrolment criteria, timing, dose, and formulation of the probiotics used. Moreover, BPD was not the primary outcome in any of the studies and the number of RCTs of probiotics reporting on BPD as secondary outcome was relatively small. In addition, none of the included studies specifically targeted the most vulnerable population for BPD (infants < 28 weeks GA).

Inflammatory processes such as NEC and LOS may increase the risk of developing BPD through direct and indirect mechanisms. Proinflammatory cytokines may exert a direct effect on lung development or sensitize the lung to the effects of oxygen, mechanical ventilation, or other stressors [8,9,15,65]. On the other hand, infants suffering from NEC and LOS often require more aggressive and prolonged mechanical ventilation, that may lead to increased lung injury [8,9,15,65]. It has been suggested that avoiding postnatal infection is more important than avoiding invasive mechanical ventilation to decrease the inflammatory response in developing lungs [65]. Studies directed at evaluating the impact of quality improvement efforts to reduce LOS in preterm infants showed that a reduction in LOS is accompanied by decreased rates of BPD [66,67]. However, BPD is a multifactorial condition in which genetic predisposition, as well as prenatal and prenatal conditions all play a role [1–4]. In an interesting study, Lapcharoensap et al. showed a positive relationship between the reduction in LOS and the reduction in BPD with a coefficient of determination (r^2) of 0.08,

suggesting that only the 8% of the reduction of BPD is attributable to the reduction in nosocomial infection rates [67].

The 15 studies included in our meta-analysis represent a subset of the larger number of RCTs included in the meta-analyses on probiotics for NEC and LOS prevention. Therefore, we analysed whether the protective effects of probiotics on NEC and/or LOS were also present in the RCTs included in our study. Pooling the 15 studies showed a significant reduction of NEC (RR 0.52, 95% CI 0.33 to 0.81) and a close to significant reduction of LOS (RR 0.82 95% CI 0.65 to 1.03) in the probiotics group. We speculated that studies with higher protective effects against NEC and/or LOS would show more effect on the development of BPD. However, meta-regression did not show a significant correlation between the RR for NEC and LOS and the RR for BPD. This suggests that the reduction in postnatal inflammatory events did not translate into a reduction of BPD.

Several meta-analyses showed that probiotics reduce mortality among VLBW infants [23,25,38]. It has been suggested that improved survival of VLBW infants may result in increased numbers of patients with BPD [68]. In the group of studies included in our meta-analysis, we could not observe a significant effect of probiotics on mortality (RR 0.80, 95% CI 0.60 to 1.06). In addition, meta-regression could not show a significant correlation between the RR for mortality and the RR for BPD. Therefore, our data suggest that the effect of probiotics on mortality did not affect the rate of BPD in the RCTs. Nevertheless, a robust conclusion from meta-regression would require a larger number of included studies [46,47].

One important limitation inherent to any meta-analysis on BPD is the heterogeneity of the definition of the condition [16,69,70]. In a systematic review which included 47 RCTs of drugs for BPD, 34% did not identify the definition of BPD that was used. Of the trials that defined BPD, 22 used oxygen dependency at 36 weeks PMA, with two trials refining that definition with a test of oxygen need [16]. Fourteen trials provided data on oxygen requirement and four trials used both oxygen supplementation at 28 days and oxygen supplementation at 36 weeks PMA [16]. Similarly, in our meta-analysis only six out of 15 RCTs reported a definition of BPD. Upon request, the authors of eight studies kindly clarified their definition. Even after clarification, there was marked heterogeneity in BPD definition. As pointed out by Jobe and Bancalari [69], current definitions of BPD lack precision and do not have good predictive values for later pulmonary and neurodevelopmental outcomes. There are substantial efforts being made to develop better diagnostic criteria for BPD [69], but it will take time before these improved definitions of BPD are reflected in RCTs and meta-analyses.

As mentioned above, the RCTs included in our analysis had important differences in the type, amount, and timing of probiotic supplementation. The choice of probiotic strain(s) is crucial and meta-analyses on probiotics have been criticized because, in most of them, probiotics administered for treatment/prevention of a specific disease or condition were all evaluated together [26,71–73]. It is now generally accepted that different bacterial strains of the same genus and species, verified also by genomic information, may exert completely different effects on the host [72]. Separate meta-analyses analysing the effects of well-defined individual, single-strain or multiple-strain probiotic preparations appear to be more appropriate, but the important heterogeneity of the RCTs makes this approach very difficult [26,71–73]. We attempted to explore whether the studies using *Lactobacillus* or *Bifidobacterium* species showed a different effect on BPD. We also performed a separate analysis for multi-strain probiotics because recent meta-analyses suggest that the use of more than one strain has a stronger effect in the prevention of NEC [74]. None of these subgroup analyses suggested a significant preventive effect of probiotics on BPD. However, the number of studies included in the subgroup analysis was low, making the results inconclusive.

Besides their effect on NEC and LOS prevention, there are some other mechanisms of action ascribed to probiotics which may directly counteract the disruption of lung development prompting to BPD [26,75]. In the first place, the immature immune system of premature infants is unable to balance pro-inflammatory responses, leading to a sustained status of inflammation that contributes significantly to several neonatal diseases, including BPD [15]. A decreased number of T regulatory

cells (Tregs), which constitute the anti-inflammatory lymphocytic subset, and higher proportions of activated pro-inflammatory T cells have been related with the development of BPD [76,77]. Probiotics seem to have a role in improving Treg generation, expansion and activity, while decreasing activation/proliferation of the pro-inflammatory lymphocytic subsets. These effects may result in the recovery of the immune homeostasis with polarization of the immune system toward an anti-inflammatory phenotype [78,79]. Secondly, it has been suggested that each additional day of antibiotic therapy in the first 2 weeks of life in VLBW infants may be associated with an increased BPD rate and severity [80]. This could be explained by the antibiotic-induced decrease in diversity of lung microbiota which has been linked to BPD development [81]. Probiotics are known to restore intestinal microbiota after antibiotic therapy [82] and to produce a strong suppressive effect on airway inflammation [83]. Lastly, poor nutrition is associated with lung underdevelopment and the occurrence of BPD [84]. In experimental NEC, probiotic supplementation reversed the detrimental effects of combined hyperoxia and suboptimal nutrition on lung vascular endothelial growth factor (VEGF) levels, suggesting that this strategy may help improve lung vasculogenesis [85].

In conclusion, our study could not demonstrate any significant effect of probiotic supplementation on the risk of developing of BPD. Given the remarkable theoretical benefits of probiotics supplementation in ameliorating several aspects of BPD pathogenesis and the limitations of the analysis, our data should be seen as a starting point rather than definitive results. The main merit of our study was to collect, for the first time, the available data on the role of probiotic supplementation in the prevention of BPD, and to revise the possible specific mechanisms of action. Nevertheless, further experimental and clinical data are needed to draw more solid conclusions. Particularly, more studies designed to select the optimal probiotic preparation, dosing, and duration of therapy are still needed [29]. These studies should compare probiotic strains that have been reported to be safe and effective in previous trials [73] and include outcomes, such as BPD, which can be indirectly affected by the changes in immunity and nutritional status induced by probiotic supplementation.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Risk of bias assessment of studies included in meta-analysis.

Study	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias
Akar 2017 [49]	LR	UR	LR	LR	UR	UR	UR
Al Hosni 2011 [48]	UR	UR	LR	LR	LR	LR	LR
Costeloe 2016 [62]	LR	LR	LR	LR	LR	LR	LR
Demirel 2013 [50]	LR	LR	LR	LR	LR	LR	LR
Dilli 2015 [51]	LR	LR	LR	LR	LR	LR	LR
Fujii 2006 [52]	UR	UR	HR	UR	LR	UR	UR
Jacobs 2013 [53]	LR	LR	LR	LR	LR	LR	LR
Lin 2008 [54]	LR	LR	LR	LR	LR	UR	LR

Table A1. *Cont.*

Study	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Blinding of Outcome Assessment	Incomplete Outcome Data	Selective Reporting	Other Bias
Manzoni 2009 [55]	LR	UR	LR	LR	LR	LR	LR
Saengtawesin 2014 [56]	UR	UR	HR	HR	UR	UR	UR
Sari 2012 [57]	LR	LR	LR	LR	LR	LR	LR
Serce 2013 [58]	LR	LR	LR	LR	UR	UR	LR
Stratiki 2007 [59]	UR	UR	LR	LR	UR	UR	LR
Totsu 2014 [60]	LR	UR	LR	LR	LR	LR	LR
Underwood 2009 [61]	LR	LR	LR	LR	LR	LR	LR

HR: High risk of bias; LR: Low risk of bias; UR: Unclear risk of bias.

Table A2. Sensitivity analyses for BPD28: results of random effects meta-analyses when removing one study.

Removed Study	Statistics with Study Removed				
	MH RR	Lower Limit 95% CI	Upper Limit 95% CI	*Z*-Score	*p*
Demirel 2013 [50]	1.01	0.92	1.12	0.21	0.832
Dilli 2015 [51]	1.02	0.92	1.13	0.37	0.708
Fujii 2006 [52]	1.01	0.91	1.11	0.15	0.878
Jacobs 2013 [53]	1.04	0.86	1.25	0.38	0.703
Saengtawesin 2014 [56]	1.00	0.90	1.10	−0.09	0.931
Totsu 2014 [60]	0.99	0.89	1.10	−0.22	0.829
Stratiki 2007 [59]	1.01	0.91	1.11	0.11	0.916

BPD28: bronchopulmonary dysplasia, defined as oxygen dependence at 28 days of life; CI: confidence interval; MH RR: Mantel–Haenszel risk ratio.

Table A3. Sensitivity analyses for BPD36: results of random effects meta-analyses when removing one study.

Removed Study	Statistics with Study Removed				
	MH RR	Lower Limit 95% CI	Upper Limit 95% CI	*Z*-Score	*p*
Akar 2017 [49]	1.09	0.97	1.21	1.45	0.146
Al Hosni 2012 [48]	1.09	0.97	1.23	1.48	0.138
Costeloe 2016 [62]	1.07	0.93	1.22	0.94	0.350
Dilli 2015 [51]	1.08	0.97	1.21	1.43	0.153
Jacobs 2013 [53]	1.09	0.96	1.24	1.32	0.188
Lin 2008 [54]	1.04	0.93	1.17	0.69	0.488
Manzoni 2009 [55]	1.08	0.96	1.20	1.32	0.187
Sari 2012 [57]	1.07	0.96	1.20	1.25	0.213
Serce 2013 [58]	1.07	0.96	1.20	1.26	0.209
Totsu 2014 [60]	1.05	0.93	1.17	0.76	0.448
Underwood 2009 (CUL) [61] [1]	1.08	0.97	1.20	1.34	0.179
Underwood 2009 (PBP) [61] [1]	1.08	0.97	1.20	1.36	0.173

[1] Culturelle (CUL) and ProBioPlus DDS (PBP) were the names assigned by the authors to the probiotic preparations. BPD36: bronchopulmonary dysplasia, defined as oxygen dependence at 36 weeks post-menstrual age; CI: confidence interval; MH RR: Mantel–Haenszel risk ratio.

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Review

Effect of Probiotics and Prebiotics on Immune Response to Influenza Vaccination in Adults: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Abstract: We conducted a meta-analysis to evaluate the effects of probiotics and prebiotics on the immune response to influenza vaccination in adults. We conducted a literature search of Pubmed, Embase, the Cochrane Library, the Cumulative Index to Nursing and Allied Health (CINAHL), Airiti Library, and PerioPath Index to Taiwan Periodical Literature in Taiwan. Databases were searched from inception to July 2017. We used the Cochrane Review risk of bias assessment tool to assess randomized controlled trial (RCT) quality. A total of 20 RCTs comprising 1979 adults were included in our systematic review. Nine RCTs including 623 participants had sufficient data to be pooled in a meta-analysis. Participants who took probiotics or prebiotics showed significant improvements in the H1N1 strain seroprotection rate (with an odds ratio (OR) of 1.83 and a 95% confidence interval (CI) of 1.19–2.82, $p = 0.006$, $I^2 = 0\%$), the H3N2 strain seroprotection rate (OR = 2.85, 95% CI = 1.59–5.10, $p < 0.001$, $I^2 = 0\%$), and the B strain seroconversion rate (OR = 2.11, 95% CI = 1.38–3.21, $p < 0.001$, $I^2 = 0\%$). This meta-analysis suggested that probiotics and prebiotics are effective in elevating immunogenicity by influencing seroconversion and seroprotection rates in adults inoculated with influenza vaccines.

Keywords: probiotics; prebiotics; seroprotection; seroconversion; influenza vaccine; systematic review; meta-analysis

1. Introduction

Influenza is an acute viral respiratory infection caused by RNA viruses and results in fever and myalgia in infected people. Although influenza is self-limited in most cases, it can cause serious diseases such as pneumonia, myocarditis, and encephalitis, which result in high morbidity and significant mortality in children, pregnant women, and the elderly. In general, epidemically seasonal influenza leads to three to five million severe illness cases and around 250,000 to 500,000 deaths in the world yearly. Even more, influenza pandemics are unpredictable and can have significant impacts on human health and the economy worldwide. Currently, annual influenza vaccines are the main intervention for minimizing both the mortality and morbidity of influenza [1].

Although vaccination in children, adolescents, and young adults can help prevent influenza infection by 70% to 90%, among people older than 65 years old its protective effects only range from 30% to 40%, according to a previous report [2]. Aging is accompanied by a decline in both innate and

adaptive immune responses. Suboptimal cytotoxicity of natural killer (NK) cells, phagocytosis, B cell antibody production, and T cell cellular immune response result in poorer responses to both infection and immunization [3,4]. This immunosenescence caused by aging limits the protective effects of vaccination in older adults. Adjuvants such as heat-labile enterotoxin have been co-administrated with the inactivated vaccine to improve potency. However, there remain some safety concerns regarding this process [5].

Probiotics such as *Lactobacillus* and *Bifidobacterium* are live bacteria that are beneficial to the host when administrated in proper amounts [6]. The use of probiotics has been shown to not only modulate both innate and adaptive immunity in the elderly, but also reduce the length of infection in children and adults [7–9]. Prebiotics like oligosaccharides are substances that stimulate the metabolism and growth of commensal enteric bacteria that benefit the host. It has been proven that prebiotics can modulate B cell response and augment the Th1-dependent immune response [10–12]. Both probiotics and prebiotics have been shown in clinical trials to have protective effects against influenza infection. In addition, there have been studies focused on the usefulness of adjuvant supplementation of probiotics or prebiotics with measles vaccination. From this point of view, probiotic or prebiotic supplementation appears to be an attractive and safe way to enhance the effectiveness of influenza vaccines.

Several randomized controlled trials (RCTs) have evaluated the influence of probiotic or prebiotic consumption on individual immune responses induced by an influenza vaccine, but no systematic review has examined the link between the consumption of probiotics or prebiotics and immunogenicity outcomes in adults vaccinated with an influenza vaccine. Furthermore, results of former studies concerning the efficacy of supplementation in relation to subsequent serum antibody changes after influenza vaccination remain inconclusive. The present systematic review and meta-analysis thus aim to explore the effectiveness of probiotics and prebiotics on immune functions in adults inoculated with an influenza vaccine.

2. Methods

This systematic review and meta-analysis were conducted in accordance with the Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) guidelines [13] (Table S1).

We searched the following databases from inception to the end of July 2017: Embase, PubMed, the Cochrane Library, the Cumulative Index to Nursing and Allied Health (CINAHL), the Airiti Library, and the PerioPath Index to the Taiwan Periodical Literature in Taiwan.

We used the keywords "influenza vaccine" AND "probiotics" OR "prebiotics" OR "synbiotics" in our search. Our strategy is shown in Table S2. To ensure a comprehensive search, we did not limit the language, year, or type of publication. Two authors (PCS and SJL) conducted the search independently, and disagreements were resolved through discussion with the third author (WTL).

2.1. Study Selection and Methodological Quality Assessment

After the initial search, two independent reviewers (PCS and TLY) assessed each publication to determine whether the article met the inclusion criteria for systematic review and meta-analysis. The RCTs included met all of the following eligibility criteria: (1) focused on human adults; (2) includes a controlled group in the study design; (3) includes inoculation of an influenza vaccine and use of probiotics, prebiotics, or synbiotics by the intervention group; and (4) reports at least one immunological response to vaccination. We excluded the following: (1) articles irrelevant to the topic, (2) duplicate publications, (3) trials of a cross-over study design, and (4) studies in which the control arm received an effective intervention rather than a placebo.

Quality assessment of all included studies was conducted independently by two researchers (WTL and TLY) using the Cochrane Review risk of bias assessment tool [14]. The adequacy of randomization, allocation concealment, blinding methods, implementation of the intention-to-treat analysis, dropout

rate, complete outcome data, selective data reporting, and other biases were assessed. Each domain was categorized as low, high, or unclear.

2.2. Data Extraction and Analysis

Three authors (T.L.Y., C.Y.L., and W.T.L.) independently extracted the data from all included studies, and the following data were collected: first author's name, year of publication, country of publications, number of patients, age of patients, sex ratio of patients, type of intervention, type of vaccine, clinical outcome measures, and severe adverse effects. To evaluate influenza vaccine immunogenicity, factors affecting antibody Geometric Mean Titer (GMT) and seroprotection and seroconversion rates were extracted from the trials. Such factors included Hemagglutination inhibition (HI) antibody titers, serum immunoglobulins, cytokine secretion, lymphocyte proliferation, immune cell phenotypes, compliance variables, biochemical markers, and episode or duration of upper respiratory tract infection or flu-like illness. Our objective was to determine the influence of probiotics and prebiotics on the seroprotection and seroconversion rates of adults after influenza vaccination. HI antibody titer equals the maximum dilution capable of inhibiting the agglutination of guinea pig red blood cells with the influenza viruses under standardized conditions [15]. Seroconversion rate is defined as the proportion of volunteers achieving at least a fourfold increase in antibody titer after vaccination. Seroprotection rate is defined as the proportion of volunteers achieving an influenza antibody titer greater than or equal to 40 in an HI test [16].

The European Committee for Proprietary Medicinal Products (CPMP) guidelines [17] set the cut-off levels of vaccine immunogenicity for a population over the age of 60 years as at least a 60% seroprotection rate, at least a 30% seroconversion rate, and an over 2.5-fold increase in antibody GMT. Each of the vaccine antigens must meet at least one of the above criteria in the CPMP guidelines.

Meta-analysis was conducted when the trials had acceptable clinical homogeneity and statistical heterogeneity. Due to the significant heterogeneity expected among the studies, a random effects model was employed using DerSimonian and Laird's method [18,19]. To evaluate the differences in immunogenicity between the intervention and the control groups, dichotomous data were analyzed using an odds ratio (OR) with 95% confidence intervals (CI). Heterogeneity was quantified using the Cochran Q TEST and I^2 statistics [19]. Potential publication bias was assessed by observing the symmetry of funnel plots and by using Egger's test [20]. Meta-analysis was performed using Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014. Comprehensive Meta-Analysis version 3 (Biostat, Englewood, NJ, USA) was used to conduct Egger's test and the meta-regression.

3. Results

3.1. Description of Studies and Quality Assessment

Figure 1 shows the search process and outcomes. A total of 19 publications with 20 RCTs were included for our systematic review [21–39]. Two trials (a pilot and a confirmatory study) with different patient numbers, treatment protocols, and years of study were published together [35]. Thirteen trials focused on probiotics [22,26–36], while the other six RCTs focused on prebiotics [23–25,37–39]. Only one study concentrated on synbiotics [21]. Akatsu et al. published a letter to the editor [28] and an original article [27] in the same year. As the study methods were different, we included both of the publications in our review.

Most of the included studies had low bias, as shown by our quality assessment using the Cochrane assessment tool. The detailed quality assessment of each included study is shown in Table S3.

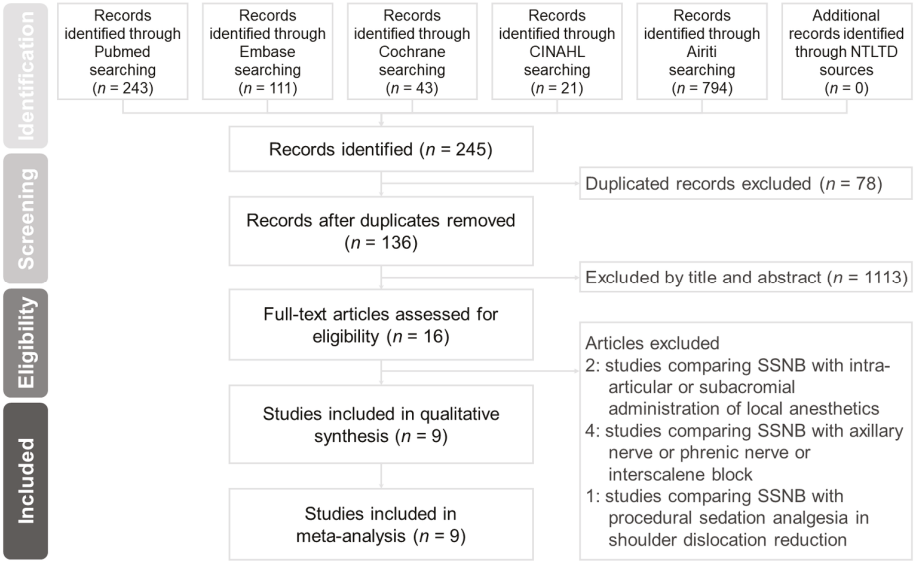


Figure 1. Preferred Reporting Items for Systematic review and Meta-Analysis (PRISMA) flow diagram.

3.2. Demographics

The characteristics of the included trials are shown in Table 1. These studies were conducted worldwide, with six trials in Japan [22–24,27,28,33], three trials in the USA [32,37,38], two studies each in Spain [31,36] and the UK [21,25], one publication in France [35], and one trial each in Australia [34], Belgium [29], Italy [30], Germany and Denmark [26], and Chile [39]. Seven RCTs enrolled healthy adults or older adults [21,25,26,30,32,34,36], and two trials enrolled healthy older adults [38,39]. In another eleven trials, subjects living in hospitals, nursing homes, or long-term care facilities were enrolled [22–24,27–29,31,33,35,37]. Participants fed by enteral tube or percutaneous endoscopic gastrostomy were enrolled in three studies conducted in Japan [23,24,27].

A total of 1979 participants with an average age of 58.1 years were enrolled. The male to female ratio was 2.2.

Table 1. Characteristics of randomized clinical trials using probiotics/prebiotics/synbiotics on Influenza-vaccinated adults.

Reference	Country (Tx Duration)	Population (M%:F%)	Age Mean	Intervention: Control	Intervention	Type of Vaccine	Outcome Measure	Severe AEs
Olivares 2007 [36]	Spain (4 weeks)	50 healthy adults (62%:38%)	33.00	25:25	*L. fermentum* CECT5716 1×10^{10} CFU daily	H1N1: New Caledonia/20/99 H3N2: A/Fujian/411/2002 B: Shanghai/361/2002	Total plasma Ig/cytokine concentration/lymphocyte subpopulation/pattern of subsequent illness	Nil
French & Penny 2009 [34]	Australia (6 weeks)	47 healthy adults (41%:59%)	31.55	15:32	*L. fermentum* VRI 003 1×10^{9} CFU	H1N1: New Caledonia/20/99 H3N2: California/7/2004 B: Malaysia/2506/2004	HI titers/titers to Measles, Varicella zoster antigens/patterns of subsequent Illness	NR
Boge 2009 [35] (pilot)	France (7 weeks)	68 healthy adults in nursing home (44%:56%)	83.64	44:42	*L. casei* DN-114 001 twice daily	H1N1: New Caledonia/20/99 H3N2: Wisconsin/67/2005 B: Malaysia2506/2004	HI titers/seroconversion rate/seroprotection rate	10
Boge 2009 [35] (confirmed)	France (13 weeks)	222 elders in nursing home (33%:67%)	84.64	113:109	*L. casei* DN-114 001 twice daily	H1N1: New Caledonia/20/99 H3N2: California/7/2004 B: Shanghai/361/2002a B: Jiangsu/10/2003a	HI titers/seroconversion rate/seroprotection rate	30
Namba 2010 [33]	Japan (2 weeks)	27 healthy elders in health care facility (11%:89%)	86.70	13:14	*B. longum* BB536 1×10^{11} CFU daily	H1N1: New Caledonia/20/99 H3N2: Wyoming/3/2003 B: Shanghai/361/2002	HI titers/NK cell activity, neutrophil bactericidal and phagocytic activity/cell-mediated immunity/pattern of subsequent illness	NR
Davidson 2011 [32]	USA (4 weeks)	42 healthy adults (38%:62%)	33.30	21:21	*L. GG* 1×10^{10} CFU twice daily	H1N1: Solomon Islands/3/2006 H3N2: Wisconsin/67/2005 B: Malaysia/2506/2004	Hi titers/seroconversion rate	1
Van Puyenboreck 2012 [29]	Belgium (3 weeks)	737 healthy adults in nursing home (25%:75%)	84.06	375:362	*L. casei Shirota* 6.5×10^{9} CFU twice daily	H1N1: Solomon Islands /3/2006 IVR-145 H3N2: Wisconsin /67/2005 B: Malaysia /2506/2004	HI titers/seroconversion rate/seroprotection rates/pattern of subsequent illness	NR
Rizzardini 2012 [30]	Italy (6 weeks)	211 healthy adults (44%:56%)	33.20	109:102	BB-12® (DSM15954) 1×10^{9} CFU *L. casei* 431® (ATCC55544) daily	H1N1: Brisbane/59/2007 H3N2: Uruguay/716/2007 B: Florida/4/2006	Total plasma Ig/vaccine-specific Ig/salivary Ig/total salivary Ig/plasma interferon-γ, IL-2, IL-10/NK cell activity/CD4+T-lymphocytes/ phagocytosis	Nil

Table 1. *Cont.*

Reference	Country (Tx Duration)	Population (M%:F%)	Age Mean	Intervention: Control	Intervention	Type of Vaccine	Outcome Measure	Severe AEs
Bosch 2012 [31]	Spain (12 weeks)	60 adults in nursing home (NR)	65–85	19:14:15 Group A: 19 Group B: 14 Control: 15	*L. plantarum* CECT7315/7316 daily Group A: 5×10^9 CFU Group B: 5×10^8 CFU	H1N1: Solomon Islands/3/2006 H3N2: Wisconsin/67/2005 B: Malaysia/2506/2004	HI titers/total plasma Ig/Influenza-specific Ig/pattern of subsequent illness/fecal Microbiota	NR
Akatsu 2013a [28] (letter)	Japan (12 weeks)	15 healthy adults in nursing home (47%:53%)	75.74	8:7	*L. paracasei* MoLac 1×10^{11} CFU	H1N1: Brisbane/59/2007 H3N2: Uruguay/716/2007 B: Brisbane/60/2008	HI titers/total serum Ig/seroconversion rate/NK cell activity and neutrophil phagocytic activity	NR
Akatsu 2013b [27] (paper)	Japan (12 weeks)	45 enteral tube feeding hospitalized adults (29%:71%)	81.70	23:22	Bifidobacterium strain, BB536 5×10^{10} CFU twice daily	H1N1: Brisbane/59/2007 H3N2: Uruguay/716/2007 B: Brisbane/60/2008	HI titers/total plasma Ig/NK cell activity/innate immunity	Nil
Jespersen 2015 [26]	German, Denmark (6 weeks)	1104 healthy adults (41%:59%)	31.45	553:551	*L. casei* 431 (ATCC55544) 1×10^9 CFU daily	H1N1: California/7/2009 H3N2: Perth/16/2009 B: Brisbane/60/2008	HI titers/influenza A-specific antibodies/seroconversion rate/pattern of subsequent illness	5
Maruyama 2016 [22]	Japan (6 weeks)	42 elders in nursing home (19%:81%)	87.15	21:21	*L. paracasei* MCC 1849 1×10^{11} CFU daily	H1N1: California/7/2009 pdm09 H3N2: Texas/50/2012 B: Massachusetts/2/2012 (Yamagata lineage)	HI titers/total plasma Ig/NK cell activity, neutrophil phagocytic and bactericidal activity/pattern of subsequent illness:	Nil
Enani 2017 [21]	UK (8 weeks)	112 healthy adults (NR)	18–35 60–85	Young group: 31:31 Older group: 29:33	*B. longum* 10^9 CFU/day with GI-OS 8 g/day	H1N1: California/7/2009 H3N2: Perth/16/2009 B: Brisbane/60/2008	B/T cell phenotyping/re-stimulation of PBMC/anti-CMV IgG Ab	NR
Bunout 2002 [39]	Chile (28 weeks)	66 healthy elders (NR, but similar %)	75.73	23:20	FOS (70% raftilose, 30% raftiline) 2 sachets daily	PPSV 23 H1N1: Caledonia A: Moscow (subtype AC3N2), Sydney B: Belgium (code 184-93)	Serum Ig/sIgA/Ab titers/cytokine secretion/lymphocyte proliferation/episode of URI	3

Table 1. *Cont.*

Reference	Country (Tx Duration)	Population (M%:F%)	Age Mean	Intervention: Control	Intervention	Type of Vaccine	Outcome Measure	Severe AEs
Langkamp-Henken 2004 [38]	USA (26 weeks)	66 healthy elders (47%:53%)	81.54	34:32	High oleic safflower oil, soybean oil, FOS, structured TG 8 oz. daily	H1N1: Beijing/262/95 H3N2: Sydney/5/97 B: Yamanashi/166/98 (B/Beijing/184/93)	Ab titers/lymphocyte proliferation/daily symptoms of URI	NR
Langkamp-Henken 2006 [37]	USA (10 weeks)	157 frail elders in LTC facilities (28%:72%)	83.36	76:72	Antioxidants, B vitamins, selenium, zinc, FOS, structured TG 240 mL daily	H1N1: Caledonia/20/99 H3N2: Panama/2007/99 B: Hong Kong/1434/2002	Cytokine studies/lymphocyte activation markers/immune cell phenotypes	NR
Nagafuchi 2015 [24]	Japan (14 weeks)	24 enteral tube feeding hospitalized elders (46%:54%)	80.30	12:12	BGS (1.65 µg/100 kcal), DHNA, GOS (0.4 g/100 kcal), fermented milk products	H1N1: California/7/2009 H3N2: Victoria/210/2009 B: Brisbane/60/2008	Ab titers/blood biochemical indices/intestinal bacterial populations	Nil
Lomax 2015 [25]	UK (8 weeks)	49 healthy adults (26%:74%)	54.98	22:21	50:50 mixture of long-chain inulin and oligofructose 8 g daily	H1N1: Brisbane/59/2007 H3N2: Brisbane/10/2007 B: Florida/4/2006	HI titers/total plasma Ig/vaccine-specific Ig/NK cell activity, immune cell phenotypes bactericidal activity, T-cell activity	NR
Akatsu 2016 [23]	Japan (8 weeks)	23 PEG-fed bedridden hospitalizedelders (13%:87%)	81.00	12:11	Heat-treated lactic acid bacteria-fermented milk products, GOS 4 g/day, BGS 0.4 g/day	H1N1: Solomon Islands/3/2006 H3N2: Hiroshima/52/2005 B: Malaysia/2506/2004	Hi titers/cytokine levels/biochemical markers	NR

L. fermentum: *Lactobacillus fermentum*; *L. casei*: *Lactobacillus casei*; *L. plantarum*: *Lactobacillus plantarum*; *L. paracasei*: *Lactobacillus paracasei*; *L.GG*: *Lactobacillus GG*; *B. longum*: *Bifidobacterium longum*; CFU: colony-forming unit; LTC: long term care facilities; FOS: fructo-oligosaccharides; GOS: galacto-oligosaccharide; TG: triglycerol; BGS: bifidogenic growth stimulator; DHNA: 1,4-dihydroxy-2-naphthoic acid; PPSV 23: pneumococcal polysaccharide vaccine 23; PEG: percutaneous endoscopic gastrostomy; Ig: immunoglobulin; sIg: specific Immunoglobulin, Ab: antibody, PBMC: peripheral blood mononuclear cells, GI-OS: gluco-oligosaccharide, AEs: adverse events, CMV: cytomegalovirus, NK cell: nature killer cell, NR: not-regulated, Tx: treatment, URI: upper respiratory tract infection, Nil: none.

3.3. Intervention

Ten RCTs used *Lactobacillus* [22,26,28–32,34–36] as a probiotic. Four studies [21,27,30,33] selected *Bifidobacterium*. *Lactobacillus casei or paracasei* were the most commonly used probiotics in the included studies [22,26,28–30,35], followed by *Lactobacillus fermentum* [34,36], *Lactobacillus rhamnosus GG* [32], and *Lactobacillus plantarum* [31]. One study compared two different probiotics, *Bifidobacterium animalis ssp. lactis* and *Lactobacillus paracasei subsp. paracasei* [30]. Another trial compared the effect of *Lactobacillus plantarum* in different doses [31].

Prebiotics were supplied in different combinations across the included studies. Fructo-oligosaccharide was the most commonly used prebiotic component [37–39] mixed with different oils [38], triglycerols, vitamins, or minerals [37,38], followed by galacto-oligosaccharides [23,24] mixed with a bifidogenic growth stimulator or fermented milk products. One trial selected long-chain inulin and oligofructose [25].

The supplementation duration ranged from 2 to 28 weeks, with an average of 7, 16, and 8 weeks in probiotics, prebiotics, and synbiotics, respectively. Out of 1979 total participants, 49 individuals had severe adverse effects.

Almost all of the included studies used a trivalent inactivated influenza vaccine. Only two RCTs selected a live attenuated influenza vaccine [23,32]. In one trial, all participants were both vaccinated with a trivalent inactivated influenza vaccine and the pneumococcal polysaccharide vaccine 23 [39].

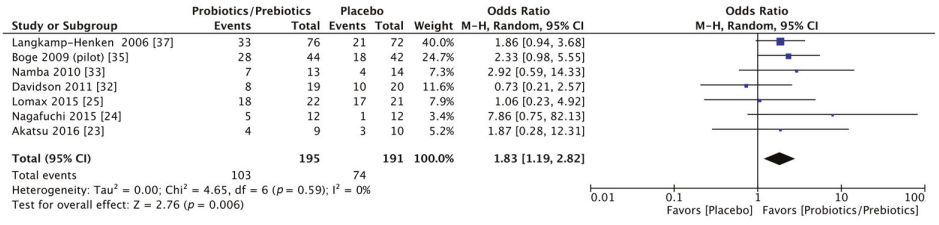
3.4. Outcome Measurement

We excluded one RCT [27] from our meta-analysis when considering seroprotection rate to prevent a possible overestimation of the real effect from the results. The excluded study had reported data in which the HI was higher than 20, which is lower than the amount required by the definition of seroprotection. After performing a thorough review of an RCT conducted in 2015 [26], we found that the numbers were not compatible with the data in the article. As we had reasonable doubts concerning the accuracy of the numbers in the article, we excluded the article from our meta-analysis.

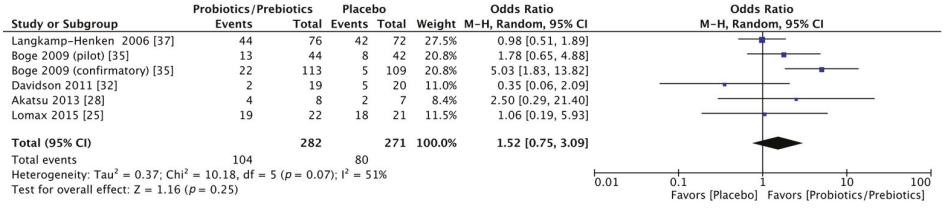
3.5. Efficacy of Probiotics and Prebiotics in Participants Inoculated with an Influenza Vaccine Compared with Controls

Seven RCTs [23–25,32,33,35,37] including 389 participants had sufficient data to be pooled for an analysis of seroprotection rate. Meanwhile, a total of six RCTs [25,28,32,35,37] including 553 participants were enrolled for our meta-analysis to determine seroconversion rate. The average age of all participants was 74.8 years old. The seroprotection rates in those who took probiotics or prebiotics with the H1N1, H3N2, and influenza B vaccines were 53%, 84%, and 53%, respectively. The overall seroconversion rates for the H1N1, H3N2, and influenza B vaccines were 37%, 65%, and 50%, respectively.

Significant immunogenicity differences were documented between those who took probiotics or prebiotics and the controls. For the H1N1 vaccine, the OR for seroprotection was 1.83, with a 95% CI of 1.19–2.82, $I^2 = 0\%$, $p = 0.006$ (Figure 2a), whereas the OR for seroconversion was 1.52, with a 95% CI of 0.75–3.09, $I^2 = 51\%$, $p = 0.25$ (Figure 2b). With regards to the H3N2 vaccine, there was a significant difference in the seroprotection rate (probiotics/prebiotics vs. controls, OR = 2.85, 95% CI = 1.59–5.10, $I^2 = 0\%$, $p < 0.001$) (Figure 3a) but not the seroconversion rate (OR = 2.54, 95% CI = 0.93–6.91, $I^2 = 83\%$, $p = 0.07$) (Figure 3b). Furthermore, for the influenza B vaccine, a significant difference was noted in the seroconversion rate (OR = 2.11, 95% CI = 1.38–3.21, $I^2 = 0\%$, $p < 0.001$) (Figure 4b) and not the seroprotection rate (OR = 0.99, 95% CI = 0.65–1.52, $I^2 = 0\%$, $p = 0.97$) (Figure 4a).

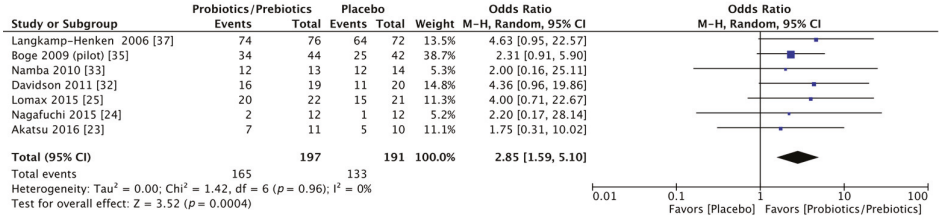


(**a**)

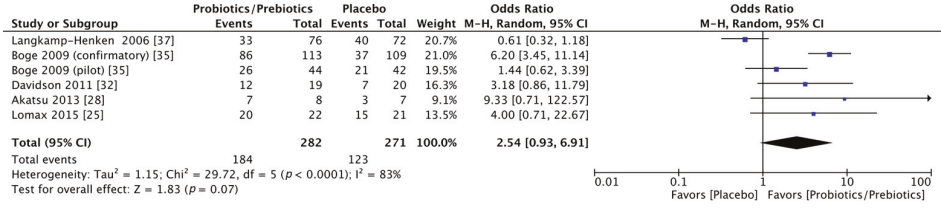


(**b**)

Figure 2. Forest plots of seroprotection and seroconversion rate of H1N1 strain. (**a**) Forest plot of seroprotection rate of H1N1 strain; (**b**) Forest plot of seroconversion rate of H1N1 strain. The bold data represents total participants of all included studies and the Odds ratio (OR) between the probiotics/prebiotics group and the placebo group. The diamond stands for the pooled OR. Weights are from random-effects model. CI: confidence interval.

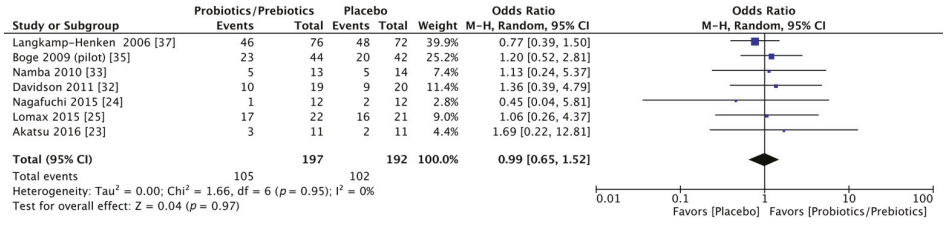


(**a**)



(**b**)

Figure 3. Forest plots of seroprotection and seroconversion rate of H3N2 strain. (**a**) Forest plot of seroprotection rate of H3N2 strain; (**b**) Forest plot of seroconversion rate of H3N2 strain. The bold data represents total participants of all included studies and the Odds ratio (OR) between the probiotics/prebiotics group and the placebo group. The diamond stands for the pooled OR. Weights are from random-effects model. CI: confidence interval.



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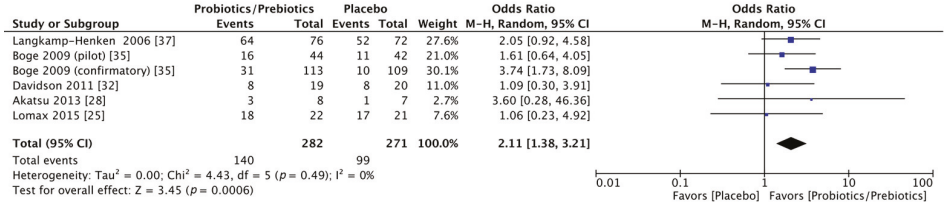
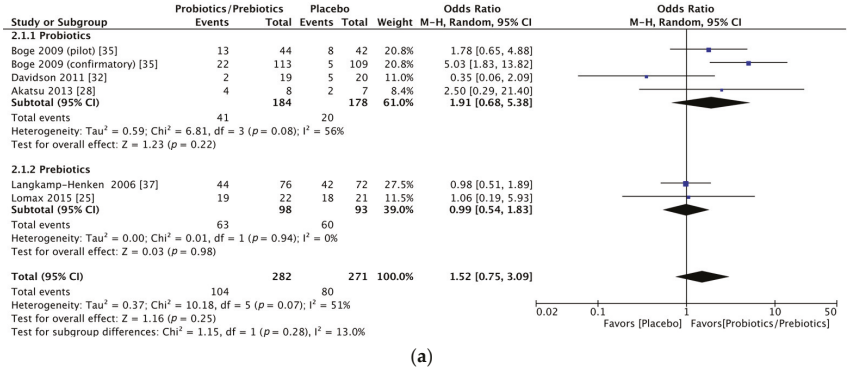


(**b**)

Figure 4. Forest plots of seroprotection and seroconversion rate of B strain. (**a**) Forest plot of seroprotection rate of B strain; (**b**) Forest plot of seroconversion rate of B strain. The bold data represents total participants of all included studies and the Odds ratio (OR) between the probiotics/prebiotics group and the placebo group. The diamond stands for the pooled OR. Weights are from random-effects model. CI: confidence interval.

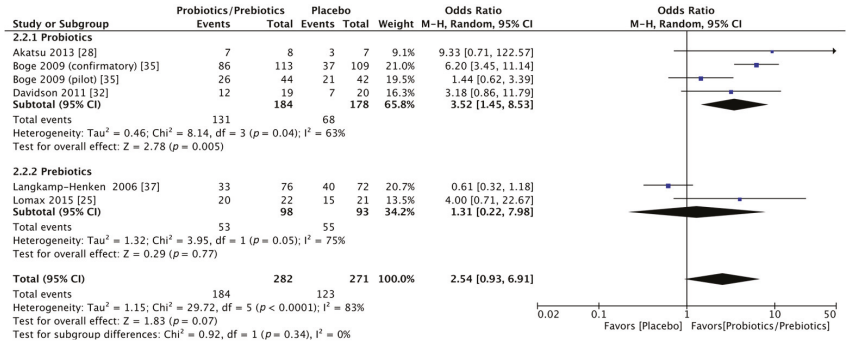
3.6. Subgroup Meta-Analysis of Influenza Vaccine Immunogenicity in Participants Supplied with Different Supplements

Due to the relatively moderate heterogeneity of seroconversion rates, we performed a subgroup analysis according to the intervention of probiotics or prebiotics. In the H1N1 seroconversion rate, the results remained unchanged except for a decrease in heterogeneity (OR = 1.91, 95% CI = 0.68–5.38, I^2 = 56%, p = 0.22; OR = 0.99, 95% CI = 0.54–1.83, I^2 = 0%, p = 0.98, forest plot in Figure 5a) after dividing all of the participants into probiotic and prebiotic groups. For the H3N2 seroconversion rate, the favorable effect was shown in the probiotics group (OR = 3.52, 95% CI = 1.45–8.53, I^2 = 63%, p = 0.005) but not in the prebiotics group (OR = 1.31, 95% CI = 0.22–7.98, I^2 = 75%, p = 0.77, forest plot in Figure 5b).



(**a**)

Figure 5. *Cont.*

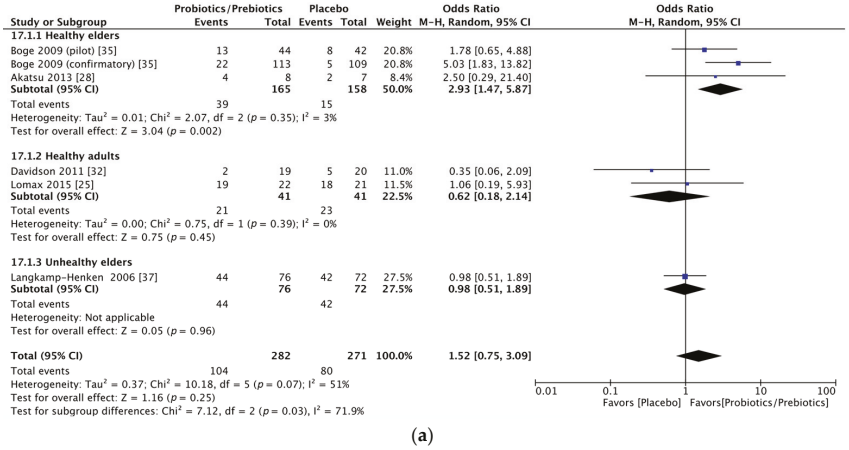


(**b**)

Figure 5. Forest plots of subgroup analysis by intervention type of seroconversion rate of H1N1 and H3N2 strains. (**a**) Forest plot of subgroup analysis by intervention type of seroconversion rate for influenza H1N1 strain; (**b**) Forest plot of subgroup analysis by intervention type of seroconversion rate for influenza H3N2 strain. The bold data represents total participants of all included studies and the Odds ratio (OR) between the probiotics/prebiotics group and the placebo group. The diamond stands for the pooled OR. Weights are from random-effects model. CI: confidence interval.

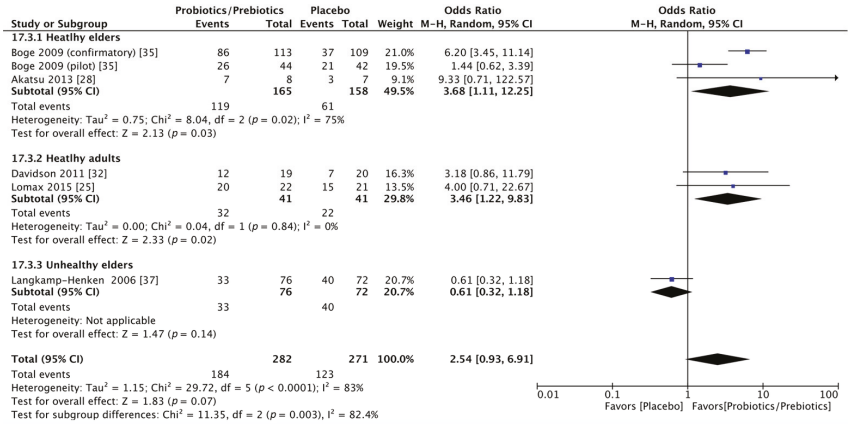
3.7. Subgroup Meta-Analysis of the Immunogenicity of the Influenza Vaccine in Participants Divided into Different Health Statuses

Given the persistent heterogeneity, we performed another subgroup meta-analysis based on the different health statuses of the participants. We found that participants in the included studies could be grouped into the following three categories: healthy young to middle-aged adults, healthy older adults, and frail or hospitalized older adults. The heterogeneity of the seroconversion rate was lowered as a result of the subgroup meta-analysis (Figure 6a,b). In addition, among the two indexes (seroprotection and seroconversion rates) used to evaluate the effects of probiotics and prebiotics in relation to the three strains of influenza vaccine, participants from the healthy older adult category had the best response to influenza vaccination followed by the healthy young to middle-aged adults and then the frail or hospitalized older adults (Table 2).



(**a**)

Figure 6. *Cont.*



(**b**)

Figure 6. Forest plots of subgroup analysis by participants of seroconversion rate of H1N1 and H3N2 strains. (**a**) Forest plot of subgroup analysis by health status of participants of seroconversion rate for influenza H1N1 strain; (**b**) Forest plot of subgroup analysis by health status of participants of seroconversion rate for influenza H3N2 strain. The bold data represents total participants of all included studies and the Odds ratio (OR) between the probiotics/prebiotics group and the placebo group. The diamond stands for the pooled OR. Weights are from random-effects model. CI: confidence interval.

Table 2. Subgroup analysis of odds ratio of seroprotection and seroconversion rate for different influenza strains based on health status of participants.

Subgroup	H1N1	H3N2	B
Health elders			
Seroprotection	2.46 (1.15–5.26) †	2.27 (0.94–5.47)	1.19 (0.56–2.50)
Seroconversion	2.93 (1.47–5.87) *	3.68 (1.11–12.25) †	2.69 (1.51–4.78) *
Health young/middle-aged adults			
Seroprotection	0.85 (0.32–2.25)	4.20 (1.34–13.16) †	1.22 (0.48–3.12)
Seroconversion	0.62 (0.18–2.14)	3.46 (1.22–9.83) †	1.08 (0.40–2.88)
Hospitalized elders			
Seroprotection	2.06 (1.11–3.82) †	2.83 (0.97–8.21)	0.80 (0.43–1.49)
Seroconversion	0.98 (0.51–1.89)	0.61 (0.32–1.18)	2.05 (0.92–4.58)

* $p < 0.005$, † $p < 0.05$.

3.8. Meta-Regression

To examine the heterogeneity of the current analysis, a meta-regression analysis was also done using the age of participants and the duration of supplementation as moderators in the single meta-regression. We found that the effect of probiotics or prebiotics on immune responses to all of the influenza vaccine strains was not significantly confounded by age. The effects of probiotics and prebiotics on the seroconversion rate against the influenza B strain (slope = 0.14, $p = 0.049$) and the seroconversion rate against the influenza H1N1 strain (slope = 0.21, $p = 0.043$) were significantly confounded by the duration of supplementation.

3.9. Assessment of Publication Bias

Our funnel plots are symmetric upon inspection (Figures S1 to S6). Egger's regression confirmed that there was no statistically significant publication bias with a p value > 0.05 (Table S4).

4. Discussion

To the best of our knowledge, this is the first systemic review and meta-analysis to be conducted on the effect of supplementary probiotic and prebiotic use on influenza vaccine efficacy in adults. In our analysis, we found that the supplementation of influenza vaccines with probiotics or prebiotics before vaccination increased the immunogenicity to specific influenza viral strains, including the H1N1, H3N2, and B strains. The current study included seven RCTs related to seroprotection rates that revealed a significantly better protective effect in those who took probiotics or prebiotics orally as an adjuvant for the parenterally administered H1N1 and H3N2 vaccines. In addition, pooled results from the six studies focused on seroconversion rate showed a significantly enhanced efficacy of the influenza B vaccine in those who consumed probiotics or prebiotics.

In our analysis, the participants supplemented with probiotics or prebiotics not only satisfied at least one of the CPMP guidelines [17] for all influenza strains (seroprotection rate against H3N2 and seroconversion rates against the H1N1, H3N2, and B strains), but also displayed higher seroprotection and seroconversion rates against the H1N1, H3N2, and B strains than those of the control group. For one RCT [37] included in our analysis, we might have underestimated the seroprotection rate against H1N1, as an HI equal to or above 100 was used as the standard. In addition, we excluded another study that defined seroprotection as HI of 20 or over [27]. We have more confidence in our results because our choice to underestimate rather than overestimate the real effects led to solid results on the benefits of probiotics and prebiotics.

Previous RCTs on the efficacy of the use of probiotics and prebiotics as supplements for amplifying the effect of influenza vaccines have reported inconsistent conclusions and a lack of evidence to support such a use of probiotics or prebiotics in clinical practice. Our results are consistent with the majority of the 20 enrolled RCT studies; only three trials showed results that were inconsistent with ours [29,36,39]. The inconsistency might be attributed to not only study design, such as the type and duration of supplementation, but also the demographic characteristics of the participants, more specifically age and health status. We tried to investigate the possible confounding effect of these variables on the probiotic or prebiotic efficacy in relation to an influenza vaccine. We found that the duration of supplementation, and not the age of participants, had a significant impact on the participants' response to probiotics or prebiotics. Longer duration of supplementation rendered participants more sensitive to vaccine stimulation. In previous reports [29,40], aging has been suggested as the reason for a poorer immune response to both influenza vaccines and probiotic stimulation. However, younger ages may not show positive effects from probiotic or prebiotic supplementation because this age group has higher possibility of an optimal response to vaccination. In our study, we found that health status plays a more important role than age. Our analysis showed that healthy older adults obtained the most benefit from probiotics and prebiotics, compared with the other two types of participants. The solid evidence from our results has clinical importance: clinicians can use our results to make tailored suggestions for specific populations to augment vaccine immunogenicity.

Compliance may also be a confounding factor in interventional studies. In current analysis, twelve studies recorded the compliance. Three studies further confirmed intake of probiotics via culture-based mechanism, using qPCR, counting fecal bacteria numbers, or detecting fecal probiotics strains (Table S5). However, only 4 of the 12 studies were included in the meta-analysis with only 1 study declaring not good compliance. We found that compliance had no impact on the current results. Moreover, the strains of probiotics may also play a vital role. In the further subgroup analysis based on different probiotics strains, we found that non-LGG strains (i.e. *L. casei*, *L. paracasei*, and *B. longum*) had positive effects on immunogenicity changes in all vaccine strains. However, LGG showed no effects in any of the three vaccine strains (Table S6). Further studies are required to clarify the influences of different probiotics strains.

The underlying mechanisms of probiotics and prebiotics in terms of their effect on immune functions may differ. Probiotics induce cellular immunity in phagocyte and NK cells [33,41] and promote IgA secretion into saliva to enhance the vaccine effects [35,42]. Furthermore, the metabolites

of probiotics, such as short-chain fatty acids, and the peptidoglycan components of probiotics appear benefits on both the host gut epithelium and microbiota by modulating the immune function [43,44]. It has also been shown that probiotics shorten the duration and decrease incidence of infections in the elderly during winter [33]. Prebiotics promote the development of the bifido flora in the intestines and enhance both the production of interferon γ and NK cell activity [45–47]. In addition, interferon γ is produced by Th1 cells and has a protective role against influenza infection through its antiviral effects.

Prebiotics are generally considered to promote the viability or the function of probiotics by their fermentation. However, no previous studies have directly compared the efficacy of prebiotics with that of probiotics in improving the immune response to an influenza vaccine. In our analysis, although the comparison was not direct, the subgroup analysis disclosed that supplementation with probiotics achieved more immunogenicity changes than supplementation with prebiotics (Table 3). Nagafuchi et al. further showed that the seroprotective effect was maintained for a longer period when fermented milk (probiotic) was given with a bifidogenic growth stimulator and galacto-oligosaccharide (prebiotic) in enterally-fed older adults vaccinated with H1N1 [24]. Therefore, a simultaneous supply of prebiotics and probiotics might be an effective method of enhancing immune reactions to an influenza vaccine.

A strength of the current study is the low heterogeneity of the pooled analysis. Furthermore, the trials included in our analysis were collected from numerous databases and comprised studies in different languages drawing from different perspectives and cultures.

There are several limitations to the present meta-analysis. First, the outcomes were the rates of seroprotection and seroconversion, not the changes in antibody geometric mean titer (GMT) due to influenza vaccination. The main reason for this was that only a few of the included studies recorded the antibody titers before and after vaccination. Second, there was only one trial with a subgroup analysis of synbiotics and no trials investigating probiotics or prebiotics versus synbiotics, thereby limiting the comparison of different supplements. Third, due to the limited number of included studies and thus insufficient data on basic immune status and original antibody titers against influenza, it was not possible to perform more subgroup analyses or meta-regressions to examine the impact of variables that may influence the heterogeneity of some observed results in our study. Finally, the medications used by the hospitalized patients in the studies might have been confounding factors, and thus require further clarification; however, none of the included studies provided data on medication records.

Table 3. Subgroup analysis of odds ratio of seroprotection and seroconversion rate for different influenza vaccine strains based on supplements.

Subgroup	H1N1	H3N2	B
Probiotics			
Seroprotection	1.73 (0.79–3.80)	2.68 (1.25–5.72) †	1.23 (0.65–2.33)
Seroconversion	1.91 (0.68–5.38)	3.52 (1.45–8.53) *	2.24 (1.24–4.06) *
Prebiotics			
Seroprotection	1.88 (1.06–3.33) †	3.11 (1.25–7.71) †	0.84 (0.48–1.48)
Seroconversion	0.99 (0.54–1.83)	1.31 (0.22–7.98)	1.78 (0.87–3.63)

* $p < 0.01$, † $p < 0.05$.

5. Conclusions

The present meta-analysis revealed that both prebiotics and probiotics can enhance the immunogenicity of a seasonal influenza vaccine in terms of the seroconversion and seroprotection rates in adults, especially in healthy older adults. Longer durations of supplementation had a linear effect on vaccine stimulation. We suggest that either prebiotics or probiotics can be used in adults, especially healthy older adults, prior to seasonal influenza vaccination. Further large RCTs focusing on the optimal dose, duration, and the synergic effect of a combination of probiotics and prebiotics are required to validate these findings.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/9/11/1175/s1, Table S1: PRISMA checklist, Table S2: Detailed searching strategy, Table S3: Quality assessment of included studies based on Cochrane risk of Bias tool, Table S4: Publication bias assessment with Egger's regression, Figure S1: Funnel plot of seroprotection rate of H1N1 strain, Figure S2: Funnel plot H3N2 seroprotection, Figure S3: Funnel plot B seroprotection, Figure S4: Funnel plot H1N1 seroconversion, Figure S5: Funnel plot H3N2 seroconversion, Figure S6 :Funnel plot B seroconversion.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

Gut Microbiota and Nonalcoholic Fatty Liver Disease: Insights on Mechanisms and Therapy

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Abstract: The gut microbiota plays critical roles in development of obese-related metabolic diseases such as nonalcoholic fatty liver disease (NAFLD), type 2 diabetes(T2D), and insulin resistance(IR), highlighting the potential of gut microbiota-targeted therapies in these diseases. There are various ways that gut microbiota can be manipulated, including through use of probiotics, prebiotics, synbiotics, antibiotics, and some active components from herbal medicines. In this review, we review the main roles of gut microbiota in mediating the development of NAFLD, and the advances in gut microbiota-targeted therapies for NAFLD in both the experimental and clinical studies, as well as the conclusions on the prospect of gut microbiota-targeted therapies in the future.

Keywords: gut microbiota; obesity; insulin resistance; NAFLD; probiotic; prebiotic; symbiotic

1. Introduction

The mammalian gastrointestinal tract is the main site for commensal bacteria. There are over 10^{14} microorganisms inside human body [1], which play important roles in maintaining human health [2]. The abundance and composition of gut microbiota are highly variable in the context of different conditions contributing to the development of various diseases [3,4]. In recent years, a huge number of studies have revealed the critical roles of gut microbiota in the development of metabolic diseases including type 1 and 2 diabetes [5,6], obesity [7–10], cardiovascular disease [11–13], and chronic liver diseases [14].

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of chronic liver diseases including simple steatosis, nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [9]. NAFLD is the most common chronic liver disease due to the prevalence of obesity worldwide [15]. In addition to the well-established "two-hit" theory [16], the alteration of gut microbiota also promotes the development of NAFLD by mediating processes of inflammation, insulin resistance, bile acids, and choline metabolism [17,18]. As a result, the elucidation on the roles of gut microbiota in NAFLD highlights the significance of gut microbiota-targeted therapies for NAFLD [19,20]. There are various ways to manipulate gut microbiota, for example through the use of probiotics, prebiotics, synbiotics, antibiotics, and some active components from herbal medicines.

In this review, we retrieved the publications on the topics of gut microbiota and NAFLD mainly published within the past 10 years through Pubmed. Based on all of the publications available, we first reviewed the main roles of gut microbiota in mediating NAFLD formation. Then, we discussed the status of gut microbiota-targeted therapies in NAFLD with both the experimental and clinical evidence, and made conclusions on the therapeutic potential of manipulating gut microbiota in the future.

2. Roles of the Gut Microbiota in NAFLD Development

Obesity is the common ground of most metabolic diseases. The gut microbiota plays critical roles in the development of obesity and obese-related metabolic diseases [21] by producing microbial metabolites like short-chain fatty acids (SCFAs) that regulate host energy harvest [22,23], or by modulating signaling pathways of host energy metabolism [24]. Study has revealed that the gut microbiota promotes the intestinal absorption of monosaccharides, accelerating de novo hepatic lipogenesis and suppressing fasting-induced adipocyte factor, resulting in the accumulation of triglycerides in adipocytes [25]. More evidence of gut microbiota affecting host energy metabolism has been acquired in numerous studies [25–27].

Insulin resistance is a basic pathophysiological process of metabolic diseases [28,29]. In NAFLD, insulin resistance accelerates the fat accumulation and inflammation in hepatocytes [30]. The enhanced inflammation and insulin resistance forms a "vicious cycle" deteriorating the development of NAFLD. The gut epithelium is a natural barrier for preventing the translocation of detrimental bacteria and harmful elements into circulation. NASH patients are typically characterized with small intestine bacterial overgrowth (SIBO) that may impair the intestinal tight junction and subsequently increase intestinal permeability. SIBO also induces hepatic expression of toll like receptor 4 (TLR4) and release of interleukin (IL)-8 that stimulates inflammatory reaction. The term "metabolic endotoxemia" was coined because of increased lipopolysaccharide (LPS) levels in the circulation in metabolic diseases [31], in which LPS combines with LPS binding protein (LBP) and then binds to themonocyte differentiation antigen(CD14)-TLR-4complex triggering an inflammatory reaction and insulin resistance [32–34]. Therefore, gut dysbiosis is causative for enhanced secretion of LPS and its mediated inflammation in NAFLD development.

Choline not only is an indispensable component of cell membrane phospholipids, but also plays important role in lipid metabolism. Choline facilitates the lipid transport in hepatocytes and prevents the abnormal accumulation of lipids in the liver, while choline deficiency usually leads to hepatic steatosis [35,36]. The gut microbiota is also involved in choline metabolism by converting it into toxic dimethylamine and trimethylamine, which are transported to liver and converted into trimethylamine oxide (TMAO) that causes liver inflammation and damage [37]. The increased production of TMAO is also the culprit for cardiovascular disease [37–39]. On the other hand, the content of dietary choline influences the composition and abundance of gut microbiota that are associated with the development of NAFLD [40]. The close relationship between gut microbiota and choline metabolism provides an important rationale for gut microbiota-targeted therapy for NAFLD.

Bile acids are synthesized from cholesterol with a wide range of physiological functions. Bile acids can not only facilitate digestion and absorption of fat-soluble food, but also preserve the intestinal barrier and prevent bacterial translocation [41,42]. Moreover, bile acids could function as signaling molecules that modulate the balance of bile acids metabolism by activating the farnesoid X receptor (FXR) and G protein-coupled bile acid receptor(TGR5) [43–46]. Studies reveal that antibiotics could attenuate the high-fat diet-induced NAFLD development by altering the composition of bile acids and inhibiting the FXR signaling pathway, whereas mice with intestine-specific FXR disruption have reduced triglyceride accumulation in the liver compared with control mice [47]. Bile acids usually have strong anti-microbial properties and gut microbiota can influence the homeostasis of bile acids pool by deconjugating and metabolizing the primary bile acids into secondary bile acids in the intestinal tract, which are involved in modulating lipids and energy metabolism pathways during NAFLD formation [44]. The crosstalk between gut microbiota and bile acids provides fundamental evidence for gut microbiota-targeted therapy of NAFLD. A schematic view on the roles of gut microbiota on NAFLD formation is summarized in Figure 1.

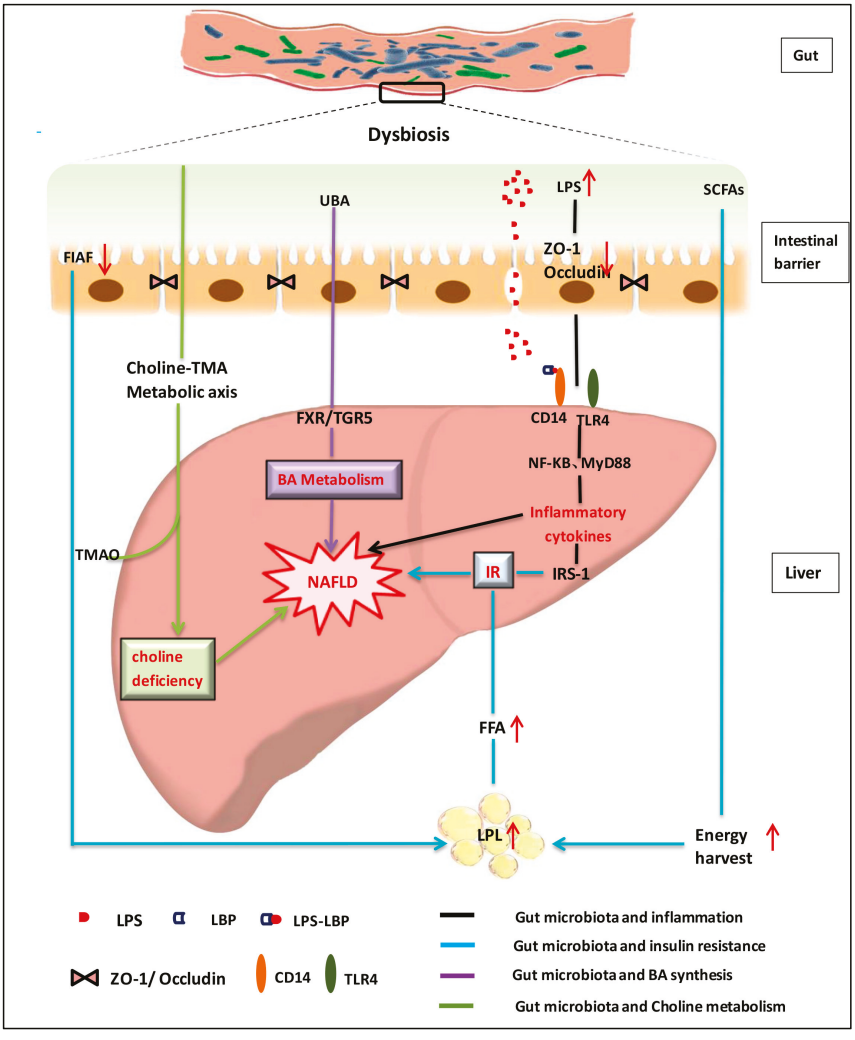

Figure 1. Schematic view on roles of gut microbiota in nonalcoholic fatty liver disease [2,48–60]. NAFLD: nonalcoholic fatty liver disease; LPS: lipopolysaccharides; LBP: LPS binding protein; SCFAs:short chain fatty acids; BA: Bile acid; TNF-α: tumor-necrosis factor alpha; TLR: Toll like receptor; CD14: monocyte differentiation antigen; UBA: unconjugated bile acid; ZO-1\Occludin: two tight junction proteins; FIAF: Fasting-induced adipocyte factor; NF-kB: Nuclear factor-κB; MyD88: myeloid differentiation factor 88; FXR: farnesoid X receptor; TGR5: Takeda G protein-coupled receptor 5; TMA: trimethylamine; TMAO: trimethylamine oxide; IR: Insulin resistance; IRS: Insulin receptor substrate; FFA: free fatty acid; LPL: lipoprteinlipase.

3. Gut Microbiota-Targeted Therapies in NAFLD

NAFLD is common with the current prevalence of obesity, however, clinical therapeutic options are still very scarce with respect to safety, effectiveness, and patient compliance [61]. As a result, the intricate relationship between gut microbiota and NAFLD opens up a new window for seeking effective and safe therapies on NAFLD by restoring gut homeostasis of NAFLD patients in various ways.

3.1. Gut Microbiota-Targeted Therapy with Probiotics

Probiotics are a collection of bacteria with a wide range of beneficial effects on host metabolism [2,62]. Bacteria of *Lactobacillus*, *Bifidobacterium* and *Satreptococcus* are most frequently used probiotics that can inhibit expansion of gram-negative pathogenic bacteria [63]. Okubo et al. investigated the effects of *Lactobacillus caseistrain* Shirota (LcS) on methionine-choline-deficient (MCD) diet-induced NASH mice [64]. They found that the MCD diet resulted in significant reduction in lactic acid bacteria (*Bifidobacterium* and *Lactobacillusin*) in feces, but values were increased by LcS supplementation. Moreover, the LcS supplement dramatically improved the symptoms of NASH induced by MCD such as hepatic histology and serum parameters triglycerides (TG), total cholesterol (TC), as well as the altered expression of hepatic genes and proteins (the mRNA levels of actin alpha/alpha-SMA(α-SMA) and tissue inhibitor of metalloproteinase 1(TIMP-1)). Meanwhile, metabolic beneficial effects of LcS supplement were observed in high-fat diet (HFD)-induced and genetic *db/db* obese mice, in which LcS supplementation significantly improved insulin resistance and lowered plasma levels of LBP [65]. Study revealed that LcS treatment protected against the fructose-induced NAFLD by suppressing the activation of the TLR4 signaling cascade in the liver [66]. Accordingly, the beneficial effect of LcS in metabolic diseases is due to the improvement of metabolic endotoxemia.

Lactobacillus is a genus of gram-positive bacteria which can convert sugars into lactic acid. Bacteria from *Lactobacillus* genus have been trialed as probiotics in studies [67–69]. Sohn et al. investigated the effects of *Lactobacillus paracasei* on NASH patients [70] and found that *L. paracasei* administration lowered inflammatory cytokines in NASH patients. However, probiotics with a single species of *Lactobacillus* bacteria did not show benefit in patients with irritable bowel syndrome or Crohn's disease [71,72]. Meanwhile, the beneficial effects of *Lactobacillus plantarum* probiotics were investigated in NAFLD models such as *L. plantarum* MA2, *L. plantarum* A7 and *L. plantarum* NCU116. Results showed that either *L. plantarum* A7 or *L. plantarum* MA2 was effective in lowering serum lipids [73,74], while *L. plantarum* NCU116 improved liver function and decreased hepatic fat accumulation as well [75]. A similar effect was observed with *L. rhamnosus* supplementation in an NAFLD model. Probiotics of *L. rhamnosus* GG (LGG) protected mice from NAFLD by increasing the abundance of beneficial bacteria, improving gut barrier function and attenuating hepatic inflammation [76], as well as the cholesterol-lowering effect through inhibition of the FXR and FGF15(fibroblast growth factor) signaling pathway [77]. In addition, several other species of *Lactobacilli* bacteria have shown potential in NAFLD prevention, including *L. johnsonii* BS15 [78], *L. reuteri* GMNL-263 [79], *L. gasseri* BNR17 [80].

Bifidobacterium (*Bif*) belongs to the *Bifidobacteria* bacteria genera in the mammalian gastrointestinal tract, and is a frequently used probiotic [81–83]. Supplementation of *Bif* significantly improved visceral fat accumulation and insulin sensitivity in HFD-fed rats [84]. Administration of *Bifidobacterium pseudocatenulatum* CECT 7765 could reduce serum cholesterol and triglycerides, and improved glucose tolerance in obese mice [85]. It is proposed that probiotic of *Bif* is superior to *Lactobacillus acidophilus* in reducing hepatic fat accumulation [86]. Compared to probiotics with a single strain of bacteria, VSL#3 is a mixed probiotic with eight types of bacteria (*Bifidobacteria* (*B. breve*, *B. longum*, *B. infantis*), *Streptococcus thermophilus*, *L. plantarum*, *L. acidophilus*, *L. paracasei* and *L. delbrueckii* subsp. *bulgaricus*) which has shown great potential in treatment of various diseases [87–91]. Experimental evidence has indicated that VSL#3 could attenuate inflammation via modulation of the nuclear factor-kB (NF-kB) pathway [92], reduce hepatic fat accumulation and ALT levels [93], improve insulin sensitivity in NAFLD models [94], and prevent against liver fibrosis in NASH patients [95]. The probiotic with combined bacteria (LGG, *Lactobacillus plantarum* WCFS1 and anthraquinone from *Cassia obtusifolia* L.) was effective in reducing blood lipid levels and improving insulin resistance in NAFLD rats [96]. Meanwhile, supplementation of combined probiotic (*Bifidobacterium infantis*, *Lactobacillus acidopilus*, and *Bacillus cereus*) could improve gut dysbiosis and liver function via suppression of the LPS/TLR4 signaling pathway [97]. Kim et al. found that consumption of kefir (a probiotic beverage containing over 50 species of lactic acid bacteria and yeast) prevented obesity and NAFLD formation by restoring the gut microbiota and

enhancing fatty acid oxidation in HFD-fed mice [98]. Further evidence of beneficial effects on NAFLD prevention has been acquired in many studies by administering probiotics with mixed bacteria [99–101]. In addition to the direct impacts on the composition of gut microbiota, the beneficial effects of probiotics on NAFLD are also associated with their metabolic activities [53]. It has been reported that probiotics of clostridium butyricum MIYAIRI 588—a butyrate-producing bacteria, decreased accumulation of lipid droplets in HFD-induced NAFLD models, improved insulin resistance [102], and reduced hepatic lipids and serum endotoxin levels in choline-deficient/L-amino acid-defined diet-induced NAFLD models [103], which may be associated with the stimulation of expression of adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK) andserine/threonine kinas (AKT) proteins, and lipogenesis- or lipolysis-related proteins.

Currently, although the beneficial effects of probiotics were mainly acquired in experimental studies, some consistent results have also been observed in clinical practice. Alisi *et al*. compared the therapeutic effects of VSL#3 in a randomized double-blind controlled study in obese children with biopsy-proven NAFLD [104]. They found that 4-month supplement of VSL#3 significantly improved liver function and increased glucagon-like peptide (GLP-1)/active glucagon-like peptide (aGLP1) levels suggesting the effects of VSL#3 might be GLP-1-dependent. Consistent effects were also observed on obese children with NAFLD by administering probiotics such as *Lactobacillus rhamnosus* strain GG [105] and mixed bacteria of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* [106]. Sepideh et al. investigated the effects of a multistrain probiotic supplementation in NAFLD patients in a RCT study, and the results showed dramatic improvement in insulin sensitivity and inflammation [107]. Moreover, synergistic effects were also observed by combining probiotics with chemical drugs such as metformin in NASH and statins in NAFLD therapy [108,109], which highlights the great potential of clinical application of probiotics either alone or combined with other drugs. Nevertheless, the clinical efficacy of probiotics still needs further validation in well-designed studies with a larger scale of participants. Solga et al. observed that 4 months of probiotic supplements not only did not reduce hepatic steatosis, but increased fat accumulation in liver of four patients [110]. In 2010, Andreasen et al. conducted a randomized-double-blinded research on effects of *L. acidophilus* NCFM on insulin sensitivity and the systemic inflammation [111]. They found that insulin sensitivity was improved in the probiotic group, but not in the placebo group, and there were no differences in systemic inflammation in either group. Meanwhile, another study indicated that an 8-week probiotic supplement did not improve total cholesterol, low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, TG, TG/ LDL and LDL/HDL ratios in diabetic patients [112]. Additionally, supplementation with *Lactobacillus acidophilus* did not improve the levels of plasma lipids in volunteers with elevated cholesterols in a double-blind placebo-controlled study [113]. A detailed summary of gut microbiota-targeted therapies on NAFLD with probiotics is provided in Table 1.

Table 1. Gut microbiota-targeted therapies of NAFLD with probiotics.

Interventions		Main Effects	Experimental Models	Ref.
Probiotic	*Lactobacillus* (LcS)	Suppressing NASH development	MCD diet-induced NASH in mice	[64]
		Improving insulin resistance and glucose intolerance	Diet-induced obesity (DIO) mice.	[65]
		Protecting against the onset of fructose-induced NAFLD	Fructose-induced NAFLD in mice	[66]
	L. paracasei	Attenuating hepatic steatosis	(High fat +10% fructose diet)-induced NASH in mice	[70]
	L. plantarum A7	Lowering serum lipids, TC, and TG levels	High-cholesterol diet-fed rats	[73]
	L. plantarum MA2	Lowering serum TC, TG and low-density lipoprotein cholesterol	Cholesterol-enriched diet-fed rats	[74]

Table 1. *Cont.*

Interventions		Main Effects	Experimental Models	Ref.
	L. plantarum NCU116	Improving liver function, oxidative stress and lipid metabolism	HFD-induced NAFLD in rats	[75]
	Lactobacillus rhamnosus GG (LGG)	Protecting mice from NAFLD attenuated liver inflammation and steatosis	High-fructose diet induced NAFLD in mice	[76]
		Improving NAFLD	HFD-induced NAFLD in rats	[82]
		Improving in alanine aminotransferase levels	20 obesity-related liver abnormalities in children	[105]
	L. johnsonii BS15	Effective in preventing NAFLD	HFD-induced NAFLD in mice	[78]
	L. reuteri GMNL-263	Ameliorating hepatic steatosis	High-fructose diet-fed rats	[79]
	L. gasseri BNR17	Inhibiting increases in body and adipocyte tissue weight	High-sucrose diet-induced obese mice.	[80]
	3 *Lactobacillus* strains	Reducing serum TC, TG, and low-density lipoprotein cholesterol	HFD-fed rats	[114]
	L. acidophilus NCFM	Inflammatory markers and the systemic inflammatory response were unaffected	45 males with T2D	[111]
	L. acidophilus	No changes in serum lipids	80 patients with elevated cholesterols	[113]
	Bifidobacterium (*Bif*)	Ameliorating visceral fat accumulation and insulin sensitivity	HFD-fed rats	[84]
		Attenuating hepatic fat accumulation	HFD-induced NAFLD in rats	[86]
		Reducing body and fat weights, blood serum levels (TC, HDL-C, LDL-C, TG, AST, ALT, and lipase levels)	HFD-induced obesity in rats	[115]
	B. pseudocatenulatum CECT 7765	Reducing serum cholesterol, TG, and insulin resistance	HFD-fed mice	[85]
	Bacteroides uniformis CECT 7771	Reducing body weight gain, liver steatosis and cholesterol and TG concentrations	HFD-induced obesity mice	[116]
Probiotic	VSL#3	Limiting oxidative and inflammatory liver damage	HFD-fed young rats	[92]
		Reducing hepatic total fatty acid content and ALT levels.	HFD-induced NAFLD in mice	[93]
		Improvements in steatosis and insulin resistance	HFD-fed mice	[94]
		Modulating liver fibrosis, without protecting from inflammation and steatosis in NASH.	MCD diet-induced NASH in mice.	[95]
		Improving the degree of liver disease in children	44 Obese children with NAFLD	[104]
		Improving plasma levels of lipid peroxidation markers: MDA(malondialdehyde), 4-HNE(4-hydroxynonenal).	22 patients with NAFLD + 20 patients with AC (alcoholic liver cirrhosis)	[117]
		Experiencing a significant increase in liver fat; no significant differences in any of the blood assays or clinical parameters	4 patients with NAFLD	[110]
	Probiotic mixtures	Improving NAFLD	HFD-induced NAFLD in rats	[96]
		Delaying the progression of NAFLD via LPS/TLR4 signaling	HSHF diet-induced NAFLD in rats	[97]
		Improving NAFLD pathogenesis and steatosis	High fat and sucrose diet (HFSD)-induced NAFLD in rats	[118]
		Influencing protein expression and decreasing steatohepatitis	MCD diet-induced NASH in rats	[99]
		Reducing obesity-related biomarkers and modulating the microbial community	Obese mice	[100]

Table 1. *Cont.*

Interventions		Main Effects	Experimental Models	Ref.
		Modulating gut microbiota and up-regulated genes related to fatty acid oxidation in both the liver and adipose tissue	HFD-induced obese mice	[98]
		Improving liver aminotransferases levels	30 patients with NAFLD	[106]
		Decreasing levels of ALT and AST and improving pediatric NAFLD	64 obese children with NAFLD	[119]
		Reducing insulin, insulin resistance, TNF-a, and IL-6	42 patients with NAFLD	[107]
		No significant changes in (LDL)-cholesterol, (HDL)-cholesterol, TG, TC TG/LDL and LDL/HDL ratios	60 patients with T2DM	[112]
		Great reductions in serum AST level and liver fat	20 patients with NASH	[120]
	MIYAIRI 588	Improving NAFLD and decreasing accumulation of lipid droplets	HFD-induced NAFLD in rats	[102]
		Improving hepatic lipid deposition and decreasing the triglyceride content, insulin resistance, serum endotoxin levels, and hepatic inflammatory indexes.	Choline-deficient/ L-amino acid-defined (CDAA)-diet-induced NAFLD in rats	[103]
	Probiotics and metformin	Improvements in liver aminotransferases, cholesterol, and TG	64 patients with NASH	[108]
	Probiotics and statins	Lowering cholesterol and products of metabolism of intestinal microflora	Patients with NAFLD	[109]
Probiotic	Probiotic yogurt	Improving hepatic enzymes, serum TC, and low-density lipoprotein cholesterol levels	72 patients with NAFLD	[121]
		Improvements in total cholesterol and LDL-C concentrations	60 people with type 2 diabetes and low-density lipoprotein cholesterol	[122]

NASH: nonalcoholic steatohepatitis; MCD: methionine-choline-deficient; HFD: high-fat diet; LDL: low-density lipoprotein; HDL-C: low density lipoprotein cholesterol; HDL: high-density lipoprotein; HDL-C: high density lipoprotein cholesterol; TG: triglycerides; TC: total cholesterol; IL: interleukin. T2D: type 2 diabetes; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LPS: lipopolysaccharides; TLR: Toll like receptor; HSHF: high sugar and high fat; TNF-α: tumor-necrosis factor alpha.

3.2. Gut Microbiota-Targeted Therapy with Prebiotic

Prebiotics are indigestible food ingredients with beneficial effects, as they selectively stimulate the growth and/or activity of "good" and suppress the "bad" bacteria resident in the colon [123]. They can be defined as a fermented ingredient that allows changes both in the composition and/or activity in the gastrointestinal microflora conferring benefits upon host well-being and health [124,125]. Evidence suggested that prebiotic supplements prevented NAFLD development in both experimental and clinical studies [126,127].

In 2009, Cani et al. found that prebiotics of oligofructose (a mixture of fermentable dietary fibers) decreased plasma LPS and cytokine levels, and hepatic expression of inflammatory and oxidative stress markers in obese mice. An improvement in intestinal permeability and production of GLP-2 was also shown [128]. In an MCD diet-induced steatohepatitis mice model, a dietary fructooligosaccharide (FOS) supplement attenuated the extent of steatohepatitis by restoring the homeostasis of gut microbiota and intestinal epithelial barrier function [129]. Pachikian et al. reported that a FOS supplement reduced hepatic triglyceride accumulation in *n*-3 PUFA (polyunsaturated fatty acid)-depleted diet-induced NAFLD model by altering microbiota composition and increasing production of GLP-1 [130]. Meanwhile, the FOS supplement stimulated fatty acid oxidation by activating peroxisome proliferator-activated receptor-alpha (PPAR-α) and reduced cholesterol accumulation by inhibiting SREBP-2 (sterol-regulatory-element-binding protein isoform 2) in liver

without affecting SREBP-1 expression and activity [130,131]. Lactulose is a prebiotic that promotes the growth of lactic acid bacteria and *Bifidobacteria* [132]. A study indicated that lactulose treatment decreased the hepatic inflammation and serum endotoxin levels in rats with steatohepatitis [133]. Chitin–glucan (CG) is another type of prebiotic from fungal source. Neyrinck et al. investigated the function of CG in HFD-induced obese mice and found CG treatment decreased body weight gain, improved glucose intolerance and hepatic triglyceride accumulation by restoring bacteria of clostridial cluster XIVa [134].

The combination of prebiotics with natural components will yield more benefits than prebiotics on their own. For example, combined therapy of isomalto-oligosaccharides (IMOs) with lycopene (an antioxidant) prevented body weight gain, enhanced adipose tissue fat mobilization, and improved insulin resistance and metabolic endotoxemia in HFD-induced NAFLD mice. The observed effects were associated with their modulation of microbial production of SCFAs [135].

In the clinic, prebiotics have also been tested for their benefits in various diseases [136–140]. Oligofructose (OFS), an inulin-type fructan, was added to diet of NASH patients in a pilot randomized double-blind study [127]. Their results showed that the OFS supplement decreased serum ALT and AST levels significantly. Prebiotics of mixed galacto-oligosaccharides and fructo-oligosaccharides (9:1) stimulated the abundance of *Bifidobacteria* bacteria in infants [141]. Similarly, administration of prebiotic inulin and oligofructose (50:50 in mixture) increased the abundance of *Bifidobacterium* and *Faecalibacterium prausnitzii*, which negatively correlated with serum LPS levels [142]. Prebiotics have shown great potential in prevention of obesity and NAFLD development by lowering the permeability of intestinal wall, attenuating metabolic endotoxemia, and reducing the accumulation of fat [143]. The gut microbiota-targeted therapies with prebiotics were summarized in Table 2.

Table 2. Gut microbiota-targeted therapies of NAFLD with prebiotics.

	Interventions	Main Effects	Experimental Models	Ref.
Prebiotic	Oligofructose (OFS)	Lowering LPS and cytokine levels, and decreasing the hepatic expression of inflammatory and oxidative stress markers	Obese and diabetic mice	[128]
		Decreasing serum ALT, AST and insulin level	Patients with NASH	[127]
	Fructooligosaccharides (FOS)	Restoring normal gastrointestinal microflora and intestinal epithelial barrier function, and decreasing steatohepatitis	MCD diet-induced NASH in mice.	[129]
		Reducing hepatic TG and TC level, modulating hepatic steatosis	*N*-3PUFA (polyunsaturated fatty acid)-depleted diet-fed mice	[130]
	Lactulose	Ameliorating the hepatic inflammation and decreasing serum levels of ALT and AST	HFD-induced NASH in rats	[133]
	Chitin–glucan (CG)	Decreasing weight gain, fat mass development, glucose intolerance, and hepatic TG accumulation	HFD-induced obese mice	[134]
	Isomalto-oligosaccharides (IMOs)	Preventing weight gain, adiposity, and improving insulin resistance.	HFD-induced NAFLD in mice	[135]
	Galacto-oligosaccharides and fructo-oligosaccharides (9:1)	Increasing abundance and proportion of bifidobacteria	Formula-fed infants (FF)	[141]
	Inulin-type fructans(ITF) prebiotics (inulin + oligofructose)	Changing the gut microbiota composition and host metabolism	30 obese women	[142]

3.3. Gut Microbiota-Targeted Therapy with Synbiotic

Synbiotics are the combination of probiotics and prebiotics [144]. Synbiotics usually produce benefits by selectively stimulating the growth and/or activating the metabolism of health-promoting bacteria [145]. Administration of synbiotics containing *Lactobacillus paracasei*

B21060 plus arabinogalactan and fructooligosaccharides attenuated hepatic inflammation and increased expression of nuclear PPARs and their targeted genes in HFD-induced NAFLD rats [146]. Synbiotics have shown various benefits in metabolic diseases, such as improvement of insulin resistance, glucose control, and inflammatory cytokine synthesis [147–149].

In the clinic, the therapeutic effect of a synbiotic containing seven probiotics and oligofructose was evaluated in patients with NAFLD in a double-blind RCT. The results showed that synbiotic therapy significantly decreased ALT levels [150]. Malaguarnera et al. observed that combination of synbiotic (*B. longum* and Fos) and lifestyle intervention in NASH patients resulted in a much greater improvement compared to lifestyle intervention alone, including reduction of serum TNFα, CRP (C-reactive protein), endotoxin, and AST levels, improvement in HOMA-IR(homeostasis model assessment of insulin resistance)and extent of NASH activity index [151]. Synbiotic therapy showed improvements in levels of fasting blood glucose, TG, and inflammatory cytokines in both obese and lean NAFLD patients [152,153]. Therefore, synbiotics are a promising gut microbiota-targeted intervention for NAFLD prevention or therapy. Nevertheless, more clinical validations are also needed. A summarized gut microbiota-targeted therapy on NAFLD with synbiotics was provided in Table 3.

Table 3. Gut microbiota-targeted therapies of NAFLD with synbiotics.

	Interventions	Main Effects	Experimental Models	Ref.
Synbiotic	*L. paracasei* B21060 + arabinogalactan + FOS	Lessening NAFLD progression, preserving gut barrier integrity and reducing the severity of liver injury and IR	HFD-induced NAFLD in rats	[146]
	Seven probiotics + OFS	Improving NAFLD and decreasing levels of ALT and AST	52 patients with NAFLD	[150]
	B. longum + FOS	Reductions in TNF-a, serum AST levels, serum endotoxins, steatosis, and the NASH activity index	66 patients with NASH	[151]
	Dietary fiber + *L. reuteri*	Improving NAFLD and reducing serum levels of most of the inflammatory mediators	50 lean patients with NAFLD	[152]
	Seven probiotics + FOS	Protecting against NAFLD progression and improving steatosis	80 NAFLD patients	[153]

3.4. Gut Microbiota-Targeted Therapies with Other Approaches

In addition to probiotics/prebiotics/synbiotics, gut microbiota-targeted interventions have also been investigated with other approaches. Butyrate is an SCFA and is an important gut microbial metabolite derived from fermentation of nondigestible polysaccharides. Butyrate has a critical role in affecting metabolic disease development in a variety of ways, including modulation on energy harvest, hepatic lipogenesis and gluconeogenesis, adipokine signaling in adipocytes, intestinal permeability, and appetite regulation in the brain [154,155]. Administration of sodium butyrate alleviated inflammation and fat accumulation in HFD-induced NAFLD mice by increasing the abundances of the beneficial bacteria *Christensenellaceae*, *Blautia* and *Lactobacillus* [156]. Therefore, appropriate approaches such as engineered bacteria could be developed to enhance the production of beneficial gut microbial metabolites (e.g., butyrate) or intervention with chemical drugs to promote the proliferation of "good" bacteria, and suppress the "bad" ones.

Antibiotics are frequently used in the clinic, although their disruption of gut microbial homeostasis is a double-edged sword [157]. On one hand, the short-term application of antibiotic can result in long-lasting impacts on host metabolism. On the other hand, administration of some kinds of antibiotics may attenuate diseases. For example, oral administration of cidomycin increased the small intestine transit rate and lowered serum ALT, AST, and TNF-α levels in NASH rats, suggesting the potential of cidomycin in alleviating the severity of NASH by intervening gut microbiota [158]. In the clinic, administration of rifaximin could decrease the circulating endotoxin and ALT levels in patients with NAFLD [159]. Although the improvement in NAFLD, especially in NASH, by short-term

administration of antibiotic (e.g., rifaximin) can be observed, the long-term application of antibiotics is not encouraged because of probable side effects [160]. Nevertheless, the changes in gut microbiota resulting from antibiotics could provide important evidence for exploring alternative ways to modulate gut microbiota in disease therapy.

Compared to antibiotics, some ingredients from herbal medicines have shown more prospects for gut microbiota modulation with minor side effects [161,162]. Berberine is a typical herbal component with potent antibacterial activity, especially bacteria in intestinal tract, because berberine can hardly be absorbed in gut [163]. Currently, increasing evidence has confirmed the therapeutic effect of berberine on metabolic diseases including obesity, NAFLD, and type 2 diabetes via modulation on gut microbiota [164–166]. It has been revealed that berberine administration restored the relative abundance of *Bifidobacteria* and the ratio of *Bacteroidetes*/*Firmicutes* in HFD-induced NASH mice resulting in significant reduction in body weight, serum levels of lipids, glucose, insulin and inflammatory cytokines [167,168]. TSG (2,3,5,4′-tetrahydroxy-stilbene-2-*O*-β-D-glucoside) is an active component of the traditional Chinese medicine (TCM) *Polygonum multiflorum* Thunb, which has shown significant effects in NAFLD prevention by modulating gut microbiota, improving the intestinal mucosal barrier, and suppressing the expression of NF-κB [169]. Resveratrol is a natural polyphenol with anti-oxidative activity [170]. Recent studies showed resveratrol was also effective in preventing metabolic diseases such as obesity and NASH by regulating gut microbiota [171]. In addition to the individual component from herbal medicines, recent investigations revealed that the efficacy of some TCM formulas was associated with the modulation on gut microbiota. For example, Qushi Huayu Fang (a mixture of five herbs including *Artemisia capillaries Thunb*, *Gardenia jasminoides Ellis*, *Fallopia japonica*, *Curcuma longa* L., and *Hypericum japonicum Thunb*) is an ancient TCM formula which has been used for NAFLD treatment. Recent studies showed that administration of Qushi Huayu Decoction (QHD) significantly decreased body weight, alleviated hepatic steatosis, and reduced the content of TG and free fatty acids in liver in HFD-induced NAFLD rats. It showed that the QHD-treated group harbored significantly different gut microbiota from that of model rats, and the bacterial profiles of NAFLD rats could be modulated by the QHD [172,173]. Recently, the anti-obesity property of daesiho-tang (DSHT) was also investigated. It was found that DSHT treatment significantly reduced levels of serum TC and TG as well as hepatic fat accumulation that were associated with the regulation on abundance of gut microbiota [174]. Although the mechanisms underlying TCM therapy are extremely complicated and largely unknown, the gut microbiota was supposed to be an important target for many TCM formulas because many kinds of chemicals derived from TCM are unabsorbable. Those unabsorbed chemicals in TCM can influence gut microbiota directly or be metabolized into absorbable or active form by gut microbiota. A summary of gut microbiota-targeted therapies on NAFLD with other approaches were provided in Table 4.

Table 4. Gut microbiota-targeted therapies of NAFLD—other approaches.

Interventions		Main Effects	Experimental Models	Ref.
Antibiotic	Cidomycin	Lowering serum levels of ALT, AST and TNF-α and alleviating the severity of NASH	Rats with NASH	[158]
	Vancomycin + Neomycin + Metronidazole + Ampicillin	Adjusting gut microecology and alleviating the lesions of NAFLD	HFD-induced NAFLD in rats	[175]
	Rifaximin	Improving NAFLD and reducing endotoxin and IL-10 levels	42 patients with NAFLD	[159]
Herbal medicine or natural active ingredient	2,3,5,4′-tetrahydroxy-stilbene-2-*O*-β-D-glucoside (TSG)	Reversing NAFLD and reducing FFA accumulation, and increasing the protein expression of ZO-1 and occludin	HFD-induced NAFLD in rats	[169]
	Resveratrol	Reducing blood glucose and lipid levels, and lowering both body and visceral adipose weights	HFD-fed mice	[171]

Table 4. *Cont.*

Interventions		Main Effects	Experimental Models	Ref.
	Qushi Huayu Fang	Reducing body weight, TG and free fatty acids, alleviating hepatic steatosis	HFD-induced NAFLD in rats	[172]
		Enhancing the hepatic anti-oxidative mechanism, decreasing hepatic lipid synthesis, and promoting the regulatory T cell inducing microbiota in the gut	HFD-induced NAFLD in rats	[173]
	Daesiho-tang (DSHT)	Ameliorating body weight gain, body fat, decreasing TC and TG	HFD-fed obese mice	[174]
	Gegen Qinlian Decoction (GQD)	Alleviating T2D, increasing the amounts of beneficial bacteria	187 patients with type 2 diabetes (T2D)	[176]

4. Conclusions and Perspectives

Currently, the gut microbiota has been recognized as a critical factor contributing to the development of NAFLD and the gut microbial-related mechanisms have also been well elucidated. As a result, the strategy of gut microbiota-targeted therapy on NAFLD is highly valued in the context of accumulating benefits of gut microbial modulation by using probiotics, prebiotics, synbiotics, antibiotics, and herbal medicines. Although many experimental reports were exciting, discrepant results were also observed in the clinic. Therefore, the clinical efficacy of gut microbiota-targeted therapies on NAFLD still need to be confirmed with large-scale and well-organized RCT studies. The main factors contributing to the variation of therapeutic outcomes in the clinic are differences in bacterial activity of probiotics or the diversified dysbiosis among NAFLD patients. In this sense, probiotics with mixed bacteria such as VSL#3 are more prospective than those with individual type of bacteria. Meanwhile, the gut microbiota-related efficacy of natural components from herbal medicines or the TCM formula itself highlighted the great potential of seeking novel medicines from TCM because some TCMs showed their effects by nourishing "good" bacteria and suppressing "bad" ones. Currently, 16S rDNA-based sequencing is still the major approach for most gut microbiota-involved studies because it is relatively affordable and applicable for most laboratories. Although 16S rDNA sequencing can provide a general description on the structural differences of the microbiome between samples, especially at the genus level, it is usually frustrating when information for specific bacteria species is necessary. Consequently, metagenomics will be more applicable for figuring out specific bacterial species that may contribute to the disease development or therapeutic efficacy, as well as the involved microbial functions.

In summary, gut microbiota-targeted therapies for diseases are still in their infancy. Nevertheless, we envision that more gut microbiota-targeted therapies will be tested in the context of accumulation of therapeutic evidence and advances in elucidation of gut microbial-related mechanisms in diseases, as well as the technological innovation of gut microbiome analysis.

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Appendix A

Search strategy

- The main source of material was pubmed, and the search keywords used were as follows: "gut microbiota", "gut flora", "nonalcoholic fatty liver disease(NAFLD)", "nonalcoholic steatohepatitis(NASH)", "steatosis","probiotic", "prebiotic", "antibiotic", "herbal medicince";

- Selected papers have no language restrictions;
- Most of the papers selected were published during the past 10 years;
- References of some identified papers were further searched for related papers to cover this topic as completely as possible.

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Review

Effects of Probiotics, Prebiotics, and Synbiotics on Human Health

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Abstract: The human gastrointestinal tract is colonised by a complex ecosystem of microorganisms. Intestinal bacteria are not only commensal, but they also undergo a synbiotic co-evolution along with their host. Beneficial intestinal bacteria have numerous and important functions, e.g., they produce various nutrients for their host, prevent infections caused by intestinal pathogens, and modulate a normal immunological response. Therefore, modification of the intestinal microbiota in order to achieve, restore, and maintain favourable balance in the ecosystem, and the activity of microorganisms present in the gastrointestinal tract is necessary for the improved health condition of the host. The introduction of probiotics, prebiotics, or synbiotics into human diet is favourable for the intestinal microbiota. They may be consumed in the form of raw vegetables and fruit, fermented pickles, or dairy products. Another source may be pharmaceutical formulas and functional food. This paper provides a review of available information and summarises the current knowledge on the effects of probiotics, prebiotics, and synbiotics on human health. The mechanism of beneficial action of those substances is discussed, and verified study results proving their efficacy in human nutrition are presented.

Keywords: probiotic bacteria; prebiotics; synbiotics; human health; gut microbiota

1. Introduction

Nowadays, besides the basic role of nutrition consisting in the supply of necessary nutrients for growth and development of the organism, some additional aspects are becoming increasingly important, including the maintenance of health and counteracting diseases. In the world of highly processed food, particular attention is drawn to the composition and safety of consumed products. The quality of food is very important because of, i.e., the problem of food poisoning, obesity, allergy, cardiovascular diseases, and cancer—the plague of the 21st century. Scientific reports point to the health benefits of using probiotics and prebiotics in human nutrition. The word "probiotic" comes from Greek, and it means "for life". Most probably, it was Ferdinand Vergin who invented the term "probiotic" in 1954, in his article entitled "Anti-und Probiotika" comparing the harmful effects of antibiotics and other antibacterial agents on the intestinal microbiota with the beneficial effects ("probiotika") of some useful bacteria [1]. Some time after that, in 1965, Lilly and Stillwell described probiotics as microorganisms stimulating the growth of other microorganisms [2]. The definition of probiotics has been modified and changed many times. To emphasise their microbial origin, Fuller (1989) stated that probiotics must be viable microorganisms and must exert a beneficial effect on their host [3]. On the other hand, Guarner and Schaafsma (1998) indicated the necessary use of an appropriate dose of probiotic organisms required to achieve the expected effect [4]. The current definition, formulated in 2002 by FAO (Food and Agriculture Organization of the United Nations) and WHO (World Health Organization) working group experts, states that probiotics are "live strains

of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [5]. The definition was maintained by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2013 [6].

Results of clinical studies confirm the positive effect of probiotics on gastrointestinal diseases (e.g., irritable bowel syndrome, gastrointestinal disorders, elimination of *Helicobacter*, inflammatory bowel disease, diarrhoeas) and allergic diseases (e.g., atopic dermatitis). Many clinical studies have proven the effectiveness of probiotics for treatment of diseases such as obesity, insulin resistance syndrome, type 2 diabetes, and non-alcoholic fatty liver disease. Furthermore, the positive effects of probiotics on human health have been demonstrated by increasing the body's immunity (immunomodulation). Scientific reports also show the benefits of the prophylactic use of probiotics in different types of cancer and side effects associated with cancer. Many clinical studies have proven the effectiveness of probiotics, and recommended doses of probiotics are those that have been used in a particular case. Keep in mind that how probiotics work may depend on the strain, dose, and components used to produce a given probiotic product.

In 1995, prebiotics were defined by Gibson and Roberfroid as non-digested food components that, through the stimulation of growth and/or activity of a single type or a limited amount of microorganisms residing in the gastrointestinal tract, improve the health condition of a host [7]. In 2004, the definition was updated and prebiotics were defined as selectively fermented components allowing specific changes in the composition and/or activity of microorganisms in the gastrointestinal tract, beneficial for host's health and wellbeing [8]. Finally, in 2007, FAO/WHO experts described prebiotics as a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota [9].

Prebiotics may be used as an alternative to probiotics or as an additional support for them. However different prebiotics will stimulate the growth of different indigenous gut bacteria. Prebiotics have enormous potential for modifying the gut microbiota, but these modifications occur at the level of individual strains and species and are not easily predicted a priori. There are many reports on the beneficial effects of prebiotics on human health.

High potential is attributed to the simultaneous use of probiotics and prebiotics. In 1995, Gibson and Roberfroid introduced the term "synbiotic" to describe a combination of synergistically acting probiotics and prebiotics [7]. A selected component introduced to the gastrointestinal tract should selectively stimulate growth and/or activate the metabolism of a physiological intestinal microbiota, thus conferring beneficial effect to the host's health [10]. As the word "synbiotic" implies synergy, the term should be reserved for those products in which a prebiotic component selectively favours a probiotic microorganism [11]. The principal purpose of that type of combination is the improvement of survival of probiotic microorganisms in the gastrointestinal tract.

Synbiotics have both probiotic and prebiotic properties and were created in order to overcome some possible difficulties in the survival of probiotics in the gastrointestinal tract [12]. Therefore, an appropriate combination of both components in a single product should ensure a superior effect, compared to the activity of the probiotic or prebiotic alone [13,14].

The aim of the review was to discuss the mechanisms of action of probiotics, prebiotics, and synbiotics, as well as the current insight into their effect on human health. The selection of probiotic strains, prebiotics, and their respective dosages is crucial in obtaining a therapeutic effect, so separate sections are dedicated to this topic. Further research into the acquisition of new probiotic strains, the selection of probiotics and prebiotics for synbiotics, dose setting, safety of use, and clinical trials documenting the desired health effects is necessary. Effects should be confirmed in properly scheduled clinical trials conducted by independent research centres.

2. Probiotics

The knowledge of the beneficial effects of lactic acid fermentation on human health dates back to ancient times. The Bible mentions sour milk several times. Ancient Romans and Greeks knew various

recipes for fermented milk. A specific type of sour milk, called "leben raib", prepared from buffalo, cow, or goat milk, was consumed in ancient Egypt. A similar "jahurt" was also commonly consumed by people inhabiting the Balkans. In India, fermented milk drinks were known already 800–300 years B.C., and in Turkey in the 8th century. A milk drink called "ajran" was consumed in Central Russia in the 12th century, and "tarho" was consumed in Hungary in the 14th century [15].

A particular interest in lactic acid fermentation was expressed in the beginning of the 20th century by the Russian scientist and immunologist working for the Pasteur Institute in Paris, awarded with the Nobel Prize in medicine for his work on immunology (in 1907), Ilia Miecznikow. Here is a quote from his book "Studies on Optimism": "with various foods undergoing lactic acid fermentation and consumed raw (sour milk, kefir, sauerkraut, pickles) humans introduced huge amounts of proliferating lactic acid bacteria to their alimentary tracts" [16].

2.1. Selection Criteria and Requirements for Probiotic Strains

According to the suggestions of the WHO, FAO, and EFSA (the European Food Safety Authority), in their selection process, probiotic strains must meet both safety and functionality criteria, as well as those related to their technological usefulness (Table 1). Probiotic characteristics are not associated with the genus or species of a microorganism, but with few and specially selected strains of a particular species [6]. The safety of a strain is defined by its origin, the absence of association with pathogenic cultures, and the antibiotic resistance profile. Functional aspects define their survival in the gastrointestinal tract and their immunomodulatory effect. Probiotic strains have to meet the requirements associated with the technology of their production, which means they have to be able to survive and maintain their properties throughout the storage and distribution processes [17]. Probiotics should also have documented pro-health effects consistent with the characteristics of the strain present in a marketed product. Review papers and scientific studies on one strain may not be used for the promotion of other strains as probiotics. It has to be considered, as well, that the studies documenting probiotic properties of a particular strain at a tested dose do not constitute evidence of similar properties of a different dose of the same strain. Also, the type of carrier/matrix is important, as it may reduce the viability of a particular strain, thus changing the properties of a product [18,19].

Table 1. Selection criteria of probiotic strains [5,20].

Criterion	Required Properties
Safety	• Human or animal origin. • Isolated from the gastrointestinal tract of healthy individuals. • History of safe use. • Precise diagnostic identification (phenotype and genotype traits). • Absence of data regarding an association with infective disease. • Absence of the ability to cleave bile acid salts. • No adverse effects. • Absence of genes responsible for antibiotic resistance localised in non-stable elements.
Functionality	• Competitiveness with respect to the microbiota inhabiting the intestinal ecosystem. • Ability to survive and maintain the metabolic activity, and to grow in the target site. • Resistance to bile salts and enzymes. • Resistance to low pH in the stomach. • Competitiveness with respect to microbial species inhabiting the intestinal ecosystem (including closely related species). • Antagonistic activity towards pathogens (e.g., *H. pylori*, *Salmonella* sp., *Listeria monocytogenes*, *Clostridium difficile*). • Resistance to bacteriocins and acids produced by the endogenic intestinal microbiota. • Adherence and ability to colonise some particular sites within the host organism, and an appropriate survival rate in the gastrointestinal system.
Technological usability	• Easy production of high biomass amounts and high productivity of cultures. • Viability and stability of the desired properties of probiotic bacteria during the fixing process (freezing, freeze-drying), preparation, and distribution of probiotic products. • High storage survival rate in finished products (in aerobic and micro-aerophilic conditions). • Guarantee of desired sensory properties of finished products (in the case of the food industry). • Genetic stability. • Resistance to bacteriophages.

2.2. Probiotic Microorganisms

Probiotic products may contain one or more selected microbial strains. Human probiotic microorganisms belong mostly to the following geni: *Lactobacillus*, *Bifidobacterium*, and *Lactococus*, *Streptococcus*, *Enterococcus*. Moreover, strains of Gram-positive bacteria belonging to the genus *Bacillus* and some yeast strains belonging to the genus *Saccharomyces* are commonly used in probiotic products [21].

Probiotics are subject to regulations contained in the general food law, according to which they should be safe for human and animal health. In the USA, microorganisms used for consumption purposes should have the GRAS (Generally Regarded As Safe) status, regulated by the FDA (Food and Drug Administration). In Europe, EFSA introduced the term of QPS (Qualified Presumption of Safety). The QPS concept involves some additional criteria of the safety assessment of bacterial supplements, including the history of safe usage and absence of the risk of acquired resistance to antibiotics [22,23]. Table 2 presents probiotic microorganisms contained in pharmaceutical products and as food additives.

Table 2. Probiotic microorganisms used in human nutrition [24–26].

Type *Lactobacillus*	Type *Bifidobacterium*	Other Lactic Acid Bacteria	Other Microorganisms
L. acidophilus [(a),*] *L. amylovorus* [(b),*] *L. casei* [(a),(b),*] *L. gasseri* [(a),*] *L. helveticus* [(a),*] *L. johnsonii* [(b),*] *L. pentosus* [(b),*] *L. plantarum* [(b),*] *L. reuteri* [(a),*] *L. rhamnosus* [(a),(b),*]	*B. adolescentis* [(a)] *B. animalis* [(a),*] *B. bifidum* [(a)] *B. breve* [(b)] *B. infantis* [(a)] *B. longum* [(a),*]	*Enterococcus faecium* [(a)] *Lactococcus lactis* [(b),*] *Streptococcus thermophilus* [(a),*]	*Bacillus clausii* [(a),*] *Escherichia coli Nissle 1917* [(a)] *Saccharomyces cerevisiae (boulardi)* [(a),*]

[(a)] Mostly as pharmaceutical products; [(b)] mostly as food additives; * QPS (Qualified Presumption of Safety) microorganisms.

2.3. Mechanism of Action of Probiotics

A significant progress has been observed lately in the field of studies on probiotics, mostly in terms of the selection and characteristics of individual probiotic cultures, their possible use, and their effect on health.

Probiotics have numerous advantageous functions in human organisms. Their main advantage is the effect on the development of the microbiota inhabiting the organism in the way ensuring proper balance between pathogens and the bacteria that are necessary for a normal function of the organism [27,28]. Live microorganisms meeting the applicable criteria are used in the production of functional food and in the preservation of food products. Their positive effect is used for the restoration of natural microbiota after antibiotic therapy [29,30]. Another function is counteracting the activity of pathogenic intestinal microbiota, introduced from contaminated food and environment. Therefore, probiotics may effectively inhibit the development of pathogenic bacteria, such as *Clostridium perfringens* [31], *Campylobacter jejuni* [32], *Salmonella* Enteritidis [33], *Escherichia coli* [34], various species of *Shigella* [35], *Staphylococcus* [36], and *Yersinia* [37], thus preventing food poisoning. A positive effect of probiotics on digestion processes, treatment of food allergies [38,39], candidoses [40], and dental caries [41] has been confirmed. Probiotic microorganisms such as *Lactobacillus plantarum* [42], *Lactobacillus reuteri* [43], *Bifidobacterium adolescentis*, and *Bifidobacterium pseudocatenulatum* [44] are natural producers of B group vitamins (B1, B2, B3, B6, B8, B9, B12). They also increase the efficiency of the immunological system, enhance the absorption of vitamins and mineral compounds, and stimulate the generation of organic acids and amino acids [18,45–47]. Probiotic microorganisms may also be able to produce enzymes, such as esterase, lipase, and co-enzymes A, Q, NAD, and NADP. Some products of probiotics' metabolism may also show antibiotic (acidophiline, bacitracin, lactacin), anti-cancerogenic, and immunosuppressive properties [45,48–50].

Molecular and genetic studies allowed the determination of the basics of the beneficial effect of probiotics, involving four mechanisms:

(1) Antagonism through the production of antimicrobial substances [51];
(2) Competition with pathogens for adhesion to the epithelium and for nutrients [52];
(3) Immunomodulation of the host [53];
(4) Inhibition of bacterial toxin production [54].

The first two mechanisms are directly associated with their effect on other microorganisms. Those mechanisms are important in prophylaxis and treatment of infections, and in the maintenance of balance of the host's intestinal microbiota. The ability of probiotic strains to co-aggregate, as one of their mechanisms of action, may lead to the formation of a protective barrier preventing pathogenic bacteria from the colonisation of the epithelium [27]. Probiotic bacteria may be able to adhere to epithelial cells, thus blocking pathogens. That mechanism exerts an important effect on the host's health condition. Moreover, the adhesion of probiotic microorganisms to epithelial cells may trigger a signalling cascade, leading to immunological modulation. Alternatively, the release of some soluble components may cause a direct or indirect (through epithelial cells) activation of immunological cells. This effect plays an important role in the prevention and treatment of contagious diseases, as well as in chronic inflammation of the alimentary tract or of a part thereof [28]. There are also suggestions of a possible role of probiotics in the elimination of cancer cells [55].

Results of in vitro studies indicate the role of low-molecular-weight substances produced by probiotic microorganisms (e.g., hydroperoxide and short-chain fatty acids) in inhibiting the replication of pathogens [28]. For example, *Lactobacillus* genus bacteria may be able to produce bacteriocins, including low-molecular-weight substances (LMWB—antibacterial peptides), as well as high-molecular-weight ones (class III bacteriocins), and some antibiotics. Probiotic bacteria (e.g., *Lactobacillus* and *Bifidobacterium*) may produce the so-called de-conjugated bile acids (derivatives of bile acids), demonstrating stronger antibacterial effect than the bile salts produced by their host [28,56]. Further studies are necessary to explain the mechanism of acquiring resistance to their own metabolites by *Lactobacillus* genus bacteria. The nutrient essential for nearly all bacteria, except for lactic acid bacteria, is iron. It turns out that *Lactobacillus* bacteria do not need iron in their natural environment, which may be their crucial advantage over other microorganisms [57]. *Lactobacillus delbrueckii* affects the function of other microbes by binding iron hydroxide to its cellular surface, thus making it unavailable to other microbes [58].

The immunomodulatory effect of the intestinal microbiota, including probiotic bacteria, is based on three, seemingly contradictory phenomena [53,59]:

(1) Induction and maintenance of the state of immunological tolerance to environmental antigens (nutritional and inhalatory);
(2) Induction and control of immunological reactions against pathogens of bacterial and viral origin;
(3) Inhibition of auto-aggressive and allergic reactions.

Probiotic-induced immunological stimulation is also manifested by the increased production of immunoglobulins, enhanced activity of macrophages and lymphocytes, and stimulation of γ-interferon production. Probiotics may influence the congenital and acquired immunological system through metabolites, components of the cellular wall, and DNA, recognised by specialised cells of the host (e.g., those equipped with receptors) [28]. The principal host cells that are important in the context of the immune response are intestinal epithelial cells and intestinal immune cells. Components of the cellular wall of lactic acid bacteria stimulate the activity of macrophages. Those, in turn, are able to destroy microbes rapidly by the increased production of free oxygen radicals and lysosomal enzymes. Probiotic bacteria are also able to stimulate the production of cytokines by immunocompetent cells of the gastrointestinal tract [60]. On the other hand, the immunological activity of yeast is associated with the presence of glucans in their cellular wall. Those compounds stimulate the response of the reticuloendothelial system [61].

The last of the abovementioned probiotic effects—inhibition of the production of bacterial toxins—is based on actions leading to toxin inactivation and help with the removal of toxins from

the body. Help in detoxification from the body can take place by adsorption (some strains can bind toxins to their cell wall and reduce the intestinal absorption of toxins), but can also result from the metabolism of mycotoxins (e.g., aflatoxin) by microorganisms [62–64]. However, not all probiotics exhibit detoxifying properties, as it is a strain-related characteristic. Studies should therefore be conducted to select strains with such characteristics. The effectiveness of some probiotics in combating diarrhoea is probably associated with their ability to protect the host from toxins. The reduction of metabolic reactions leading to the production of toxins is also associated with the stimulation of pathways leading to the production of native enzymes, vitamins, and antimicrobial substances [28].

Gut microbiota play a significant role in host metabolic processes (e.g., the regulation of cholesterol absorption, blood pressure (BP), and glucose metabolism), and recent metagenomic surveys have revealed that they are involved in host immune modulation and that they influence host development and physiology (organ development) [65–67]. Nutritional programming to manipulate the composition of the intestinal microbiota through the administration of probiotics continues to receive much attention for the prevention or attenuation of the symptoms of metabolic-related diseases. Currently, studies are exploring the potential for expanded uses of probiotics for improving health conditions in metabolic disorders that increase the risk of developing cardiovascular diseases such as hypertension. Further investigations are required to evaluate the targeted and effective use of the wide variety of probiotic strains in various metabolic disorders to improve the overall health status of the host [65].

In order to confirm the beneficial role of probiotics in improving cardiovascular health and in the reduction of BP, more extensive studies are needed to understand the mechanisms underlying probiotic action. Most probably, all of the abovementioned mechanisms of probiotic action have an effect on the protection against infections, cancer, and the stabilization of balance of the host's intestinal microbiota. However, it seems unlikely that each of the probiotic microorganisms has properties of all four aspects simultaneously and constitutes a universal remedy to multiple diseases. An important role in the action of probiotics is played by species- and strain-specific traits, such as: cellular structure, cell surface, size, metabolic properties, and substances secreted by microorganisms. The use of a combination of probiotics demonstrating various mechanisms of action may provide enhanced protection offered by a bio-therapeutic product [68]. Figure 1 summarises the mechanisms and effects of action of probiotics.

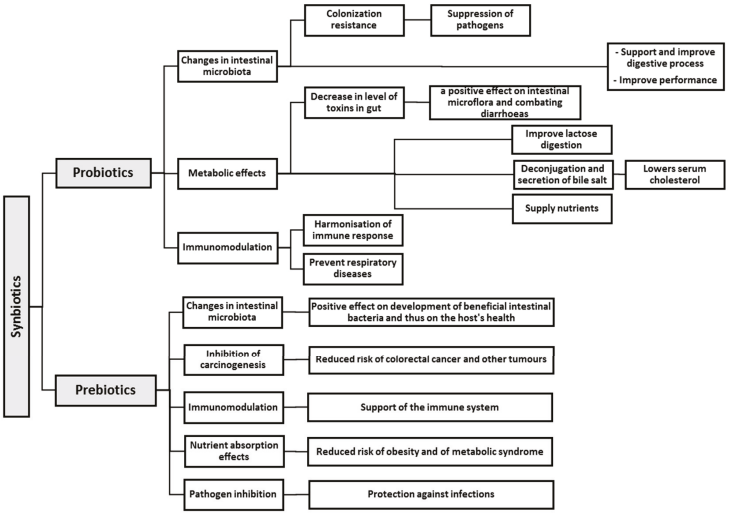

Figure 1. Mechanisms of action of synbiotics and their effects.

2.4. Probiotics for Humans

In the face of widespread diseases and ageing societies, the use of knowledge on microbiocenosis of the gastrointestinal tract and on the beneficial effect of probiotic bacteria is becoming increasingly important. The consumption of pre-processed food (fast food), often containing excessive amounts of fat and insufficient amounts of vegetables, is another factor of harmful modification of human intestinal microbiota. There is currently no doubt about the fact that the system of intestinal microorganisms and its desirable modification with probiotic formulas and products may protect people against enteral problems, and influence the overall improvement of health.

Probiotics may be helpful in the treatment of inflammatory enteral conditions, including ulcerative colitis, Crohn's disease, and non-specific ileitis. The aetiology of those diseases is not completely understood, but it is evident that they are associated with chronic and recurrent infections or inflammations of the intestine. Clinical studies have demonstrated that probiotics lead to the remission of ulcerative colitis, but no positive effect on Crohn's disease has been observed [69,70]. Numerous studies assessed the use of probiotics in the treatment of lactose intolerance [71,72], irritable bowel syndrome, and the prevention of colorectal cancer [73] and peptic ulcers [74].

Considering their role in the inhibition of some bacterial enzymes, probiotics may reduce the risk of colorectal carcinoma in animals. However, the same effect in humans has not been confirmed in clinical trials [75]. On the other hand, a positive effect on the urogenital system (prevention and treatment of Urinary Tract Infections (UTIs) and bacterial vaginitis) constitutes an excellent example of the benefits associated with the use of probiotics [76–78]. There were attempts to apply probiotics to pregnant women and neonates in order to prevent allergic diseases such as atopic dermatitis. However, the scope of action is controversial in this kind of case [79]. There is evidence that the consumption of probiotics-containing dairy products results in the reduction of blood cholesterol, which may be helpful in the prevention of obesity, diabetes, cardiovascular diseases, and cerebral stroke [80]. The reduction of cholesterol level achieved due to probiotics is less pronounced compared to the effect of pharmaceutical agents, but leads to a significant minimisation of side effects [80]. Other studies confirmed the effect of the probiotic formula VSL#3 and of the *Oxalobacter formigenes* bacterial strain on the elimination of oxalates with urine, which may potentially reduce the risk of urolithiasis [81]. Studies on animals demonstrated that orally administered *Lactobacillus acidophilus* induces expression of μ-opioid and cannabinoid receptors in intestinal cells and mediate analgesic functions in the intestine, and that the observed effect is comparable to the effect of morphine [82]. However, the effect has not been demonstrated in humans.

There are many reports on the application of probiotics in the treatment of diarrhoea. The application of *Saccharomyces boulardii* yeast to patients with acute, watery diarrhoea resulted in the cure and reduced frequency of that type of complaints in two subsequent months [83]. The efficacy of probiotic strains in the therapy of nosocomial, non-nosocomial, and viral diarrhoeas has also been documented. It turns out that probiotics may increase the amount of IgA antibodies, which leads to the arrest of a viral infection [84].

Antibiotic-associated diarrhoea (AAD) is a common complication of most antibiotics and *Clostridium difficile* disease (CDD), which also is incited by antibiotics, and is a leading cause of nosocomial outbreaks of diarrhoea and colitis. The use of probiotics for these two related diseases remains controversial. A variety of different types of probiotics show promise as effective therapies for these two diseases. Using meta-analyses, three types of probiotics (*Saccharomyces boulardii*, *Lactobacillus rhamnosus* GG, and probiotic mixtures) significantly reduced the development of antibiotic-associated diarrhoea. Only *S. boulardii* was effective for CDD [85].

Studies performed in a foster home in Helsinki (Finland) demonstrated that the regular use of *Lactobacillus rhamnosus* GG in the form of a probiotic resulted in a reduced number of respiratory tract infections [86]. Other studies demonstrated that the application of a diet depleted of fermented foods caused a reduction of congenital immunological response, as well as a significant reduction of stool *Lactobacillus* count and of the stool amount of short-chain fatty acids. Moreover, the reduction

of phagocytic activity of leukocytes was observed after two weeks of the diet, which could have a negative impact on the organism's ability to protect against infections [87]. The effect of a fermented product containing *Lactobacillus gasseri* CECT5714 and *Lactobacillus coryniformis* CECT5711 strains on blood and stool parameters was studied in a randomised, double-blind trial on 30 healthy volunteers. No negative effects were observed in the group of subjects receiving the probiotic strains. Some positive effects were observed, including: the production of short-chain fatty acids, humidity, frequency and volume of stools, and subjective improvement of intestinal function [88]. Studies by Alvaro et al. (2007) demonstrated a significant reduction of *Enterobacteriaceae* count and increased galactosidase activity in the alimentary tract of yoghurt consumers, compared to those who did not eat yoghurt [89]. Table 3 lists the results of studies focusing on the effect of probiotics on human health. There are examples of clinical trials during which the probiotics group received the probiotic prophylactically or in addition to the standard therapy.

Table 3. Examples of clinical trials regarding the effect of probiotics on human health.

References	Subjects	Microorganism	Time of Administration	Main Outcome
		Obesity		
[90]	50 obese adolescents	*L. salivarius* Ls-33	12 weeks	Increase in the ratios of *Bacteroides*, *Prevotellae*, and *Porphyromonas*.
[91]	50 adolescents with obesity	*L. salivarius* Ls-33	12 weeks	No effect.
[92]	87 subjects with high BMI	*L. gasseri* SBT2055	12 weeks	Reduction in BMI, waist, abdominal VFA, and hip circumference.
[93]	210 adults with large VFA	*L. gasseri* SBT2055	12 weeks	Reduction in BMI and arterial BP values.
[94]	40 adults with obesity	*L. plantarum*	3 weeks	Reduction in BMI and arterial BP values.
[95–97]	75 subjects with high BMI	*L. acidophilus* La5, *B. lactis* Bb12, *L. casei* DN001	8 weeks	Changes in gene expression in PBMCs as well as BMI, fat percentage, and leptin levels.
[98]	70 overweight and obese subjects	*E. faecium* and 2, *S. thermophilus strains*	8 weeks	Reduction in body weight, systolic BP, LDL-C, and increase in fibrinogen levels.
[99]	60 overweight subjects	*Bifidobacterium*, *Lactobacillus*, *S. thermophilus*	6 weeks	Improvement in lipid profile, insulin sensitivity, and decrease in CRP.
[100]	58 obese PM women	*L. paracasei* N19	6 weeks	No effect.
[101]	156 overweight adults	*L. acidophilus* La5, *B. animalis* subsp. *lactis* Bb12	6 weeks	Reduction in fasting glucose concentration and increase in HOMA-IR.
		Insulin resistance syndrome		
[102]	28 patients with IRS	*L. casei* Shirota	12 weeks	No effect.
[103]	30 patients with IRS	*L. casei* Shirota	12 weeks	Significant reduction in the VCAM-1 level.
[104]	24 PM women with IRS	*L. plantarum*	12 weeks	Glucose and homocysteine levels were significantly reduced.
		Type 2 diabetes		
[105]	40 patients with T2D	*L. planatarum* A7	8 weeks	Decreased methylation process, SOD, and 8-OHDG.
[106]	45 patients with T2D	*L. acidophilus* La-5, *B. animalis* subsp. *lactis* BB-12	6 weeks	Significant difference between groups concerning mean changes of HbA1c, TC, and LDL-C.

Table 3. *Cont.*

References	Subjects	Microorganism	Time of Administration	Main Outcome
[107]	44 patients with T2D	*L. acidophilus* La-5, *B. animalis* subsp. *lactis* BB-12	8 weeks	Increased HDL-C levels and decreased LDL-C/HDL-C ratio.
[108]	64 patients with T2D	*L. acidophilus* La5, *B. lactis* Bb12	6 weeks	Reduced fasting blood glucose and antioxidant status.
[109]	60 patients with T2D	*L. acidophilus* La5, *B. lactis* Bb12	6 weeks	TC and LDL-C improvement.
[110]	45 males with T2D	*L. acidophilus* NCFM	4 weeks	No effect.
Non-alcoholic fatty liver disease				
[111]	20 obese children with NAFLD	*L. rhamnosus* GG	8 weeks	Decreased ALT and PG-PS IgAg antibodies.
[112]	28 adult individuals with NAFLD	*L. bulgaris*, *S. thermophilus*	12 weeks	Decreased ALT and γ-GTP levels.
[113]	72 patients with NAFLD	*L.acidophilus* La5, *B. breve* subsp. *lactis* Bb12	8 weeks	Reduced serum levels of ALT, ASP, TC, and LDL-C.
[114]	44 obese children with NAFLD	*Bifidobacterium*, *Lactobacillus*, *S. thermophilus*	16 weeks	Improved fatty liver severity, decreased BMI, and increased GLP1/aGLP1.
Irritable bowel syndrome (IBS), gastrointestinal disorders, elimination of *Helicobacter*, inflammatory bowel disease (IBD), diarrhoeas				
[115]	59 adults infected with *H. pylori*	*L. acidophilus* La5, *B. lactis* Bb12	6 weeks	Inhibitory effect against *Helicobacter pylori*.
[116]	16 patients infected with *H. pylori*	*L. casei* Shirota	6 weeks	Inhibited growth of *Helicobacter pylori* (by 64% in the probiotic group, and by 33% in the control).
[117]	269 children with otitis media and/or respiratory tract infections	*S. cerevisiae* (*boulardii*)	No data	Diarrhoea was less common in children receiving probiotic yeast (7.5%) compared to those receiving placebo (23%). No negative side effects were observed.
[118]	77 patients with ulcerative colitis	Probiotic VSL#3	12 weeks	Remission in 42.9% of patients in the probiotic group, and in 15.7% of patients in the placebo group.
[119]	90 breastfed neonates with intestinal colic	*L. reuteri* ATCC 55730	6 months	Elimination of pain and symptoms associated with intestinal colic already after one week of the use of the probiotic.
Atopic dermatitis				
[120]	512 pregnant women and 474 their newborn infants	*L. rhamnosus* HN001	women—from 35 weeks gestation until 6 months if breastfeeding, infants—from birth to 2 years	Substantially reduced the cumulative prevalence of eczema in infants.
[121]	53 children with moderate of severe atopic dermatitis	*L. fermentum* VRI 033 PCC™	8 weeks	Reduction in SCORAD.
[122]	156 mothers of high-risk children (i.e., positive family history of allergic disease) and their offspring	*B. bifidum*, *B. lactis*, *L. lactis*	Mothers—the last 6 weeks of pregnancy, offspring—12 months	Significantly reduction eczema in high-risk for a minimum of 2 years provided that the probiotic was administered to the infant within 3 months of birth.
[123]	50 children with AD	*B. animalis* subsp *lactis*	8 weeks	Significant reduction in the severity of AD with an improved ration of IFN-γ and IL-10.

Table 3. *Cont.*

References	Subjects	Microorganism	Time of Administration	Main Outcome
Alleviation of lactose intolerance				
[124]	15 healthy, free-living adults with lactose maldigestion	*S. lactis*, *L. plantarum*, *S. cremoris*, *L. casei*, *S. diacetylactis*, *S. florentinus*, *L. cremoris*	1 day	Improved lactose digestion and tolerance.
[125]	44 patients	*B. animalis* subsp. *animal*is IM386 (DSM 26137), *L. plantarum* MP2026 (DSM 26329)	6 weeks	A significant lowering effect on diarrhoea and flatulence.
Different types of cancer and side effects associated with cancer				
[126]	100 patients with colorectal carcinoma	*L. plantarum* CGMMCC No 1258, *L. acidophilus* LA-11, *B. longum* BL-88	16 days	Improvement in the integrity of gut mucosal barrier and decrease in infections complications.
[127]	63 patients with diarrhoea during radiotherapy in cervical cancer	*L. acidophilus*, *B. bifidum*	7 weeks	Reduction in incidence of diarrhoea and better stool consistency.
[128]	150 patients diagnosed with colorectal cancer	*L. rhamnosus* 573	24 weeks	Patients had less grade 4 or 4 diarrhoea, less abdominal discomfort, needed less hospital care, and had fewer chemo dose reductions due to bowel toxicity.

Abbreviations: AD—atopic dermatitis; ALT—alanine amino transferase; ASP—aspartate amino transferase; BMI—body mass index; BP—blood pressure; CRP—C-reactive protein; γ-GTP—γ-glutamyltranspeptidase; GLP1—glucagon-like peptide 1; HDL-C—high-density lipoprotein cholesterol; HOMA-IR—homeostasis model assessment of insulin resistance; IL-10—interleukin 10; LDL-C—low-density lipoprotein cholesterol; NAFLD—non-alcoholic fatty liver disease; PBMC—peripheral blood mononuclear cell; PM—postmenopausal; SCORAD—SCORing Atopic Dermatitis; SOD—superoxide dismutase, sVCAM-1—soluble vascular cell adhesion molecule-1; TC—total cholesterol; T2D—type 2 diabetes; VFA—visceral fat area; 8-OHDG—8-hydroxy-2′-deoxyguanosine.

3. Prebiotics

Different prebiotics will stimulate the growth of different indigenous gut bacteria. Prebiotics have enormous potential for modifying the gut microbiota, but these modifications occur at the level of individual strains and species and are not easily predicted a priori. Furthermore, the gut environment, especially pH, plays a key role in determining the outcome of interspecies competition. Both for reasons of efficacy and of safety, the development of prebiotics intended to benefit human health has to take account of the highly individual species profiles that may result [129].

Fruit, vegetables, cereals, and other edible plants are sources of carbohydrates constituting potential prebiotics. The following may be mentioned as such potential souces: tomatoes, artichokes, bananas, asparagus, berries, garlic, onions, chicory, green vegetables, legumes, as well as oats, linseed, barley, and wheat [130]. Some artificially produced prebiotics are, among others: lactulose, galactooligosaccharides, fructooligosaccharides, maltooligosaccharides, cyclodextrins, and lactosaccharose. Lactulose constitutes a significant part of produced oligosaccharides (as much as 40%). Fructans, such as inulin and oligofructose, are believed to be the most used and effective in relation to many species of probiotics [131].

3.1. Prebiotic Selection Criteria

According to Wang (2009), there are five basic criteria for the classification of food components such as prebiotics (Figure 2) [132]. The first criterion assumes that prebiotics are not digested (or just partially digested) in the upper segments of the alimentary tract. As a consequence, they reach the colon, where they are selectively fermented by potentially beneficial bacteria (a requirement of the

second criterion) [133]. The fermentation may lead to the increased production or a change in the relative abundance of different short-chain fatty acids (SCFAs), increased stool mass, a moderate reduction of colonic pH, reduction of nitrous end products and faecal enzymes, and an improvement of the immunological system [134], which is beneficial for the host (the requirement of the third criterion). Selective stimulation of growth and/or activity of the intestinal bacteria potentially associated with health protection and wellbeing is considered another criterion [8]. The last criterion of the classification assumes that a prebiotic must be able to withstand food processing conditions and remained unchanged, non-degraded, or chemically unaltered and available for bacterial metabolism in the intestine [132]. Huebner et al. (2008) tested several commercially available prebiotics using various processing conditions. They found no significant changes of the prebiotic activity of the tested substances in various processing conditions [135]. Meanwhile, Ze et al. (2012) showed that it was possible to alter the ability of gut bacteria by utilising starch in vitro [136]. The structure of prebiotics should be appropriately documented, and components used as pharmaceutical formulas, food, or feed additives should be relatively easy to obtain at an industrial scale [137].

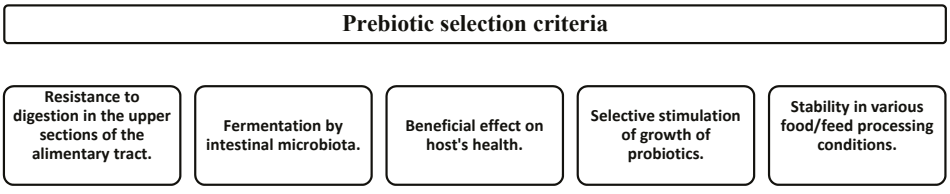

Figure 2. Requirements for potential prebiotics [132,138].

Prebiotics may be used as an alternative to probiotics or as an additional support for them. Long-term stability during the shelf-life of food, drinks, and feed, resistance to processing, and physical and chemical properties that exhibit a positive effect on the flavour and consistence of products may promote prebiotics as a competition to probiotics. Additionally, resistance to acids, proteases, and bile salts present in the gastrointestinal tract may be considered as other favourable properties of prebiotics. Prebiotic substances selectively stimulate microorganisms present in the host's intestinal ecosystem, thus eliminating the need for competition with bacteria. Stimulation of the intestinal microbiota by prebiotics determines their fermentation activity, simultaneously influencing the SCFA level, which confers a health benefit on the host [139,140]. Moreover, prebiotics cause a reduction of intestinal pH and maintain the osmotic retention of water in the bowel [134]. However, it should be considered that an overdose of prebiotics may lead to flatulence and diarrhoea—these effects are absent in the case of excessive consumption of probiotics. Prebiotics may be consumed on a long-term basis and for prophylactic purposes. Moreover, when used at correct doses, they do not stimulate any adverse effects, such as diarrhoea, susceptibility to UV light, or hepatic injuries caused by antibiotics. Prebiotic substances are not allergenic and do not proliferate the abundance of antibiotic-resistance genes. Of course, the effect of the elimination of selected pathogens achieved by the use of prebiotics may be inferior to antibiotics, but the properties mentioned above make them a natural substitute for antibiotics [134].

3.2. Prebiotic Substances

The majority of identified prebiotics are carbohydrates of various molecular structures, naturally occurring in human and animal diets. The physiological properties of potential prebiotics determine their beneficial effect on the host's health. Prebiotics may be classified according to those properties as [134]:

- not digested (or only partially digested);
- not absorbed in the small intestine;
- poorly fermented by bacteria in the oral cavity;
- well fermented by seemingly beneficial intestinal bacteria;
- poorly fermented by potential pathogens in the bowel.

Carbohydrates, such as dietary fibre, are potential prebiotics. Prebiotic and dietary fibre are terms used alternatively for food components that are not digested in the gastrointestinal tract. A significant difference between those two terms is that prebiotics are fermented by strictly defined groups of microorganisms, and dietary fibre is used by the majority of colonic microorganisms [141]. Therefore, considering one of the basic classification criteria, it turns out that using those terms alternatively is not always correct. Prebiotics may be a dietary fibre, but dietary fibre is not always a prebiotic [138]. The following non-starch polysaccharides are considered to be dietary fibre: cellulose, hemicellulose, pectins, gums, substances obtained from marine algae, as well as lactulose, soy oligosaccharides, inulins, fructooligosaccharides, galactooligosaccharides, xylooligosaccharides, and isomaltooligosaccharides. Based on the number of monomers bound together, prebiotics may be classified as: disaccharides, oligosaccharides (3–10 monomers), and polysaccharides. The most promising and fulfilling criteria for the classification of prebiotic substances, as evidenced by in vitro and in vivo studies, are oligosaccharides, including [142,143]: fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO), xylooligosaccharides (XOS), transgalactooligosaccharides (TOS), and soybean oligosaccharides (SBOS).

Also, polysaccharides such as inulin, reflux starch, cellulose, hemicellulose, or pectin may potentially be prebiotics. Examples of prebiotics that are most commonly used in human nutrition are presented in Table 4. The use of glucooligosaccharides, glicooligosaccharides, lactitol, izomaltooligosaccharides, stachyose, raffinose, and saccharose as prebiotics requires further studies [144].

Table 4. Examples of prebiotics and synbiotics used in human nutrition [134,145,146].

Human Nutrition	
Prebiotics	**Synbiotics**
FOS	
GOS	
Inulin	*Lactobacillus* genus bacteria + inulin
XOS	*Lactobacillus*, *Streptococcus* and *Bifidobacterium* genus bacteria + FOS
Lactitol	*Lactobacillus*, *Bifidobacterium*, *Enterococcus* genus bacteria + FOS
Lactosucrose	*Lactobacillus* and *Bifidobacterium* genus bacteria + oligofructose
Lactulose	*Lactobacillus* and *Bifidobacterium* genus bacteria + inulin
Soy oligosaccharides	
TOS	

Abbreviations: FOS—fructooligosaccharides; GOS—galactooligosaccharides; TOS—transgalactooligosaccharides; XOS—xylooligosaccharides.

3.3. Mechanism of Action of Prebiotics

Prebiotics are present in natural products, but they may also be added to food. The purpose of these additions is to improve their nutritional and health value. Some examples are: inulin, fructooligosaccharides, lactulose, and derivatives of galactose and β-glucans. Those substances may serve as a medium for probiotics. They stimulate their growth, and contain no microorganisms.

Figure 2 presents the principal mechanisms of prebiotic action and some of their effects on the host's health. Prebiotics are not digested by host enzymes and reach the colon in a practically unaltered form, where they are fermented by saccharolytic bacteria (e.g., *Bifidobacterium* genus). The consumption of prebiotics largely affects the composition of the intestinal microbiota and its metabolic activity [147]. This is due to the modulation of lipid metabolism, enhanced absorbability of calcium, effect on

the immunological system, and modification of the bowel function [147]. It is highly probable that providing an energy source that only specific species in the microbiota can utilize has a greater impact on microbiota composition and metabolism than these other factors. The molecular structure of prebiotics determines their physiological effects and the types of microorganisms that are able to use them as a source of carbon and energy in the bowel [134]. It was demonstrated that, despite the variety of carbohydrates that exhibit the prebiotic activity, the effect of their administration is an increased count of beneficial bacteria, mostly of the *Bifidobacterium* genus [148,149].

The mechanism of a beneficial effect of prebiotics on immunological functions remains unclear. Several possible models have been proposed [150]:

(1) Prebiotics are able to regulate the action of hepatic lipogenic enzymes by influencing the increased production of short-chain fatty acids (SCFAs), such as propionic acid.
(2) The production of SCFAs (especially of butyric acid) as a result of fermentation was identified as a modulator of histone acetylation, thus increasing the availability of numerous genes for transcription factors.
(3) The modulation of mucin production.
(4) It was demonstrated that FOS and several other prebiotics cause an increased count of lymphocytes and/or leukocytes in gut-associated lymphoid tissues (GALTs) and in peripheral blood.
(5) The increased secretion of IgA by GALTs may stimulate the phagocytic function of intra-inflammatory macrophages.

The main aim of prebiotics is to stimulate the growth and activity of beneficial bacteria in the gastrointestinal tract, which confers a health benefit on the host. Through mechanisms including antagonism (the production of antimicrobial substances) and competition for epithelial adhesion and for nutrients, the intestinal microbiota acts as a barrier for pathogens. Final products of carbohydrate metabolism are mostly SCFAs, namely: acetic acid, butyric acid, and propionic acid, which are subsequently used by the host as a source of energy [151]. As a result of the fermentation of carbohydrates, *Bifidobacterium* or *Lactobacillus* may produce some compounds inhibiting the development of gastrointestinal pathogens, as well as cause a reduction in the intestinal pH [152]. Moreover, *Bifidobacterium* genus bacteria demonstrate tolerance to the produced SCFAs and reduced pH. Therefore, due to their favourable effect on the development of beneficial intestinal bacteria, the administration of prebiotics may participate in the inhibition of the development of pathogens. There are very few documented study results regarding the inhibition of the development of pathogens by prebiotics. In 1997 and 2003, Bovee-Oudenhoven et al. studied the use of lactulose in the prevention of *Salmonella* Enteritidis infections on a rat model. Their results indicated that the acidification of the intestine occurring as a result of lactulose fermentation caused the reduced development of pathogens and increased translocation of pathogens from the bowel [153]. It was also demonstrated that the administration of prebiotics increases the absorption of minerals, mostly of magnesium and calcium [154,155].

3.4. Prebiotics for Humans

The presence of prebiotics in the diet may lead to numerous health benefits. Studies on colorectal carcinoma demonstrated that the disease occurs less commonly in people who often eat vegetables and fruit. This effect is attributed mostly to inulin and oligofructose [156]. Among the advantages of those prebiotics, one may also mention the reduction of the blood LDL (low-density lipoprotein) level, stimulation of the immunological system, increased absorbability of calcium, maintenance of correct intestinal pH value, low caloric value, and alleviation of symptoms of peptic ulcers and vaginal mycosis [157]. Other effects of inulin and oligofructose on human health are: the prevention of carcinogenesis, as well as the support of lactose intolerance or dental caries treatment [131]. Rat studies demonstrated that administration of inulin for five weeks caused a significant reduction of blood triacylglycerol levels [156]. Human studies demonstrated that the daily use of 12 g of inulin for one

month led to the reduction of blood VLDL (very low-density lipoprotein) levels (the reduction of triacylglycerols by 27%, and of cholesterol by 5%). This effect is associated with the effect of the prebiotic on hepatic metabolism and the inhibition of acetyl-CoA carboxylase and of glukose-6-phosphate dehydrogenase. It is also supposed that oligofructose accelerates lipid catabolism [157].

Asahara et al. (2001) demonstrated a protective effect of galactooligosaccharides (GOS) in the prevention of *Salmonella* Typhimurium infections in a murine model [158]. Buddington et al. (2002) confirmed a positive effect of fructooligosaccharides (FOS) on protection against *Salmonella* Typhimurium and *Listeria monocytogenes* infections [159]. Moreover, prebiotics are helpful in combating pathogenic microorganisms, such as *Salmonella* Enteritidis and *Escherichia coli*, and reduce odour compounds [160]. There are many reports regarding the positive effect of prebiotics on the carcinogenesis process. Results of rat studies proved that a prebiotic-enriched diet leads to significantly reduced indexes of carcinogenesis. Scientific research demonstrated that butyric acid may be a chemopreventive factor in carcinogenesis [161], or an agent protecting against the development of colorectal carcinoma through the promotion of cell differentiation [162]. Besides butyric acid, propionic acid also may possess anti-inflammatory properties in relation to colorectal carcinoma cells. In vitro studies on human L97 and HT29 cell lines (representing early and late stages of colorectal carcinoma) demonstrated that inulin fractions in plasma supernatant caused a significant inhibition of growth and induction of apoptosis in human colorectal carcinoma [163]. According to scientific reports, the administration of inulin and oligofructose to rats caused the inhibition of azoxymethane-induced colorectal carcinoma at the growth stage [164]. The supplementation of inulin and oligofructose at the dose of 5%–15% had also an effect on reduced occurrence of breast cancer in rats and of metastases to lungs [165]. However, those results have to be confirmed in humans. Table 5 lists the results of studies focusing on the effect of prebiotics on human health. There are examples of clinical trials during which the prebiotics group received the prebiotic prophylactically or in addition to the standard therapy.

Table 5. Examples of clinical trials regarding the effect of prebiotics on human health.

References	Subjects	Prebiotic	Time of Administration	Main Outcome
			Obesity	
[166]	48 healthy adults with a body mass index (in kg/m^2) >25	OFS	12 weeks	There was a reduction in body weight of 1.03 ± 0.43 kg with oligofructose supplementation, whereas the control group experienced an increase in body weight of 0.45 ± 0.31 kg over 12 weeks ($p = 0.01$). Glucose decreased in the oligofructose group and increased in the control group between the initial and final tests ($p \leq 0.05$). Insulin concentrations mirrored this pattern ($p \leq 0.05$). Oligofructose supplementation did not affect plasma active glucagon-like peptide 1 secretion. According to a visual analogue scale designed to assess side effects, oligofructose was well tolerated.
			Insulin resistance syndrome	
[167]	10 patients with type 2 diabetes	FOS	4 weeks (double repetition)	The plasma glucose response to a fixed exogenous insulin bolus did not differ at the end of the two periods. FOS had no effect on glucose and lipid metabolism in type 2 diabetics.
			Type 2 diabetes	
[168]	15 subjects with type 2 diabetes	AX	5 weeks (double repetition)	A supplement of 15 g/day of AX-rich fibre can significantly improve glycaemic control in people with type 2 diabetes.
[169]	11 patients with impaired glucose tolerance	AX	6 weeks	No effects of arabinoxylan were observed for insulin, adiponectin, leptin, or resistin as well as for apolipoprotein B, and unesterified fatty acids. In conclusion, the consumption of AX in subjects with impaired glucose tolerance improved fasting serum glucose and triglycerides. However, this beneficial effect was not accompanied by changes in fasting adipokine concentrations.
			Non-alcoholic fatty liver disease	
[170]	7 patients with non-alcoholic steatohepatitis	OFS	8 weeks	Compared to placebo, OFS significantly decreased serum aminotransferases, aspartate aminotransferase after 8 weeks, and insulin level after 4 weeks, but this could not be related to a significant effect on plasma lipids.

Table 5. *Cont.*

References	Subjects	Prebiotic	Time of Administration	Main Outcome
Irritable bowel syndrome (IBS), gastrointestinal disorders, elimination of *Helicobacter*, inflammatory bowel disease (IBD), diarrhoeas				
[171]	281 healthy infants (15 to 120 days)	GOS, FOS	12 months	Fewer episodes of acute diarrhoea, fewer upper respiratory tract infections.
[172]	160 healthy bottle-fed infants within 0–14 days after birth	GOS, FOS	3 months	Prebiotic formula well tolerated, normal growth trend toward a higher percentage of *Bifidobacterium* and a lower percentage of *E. coli* in stool, suppresses *Clostridium* in stool.
[173]	215 healthy infants	GOS, FOS	27 weeks	The concentration of secretory IgA was higher in the prebiotic group than the control; also, *Bifidobacterium* percentage was higher than the control and *Clostridium* was lower.
[174]	24 patients with chronic pouchitis	inulin	3 weeks	Inulin treatment resulted in decreased endoscopic and histological inflammation. This effect was associated with increased intestinal butyrate, lowered pH, and significantly decreased numbers of *Bacteroides fragilis*.
[175]	10 Crohn's disease patients	FOS	3 weeks	Reduced disease activity index.
Atopic dermatitis				
[176]	259 infants at risk for atopy	GOS, FOS	6 months	Significant reduction of frequency of AD.
[177]	259 healthy term infants with a parental history of atopy	GOS, FOS	6 months	Prebiotic group had significantly lower allergic symptoms—AD, wheezing, urticaria, and fewer upper respiratory infections than controls during the first 2 years.
Alleviation of lactose intolerance				
[178]	85 lactose intolerant participants	GOS	36 days	71% of subjects reported improvements in at least one symptom (pain, bloating, diarrhoea, cramping, or flatulence). Also on day 36, populations of bifidobacteria significantly increased by 90% in 27 of the 30 non-lactose tolerant participants who took GOS. Lactose fermenting *Bifidobacterium*, *Faecalibacterium*, and *Lactobacillus* were all significantly increased.
Different types of cancer and side effects associated with cancer				
[163]	Human L97 and HT29 cell lines (representing early and late stages of colorectal carcinoma)	inulin	No data	Growth inhibition and induction of apoptosis in human colorectal carcinoma.

Abbreviations: AD—atopic dermatitis; AX—arabinoxylan; FOS—fructooligosaccharides; GOS—galactooligosaccharides; IgA—immunoglobulin A; OFS—oligofructose.

4. Synbiotics

Synbiotics are used not only for the improved survival of beneficial microorganisms added to food or feed, but also for the stimulation of the proliferation of specific native bacterial strains present in the gastrointestinal tract [179]. The effect of synbiotics on metabolic health remains unclear. It should be mentioned that the health effect of synbiotics is probably associated with the individual combination of a probiotic and prebiotic [180]. Considering a huge number of possible combinations, the application of synbiotics for the modulation of intestinal microbiota in humans seems promising [181].

4.1. Synbiotic Selection Criteria

The first aspect to be taken into account when composing a synbiotic formula should be a selection of an appropriate probiotic and prebiotic, exerting a positive effect on the host's health when used separately. The determination of specific properties to be possessed by a prebiotic to have a favourable effect on the probiotic seems to be the most appropriate approach. A prebiotic should selectively stimulate the growth of microorganisms, having a beneficial effect on health, with simultaneous absent (or limited) stimulation of other microorganisms.

4.2. Synbiotics in Use

Previous sections discussed probiotic microorganisms and prebiotic substances most commonly used in human nutrition. A combination of *Bifidobacterium* or *Lactobacillus* genus bacteria with fructooligosaccharides in synbiotic products seems to be the most popular. Table 4 presents the most commonly used combinations of probiotics and prebiotics.

4.3. Mechanism of Action of Synbiotics

Considering the fact that a probiotic is essentially active in the small and large intestine, and the effect of a prebiotic is observed mainly in the large intestine, the combination of the two may have a synergistic effect [182]. Prebiotics are used mostly as a selective medium for the growth of a probiotic strain, fermentation, and intestinal passage. There are indications in the literature that, due to the use of prebiotics, probiotic microorganisms acquire higher tolerance to environmental conditions, including: oxygenation, pH, and temperature in the intestine of a particular organism [183]. However, the mechanism of action of an extra energy source that provides higher tolerance to these factors is not sufficiently explained. That combination of components leads to the creation of viable microbiological dietary supplements, and ensuring an appropriate environment allows a positive impact on the host's health. Two modes of synbiotic action are known [184]:

(1) Action through the improved viability of probiotic microorganisms;
(2) Action through the provision of specific health effects.

The stimulation of probiotics with prebiotics results in the modulation of the metabolic activity in the intestine with the maintenance of the intestinal biostructure, development of beneficial microbiota, and inhibition of potential pathogens present in the gastrointestinal tract [180]. Synbiotics result in reduced concentrations of undesirable metabolites, as well as the inactivation of nitrosamines and cancerogenic substances. Their use leads to a significant increase of levels of short-chain fatty acids, ketones, carbon disulphides, and methyl acetates, which potentially results in a positive effect on the host's health [184]. As for their therapeutic efficacy, the desirable properties of synbiotics include antibacterial, anticancerogenic, and anti-allergic effects. They also counteract decay processes in the intestine and prevent constipation and diarrhoea. It turns out that synbiotics may be highly efficient in the prevention of osteoporosis, reduction of blood fat and sugar levels, regulation of the immunological system, and treatment of brain disorders associated with abnormal hepatic function [185]. The concept of mechanisms of synbiotic action, based on the modification of intestinal microbiota with probiotic microorganisms and appropriately selected prebiotics as their substrates, is presented in Figure 1.

4.4. Synbiotics for Humans

Synbiotics have the following beneficial effects on humans [186]:

(1) Increased *Lactobacillus* and *Bifidobacterium* genus count and maintenance of balance of the intestinal microbiota;
(2) Improved hepatic function in patients suffering from cirrhosis;
(3) Improved immunomodulative abilities;
(4) Prevention of bacterial translocation and reduced incidence of nosocomial infections in patients' post-surgical procedures and similar interventions.

The translocation of bacterial metabolism products, such as lipopolysaccharides (LPSs), ethanol, and short-chain fatty acids (SFCAs), leads to their penetration of the liver. SCFAs also stimulate the synthesis and storage of hepatic triacylglycerols. Those processes may intensify the mechanisms of hepatic detoxication, which may result in hepatic storage of triacylglycerol (IHTG), and intensify steatosis of the organ. A randomised trial on the use of a synbiotic containing five probiotics (*Lactobacillus plantarum*, *Lactobacillus delbrueckii* spp. *bulgaricus*, *Lactobacillus acidophilus*,

Lactobacillus rhamnosus, *Bifidobacterium bifidum*) and inulin as a prebiotic in adult subjects with NASH (non-alcoholic steatohepatisis) demonstrated a significant reduction of IHTG (intrahepatic triacylglycerol) within six months [187]. It is also known that LPSs induce proinflammatory cytokines, such as the tumour necrosis factor alpha (TNF-α), playing a crucial role in insulin resistance and inflammatory cell uptake in NAFLD (non-alcoholic fatty liver disease). In the study on the effect of the synbiotic product containing a blend of probiotics (*Lactobacillus casei*, *Lactobacillus rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium breve*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus bulgaricus*) and fructooligosccharides, 52 adults participated for 28 weeks. It was found that supplementation with the synbiotic resulted in the inhibition of NF-κB (nuclear factor κB) and reduced production of TNF-α (tumour necrosis factor α) [188].

In rat studies, an increased level of intestinal IgA was found, following the introduction of the synbiotic product containing *Lactobacillus rhamnosus* and *Bifidobacterium lactis*, and inulin and oligofructose as prebiotics to the diet. Synbiotics lead to reduced blood cholesterol levels and lower blood pressure [157]. Moreover, synbiotics are used in the treatment of hepatic conditions [189] and improve the absorption of calcium, magnesium, and phosphorus [190].

Danq et al. (2013), in a meta-analysis, evaluated published studies on pro/prebiotics for eczema prevention, investigating bacterial strain efficacy and changes to the allergy status of the children involved. This meta-analysis found that probiotics or synbiotics may reduce the incidence of eczema in infants aged <2 years. Systemic sensitization did not change following probiotic administration [191].

Studies carried out within the framework of the SYNCAN project funded by the European Union verified the anti-carcinogenic properties of synbiotics. The effect of fructooligosaccharides (SYN1) combined with two probiotic strains (*Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* Bb12) on the health of patients at risk of colorectal cancer was studied. As a result, a change of biomarkers (genotoxicity, labelling index, labelled cells/crypt, transepithelial resistance, necrosis, interleukin 2, interferon γ) indicating the development of the disease in cancer patients, and in patients post polyp excision, was observed [192]. It was concluded that the application of the studied synbiotic may reduce the risk of colorectal carcinoma. A lower level of DNA damage was also observed, as well as a lower colonocyte proliferation ratio [147]. Table 6 lists the results of studies focusing on the effect of synbiotics on human health. There are examples of clinical trials during which the synbiotics group received the synbiotic prophylactically or in addition to the standard therapy.

Table 6. Examples of clinical trials regarding the effect of synbiotics on human health.

References	Subjects	Composition of Synbiotic	Time of Administration	Main Outcome
		Obesity		
[193]	153 obese men and women	*L. rhamnosus* CGMCC1.3724, inulin	36 weeks	Weight loss and reduction in leptin. Increase in Lachnospiraceae.
[194]	70 children and adolescents with high BMI	*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum*, *L. bulgaricus*, FOS	8 weeks	Decrease in BMI z-score and waist circumference.
[195]	77 obese children	*L. acidophilus*, *L. rhamnosus*, *B. bifidum*, *B. longum*, *E. faecium*, FOS	4 weeks	Changes in anthropometric measurements. Decrease in TC, LDL-C, and total oxidative stress serum levels.
		Insulin resistance syndrome		
[196]	38 subjects with IRS	*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum*, *L. bulgaricus*, FOS	28 weeks	The levels of fasting blood sugar and insulin resistance improved significantly.

Table 6. *Cont.*

References	Subjects	Composition of Synbiotic	Time of Administration	Main Outcome
Type 2 diabetes				
[197]	54 patients with T2D	*L. acidophilus*, *L. casei*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *B. longum*, *S. thermophilus*, FOS	8 weeks	Increased HOMA-IR and TGL plasma level; reduced CRP in serum.
[198]	81 patients with T2D	*L. sporogenes*, inulin	8 weeks	Significant reduction in serum insulin levels, HOMA-IR, and homeostatic model assessment cell function.
[199]	78 patients with T2D	*L. sporogenes*, inulin	8 weeks	Decrease in serum lipid profile (TAG, TC/HDL-C) and a significant increase in serum HDL-C levels.
[200]	20 patients with T2D	*L. acidophilus*, *B. bifidum*, oligofructose	2 weeks	Increased HDL-C and reduced fasting glycaemia.
Non-alcoholic fatty liver disease				
[187]	20 individuals with NASH	*L. plantarum*, *L. delbrueckii* spp. *bulgaricus*, *L. acidophilus*, *L. rhamnosus*, *B. bifidum*, inulin	26 weeks	Decreased IHTG content.
[188]	52 adult individuals with NAFLD	*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. longum*, *L. bulgaricus*, FOS	30 weeks	Inhibition of NF-κB and reduction of TNF-α.
Irritable bowel syndrome (IBS), gastrointestinal disorders, elimination of *Helicobacter*, inflammatory bowel disease (IBD), diarrhoeas				
[201]	76 patients with IBS	*L. acidophilus La-5*®, *B. animalis* ssp. *lactis* BB-12®, dietary fibres (Beneo)	4 weeks	On average, an 18% improvement in total IBS-QoL score was reported and significant improvements in bloating severity, satisfaction with bowel movements, and the severity of IBS symptoms' interference with patients' everyday life were observed. However, there were no statistically significant differences between the synbiotic group and the placebo group.
[202]	69 children aged 6–16 years who had biopsy proven *H. pylori* infection	*B. lactis* B94, inulin	14 days	From a total of 69 *H. pylori*-infected children (female/male = 36/33; mean ± SD = 11.2 ± 3.0 years), eradication was achieved in 20 out of 34 participants in the standard therapy group and 27/35 participants in the synbiotic group. There were no significant differences in eradication rates between the standard therapy and the synbiotic groups.
[203]	40 patients with UC	*B. longum*, psyllium	4 weeks	Patients with UC on synbiotic therapy experienced greater quality-of-life changes than patients on probiotic or prebiotic treatment.
Atopic dermatitis				
[204]	90 infants with AD	*B. breve* M-16V, GOS and FOS mixture (Immunofortis®)	12 weeks	This synbiotic mixture did not have a beneficial effect on AD severity in infants, although it did successfully modulate their intestinal microbiota.
[205]	40 infants and children aged 3 months to 6 years with AD	*L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. infantis*, *L. bulgaricus*, FOS	8 weeks	A mixture of seven probiotic strains and FOS may clinically improve the severity of AD in young children.

Table 6. *Cont.*

References	Subjects	Composition of Synbiotic	Time of Administration	Main Outcome
Alleviation of lactose intolerance				
[206]	20 females and males	*Lactobacillus*, *Bifidobacterium*, FOS	5 weeks	Consumption of the probiotic mixture improved the gastrointestinal performance associated with lactose load in subjects with LI. Symptoms were additionally reduced by the addition of prebiotics. The supplementation was safe and well tolerated, with no significant adverse effect observed.
Different types of cancer and side effects associated with cancer				
[192]	43 polypeptomized and 37 colon cancer patients	*L. rhamnosus* GG, *B. lactis* Bb12, inulin	12 weeks	Increased *L. rhamnosus* and *B. lactis* in faeces, reduction in *C. perfringens*, prevents increased secretion of IL-2 in polypectomized patients, increased production of interferon-γ in cancer patients.

Abbreviations: BMI—body mass index; CFU—colony-forming-unit; CRP—C-reactive protein; FOS—fructo-oligossacharides; IBS-QoL—quality of life with IBS; HDL-C—high-density lipoprotein cholesterol; HOMA-IR—homeostasis model assessment of insulin resistance; IHTG—intrahepatic triacylglycerol; IRS—insulin resistance syndrome; LDL-C—low-density lipoprotein cholesterol; LI—lactose intolerance; NAFLD—non-alcoholic fatty liver disease; NF-κB—nuclear factor κB; T2D—type 2 diabetes; TAG—triacylglycerols; TC—total cholesterol; TGL—total glutathione levels; TNF-α—tumour necrosis factor α; UC—ulcerative colitis.

5. Summary

Probiotic organisms are crucial for the maintenance of balance of human intestinal microbiota. Numerous scientific reports confirm their positive effect in the host's health. Probiotic microorganisms are attributed a high therapeutic potential in, e.g., obesity, insulin resistance syndrome, type 2 diabetes, and non-alcohol hepatic steatosis [207]. It seems also that probiotics may be helpful in the treatment of irritable bowel syndrome, enteritis, bacterial infections, and various gastrointestinal disorders and diarrhoeas. Probiotic microorganisms are also effective in the alleviation of lactose intolerance and the treatment of atopic dermatitis. A positive effect of probiotics in the course of various neoplastic diseases and side effects associated with anti-cancer therapies is also worth noting. Prebiotics may be used as an alternative to probiotics, or as an additional support for them. It turns out that the development of bio-therapeutic formulas containing both appropriate microbial strains and synergistic prebiotics may lead to the enhancement of the probiotic effect in the small intestine and the colon. Those "enhanced" probiotic products may be even more effective, and their protective and stimulatory effect superior to their components administered separately [208]. It seems that we will see further studies on combinations of probiotics and prebiotics, and further development of synbiotics. Future studies may explain the mechanisms of actions of those components, which may confer a beneficial effect on human health.

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Review

Effect of Probiotics on Metabolic Outcomes in Pregnant Women with Gestational Diabetes: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Abstract: The metabolic effects of probiotic administration in women with gestational diabetes mellitus (GDM) is unknown. The objective of this review was to investigate the effect of probiotics on fasting plasma glucose (FPG), insulin resistance (HOMA-IR) and LDL-cholesterol levels in pregnant women diagnosed with GDM. Seven electronic databases were searched for RCTs published in English between 2001 and 2017 investigating the metabolic effects of a 6–8 week dietary probiotic intervention in pregnant women following diagnosis with GDM. Eligible studies were assessed for risk of bias and subjected to qualitative and quantitative synthesis using a random effects model meta-analyses. Four high quality RCTs involving 288 participants were included in the review. Probiotic supplementation was not effective in decreasing FBG (Mean Difference = −0.13; 95% CI −0.32, 0.06, $p = 0.18$) or LDL-cholesterol (−0.16; 95% CI −0.45, 0.13, $p = 0.67$) in women with GDM. However, a significant reduction in HOMA-IR was observed following probiotic supplementation (−0.69; 95% CI −1.24, −0.14, $p = 0.01$). There were no significant differences in gestational weight gain, delivery method or neonatal outcomes between experimental and control groups, and no adverse effects of the probiotics were reported. Probiotic supplementation for 6–8 weeks resulted in a significant reduction in insulin resistance in pregnant women diagnosed with GDM. The use of probiotic supplementation is promising as a potential therapy to assist in the metabolic management of GDM. Further high quality studies of longer duration are required to determine the safety, optimal dose and ideal bacterial composition of probiotics before their routine use can be recommended in this patient group.

Keywords: probiotics; gut microbiota; Gestational Diabetes Mellitus; pregnancy; insulin resistance

1. Introduction

The gut microbiota is a collective term used to refer to the microorganisms colonizing the human gastrointestinal tract [1]. It is an important ecosystem consisting of both residential and pathogenic bacteria [2,3]. Residential intestinal microbes coexist in a symbiotic relationship with their host by extracting energy from dietary components which humans lack the enzymes to digest. In return, the microbiota produce bioactive compounds shown to benefit host metabolism. Manipulation of the gut microbiome and its fermentation by-products is emerging as a promising therapeutic treatment strategy for many chronic medical conditions [4]. A variety of factors influence the gut microbiome, including host genetics, illness, antibiotic use, dietary patterns, weight loss and pregnancy [5–8].

Perturbations in the composition of the gut microbiota have been hypothesized to contribute to the pathogenesis of obesity, inflammation and insulin resistance [9].

Throughout pregnancy the gut microbiota undergoes significant changes. From the first (T1) to the third trimester (T3), the species richness of the gut microbiome decreases [8], although this has not been observed in all studies [10]. There is an increase in Proteobacteria and Actinobacteria phyla and a reduction in beneficial bacterial species *Roseburia intestinalis* and *Faecalibacterium prausnitzii* [8,11]. These changes in gut microbial composition cause inflammation and correlate with increases in fat mass, blood glucose, insulin resistance and circulating pro-inflammatory cytokines in the expectant mother [12]. This "diabetic-like" state observed during the later stages of all healthy pregnancies is thought to maximize nutrient provision to the developing fetus [13]. However, increased insulin resistance combined with an inability to secrete the additional insulin required to maintain glucose homeostasis can result in the development of gestational diabetes mellitus (GDM) in the mother and macrosomia in the baby.

GDM is defined as the development of glucose intolerance, with first onset during pregnancy [14]. This condition is associated with adverse maternal and infant health outcomes during gestation, childbirth and postpartum. Maternal comorbidities include pre-eclampsia and increased risk of infection throughout pregnancy [15]. A seven-fold increased risk of the mother developing type 2 diabetes (T2DM) postpartum has also been reported [16]. Infant morbidity includes risk of fetal malformations and diabetic fetopathy which may cause macrosomia and subsequent mechanical complications during labor [17,18]. Additionally, studies suggest that children born to mothers with GDM have an increased risk of diabetes mellitus and metabolic dysfunction later in life [19,20]. In order to reduce the risk of such adverse health outcomes, current best practice GDM management requires modification of the maternal diet with or without pharmacological treatment such as Metformin and/or insulin [21]. Despite its benefits, pharmacotherapy may result in significant side effects including abdominal discomfort, dizziness, diarrhea and hypoglycemia [22]. As research suggests that probiotic interventions may attenuate some of the adverse metabolic effects of type 2 diabetes [23,24], probiotics may also provide an acceptable treatment option in women with GDM.

Probiotics have been defined by the World Health Organization (WHO) as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' [25]. Regular consumption of probiotics have been found to beneficially modulate the composition of the gut microbiota [26]. Increases in colonic microbial diversity have been linked to improved glucose homeostasis, attenuation of inflammation, regulation of insulin production, maintenance of the integrity of the gastrointestinal lining, and the harvesting of nutrients from the host diet [7,8,27]. Safe and effective evidence-based interventions are vital for both the prevention and optimal management of GDM. A recent RCT conducted in healthy pregnant women suggest that probiotic supplementation may improve blood glucose control during the third trimester [28] and potentially reduce the risk of developing GDM [29]. To our knowledge, no systematic reviews have investigated the effect of maternal probiotic supplementation on the metabolic health of women with established GDM, highlighting the need for further exploration of this topic. The aim of this review was to determine the effect of 6–8 week probiotic supplementation versus placebo on glucose homeostasis, lipid levels and gestational weight gain in pregnant women diagnosed with GDM.

2. Methods

This review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement [30]. A systematic computer search of the databases Proquest, Scopus, Embase, Ovid MEDLINE, Web of science, CINAHL, and the Cochrane Database of Systematic Reviews was performed for the period between 1 January 2001 and 1 January 2017. In 2001, the WHO recognized the need for guidelines to evaluate probiotic use and to substantiate health claims. The rationale for excluding papers before 2001 was due to a lack of international guidelines and criteria regulating the use of probiotics prior to this date [31]. The following search terms were used:

(1) (pregnan * OR Gestation * OR Matern * OR Obstetric * OR expectan * OR "gestational diabetes" OR "gestational diabetes mellitus"), (probiotic * OR Lactobacill * OR bacteria * OR ferment * OR microorganism * OR acidophilus OR streptococc *), and (glucose OR "blood glucose" OR insulin OR HbA1c OR "birth weight" OR metabol * OR intervention * OR "pharmaceutical Intervention"); (2) limit 1 to year '2001–2017'; limit 2 to humans; limit 3 exclude males and non-pregnant subjects. Trials were included if they were published in English, utilized an RCT study design, involved human participants diagnosed with GDM by OGTT and if at least one group of participants were randomized to receive a dietary probiotic supplement for a period of 6–8 weeks.

Studies in patients with pre-existing conditions such as type 1 diabetes, type 2 diabetes or gastrointestinal pathologies were considered beyond the scope of this review, and were therefore excluded. Intervention trials involving the administration of fermented foods (which contain unknown quantities of bacteria), prebiotics (which contain no live bacteria) or synbiotics (which contain both pre- and probiotics) were also excluded.

Resultant studies were combined and duplicates removed. All articles were independently screened for eligibility by two authors based on title and abstract. Articles were excluded if they reported a non-RCT study design, subjects were not diagnosed with GDM or there was no probiotic intervention. The reference lists of included studies were hand-searched to identify additional relevant trials. The methodological quality of all included trials was independently assessed by two authors using the Cochrane Risk of Bias tool for Quality Assessment of Randomized Controlled Trials [32]. This tool rates primary research based on the use of sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessors, completeness of outcome data, non-selective outcome reporting, and other measures of bias. Trials were assessed as satisfying each of the quality criteria using "yes", "no", or "unsure", with studies meeting the majority of quality criteria considered to have a low risk of bias, while those assigned "no" or "unsure" for most criteria were designated as moderate or high risk of bias. Discrepancies between authors risk of bias assessments were resolved through collaborative discussion until consensus was reached.

Data was independently extracted from each article by two authors using a data collection form. Data items collected included first author, article title, journal name, year of publication, country in which trial was conducted, number of trial participants: intervention group (*n*) and control group (*n*), mean participant age (year), mean participant BMI (kg/m^2), mean gestational age (week), mean length of intervention (week), composition of probiotic supplement (genus, species), number of micro-organisms in probiotic supplement (CFU/g), mean gestational weight gain (kg), mean fasting blood glucose (mmol/L), mean Homeostasis Model Assessment—Insulin Resistance (HOMA-IR) (units), mean LDL-cholesterol (mmol/L), mean number of normal deliveries (*n*), mean number of caesarian sections (*n*), mean number of interventions during delivery (*n*), maternal complications (*n*), infant complications (*n*), and infant birth weight (g).

Trials measuring FBG, LDL-cholesterol and insulin resistance (HOMA-IR) in pregnant women with GDM were subjected to a random-effects model meta-analysis using Revman 5.1 (The Cochrane Collaboration, Copenhagen, Denmark, 2014). Treatment effects and 95% CI were calculated using the Mean Difference (MD). Limited numbers of studies investigating comparable outcomes, small sample sizes and heterogeneity among prebiotic supplements and outcome measures limited the majority of data synthesis to a narrative analysis.

3. Results

3.1. Description of Selected Trials

A total of 944 citations were identified at the time of the initial database search based on the predefined inclusion and exclusion criteria. After removal of duplicate publications and exclusion of irrelevant articles, four articles [33–36] reporting on four randomized controlled trials involving 288 participants were ultimately included (Figure 1). Characteristics of the included studies are

shown in Table 1. All studies included otherwise healthy pregnant women diagnosed with GDM at 24–30 weeks gestation by oral glucose tolerance test. Participant ages ranged between 18–40 years and pre-pregnancy BMI from 26–32 kg/m^2. All trial participants were randomized to receive either a daily probiotic supplement or a placebo. Probiotic composition varied between studies, but all trials provided *Lactobacillus* spp., and three [34–36] provided *Bifidobacterium* spp. The duration of intervention ranged from 6–8 weeks. A variety of post-intervention outcome measures were reported including fasting plasma glucose, fasting insulin, C-peptide, HOMA-IR, lipid studies, inflammatory markers, pro-inflammatory cytokines, gestational weight gain, requirement for glucose-lowering pharmacotherapy, interventions required during childbirth, infant birthweight, incidence of macrosomia (birthweight > 4 kg), fetal anomalies, admissions to the neonatal intensive care unit, and 5-min Apgar score.

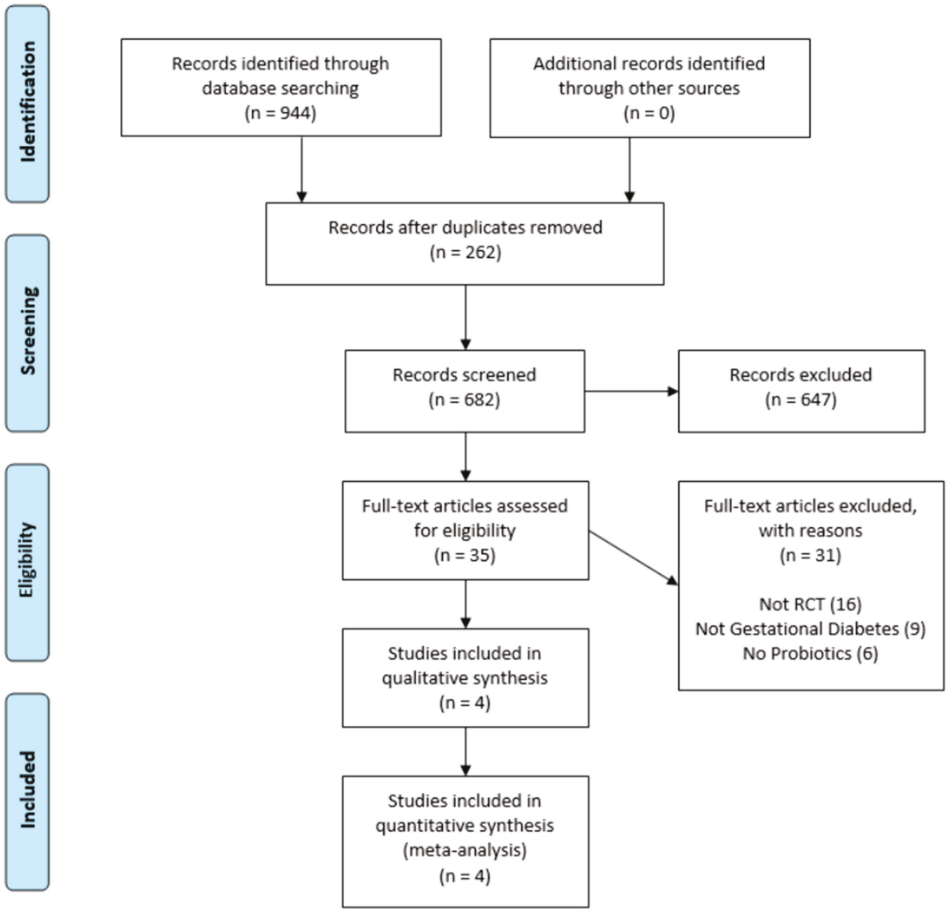

Figure 1. PRISMA flowchart showing the progression of trials through each stage of the selection process.

Table 1. Characteristics of randomised controlled trials included in the review.

Study Author/Year	Participants	Study Design/Blinding	Dietary Probiotic Intervention	Effect of Dietary Probiotic Supplement on Metabolic Outcomes
Karamali et al. (2016) [34]	Iran, *n* 60 pregnant women with GDM in third trimester (age range 18–40 years)	Parallel RCT, double-blinded	Random assignment to 6-week probiotic or placebo capsules. Each probiotic capsule contained *L. acidophilus* (2×10^9 CFU/g), *L. casei* (2×10^9 CFU/g) and *B. bifidum* (2×10^9 CFU/g)	↓ Fasting plasma glucose ↓ HOMA-IR ↔ Total cholesterol ↔ LDL cholesterol ↓ VLDL cholesterol ↓ Triglyceride ↔ gestational weight gain
Dolatkhah et al. (2015) [36]	Iran, *n* 64 pregnant women with GDM (mean age intervention 28.1 years, control 26.5 years; mean BMI intervention 31.4 kg/m^2, control 29.9 kg/m^2)	Parallel RCT, double-blinded	Random assignment to 8-week probiotic capsule with dietary advice or placebo capsule with dietary advice. Each probiotic capsule contained *L. acidophilus* LA-5, *Bifidobacterium* BB-12, *S.* thermophilus STY-31 and *L. delbrueckii* subsp. Bulgaricus LBY-27 (>4×10^9 CFU/g)	↓ Fasting plasma glucose ↓ HOMA-IR ↓ gestational weight gain
Jafarnejad et al. (2016) [35]	Iran, *n* 82 pregnant women with GDM (mean age intervention 32.4 years control 31.9 years; mean BMI intervention 26.8 kg/m^2, control 27.4 kg/m^2)	Parallel RCT, double-blinded	Random assignment to 8-week probiotic or placebo capsules. Each probiotic capsule contained VSL#3 (*S. thermophilus*, *B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii* subsp. Bulgaricus, 15×10^9 CFU/g)	↓ Fasting plasma glucose ↔ gestational weight gain ↓ HOMA-IR ↓ Interleukin-6 ↓ Tumor Necrosis Factor-aplha ↓ hs-CRP
Lindsay et al. (2015) [33]	Ireland, *n* 149 women with GDM (mean age intervention 33.5 years control 32.6 years; mean BMI intervention 29.1 kg/m^2, control 29.0 kg/m^2)	Parallel RCT, double-blinded	Random assignment to 6-week probiotic or placebo capsules. Each capsule contained *L. salivarius* UCC118 (1×10^9 CFU/g)	↔ Fasting plasma glucose ↔ HOMA-IR ↔ C-peptide ↓ Total cholesterol ↔ CRP ↔ Triglyceride ↓ LDL cholesterol ↔ HDL cholesterol ↔ gestational weight gain

RCT: Randomised Controlled Trial; GDM: Gestational Diabetes Mellitus; HOMA-IR score: homeostatic model of assessment of insulin resistance; hs-CRP: High sensitivity C-reactive protein; HDL cholesterol: High density lipoprotein; LDL cholesterol: Low density lipoprotein; HbA1c: Glycosylated haemoglobin; *n*: number of participants randomised; ↓ significantly lower than that in the comparison control group after intervention; ↑ significantly higher than that in the comparison control group after intervention; ↔ no significant difference between the probiotic-supplemented and control groups after intervention.

All of the studies included in the present review had a low risk of bias, as assessed using the Cochrane Collaboration Risk of Bias tool (Table 2). Methodological strengths of the trials included double-blinding and randomization of participants to intervention and control groups. Methodological limitations of the trials included small sample sizes and short study duration. Additionally, one of the trials had not been registered on a clinical trials registry prior to commencement, so it could not be determined whether primary outcomes reported were pre-specified before the trial began [35].

Table 2. Risk of bias summary for included studies.

Author/Year	Risk of Bias [a]	Bias Minimisation Items [b]						
		1	2	3	4	5	6	Other
Dolatkhah, 2015 [36]	Low	+	+	+	+	+	?	Funding & sponsorship free from bias, statistical analysis appropriate
Lindsay, 2015 [33]	Low	+	+	+	+	+	?	Funding & sponsorship free from bias
Jafarnejad, 2015 [35]	Low	+	+	+	+	?	?	Funding & sponsorship free from bias
Karamali, 2015 [34]	Low	+	+	+	+	+	?	Funding & sponsorship free from bias

"+" = response of "yes" to use of the bias minimization item; "?" = response of "uncertain" to the use of the bias minimization item; [a] Assessed using the Cochrane Collaboration tool for assessing risk of bias in RCTs (ref); [b] Bias minimization items: 1. Random sequence generation (selection bias); 2. Allocation concealment (selection bias); 3. Blinding of participants and personnel (performance bias); 4. Blinding of outcome assessment (detection bias); 5. Complete outcome data (attrition bias); 6. Non-selective reporting (reporting bias). Trials receiving a + response for most items are likely to have a low risk of bias.

3.2. Fasting Blood Glucose

Four studies investigated the effect of probiotic supplementation on FBG levels in pregnant women with GDM [32–35]. Two [33,35] of the four studies reported statistically significant reductions in FBG levels in the groups receiving probiotics in comparison to the groups receiving the placebo. However, a meta-analysis of all four trials (n = 288) indicated no significant reduction in FBG following probiotic supplementation (Mean Difference = −0.13; 95% CI −0.32, 0.06, p = 0.18) (Figure 2). While each of the studies included in the pooled analysis had a low risk of bias and administered probiotic supplements to women with GDM over a similar intervention period, significant interstudy heterogeneity was observed (I^2 = 88%, p < 0.001), so the calculated mean difference should be interpreted as an average intervention effect.

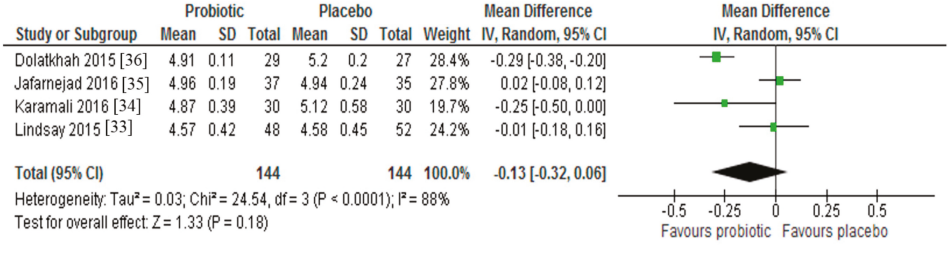

Figure 2. Effect of probiotic supplementation on fasting plasma glucose (mmol/L) in pregnant women with gestational diabetes.

3.3. Insulin Resistance

Four trials estimated insulin resistance in study participants by calculating HOMA-IR from fasting glucose and insulin values [33–36]. While one study found no change in insulin resistance between intervention and control groups following probiotic supplementation [33], three studies reported significant reductions in insulin resistance in the women receiving probiotics [34–36]. After meta-analysis (n = 288), the pooled mean difference in HOMA-IR was −0.69 (95% CI −1.24,

−0.14, $p = 0.01$), indicating a statistically significant effect favoring probiotic supplementation over placebo (Figure 3). Significant evidence of interstudy heterogeneity was observed across studies ($I^2 = 79\%$, $p < 0.01$).

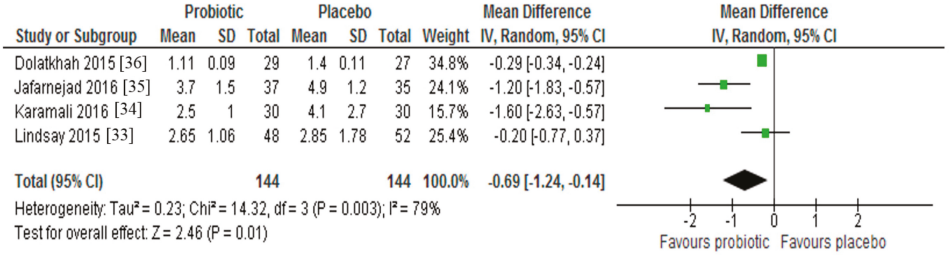

Figure 3. Effect of probiotic supplementation on HOMA-IR in pregnant women with gestational diabetes.

3.4. LDL-Cholesterol

Lindsay et al. [33] found that the usual rise in total cholesterol and LDL-cholesterol usually observed during the late stages of pregnancy was significantly attenuated in the probiotic group (both $p < 0.05$). In contrast, the study by Karamali et al. [34] reported no change in total cholesterol ($p = 0.33$) and LDL-cholesterol ($p = 0.07$) between treatment and control groups, but described significant reductions in VLDL-cholesterol and serum TG in the probiotic group (both < 0.05). When the data from both studies were pooled for meta-analysis ($n = 160$), there was no significant reduction in LDL-cholesterol following probiotic supplementation (MD = −0.16; 95% CI −0.45, 0.13, $p = 0.67$) (Figure 4).

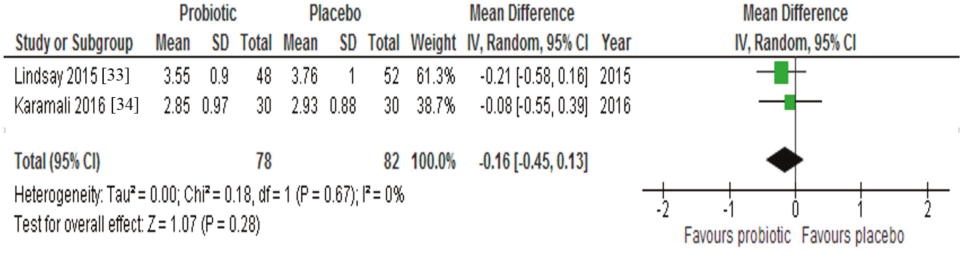

Figure 4. Effect of probiotic supplementation on LDL-cholesterol (mmol/L) in pregnant women with gestational diabetes.

3.5. Gestational Weight Gain

During the final 2 weeks of an 8-week intervention, one study reported that the weight gain of the women in the probiotic group was significantly less than the weight gain of those receiving the placebo (0.74 ± 0.14 kg vs. 1.22 ± 0.11 kg respectively), which remained significant after adjusting for daily energy intake ($p < 0.05$) [36]. However, the remaining three studies found no differences in gestational weight gain between intervention and control groups [33–35]. Two studies also reported no significant differences in infant birthweights between those born to mothers receiving the probiotic and those whose mothers received the placebo [33,34].

3.6. Obstetric Outcomes

No significant differences were found between probiotic and control groups for rates of pregnancy-induced hypertension, requirement for labor induction, commencement of glucose-lowering medications, blood loss at delivery, postpartum hemorrhage, fetal anomalies, admission of the infant to neonatal intensive care [33], and rates of delivery by caesarian section [33,34]. No adverse outcomes related to use of the probiotics were reported in any of the trials.

4. Discussion

Fasting hyperglycemia in women with GDM is associated with increased short and long-term morbidity in the offspring [37]. There is a clear need for safe, low-cost therapies to assist in the prevention and management of GDM. The gut microbial composition is altered during pregnancy, and given that specific micro-organisms in the gastrointestinal tract are able to positively influence host metabolism, probiotic supplements may contribute to the maintenance of bacterial diversity and glucose homeostasis in individuals with metabolic disturbances [38,39]. Research investigating probiotic use during pregnancy and its effect on the outcomes of GDM is an emerging area of interest. This systematic literature review aimed to explore the current evidence regarding the effect of probiotic supplementation on glucose and lipid homeostasis in pregnant women with GDM. Assessment of four randomized controlled trials in this review involving 288 pregnant women with GDM found that a 6–8 week probiotic intervention did not improve FBG or LDL-cholesterol levels. However, probiotic supplementation in women with GDM was associated with significant reductions in insulin resistance which could potentially reduce their requirement for glucose-lowering medication later in their pregnancy.

The mechanisms whereby probiotics alter glucose homeostasis are not completely understood. One proposed method is by the production of short chain fatty acids (SCFAs), generated as a by-product of bacterial fermentation of dietary fibers. SCFAs act as an energy source for intestinal cells and have been found to regulate the production of hormones effecting energy intake and expenditure such as leptin and grehlin [40]. The binding of SCFAs to G protein-coupled receptors GPR41 and GPR43 increases the intestinal expression of Peptide YY and Glucagon-like peptide-1 (GLP-1) hormones which act to reduce appetite by slowing intestinal transit time and increasing insulin sensitivity [11]. Another hypothesized mechanism of SCFA action includes reducing gastrointestinal permeability by upregulating transcription of tight junction proteins, enhancing production of Glucagon-like peptide-2 (GLP-2) which promotes crypt cell proliferation, and reducing inflammation in colonic epithelial cells by increasing PPAR-gamma activation [41]. Maintenance of the integrity of the gut barrier minimizes the concentration of lipopolysaccharide (LPS) in circulation. LPS is a structural component of gram negative bacterial cell walls, which induces an immune-cell response upon absorption into the human bloodstream, stimulating proinflammatory cytokine production and the onset of insulin resistance and hyperglycemia [42]. In support of this mechanism of probiotic action, Jafarnejad et al. [35] demonstrated probiotic-induced reductions in high sensitivity CRP, IL-6 and TNFα in their 8-week trial in women with GDM.

While this review found no significant effect of probiotic supplementation on FBG in women with GDM, a number of studies have reported positive outcomes. A study conducted in 256 pregnant women with normal glucose tolerance found significant reductions in FBG, insulin concentrations and insulin resistance following probiotic supplementation, potentially reducing participants' GDM risk [28]. However, the length of the intervention (18 months) was significantly longer than the trials included in the current meta-analysis, and fasting glucose and insulin levels were measured during pregnancy and up to 12 months postpartum in the study subjects. A Cochrane systematic review exploring the effect of probiotic supplementation during normal pregnancies concluded that although there was a reduction in the incidence of GDM in one trial, there were insufficient studies to perform a quantitative meta-analysis [43]. Further research is therefore required before probiotics can be recommended to pregnant women to reduce their risk of GDM. A meta-analysis

of six RCTs demonstrated a significant reduction in FBG in 252 subjects with type 2 diabetes [44], however changes in HbA1c, inflammatory markers, fasting insulin and HOMA-IR were inconclusive, possibly due to the brief duration of the intervention (4–8 weeks). It was also unknown whether trial participants were also receiving pharmacological therapy such as Metformin, which can influence the composition of the gut microbiota. The authors postulated that probiotics may elicit hypoglycemic effects by increasing the level of antioxidative enzymes capable of scavenging reactive oxygen species, thereby reducing oxidative stress levels [44]. Similarly, a systematic review of 12 RCTs explored the effect of probiotics on glucose tolerance in people with type 2 diabetes, concluding that probiotic supplementation significantly reduced FBG [45]. This review included trials which varied substantially in methodological quality, and a number of the probiotic treatments included yoghurts or other foodstuffs containing unknown quantities of uncertain bacterial species. Finally, a systematic review of 17 RCTs reported significant reductions in FBG, fasting insulin and HOMA-IR [46]. Trial participants represented a range of demographics with various forms of metabolic disease including GDM, hypercholesterolemia and T2DM [46], which was likely to have contributed to the large interstudy heterogeneity observed.

The contradictory findings of this review in comparison to other published reviews investigating the effect of probiotics on FBG may be related to the small sample sizes (n = 60–149) and short study durations (6–8 weeks) in the women with GDM. Moreover, the current review included trials involving only participants with GDM, which may be more resistant to the effects of probiotic supplementation than the variety of other forms of glucose intolerance included in the other reviews. Indeed, increased insulin resistance is considered a normal consequence of all healthy pregnancies [13]. As there is currently no consensus on the ideal bacterial composition and dose of probiotics for the management of glucose tolerance, the microbial components of the probiotics used in the GDM trials may not have been sufficient to effect FBG levels.

Two RCTs included in this review investigated the effect of probiotic supplementation on maternal lipid levels in GDM, with both reporting conflicting results [33,34]. While one study demonstrated that probiotic treatment may have mitigated the expected increase in total and LDL-cholesterol during pregnancy [33], the other trial reported significant reductions in VLDL-cholesterol and triglyceride, while a decrease in LDL-cholesterol approached significance [34]. Beneficial gut bacteria have been hypothesized to positively influence lipid metabolism by producing secondary bile acids which are unavailable for enterohepatic recirculation. The liver must then synthesize replacement bile acids from circulating cholesterol [47]. In the present review, a pooled analysis of LDL-cholesterol data from both studies in women with GDM was not significant. Trials of longer duration (>8 weeks) may have generated outcomes with larger effect sizes.

Three of the four studies included in this review [34–36] reported significant reductions in insulin resistance (as measured by HOMA-IR) following probiotic supplementation in women diagnosed with GDM. This did not appear to result in subsequent decreases in FBG, gestational weight gain or a reduced requirement for blood glucose-lowering medication in the intervention group, but further studies of longer duration should explore this. When all four studies were combined, there was a significant reduction in insulin resistance (MD = −0.69; 95% CI −1.24, −0.14, p = 0.01). The studies which found significant reductions in insulin resistance used *Bifidobacterium* spp. in their probiotic [34–36], whereas the study with non-significant findings did not [33]. *Bifidobacterium* spp. have been reported to play a protective role in the prevention of metabolic perturbations by reducing LPS-induced oxidative stress and low grade chronic inflammation [48].

The current management for GDM involves lifestyle changes through dietary modification and physical activity, and pharmacological intervention with metformin and/or insulin if required in order to achieve target blood glucose levels [49–51]. Metformin reduces hyperglycemia by increasing insulin sensitivity and reducing excessive hepatic gluconeogenesis [49]. Both insulin and metformin are considered safe for use in pregnancy, but can be associated with unwanted side effects such as gastrointestinal disturbances and hypoglycemia [52,53]. Metformin contributes to a healthy gut

microbiome by increasing the growth of *Akkermansia muciniphila* and *Lactobacillus* spp. in murine studies, but further research in humans is required to confirm this [54]. Metformin-induced expansion of the *Akkermansia muciniphila* population has been shown to modulate glucose homeostasis in obese mice fed a high fat diet [55]. *Akkermansia muciniphilia* is a mucin-degrading bacterium important for the regulation of the thickness of the mucin layer lining the host gastrointestinal tract, thus protecting the integrity of the gut barrier [56]. Human studies are now required to determine whether metformin-induced improvements in gut microbial diversity contribute to improvements in glucose tolerance. The studies included in this review were not affected by participant use of metformin, as the women required to commence metformin or insulin during the course of the trials were excluded from the final analyses.

All of the RCTs in this review were determined to have a low risk of bias, with most authors publishing their study protocol in a clinical trials registry with pre-specified primary and secondary outcomes prior to study commencement. However, a limitation of these trials were their short duration and small sample sizes. Constraints associated with this systematic review include the substantial interstudy statistical heterogeneity observed, and the use of probiotics of differing microbial composition between trials. The metabolic benefits of probiotics may be strain specific, so the optimal species, dose and duration of treatment in GDM requires further elucidation.

5. Conclusions

The present review found that while probiotic supplementation resulted in a significant reduction in insulin resistance in pregnant women with GDM, there was no significant effect on fasting blood glucose or LDL-cholesterol levels. Further high quality studies using defined doses of specific bacterial species are required to confirm these findings and their clinical relevance before their routine use can be recommended in this patient group.

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Conflicts of Interest: The authors declare that there was no personal or financial conflicts of interest.

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nutrients

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Review

Consumption of Yogurt and the Incident Risk of Cardiovascular Disease: A Meta-Analysis of Nine Cohort Studies

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Abstract: Previous systematic reviews and meta-analyses have evaluated the association of dairy consumption and the risk of cardiovascular disease (CVD). However, the findings were inconsistent. No quantitative analysis has specifically assessed the effect of yogurt intake on the incident risk of CVD. We searched the PubMed and the Embase databases from inception to 10 January 2017. A generic inverse-variance method was used to pool the fully-adjusted relative risks (RRs) and the corresponding 95% confidence intervals (CIs) with a random-effects model. A generalized least squares trend estimation model was used to calculate the specific slopes in the dose-response analysis. The present systematic review and meta-analysis identified nine prospective cohort articles involving a total of 291,236 participants. Compared with the lowest category, highest category of yogurt consumption was not significantly related with the incident risk of CVD, and the RR (95% CI) was 1.01 (0.95, 1.08) with an evidence of significant heterogeneity (I^2 = 52%). However, intake of ≥200 g/day yogurt was significantly associated with a lower risk of CVD in the subgroup analysis. There was a trend that a higher level of yogurt consumption was associated with a lower incident risk of CVD in the dose-response analysis. A daily dose of ≥200 g yogurt intake might be associated with a lower incident risk of CVD. Further cohort studies and randomized controlled trials are still demanded to establish and confirm the observed association in populations with different characteristics.

Keywords: yogurt intake; stroke; coronary heart disease; cardiovascular disease; meta-analysis

1. Introduction

Cardiovascular disease (CVD) is still an important public health problem around the world [1]. The prevalence of coronary heart disease (CHD) and stroke has progressively increased during the past decades [1–3]. Given the very large social and economic burden of the treatment of CVD [4], identifying modifiable factors is imperative and feasible for preventing the progress of CVD.

Several dietary patterns and individual foods have been demonstrated to exert preventive effects on CVD risk [5–7]. In particular, the benefits of yogurt intake have recently drawn a lot of attention [8,9]. Yogurt is defined as the product of fermentation of the *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus* [10]. Being an important component of the human diet for several millennia [10], it will be a major public health implication if yogurt consumption is demonstrated to have a protective role in delaying the development of CVD.

Previous systematic reviews and meta-analyses have evaluated the association of dairy consumption and the risk of CVD [11–15]. However, the conclusions were inconsistent. In the

subgroup analysis of yogurt intake, no association was established between yogurt intake and CVD or stroke in three previous meta-analyses [11–13]. Soedamah-Muthu et al. did not separately estimate the effect of yogurt intake apart from other dairy products [14]. Hu et al. observed a protective role of yogurt intake on stroke, but only three studies were included in the pooled analysis [15].

To the best of our knowledge, no quantitative analysis has specifically assessed the effect of yogurt intake on the risk of incident CVD. Moreover, whether different amounts of yogurt consumption present different impacts on CVD risk is still uncertain. Therefore, we performed a systematic review and meta-analysis to pool the evidence from prospective cohort studies on the relationship of yogurt intake and the incident risk of CVD. Furthermore, we attempted to evaluate the potential dose-response pattern of the association.

2. Materials and Methods

2.1. Literature Search

The present systematic review and meta-analysis was carried out according to a standard process [16,17]. We searched the PubMed and the Embase databases from inception to 10 January 2017 for records relevant to yogurt consumption and risk of incident CVD. Our search included terms as "fermented dairy", "yogurt", "yoghurt", "sour milk", "fermented milk", "cultured milk", "probiotic", etc. Language restriction was not set. A detailed search strategy is presented in Supplementary Table S1. The references of the relevant reviews and original articles were manually searched to find out more potential eligible studies. When multiple published articles were found from an identical study, the one with the longest follow-up duration was included in the present analysis.

2.2. Selection Criteria and Data Extraction

The authors independently conducted the initial screening process. After removing the duplicate records, we identified the title and the abstract of each eligible article. Unrelated articles were excluded, and articles of interest were included as further evaluation. Any disagreements were resolved by discussion between the two authors.

Inclusive criteria: (1) studies reporting the relationship of yogurt intake and the incident risk of CVD (CHD or stroke) by using adjusted relative risks (RRs), hazard ratios (HRs), or odds ratios (ORs) and their corresponding 95% confidence intervals (CIs); (2) studies in which exposures were the fermented milk, but yogurt was the largest contribution to the total fermented milk; and (3) the study design was based on prospective cohort. Exclusive criteria: (1) the data reported individual components of yogurt, such as protein or probiotics; (2) studies that only reported results for total dairy/milk products or combined non-fermented and fermented milk; (3) the studies in which the participants are aged <18 years; and (4) the studies in which the participants are pregnant or lactating females.

Data extraction was independently implemented by two authors. Data were extracted from each eligible article including the first author, the published year, the study location, the number of cases and total participants, baseline age and gender of participants, the method of exposure and the outcome measurements, the type of exposure and outcome, duration of follow-up, adjusted variables, and the largest number of adjusted ORs, RRs, or HRs with their corresponding 95% CIs of incident CVD for all categories of yogurt consumption.

2.3. Quality Assessment

The Newcastle-Ottawa quality scale (NOS) [18] was used to estimate the quality assessment of all eligible articles. Higher points indicated higher study quality, and the scale ranged between 0 and 9 points. Three domains were assessed: (1) the basis of the cohort selection (0–4 points); (2) the comparability of the cohort design and analysis (0–2 points); (3) and the adequacy of measurements including exposure and outcome variables (0–3 points).

2.4. Statistical Analysis

Statistical analyses were conducted using two sorts of software: Stata (12.0, StataCorp LP, College Station, TX, USA) and Review Manager (5.2, The Nordic Cochrane Centre, Copenhagen, Denmark). We applied RRs to measure the effect size for articles using the incident cases of CVD as an outcome. An approach of generic inverse-variance was used to pool the outcome data for the yogurt intake of highest vs. lowest category with a random-effects model. The *p*-values less than 0.05 were regarded as statistical significant. Heterogeneity across studies was examined by the I^2 statistic which, when greater than 50%, indicated significant results [19]. Additionally, we conducted a stratified analysis based on pre-specified characteristics including the type of CVD (CHD or stroke), study location (North America or Europe), age (<40 or ≥40 years), gender (male, female or both sexes), the exposure type (yogurt or combined with other dairy products), and the exposure dose (<200 or ≥200 g/day). Furthermore, meta-regression analysis was used to identify the potential difference of the two groups, *p*-values of less than 0.1 were judged as significant. To evaluate the effect of an individual article on the overall pooled results, a sensitivity analysis was conducted by omitting each article from the overall analysis in every turn. The publication bias was examined through the tests of Begg's and Egger's [20,21].

Generalized least squares trend (GLST) estimation model was used to compute the specific slopes in the dose-response analysis [22,23]. For categories (at least three) of yogurt consumption that were open (e.g., 30–69 g/day), we assigned the median value as the homologous category of yogurt intake. If the maximum dose was unlimitedly fixed (e.g., >200 g/day), we assumed that the mean was 25% larger than the lower level of the specific category [24]. When the number of cases for each category was not available, the RRs were acquired with a general estimate [25]. When studies reported yogurt intake in serving/day, we converted the intake to g/day using a standard unit of 244 g [26]. The results of the dose-response analysis were shown for each gram increased in daily yogurt intake. A restricted cubic spline model (four-knot) was applied for the assessment of non-linearity hypothesis in the association between yogurt intake and the incident risk of CVD.

3. Results

3.1. Article Identification and Selection

Figure 1 presents a flow diagram of articles included in the present study. In the initial search process, 1348 studies were identified from the Pubmed and the Embase databases. After removing the duplicated articles, 1161 studies were included for further assessment. A total of 1138 studies were excluded after reading the titles and the abstracts. The remaining 23 studies were evaluated to assess for eligibility after reading the full-text. Finally, nine cohort studies were eligible for inclusion in our meta-analysis [27–35]. One of the nine articles was identified from references of a full-text article [29].

3.2. Study Characteristics

Characteristics of each included article are shown in Table 1. Publication years were ranged between 1999 and 2015. Two articles were conducted in North America [27,29] and the remaining seven articles were performed in Europe [28,30–35]. Follow-up durations ranged between 10.2 [31] and 17.3 years [33]. Six articles included both men and women [27,28,31,33–35], one article included only men [30], and two articles included only women [29,32]. The baseline age of the participants ranged from ≥21 [28] to ≥55 years [33]. The number of study participants ranged from 1759 [27] to 85,764 [29] for a total number of 291,236. Yogurt intake was assessed by a food-frequency questionnaire (FFQ), and the incidences of CVD were ascertained from medical records or registries in all included articles.

Table 1. Characteristics of the included articles.

First Author, Published Year	Study Location	Follow-Up (Years)	Male (%)	Baseline Age (Years) (Minimum-)	Participants, No.	Exposure		Outcome			Adjustment *
						Type	Category	Method of Ascertainment	Type	Case, No.	
Avalos, 2013 [27]	US	16.3	42.7	49-	1759	Yogurt	Never/rarely, sometimes/often	Medical records	CHD	454	1–6
Dalmeijer, 2013 [28]	Netherland	13.1	25.5	21-	33,625	Buttermilk, yogurt, cheese	Per SD of the mean g/day	Registries	CHD, Total stroke	1648, 531	1, 2, 7–18
Iso, 1999 [29]	US	13.6	0.0	34-	85,764	Yogurt	≥5 times/week, never	Registries and medical records	Ischemic stroke	347	1, 10
Larsson, 2009 [30]	Finland	13.6	100	50-	26,556	Yogurt	Quintile	Registries	Cerebral Infarction, Intracerebral Hemorrhage, Subarachnoid Hemorrhage	1950, 277, 114	1, 2, 8–11, 13–17, 19–29
Larsson, 2016 [31]	Sweden	10.2	53.8	45-	74,961	Yogurt, sour milk	Quintile	Registries	Total stroke	4089	1, 2, 7–11, 13–17, 22, 24, 30–32
Patterson, 2013 [32]	Sweden	11.6	0.0	48-	33,636	Yogurt	Quintile	Registries	Myocardial infarction	1392	8–11, 14, 15, 21, 24, 28, 30–34
Praagman, 2015 [33]	Dutch	17.3	37.9	55-	4235	Yogurt	<50, 50–100, >100 g/day	Registries	CHD, Total stroke	564, 567	1, 2, 7, 8, 10, 11, 13–18, 24, 35
Soedamah-Muthu, 2013 [34]	UK	10.8	72.0	35-	4255	Yogurt	Tertiles	Registries	CHD	323	1, 2, 8–11, 13–17, 24, 32, 35
Sonestedt, 2011 [35]	Sweden	12.0	38.1	44-	26,445	Fermented milk	Quartile	Registries	CVD	2520	1, 2, 7–11, 13–16, 24, 28, 36–38

* 1 = age, 2 = body mass index (BMI), 3 = diabetes, 4 = hypertension, 5 = LDL-cholesterol, 6 = estrogen use, 7 = gender, 8 = total energy intake, 9 = physical activity, 10 = smoking, 11 = education, 12 = ethanol intake, 13 = coffee intake, 14 = fruit intake, 15 = vegetables intake, 16 = fish intake, 17 = meat intake, 18 = bread intake, 19 = supplementation group, 20 = cholesterol, 21 = serum HDL cholesterol, 22 = diabetes, 23 = heart disease, 24 = alcohol intake, 25 = sugar intake, 26 = poultry intake, 27 = potatoes intake, 28 = whole grains intake, 29 = refined grains intake, 30 = aspirin use, 31 = hypertension, 32 = family history of myocardial infarction, 33 = waist-to-hip ratio, 34 = hormone therapy usage, 35 = tea intake, 36 = ethnicity, 37 = employment grade, 38 = season method. CHD, coronary heart disease; CVD, cardiovascular disease; SD, standard deviation.

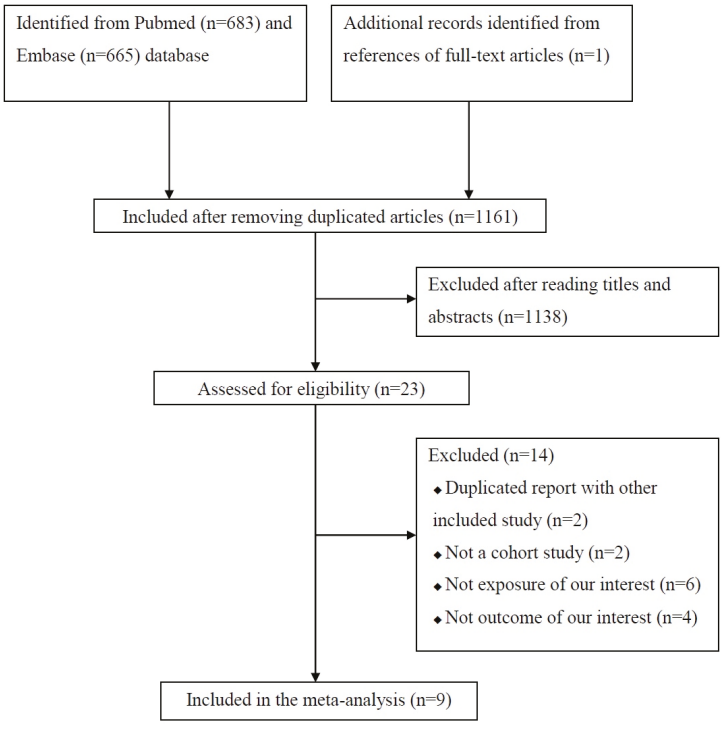

Figure 1. Flow diagram of articles included in the present study.

3.3. Quality Assessment

All studies received a quality score of 8–9 stars (Supplementary Table S2). All articles measured yogurt intake by FFQ. The diagnoses of CVD were ascertained from medical records or registries in all included articles. The follow-up duration of all articles was greater than 10 years. One article did not exclude participants with a history of CHD events [27], and one study only included the elderly male smokers (at a higher risk of CVD) [30]. One article only adjusted for age and smoking status in the statistical model [29].

3.4. Yogurt Consumption and the Occurrence of CVD

Figure 2 shows the forest plot of RRs (95% CIs) for the relationship of yogurt consumption (highest vs. lowest dose) and the occurrence of CVD by type of outcome. Yogurt consumption was not significantly associated with the developing of CVD in the pooled analysis of 14 comparatives, and the RR (95% CI) was 1.01 (0.95, 1.08) with an evidence of significant heterogeneity (I^2 = 52%). In the stratified analysis by type of outcome, the pooled RRs (95% CIs) of yogurt consumption were 1.04 (0.95, 1.15) for CHD, 1.02 (0.92, 1.13) for stroke, and 0.87 (0.77, 0.98) for the incident CVD events. Sensitivity analysis showed that further exclusion of any individual comparative did not significantly alter the pooled RR, and the RRs (95% CIs) ranged between 0.99 (0.94, 1.07) and 1.03 (0.96, 1.09). Exclusion the study by Larsson et al. [30] reduced the heterogeneity to 40%. No publication bias was observed among 14 comparatives (Supplementary Figure S1, Egger's test: p-value = 0.228, Begg's test: p-value = 0.254).

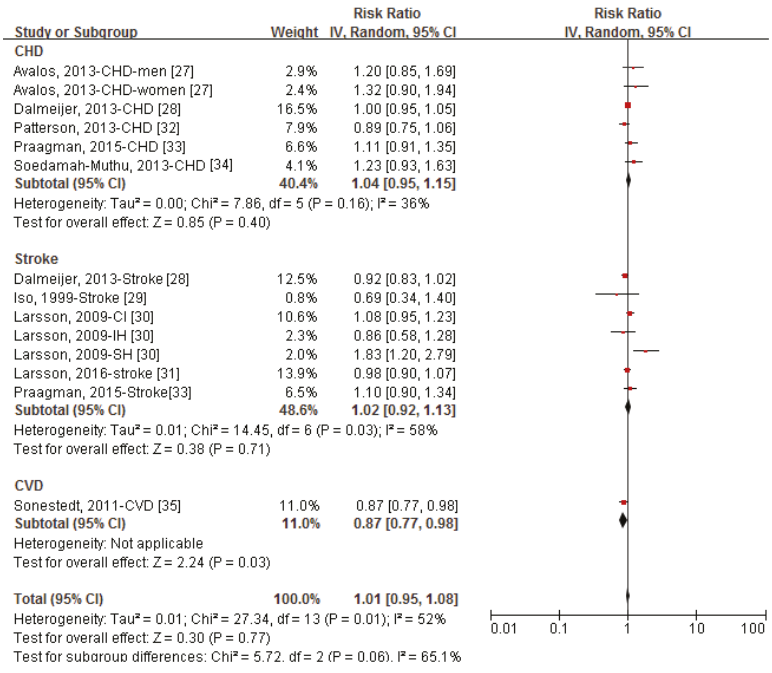

Figure 2. Forest plot of relative risks (RRs) and 95% confidence intervals (CIs) for the association between category of yogurt intake (highest vs. lowest) and the incident risk of coronary heart disease (CHD), stroke, and cardiovascular disease (CVD). CI, cerebral infarction; IH, intra-cerebral hemorrhage; SH, subarachnoid hemorrhage.

3.5. Subgroup Meta-Analysis

As shown in Table 2, analyses by study location, age, gender, and the type of exposure did not significantly affect the associations between yogurt intake and the incident risk of CVD (*p*-value for difference >0.1 for each group). Stratified analysis by yogurt dose of the highest category (<200 or ≥200 g/day) significantly affected the association (*p*-value for difference = 0.09), and ≥200 g/day yogurt intake was significantly associated with lower risk of CVD compared with the reference category, and the RR (95% CI) was 0.92 (0.85, 1.00).

Table 2. Stratified analysis of the association between yogurt consumption and the incident risk of cardiovascular disease.

	Comparisons, No.	Relative Risk (95% Confidence Interval)
Total	14	1.01 (0.95, 1.08)
Study location		
North America	3	1.15 (0.87, 1.52)
Europe	11	1.00 (0.94, 1.07)
p-value for difference		0.36
Age		
<40 years	4	0.98 (0.90, 1.08)
≥40 years	10	1.04 (0.94, 1.14)
p-value for difference		0.66

Table 2. *Cont.*

	Comparisons, No.	Relative Risk (95% Confidence Interval)
Gender		
Male	4	1.17 (0.92, 1.48)
Female	3	0.97 (0.72, 1.32)
Both sexes	7	0.98 (0.93. 1.05)
p-value for difference		0.26
Exposure type		
Yogurt	11	1.06 (0.96, 1.18)
Yogurt combined with other dairy products	3	0.96 (0.89, 1.04)
p-value for difference		0.25
Dose of the highest category		
<200 g/day	11	1.06 (0.98, 1.15)
≥200 g/day	3	0.92 (0.85, 1.00)
p-value for difference		0.09

3.6. Dose-Response Analysis

After excluding three articles [27–29] that reported fewer than three categories of yogurt consumption, the remaining six studies were included in the dose-response analysis. Although the association was not significant, there was a trend that higher level of yogurt consumption was associated with a lower risk of incident CVD events (Figure 3).

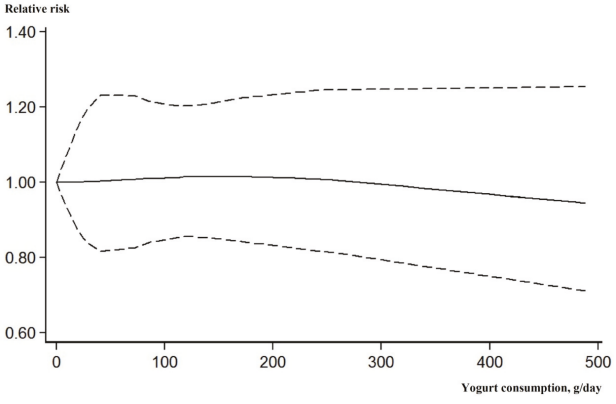

Figure 3. Dose-response association between yogurt consumption (g/day) and the incident risk of cardiovascular disease. Solid line, best-fitting restricted cubic spine; dotted line, 95% confidence interval.

4. Discussion

The present systematic review and meta-analysis identified nine cohort articles involving a total of 291,236 participants. Compared with the lowest category, the highest category of yogurt consumption was not significantly related with the incident risk of CVD; however, intake of ≥200 g/day yogurt was significantly associated with a lower risk of CVD in the subgroup analysis. There was a trend that a higher level of yogurt consumption was associated with a lower incident risk of CVD in the dose-response analysis.

An increasing number of epidemiological studies have supported the beneficial effects of yogurt consumption on lowering blood pressure, total cholesterol concentrations, total cholesterol, and plasma glucose [9,36,37]. Although the findings remain controversial, several epidemiological and clinical studies have suggested the potential role of yogurt intake in weight management [38,39]. Obesity, hyperlipoidemia, and high blood pressure are well-known risk factors of CVD and, thus, the

above studies indirectly support the beneficial role of yogurt consumption on CVD risk. However, in agreement with several previous meta-analyses [11–13], we did not observe a significant association between yogurt intake and CVD. We have obtained a significantly reverse association when only including the highest yogurt consumption of ≥200 g/day, indicating that lower consumption of yogurt (<200 g/day) may represent a missed opportunity to contribute to a lower risk of CVD. Yogurt provides a good source of active components, such as calcium, vitamin D, sphingolipids, and probiotics [40]. Among these, probiotic micro-organisms have an effect on weight reduction, which may translate to a reduced risk of CVD through supporting a healthy gut microbiota composition [41]. In addition, some studies revealed that yogurt might interfere with cholesterol synthesis. However, whether the effects on lipids could be translated to a decreasing risk of CVD is warranted to confirm in the future [6].

Compared with previously published meta-analyses relevant to this topic [11–15], this is the first systematic review and meta-analysis to specifically evaluate the association between yogurt intake and CVD risk. All included studies were of high-quality, and the follow-up durations were long enough for outcomes to occur. Furthermore, the larger sample size (12,262 cases among 291,236 participants) enabled us to perform stratified and dose-response analyses to explore the potential association. Finally, unlike the study by Qin [11] and de Goede et al. [13], only CVD incidence, but not mortality case, was accepted as an outcome.

Limitations of our meta-analysis should be mentioned. First, considerable heterogeneity has been observed across studies. This is not surprising given the diversity in study characteristics of participants, various doses of yogurt consumption, different type of outcome, and adjusted confounders. Further sensitivity analysis revealed that the study by Larsson et al. [30] might be the source of heterogeneity. Different from other studies, participants with a higher risk of stroke were included, and three types of stroke were separately reported in the work by Larsson et al. [30]. Second, detailed data relevant to the patterns of yogurt, such as species of the lactic acid bacteria (*Lactobacillus sp.*, *Enterococcus sp.*, or *Streptococcus sp.*), consuming time and fat-containing (skim fat, whole-fat, or low-fat yogurt) were not illustrated in all included studies. Some RCTs have indicated that intake of low-fat and whole-fat dairy products may cause different effects on blood pressure, weight and depressive symptoms [42,43]. Therefore, further studies are needed to resolve this issue. Third, only baseline dietary habits were collected and analyzed in all included studies. Participants may have changed their lifestyle and dietary patterns during the long follow-up period. Fourth, although most studies adjusted for nearly all the important covariates, other potential unmeasured confounders may have influence our findings. Fifth, all studies were conducted in Western developed countries, limiting the generalizability of the results to a broader demographic. Considering that the consumption and making methods of yogurt vary greatly from country to country [10], region-difference should be considered. Finally, our findings are based on observational studies and, thus, causal association cannot be established.

5. Conclusions

In conclusion, the present meta-analysis based on nine independent cohort studies provides a non-significant association between yogurt intake and CVD risk. Daily dose of ≥200 g yogurt intake might be associated with a lower risk of CVD. Further cohort studies and randomized controlled trials are still demanded to establish and confirm the observed association in populations with different characteristics.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/3/315/s1, Figure S1: Funnel plot of the 14 comparatives, Table S1. Search strategy, Table S2: Study quality of each included article (maximum: 9 stars).

Author Contributions: Wu Lei was responsible for the study design. Data were analyzed by Wu Lei and Sun Dali. All authors have taken part in the writing and the revising of the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

The Effect of *Bifidobacterium animalis* ssp. *lactis* HN019 on Cellular Immune Function in Healthy Elderly Subjects: Systematic Review and Meta-Analysis

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Abstract: Elderly people have increased susceptibility to infections and cancer that are associated with decline in cellular immune function. The objective of this work was to determine the efficacy of *Bifidobacterium* (*B.*) *animalis* ssp. *lactis* HN019 (HN019) supplementation on cellular immune activity in healthy elderly subjects. We conducted a systematic review of Medline and Embase for controlled trials that reported polymorphonuclear (PMN) cell phagocytic capacity or natural killer (NK) cell tumoricidal activity following *B. lactis* HN019 consumption in the elderly. A random effects meta-analysis was performed with standardized mean difference (SMD) and 95% confidence interval between probiotic and control groups for each outcome. A total of four clinical trials were included in this analysis. *B. lactis* HN019 supplementation was highly efficacious in increasing PMN phagocytic capacity with an SMD of 0.74 (95% confidence interval: 0.38 to 1.11, $p < 0.001$) and moderately efficacious in increasing NK cell tumoricidal activity with an SMD of 0.43 (95% confidence interval: 0.08 to 0.78, $p = 0.02$). The main limitations of this research were the small number of included studies, short-term follow-up, and assessment of a single probiotic strain. In conclusion, daily consumption of *B. lactis* HN019 enhances NK cell and PMN function in healthy elderly adults.

Keywords: aging; *Bifidobacterium*; elderly; immunity; probiotic

1. Introduction

Globally, elderly people represent the fastest growing population. Older individuals have typically weaker immune responses to vaccination and elevated risk for infections, certain autoimmune diseases, and cancer [1]. Many of these health risks are a consequence of declining immune function associated with the aging process, i.e., immunosenescence [2]. Traditional hallmarks of immunosenescence include adaptive immunity components such as lower number and/or proportions of peripheral blood naïve T cells (cluster of differentiation 8 (CD8+)), increased number of memory/effector cytotoxic T cells (CD8+) [3], as well as altered capacity of peripheral blood T cells to proliferate and secrete cytokines [1]. Furthermore, B-cell function and quantity appear to decline with age [4]. Age-related alterations have also been reported for innate immune system and specifically for function of neutrophils and natural killer (NK) cells. Neutrophils, which are important for early immune response for infections, show decreased chemotaxis, phagocytic activity, and declined superoxide generation in the elderly [5,6]. Polymorphonuclear (PMN) cells in the blood are mostly neutrophils (90%–95%), together with smaller fraction of eosinophils and basophils. The number of NK-cells increases with age, but their signaling efficiency, cytokine production, and up regulation of co-stimulatory molecules is suboptimal, leading to a net decrease in function [7–9]. It has been suggested that underlying defect of the declined innate

cellular function in the elderly may be a result of decreased sensitivity of toll like receptor (TLR) signaling that is important in recognizing microbial structures [10]. These changes in immune function may compromise the early recognition and elimination of virus infected and malignant cells [2].

Gut microbiota plays an important role in immunosenescence and is influenced by physiological aging process, lifestyle, and diet [11–14]. It has been shown that aging gut microbiota has specific features compared to microbiota of younger adults—like lower levels of bifidobacteria and higher levels of *Bacteroidetes* spp. [12,14]. The above changes in microbiota composition may be indicative of dysbiosis and poorer health. For instance, lower bifidobacteria levels have been found to associate with increased risk of *Clostridium difficile* associated diarrhea [15], hospitalization [16], antibiotic treatment [17], and frailty [18]. Thus, targeted dietary interventions that restore composition of the microbiota could reduce the risk of age related morbidities and improve the quality of the life of the elderly.

It has been suggested that probiotic bacteria could have potential for improving immune system function in the elderly [19]. Probiotics are known to interact with TLRs and other microbial pattern recognition receptors on immune system cells in intestinal mucosa and thus directly influence their functions [20]. Furthermore, specific probiotic strains may induce beneficial changes in the gut microbiota that have an impact on immune status. For instance, *B. longum* strains induced changes in the bifidobacteria population, which correlated with tumor necrosis factor (TNF)-α and interleukin (IL)-10 levels in plasma [21], and *Lactobacillus (L.) casei* and *L. plantarum* strains improved influenza virus vaccination responses in the elderly [22,23]. Although there are some clinical trials investigating probiotics and their effectiveness in improving immune responses in the elderly [19], systematic information on strain-specific effects of probiotics on the immunity in this population is lacking. One of the probiotics applied in clinical studies in this area is *Bifidobacterium (B.) animalis* ssp. *lactis* HN019. The objective of this systematic review and meta-analysis of controlled studies was to evaluate the effect of *B. lactis* HN019 versus non-probiotic control on cellular innate immune activity of healthy elderly subjects.

2. Materials and Methods

2.1. Literature Search

The study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [24]. Medline and Embase databases were searched for randomized or non-randomized controlled studies published in English-language journals that reported PMN cell phagocytosis activity or NK cell tumoricidal activity following *B. lactis* HN019 consumption in reportedly healthy, elderly (≥60 years) adults. No date restrictions were applied to the searches. The details of the Medline search strategy are listed in Table 1. The syntax for Embase was similar, but adapted as necessary. Additionally, manual searches were conducted using the Directory of Open Access Journals, Google Scholar, and the reference lists of included papers and relevant meta-analyses. The final search was conducted in June 2016.

2.2. Study Selection

One reviewer selected studies for inclusion in the review. Articles were then independently assessed by a second reviewer, who confirmed eligibility. Titles and abstracts were initially screened to exclude non-English manuscripts, review articles, commentaries, letters, case reports, animal studies, and obvious irrelevant studies. Full-texts of the remaining articles were retrieved and reviewed.

Table 1. Medline search strategy.

Intervention Search Terms
1. Probiotic
2. Synbiotic
3. Bifidobacteri *
4. Lactis
5. B. lactis
6. HN019
7. Yogurt (yoghurt)
8. Fermented milk
Outcomes Search Terms
9. Phagocyt *
10. Polymorphonuclear
11. PMN
12. Natural killer cell
13. NK cell
14. Tumoricidal
15. Immun *
Combination Terms
16. or/1–8
17. or/9–15
18. and/16–17

An asterisk represents a wildcard symbol used in a search query to represent end truncation. NK: natural killer; PMN: polymorphonuclear.

2.3. Data Extraction

Data were independently extracted from eligible articles by two reviewers. Data extraction discrepancies between the reviewers were resolved by consensus. The types of data recorded in the standardized data extraction forms included general manuscript information, subject characteristics, study characteristics, PMN phagocytic capacity, and NK cell tumoricidal activity. Potential sources of bias were assessed by evaluating randomization, blinding, type of control, and main outcome definitions among studies.

2.4. Data Synthesis

A random effects meta-analysis model was developed based on the a priori assumption that treatment effects would be heterogeneous among studies. For each outcome, a pooled standardized mean difference (SMD) and 95% confidence interval were calculated. For reference, SMD values of 0.2, 0.5, 0.8, and 1.0 are defined as small, medium, large, and very large effect sizes, respectively [25]. Forest plots were used to illustrate individual study findings and pooled meta-analysis results. Heterogeneity of effects across studies was planned to be estimated using the I^2 statistic if at least 10 studies were included in the meta-analysis. p-values were two-sided with a significance level <0.05. Statistical analyses were performed using Comprehensive Meta-analysis version 2.2 (Biostat, Englewood, NJ, USA).

3. Results

The initial database search retrieved 82 titles and abstracts; hand searching relevant bibliographies identified three additional records. After screening records for inclusion criteria, 74 records were deemed irrelevant and 11 full text articles were reviewed for eligibility. Ultimately, four studies [26–29] were included in the final analysis. A flow diagram of study identification and selection is shown in Figure 1.

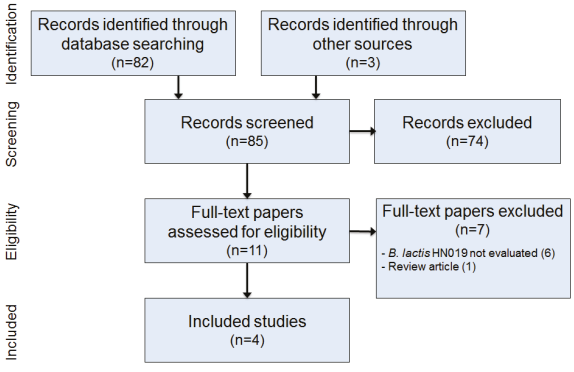

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) study flow diagram.

Regarding the evaluation of *B. lactis* HN019 relative to control, one study [28] was a randomized trial with a parallel control group and three studies [26,27,29] used a 3-week run-in period as the control. Subjects in each study were healthy elderly adults with a median age between 60 and 70 years. The interventions evaluated were daily consumption of low-fat milk, with or without *B. lactis* HN019. The daily dosages of *B. lactis* HN019 ranged from 5×10^9 to 3×10^{11} colony forming units (cfu) and the treatment durations ranged from 3 to 6 weeks (Table 2). An assessment of potential sources of bias are listed in Table 3. One study [28] utilized random allocation and two studies [26,28] blinded subjects to the allocated intervention. The main outcome definitions were consistent across studies and, therefore, pooling data among studies was appropriate.

Table 2. Study characteristics.

Study	No. Subjects (HN019:Control)	Female (%)	Age (Median, Range)	Delivery Vehicle	HN019 Daily Dose (cfu)	Intervention Duration [c]
Arunachalam, 2000 [28]	13:12	72	69 (60–83)	Low-fat milk	3×10^{11}	6 weeks
Chiang, 2000 [29]	27 [a]	70	60 (41–81)	Low-fat milk	5×10^{10}	3 weeks
Gill, 2001a [27]	15 [a,b]	60	69 (63–84)	Low-fat milk	5×10^{10} [b]	3 weeks
Gill, 2001b [26]	14 [a]	57	70 (60–84)	Low-fat milk	5×10^9	3 weeks

cfu: colony forming units. [a] All subjects completed run-in control and *B. lactis* HN019 intervention; [b] Study randomized subjects to high dose (5×10^{10} cfu) or low dose (5×10^9 cfu) *B. lactis* HN019; data from high-dose group only were used for analyses; [c] Represents duration of each intervention, not duration of entire study.

Table 3. Bias assessment.

Study	Randomization	Blinding	Control	Definitions	
				Phagocytic Capacity	NK Cell Tumoricidal Activity
Arunachalam, 2000 [28]	Yes	Yes	Parallel group	Relative increase in % PMN cells showing phagocytic activity	–
Chiang, 2000 [29]	No	No	3-week run-in period	% PMN cells showing phagocytic activity	% NK cell tumor killing activity
Gill, 2001a [27]	No	No	3-week run-in period	% PMN cells showing phagocytic activity	% NK cell tumor killing activity
Gill, 2001b [26]	No	Yes	3-week run-in period	–	% NK cell tumor killing activity

Dashed line indicates data not reported; NK: natural killer; PMN: polymononuclear.

Phagocytic capacity outcomes were consistent among individual studies (SMD range: 0.60 to 1.01). Furthermore, each study reported statistically significant improvements in PMN phagocytic capacity with *B. lactis* HN019 relative to control. The pooled SMD for PMN phagocytic capacity was 0.74 (95% CI: 0.38 to 1.11, $p < 0.001$), representing a large treatment effect in favor of *B. lactis* HN019 (Figure 2). Heterogeneity in phagocytic capacity was not formally assessed given the small number of studies.

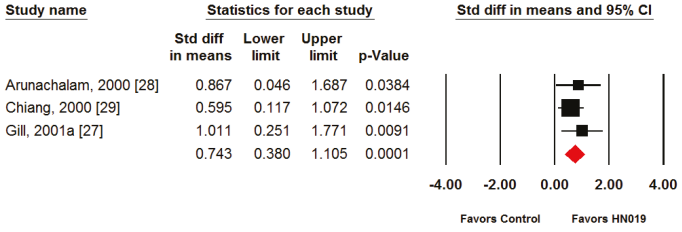

Figure 2. Forest plot of polymorphonuclear phagocytic capacity with consumption of *B. lactis* HN019 vs. control. Random effects meta-analysis using the standardized mean difference (SMD) statistic. The SMD of *B. lactis* HN019 relative to control is plotted for each study. A pooled estimate of SMD (diamond) and 95% confidence interval (diamond width) summarizes the effect size. Effects to the left of 0 indicate greater polymorphonuclear (PMN) phagocytic capacity with control; effects to the right of 0 indicate greater capacity with *B. lactis* HN019. When the horizontal bars of an individual study, or the pooled diamond width, cross 0, the effect is not significantly different. The pooled SMD was 0.74 (95% CI: 0.38 to 1.11, $p < 0.001$), representing a large treatment effect in favor of *B. lactis* HN019.

NK cell tumoricidal activity outcomes were consistent among individual studies (SMD range: 0.36 to 0.63). Although no individual study reported statistically significant differences between the groups, the pooled SMD for NK cell tumoricidal activity was 0.43 (95% CI: 0.08 to 0.78, $p = 0.02$), representing a statistically significant moderate treatment effect in favor of *B. lactis* HN019 (Figure 3). Heterogeneity in NK cell tumoricidal activity was not formally assessed given the small number of studies.

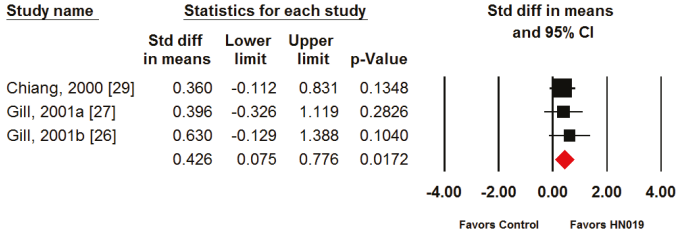

Figure 3. Forest plot of natural killer (NK) cell tumoricidal activity with consumption of *B. lactis* HN019 vs. control. Random effects meta-analysis using the standardized mean difference (SMD) statistic. The SMD of *B. lactis* HN019 relative to control is plotted for each study. A pooled estimate of SMD (diamond) and 95% confidence interval (diamond width) summarizes the effect size. Effects to the left of 0 indicate greater NK cell tumoricidal activity with control; effects to the right of 0 indicate greater activity with *B. lactis* HN019. When the horizontal bars of an individual study, or the pooled diamond width, cross 0, the effect is not significantly different. The pooled SMD was 0.43 (95% CI: 0.08 to 0.78, $p = 0.02$), representing a moderate treatment effect in favor of *B. lactis* HN019.

4. Discussion

With modern life-style the human life expectancy is increasing and the health in old age is of increasing concern to individuals and society. It has been suggested that probiotic supplementation

could offer means to reverse some age-related changes in intestinal microflora composition and to help maintain the aging immune system that are associated with age-related morbidities [30]. Despite the potential benefits of the probiotics for the elderly, the efficacy of only few probiotic strains on immune function in this population has been tested in clinical studies. This systematic review and meta-analysis reports the efficacy of *B. lactis* HN019 on PMN phagocytic capacity and NK cell tumoricidal activity in healthy elderly subjects. The final analysis included four controlled clinical trials with variable designs, doses, and treatment durations. The outcomes in these studies included either PMN phagocytic capacity or NK cell tumor killing activity or both. The pooled data showed that short-term, 3 to 6 weeks, consumption of *B. lactis* HN019 resulted in significantly enhanced PMN phagocytic capacity and NK cell tumoricidal activity in the healthy elderly population.

Three independent clinical studies assessing the effect of *B. lactis* HN019 on PMN phagocytic capacity were included in this study [27–29]. Although the number of subjects in the trials was modest (Table 2), all consistently showed statistically significant effect ($p < 0.05$) on phagocytic capacity. The meta-analysis of the trials showed that HN019 had a large treatment effect ($p < 0.001$) on phagocytic capacity of PMN cells (Figure 2), confirming the original study findings. Whether the improvement in PMN function by HN019 leads to better resistance to infections was not studied in the original publications. However, it is recognized that phagocytosis is a first-line defense function of PMN cells against infectious diseases that are found more prevalent in the elderly population [31,32].

Previously published studies on the effect of probiotics on phagocytosis have reported that daily consumption of *B. lactis* Bi-07 improved the phagocytic activity of monocytes and granulocytes in healthy elderly adults [33], but in contrast, supplementation with *L. johnsonii* La1 decreased phagocytic activity of neutrophils in the elderly [34]. As intestinal bifidobacteria count decreases with age [12,14], it is noteworthy that two different bifidobacteria strains Bi-07 and HN019 were efficacious whereas the lactobacillus strain was not, however, the data is very limited to make any conclusions on the differences in the efficacy on the genera level. In fact, a recent consensus paper concluded that the effects of probiotics on immune function are strain-specific [35].

Whereas PMN phagocytosis and NK-cell cytotoxicity are key immune functions against infections, NK cells function also in the elimination of cancerous cells. This study included three clinical trials that investigated the effect of *B. lactis* HN019 on NK-cell tumoricidal activity, but none showed statistically significant results in the original publications [26,27,29]. However, the group sizes were relatively small and pooling the data in this meta-analysis resulted in a statistically significant ($p = 0.017$) treatment effect for improving NK-cell activity in the elderly (Figure 3). Although the result shows improvement in NK-cell activity, it remains to be studied if the improved function by *B. lactis* HN019 results in health benefit for the elderly. Nevertheless, research in the elderly shows that low NK cell activity is associated with the development of infectious diseases [7,36]. Also, the incidence of cancer and mortality rate has been found higher in populations with a low NK cell activity compared with those with higher NK cell activities [36–38].

Effect of other probiotics than HN019 studies have also been investigated on NK cell activity. A combination of *L. rhamnosus* HN001 and *L. acidophilus* NCFM resulted in a significantly increased cytotoxicity of the NK cells [39]. In contrast, *B. longum* BB536 intake for 12 weeks had no effect on NK cell activity in elderly patients fed by enteral tube feeding [40] and *L. gasseri* TMC0356 supplementation for 4 weeks had no significant impact on NK cell counts or NK cell activity [41]. Altogether three studies have assessed the effect of *L. casei* Shirota on NK cell function: (i) Takeda and colleagues showed that *L. casei* Shirota may elevate NK cell activity after 4 weeks when compared to placebo [42]; (ii) the consumption of *L. casei* Shirota was associated with a significant increase in NK cell activity relative to baseline, however the difference was not significant when compared with placebo [43]; and (iii) a study showed that NK-cell activity was not significantly augmented by a 3-week intake of *L. casei* Shirota [44]. In summary, the results on probiotic efficacy on NK cell activity in the elderly vary and may be strain and study dependent.

Although the impact of *B. lactis* HN019 on specific cellular immune parameters seems relatively clear there were still limitations inherent in the findings of this meta-analysis, including a potential for bias due to relatively small study populations. In addition, the study outcomes may have been affected by other factors such as the length of intervention, the formula, and the supplementation dose. In the studies included in this meta-analysis, the *B. lactis* HN019 supplementation was given to healthy elderly adulty for 3–6 weeks suggesting that relatively short term consumption of *B. lactis* HN019 has an impact on immune cell function in blood; however, it remains unknown if the stimulatory effect would have been maintained in longer term consumption [45]. The daily *B. lactis* HN019 doses were between 5×10^{10} and 3×10^{11} cfu/day in PMN studies and 5×10^{9}–5×10^{10} cfu/day in NK cell studies, suggesting that HN019 is efficacious with a range of doses typically used in dietary supplements. In all the *B. lactis* HN019 clinical trials low-fat milk was used as a delivery vehicle, and as such, no firm conclusions on the role of the vehicle on these immune outcomes can be drawn. A further consideration relates to type of control. The study by Arunachalam et al. [28] used a parallel group assigned to placebo, whereas the other three studies [26,27,29] utilized a run-in period control. Due to a small number of included studies, subgroup analysis and meta-regression to investigate sources of heterogeneity in outcomes (e.g., type of control, study duration, patient age) was not possible. Finally, since this report was focused on a single probiotic strain, the results cannot be generalized to probiotics as a whole.

5. Conclusions

The findings from this meta-analysis suggest that daily short-term consumption of probiotic *B. lactis* HN019 enhances PMN phagocytic capacity and NK cell tumoricidal activity in healthy elderly adults. As the health of the elderly was not assessed in the original studies, the correlation between *B. lactis* HN019 mediated PMN and NK cell activity improvement and resistance to infection and diseases remains to be confirmed in future trials.

Supplementary Materials: The supplementary materials are available online at http://www.mdpi.com/2072-6643/9/3/191/s1.

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Conflicts of Interest: L.E.M. is a consultant to Bio-K Plus, DuPont, Fonterra, Natren, and United Agricultural Services (UAS) Laboratories; L.L. and M.J.L. are employees of DuPont Nutrition and Health.

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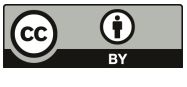

nutrients

MDPI

Commentary

Para-probiotics for Preterm Neonates—The Next Frontier

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Abstract: Current evidence supports the use of probiotics in preterm neonates for prevention of necrotizing enterocolitis, mortality and late onset sepsis. Despite the strong evidence, the uptake of this intervention has not been universal due to concerns including probiotic sepsis, pro-inflammatory response and transmission of antibiotic resistance. Critically ill extremely preterm neonates with potentially compromised gut integrity are at higher risk of probiotic sepsis due to translocation. In most countries, probiotics are sold as food supplements with poor quality control. The traditional definition of probiotics as "live microorganisms" has been challenged as many experts have questioned the importance of viability in the context of the beneficial effects of probiotics. Paraprobiotics (ghost probiotics), are defined as non-viable microbial cells (intact or broken) or crude cell extracts (i.e., with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer. Current evidence indicates that paraprobiotics could be safe alternatives to probiotics in preterm neonates. High-quality pre-clinical and clinical studies including adequately powered randomised controlled trials (RCTs) are warranted in preterm neonates to explore this new frontier.

Keywords: infant; newborn; probiotics; review; sepsis

1. Introduction

Despite the recent advances in technology and progress in research, necrotising enterocolitis (NEC), mortality, late onset sepsis (LOS), and long-term neurodevelopmental impairment (NDI) continue to remain a major health burden in preterm neonates [1,2]. NEC continues to be major gastrointestinal emergency in ~7% of preterm very low birth weight (VLBW: Birth weight < 1500 g) neonates. Mortality is high, up to 20 to 30%, with the highest rate among neonates requiring surgery [3–6]. The morbidity of definite (≥Stage II) NEC is significant and includes prolonged hospitalisation, recurrent infections, long-term dependence on parenteral nutrition, and survival with intestinal failure, and long-term NDI, especially in extremely low birth weight (ELBW: Birth weight < 1000 g) neonates needing surgery for the illness [7]. Stey et al. have estimated the cost of treatment of one case of NEC requiring surgery, close to $400,000 [8].

The World Health Organisation (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." [9]. Bacteria such as bifidobacteria, and lactobacilli, and non-pathogenic fungi such as Saccharomyces, are the most common type of microorganisms used as probiotics. The mechanisms for the benefits of probiotics include gut barrier enhancement, immune response modulation (e.g., TLR4 receptor, nuclear factor-B, inflammatory cytokines), and competitive inhibition of gut colonisation by pathogens [10,11].

Systematic reviews of randomised controlled trials (RCTs) have shown that prophylactic administration of enteral probiotics significantly reduces the risk of ≥Stage II NEC, mortality, LOS and the time to full enteral feeding in preterm VLBW neonates [12,13]. Many developed countries including Australia, Canada, Finland, Denmark, and Germany, are currently using probiotics as standard practice for prevention of NEC in preterm VLBW neonates [14–18]. A systematic review of observational studies has confirmed the benefits of enteral probiotics in preterm neonates [19].

Despite the various systematic reviews of RCTs and 'before vs. after' routine use studies (Non-RCTs) reporting significant benefits of probiotics in reducing the risk of ≥Stage II NEC, LOS, all-cause mortality, and feeding intolerance, the uptake of this intervention has been relatively slow, and not universal. The reasons for this slow uptake include the concerns about probiotic sepsis, development and transmission of antibiotic resistance, possibility of an exaggerated pro-inflammatory response, and non-availability of or difficulties in accessing high quality, safe and effective products [20]. In most countries, probiotics are sold as food supplements with poor quality control. The death of a preterm neonate from a contaminated probiotic product that was used in a large RCT by Jacobs et al., has highlighted the issue of poor quality control for probiotic products [21,22].

The risk of probiotic translocation and sepsis is higher; especially in critically ill and/or extremely preterm neonates (e.g., suspected/proven sepsis or NEC) with potentially compromised gut integrity [14,23–26]. Although none of the RCTs have reported probiotic sepsis, there are reports of the administered probiotic causing serious infections such as septicemia, pneumonia and meningitis [24–29]. Kopp et al. reported that *L. rhamnosus* GG (LGG) supplementation during pregnancy and early infancy did not reduce the incidence or severity of atopic dermatitis in children, but was associated with an increased rate of recurrent episodes of wheezing bronchitis [30]. It is important to note that probiotic strains may express virulence factors or acquire antibiotic resistance genes via horizontal gene transfer [31]. However, advances in technology have allowed the removal of plasmid for antibiotic resistance from maternal strain [32]. Zheng et al. have emphasised the importance of broader screening of antibiotic resistance in commercially manufactured probiotic supplements. They recommend techniques such as computational simulations, live imaging and functional genomics to study the evolutionary behaviour, adaptations and dynamics of commercially manufactured probiotic for optimising their safety [33].

2. Para-probiotics

Probiotic products contain a mix of live and dead cells; however, the population of dead cells at any given time during the shelf life of such products is virtually unknown [34–36]. The dead cell preparations from probiotics have been fractionated and various cellular components and metabolites have been shown to produce a range of biological responses [34]. Taverniti et al. propose the term 'paraprobiotic' (ghost probiotics), to define non-viable microbial cells (intact or broken) or crude cell extracts (i.e., with complex chemical composition), which, when administered (orally or topically) in adequate amounts, confer a benefit on the human or animal consumer [37].

Several methods of inactivation of probiotics have been studied including the use of heat, chemicals (e.g., formalin), gamma or ultraviolet rays, and sonication. Different methods of inactivation may affect structural components of the cell differently, and influence its immunomodulatory activity [37]. Heat treatment seems to be the method of choice for inactivation of probiotic strains in majority of studies [37,38].

3. Mechanisms of Action of Para-probiotics

The mechanisms of action of heat-inactivated/killed probiotics are poorly understood. A variety of biological responses have been reported after administering killed (mostly heat-killed) probiotics to various mammalian and avian species [37]. Animal studies in gastrostomy-fed infant rats show that live and heat-killed *L. rhamnosus GG* decreases LPS-induced pro-inflammatory mediators and increases anti-inflammatory mediators [39]. Bloise et al. reported similar results on human placental trophoblast

cells [40]. Studies in epithelial cells and in an infant formula-fed rodent model suggest that killed microbes may be as effective as live microbes in modulating additional inflammatory stimuli [41,42]. Other potential mechanisms of action of killed/inactivated probiotics include adhesive properties of BoPA a cell surface lipoprotein identified in *Bifidobacterium* (*B.*) *bifidum* and anti-inflammatory effects of *L. acidophilus* and *L. plantarum* [37,43]. Other killed probiotics with beneficial immunomodulatory responses in laboratory settings include *L. rhamnosus GG*, *L. plantarum* L-137, *B. breve*, *Escherichia coli Nissle* 1917, *B. bifidum*, *L. acidophilus*, *L. helveticus*, *B. bifidum* and *L. casei*. [37]. Sakai et al. reported that killed *Enterococcus faecalis* (EC-12) prevented vancomycin-resistant enterococci colonization in the cecum of newly hatched chicks [44]. Reduced capacity for mucosal adhesion is a potential adverse effect of heat inactivation of a probiotic strain. However, contrary to the expectation, strain *P. freundereichii* has been shown to have an increased ability for adhesion after heat inactivation [45]. Reduced ability to exert an anti-inflammatory effect after heat inactivation is another concern. However, heat-inactivated strains *L. casei* strain Shirota or *L. fermentum* MS15 [46] have been shown to modulate inflammatory response by regulating IL-10, human B-defensin and other pro-inflammatory cytokines and *B. breve* and *B. bifidum* have improved ability to increase the secretion of IL-10, an anti-inflammatory cytokine [47].

Adams and Kataria et al. have also explored the field of non-viable versus viable probiotic strains. Adams reviewed the 'probiotic paradox', and beneficial biological responses of live and dead probiotic bacteria [34]. Enhanced safety and longer shelf life were considered as the advantages of 'dead' probiotic bacteria. Dead probiotics had various biological responses including anti-inflammatory effects, attenuation of colitis, reduction of IL-8 production, stimulation of gut immune system, and stimulation of IL-6 production, in pre-clinical studies [34]. Kataria et al. summarised the mechanisms of action of "dead" probiotics or their components, and reported that dead microbes could modulate anti-inflammatory effects as effectively as live probiotics [20].

4. Which Probiotic Species Can Be Used in Their Heat-Inactivated form as Para-probiotics?

The *Lactobacillus* and *Bifidobacterium* species are commonly used probiotics. Emerging evidence indicates that strains of both *Lactobacillus* [45,48,49] and *Bifidobacterium* [50–54] species are capable of beneficial effects in their heat-inactivated form. The case of *B. breve* M-16V is worth noting, as this probiotic strain is being used routinely in preterm infants for prevention of NEC [55]. Sugahara et al. have investigated the differences between live and heat-killed *B. breve* M-16V, in the regulation of immune function, intestinal metabolism and intestinal gene expression using gnotobiotic mouse model and omics approaches [50]. Both live and heat-killed forms of *B. breve* M-16V showed immune-modulating effects that suppressed pro-inflammatory cytokine production in spleen cells and affected intestinal metabolism. However, live strains exhibited more significant effects in the regulation of intestinal metabolism and intestinal gene expression involved in nutrient metabolism [50]. Athalye-Jape et al. have reported a strain specific systematic review of RCTs and non-RCTs of *B. breve* M-16V in preterm infants [55]. A total of 5 RCTs (n = 482) and 4 non-RCTs (n = 2496) were included. Data from the 3 small RCTs (n = 386) reporting on clinically important outcomes was inadequate to derive firm conclusions [18,56,57]. Meta-analysis of data from non-RCTs showed significant benefits on LOS, mortality, and the postnatal age at full feeds. There were no *B. breve* M-16V related adverse effects [55]. The findings reported by Sugahara et al., and Athalye-Jape et al. suggest that inactivated *B. breve* M-16V may be a suitable paraprobiotic strain for assessment in clinical trials [50,55]. Research on other paraprobiotics (developed from other probiotic strains that have been shown to be effective in RCTs and/or non-RCTs) is important to study their safety and efficacy against placebo and/or probiotics in preterm VLBW neonates.

5. Clinical and Pre-Clinical Studies of Para-probiotics

Lahtinen has recently reviewed the pre-clinical and clinical studies on live versus inactivated probiotics [35]. Clinical studies comparing non-viable versus viable probiotic strains for various conditions (e.g., diarrhoea, irritable bowel syndrome, eradication of H. pylori, cow's milk protein

intolerance) were few, and showed comparable, high or lower efficacy of non-viable versus viable probiotics. Small sample sizes, lack of a placebo group, and use of non-standardised strains were some of the limitations of these studies. Findings from various preclinical studies included the following: (1) Viable and non-viable lactobacilli had equal ability for adherence to gut mucosa [58], and heat-killing and protease treatments impaired the mucus adherent property [59]; (2) Heat-killing changed the intestinal location of the bacteria. Live bacteria were seen in Peyer's patches and lamina propria whereas most heat-killed bacteria were in the lumen and cleared rapidly [60]; (3) Heat-killed lactobacilli inhibited pathogen adhesion to the gut mucosa by competitive exclusion [61]; (4) Inactivated lactobacilli enhanced gut epithelial barrier [62]; (5) Non-viable probiotic components such as cell wall extracts [63], lipoteichoic acid [64], bacterial DNA [65,66], and surface (S)-layer proteins [67] can have immunomodulatory effects by various mechanisms including increased salivary IgA production [68], modulation of host T-cell responses [69] and gene expression [70]; (6) Live and inactivated probiotics had comparable effects on innate immunity [71–73]; (7) Live as well as killed *B. lactis* HN019 enhanced phagocytic responses in peripheral blood cells; however, only viable bacteria increased the phagocytic activity of peritoneal cells [74]; (8) As for adaptive immunity, many studies favoured live over non-viable bacteria [60,75–78], but some showed that both forms had similar effects on the phenotype and functions of human myeloid dendritic cells [79]. Overall, the evidence from pre-clinical and clinical studies suggested that "in some situations, depending on the mechanism of action, probiotic effects are not dependent on cell viability". The need for clinical studies and consideration of the differences in the effects of dormant (during storage), inactivated and live bacteria was emphasised [80].

6. Systematic Review of Studies of Modified Probiotics for Prevention and Treatment of Various Diseases

Zorzela et al. have reported a systematic review of trials of dead bacteria/yeasts ('Modified microbes') inactivated by heating/sonication of probiotic strains, for prevention (n = 14) or treatment (n = 26) of various diseases, mainly in adults and children [38]. The trials compared modified microbes with either placebo (44%) or the same probiotic strain (39%) or standard treatment (17%). Compared with probiotics, the modified microbes were not significantly more or less effective in 86% of prevention and 69% of treatment trials. Meta-analysis of data from 5 RCTs showed significant benefits of modified L. acidophilus (Standard mean difference: −0.81, 95% CI: −1.44, −0.17) as an adjuvant in treatment of acute diarrhoea; however, there was significant heterogeneity (I^2 = 86%). The incidence of adverse events was comparable for modified microbes, probiotics and other controls but many trials did not report adequate data on safety. Overall, there was some evidence that modified microbes may be useful for few conditions. The limitations of this review include the heterogeneity of methodology and the tested strains in the included studies and their small sample sizes [38].

In summary, current evidence indicates that paraprobiotics could be safe alternatives to probiotics in preterm neonates. High quality pre-clinical as well as clinical studies including adequately powered RCTs are warranted in preterm neonates to explore this new frontier. A cluster RCT design is appropriate to avoid the issue of cross-contamination. A non-inferiority design will be acceptable for this purpose. However, deciding the clinically acceptable margin of inferiority will be an important issue considering the effect size for benefits of probiotics for NEC, all-cause mortality, late onset sepsis, and time to full feeding in preterm infants. Considering that effects of probiotic strains are 'strain-specific', rigorous assessment of specific effects of different paraprobiotic strains are important. This issue is also relevant when a mixture of strains is used. Assessment of the effects of added prebiotic oligosaccharides is another important issue. Finally, the significance of the utility of paraprobiotics beyond the preterm neonatal population cannot be ignored.

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