New Insights in Oral Health and Diets

Edited by
Theodoros Varzakas and Maria Antoniadou

Printed Edition of the Special Issue Published in *Applied Sciences*

www.mdpi.com/journal/applsci

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Editors

Theodoros Varzakas
Maria Antoniadou

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Editors

Theodoros Varzakas	Maria Antoniadou
Food Science and Technology	Dental School
University of the Peloponnese	National and Kapodistrian
Kalamata	University of Athens
Greece	Athens
	Greece

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Applied Sciences* (ISSN 2076-3417) (available at: www.mdpi.com/journal/applsci/special_issues/Oral_Diets).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

LastName, A.A.; LastName, B.B.; LastName, C.C. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-0365-2641-6 (Hbk)
ISBN 978-3-0365-2640-9 (PDF)

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About the Editors

Theodoros Varzakas

Theodoros Varzakas has a Bachelor (Honours) in Microbiology and Biochemistry (1992), a Ph.D. in Food Science and Technology, and an MBA in Food and Agricultural Management from Reading University, UK (1998). He has also worked as a postdoctoral research staff at the same university. He has worked in large pharmaceutical and multinational food companies in Greece for 5 years and has at least 20 years of experience in the public sector. Since 2005, he has served as Assistant, Associate, and Full Professors at the Department of Food Science and Technology, University of Peloponnese, ex Technological Educational Institute of Peloponnese, Greece, specializing in issues of food technology, food processing/engineering, and food quality and safety. He is also a Section Editor in Chief for the Journal *Foods in Food Security and Sustainability* (2020-), was an ex Editor in Chief for *Current Research in Nutrition and Food Science* (2015-2019), and is a reviewer and member of the editorial board in many international journals. He has written more than 200 research papers and chapters in books and has presented more than 160 papers and posters at national and international conferences. He has written and edited 10 books in Greek and 11 in English on sweeteners, biosensors, food engineering, and food processing, published by CRC. He has participated in many European and national research programs as a coordinator or scientific member.

Maria Antoniadou

Dr. Maria Antoniadou is an Assistant Professor at the Dental School of the National and Kapodistrian University of Athens, (NKUA) with experience in the clinical and managerial practice of dentistry. She has been a research and clinical member in the clinic of perio-prosthetics of the University of Freiburg, Germany. She holds a master's degree in aesthetic dentistry from the University of Athens, while her doctoral thesis was on the application of dental biomaterials in the modern practice of dentistry. She is specialized in dental psychology and teaching on dental counseling about diet for better oral health, patient management (especially disadvantaged population groups) and dental strategic, marketing and management of health units. She has completed the "Coaching-AC Accredited" program of the NKUA (for certification by the European Organization Association for Coaching) and the program "Coaching-Skills and Practice" by HRE (ACSTH by ICF-International Coaching Federation). She has written more than 70 papers and chapters in peer-reviewed journals and books and presented more than 30 papers in national and international conferences. She has written and edited an innovative book in Greek, about "Humanities and basic principles of coaching in Dentistry".

Editorial

New Insights in Oral Health and Diets

Maria Antoniadou [1] and Theodoros Varzakas [2,*]

[1] Dental School, National and Kapodistrian University of Athens, Thivon 2, Goudi, 11527 Athens, Greece; mantonia@dent.uoa.gr
[2] Department of Food Science and Technology, University of Peloponnese, Antikalamos, 24100 Kalamata, Greece
* Correspondence: t.varzakas@uop.gr

Citation: Antoniadou, M.; Varzakas, T. New Insights in Oral Health and Diets. *Appl. Sci.* **2021**, *11*, 11397. https://doi.org/10.3390/app112311397

Received: 12 November 2021
Accepted: 29 November 2021
Published: 1 December 2021

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The aim of this Special Issue is to bring the most updated information on the innovative field of oral and general health coaching and nutritional education strategies for better oral and general health. In this direction we have seven published papers.

Voidarou et al. [1] deals with the antibacterial effect of different Greek honeys showing an outperformance of the antibacterial activity compared to manuka honey against all tested bacteria. This performance was due to the hydrogen peroxide as well peptides and phenolic compounds and flavonoids. Moreover, the antibacterial effect of the honeys in comparison to distilled water was enhanced by artificial saliva. Greek honey seems promising in controlling dental caries but further research is needed.

Syed et al. [2] determined the cytotoxicity of Khat (*Catha edulis (Vahl) Forssk. ex Endl*) on normal oral fibroblasts (NOFs) and squamous carcinoma cells (SCC4) along with expression of α-smooth muscle actin (α-SMA) in fibroblasts. It was found that Khat is cytotoxic to NOF and SCC4 cells and can also cause activation and phenotypic changes in oral fibroblasts, indicating a probable progression of oral squamous cell carcinoma.

Formoso et al. [3] deals with consumption of healthy food and proposes a small-scale approach called SANI (Italian for "healthy") which involves experts in science and marketing. It promotes two typical agri-foods of the Abruzzo area (center of Italy), tomato sauce and extra virgin olive oil, characterized as high-quality products in terms of their quality characteristics and ecological footprint. Hence, their consumption is promoted, with reference to food manufacturing issues along with marketing strategies and dissemination activities.

In the paper published by Selvamani et al. [4], an insight in probiotics bioroute from the maternal gut to mammary gland is given, suggesting an occurrence through the entero-mammary pathway. It involves many probiotic microorganisms from the gut, and gastrointestinal lymphatic vessels, macrophages, and dendritic cells are shown to play a significant role in this microbial transmission. Moreover, the distinct role of microbial factors in the development of neonatal immunity and translocation of secretory IgA (SIgA) cells from the intestinal lumen to GALT and finally to mammary glands via this entero-mammary pathway is discussed.

Amargianitakis et al. [5] address the effect of probiotic therapy as a new strategy for dental caries prevention. Probiotics can displace cariogenic microorganisms and colonize the oral cavity producing various antimicrobial substances such as bacteriocins, bacteriocin-like peptides, lactic acid, and hydrogen peroxide. Probiotic administration in dental patients involves dairy products. In caries prevention, the concept of the effector strain is already considered as a prevention strategy in adults. Finally, the modes of action of probiotics, their use in the cariology field and their clinical potential are well described along with proposed options to prevent caries using a patient coaching approach for the daily dental practice.

Fernandes et al. [6] deal with mushroom nutrition in Sub-Saharan Africa (SSA). This article encompasses the valorization of traditional African foodstuffs and ingredients. This will lead to the enhancement of the importance of establishing food-based dietary

guidelines per country. This review highlights the crucial potential of mushrooms, as one of the greatest untapped resources for feeding and providing income for Africa's growing population, protecting Sub-Saharan Africans against unhealthy stylish diets.

Antoniadou et al. [7] address diet and oral health coaching methods and models for the independent elderly. The health and oral health of the independent elderly should shift from disease management and therapy to integral customized and personal treatment plans, including lifestyle, psychological, nutritional, and oral health coaching approaches. In this paper, health coaching approaches in medical and dental settings are valued regarding their effectiveness in older adults. Diet and oral health coaching should aim to empower older adults in co-management of their oral diseases or bad diet habits affecting their oral health following an incorporated educational plan.

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

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Review

An Insight into Probiotics Bio-Route: Translocation from the Mother's Gut to the Mammary Gland

Shanmugaprakasham Selvamani [1,2], Daniel Joe Dailin [1,2], Vijai Kumar Gupta [3], Mohd Wahid [4], Ho Chin Keat [1], Khairun Hani Natasya [1], Roslinda Abd Malek [1], Shafiul Haque [4,5], R. Z. Sayyed [6], Bassam Abomoelak [7], Dalia Sukmawati [8], Theodoros Varzakas [9] and Hesham Ali El Enshasy [1,2,10,*]

[1] Institute of Bioproduct Development, Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru 81310, Malaysia; anazputra@gmail.com (S.S.); jddaniel@utm.my (D.J.D.); jared_ho@live.com (H.C.K.); haninatasya29@gmail.com (K.H.N.); roslinda@ibd.utm.my (R.A.M.)
[2] School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru 81310, Malaysia
[3] Center for Safe and Improved Food & Biorefining and Advanced Materials Research Center, Scotland's Rural College (SRUC), Kings Building, West Mains Road, Edinburgh EH9 3 JG, UK; vijai.gupta@sruc.ac.uk
[4] Research & Scientific Studies Unit, College of Nursing and Allied Health Sciences, Jazan University, Jazan 45142, Saudi Arabia; wahidbiochem@gmail.com (M.W.); Shafiul.haque@hotmail.com (S.H.)
[5] Faculty of Medicine, Bursa Uludağ University, Görükle Campus, Nilüger, Bursa 16059, Turkey
[6] Department of Microbiology, P.S.G.V.P. Mandal's, Arts, Science, and Commerce College, Shahada 425409, India; sayyedrz@gmail.com
[7] Arnold Palmer Hospital Pediatric Speciality Diagnostic Laboratory, Orlando, FL 32806, USA; Bassam.Abomoelak@orlandohealth.com
[8] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jakarta 13220, Indonesia; Dalia-Sukmawati@unj.ac.id
[9] Department Food Science and Technology, University of the Peloponnese (TV), 24100 Kalamata, Greece; t.varzakas@uop.gr
[10] City of Scientific Research and Technology Applications (SRTA), New Burg Al Arab 21934, Alexandria, Egypt
* Correspondence: henshasy@ibd.utm.my

Citation: Selvamani, S.; Dailin, D.J.; Gupta, V.K.; Wahid, M.; Keat, H.C.; Natasya, K.H.; Malek, R.A.; Haque, S.; Sayyed, R.Z.; Abomoelak, B.; et al. An Insight into Probiotics Bio-Route: Translocation from the Mother's Gut to the Mammary Gland. *Appl. Sci.* **2021**, *11*, 7247. https://doi.org/10.3390/app11167247

Academic Editor: Alessandra Durazzo

Received: 25 May 2021
Accepted: 26 July 2021
Published: 6 August 2021

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Abstract: Human breast milk (HBM) is unique in its composition as it is adapted to fulfil the newborns' nutritional requirement and helps in improving the health of newborns. Besides various nutrients, the human milk also contains diverse group of microbiotas. The human milk microbiota has a remarkable impact on the growth and development of a newborn. Additionally, the human milk microbiota enhances the colonization of microbes in the gut of infants. Debates about the origin of HBM microbial flora remain premature and contradictory in some cases. Recent data suggest that the maternal gut microbiota has a major impact on microbial composition, areolar skin, and from the infant's oral cavity. The current review investigates the possible route of microbial transfer from the maternal gut to mammary gland and suggests that it might occur through the entero-mammary pathway. It involves precise selection of probiotic microorganisms from the gut, as the human gut hosts trillions of microorganisms involved in gut homeostasis and other metabolic pathways. Gastrointestinal lymphatic vessels, macrophages, and dendritic cells are shown to play a significant role in the microbial transmission. Furthermore, the role of microbial factors in the development of neonatal immunity and translocation of secretory IgA (SIgA) cells from the intestinal lumen to GALT and finally to mammary glands via entero-mammary link are discussed.

Keywords: breast milk; microbiome; probiotics; lactic acid bacteria; entero-mammary pathway

1. Introduction

Human breast milk (HBM) is the gold standard in care for all infants and children. There are various nutritional and non-nutritional bioactive components in human milk. To ensure survivability and healthy development, their compositions are dynamic and

gradually altered as the newborn grows [1,2]. Human milk also harbors a unique microbial population that naturally develops from the first breastfeed. Due to its dynamic nature and high heterogeneity, the human milk microbiota is also a complex and variable ecosystem [3]. For many years, human milk was considered sterile, and the presence of microorganisms was considered as a contamination or due to infection such as mastitis. Numerous approaches, both culture-dependent and culture-independent, have revealed the existence of diverse bacterial communities. The bacterial communities include *Bifidobacteria*, *Staphylococci*, *Streptococci*, and other lactic acid bacteria [4–6].

Reports differ regarding the source of human milk microbiota. Their existence in the secreted milk is expected to onset during the third trimester of pregnancy and continue throughout the lactation period [7]. Studies have demonstrated that human milk microbiota continuously supply beneficial bacteria into the newborns' gut environment, contributing to the maturation of the digestive and immune functions of the growing infant [8]. Although human milk microbiota is dominated by skin microflora, the population of the beneficial microbiome in the human milk are similar to the composition of gut microbiota. Probiotic bacterial genera including lactic acid bacteria and *Bifidobacteria* are expected to originate from the maternal gut environment [8,9]. The relationship between the gut and mammary glands was confirmed in animal models [6]. In humans, studies have focused primarily on describing the composition, functions, and factors affecting milk microbiota rather than understanding the origin of milk microbiota from the maternal gut [8,9]. To address this question, this review explores the possible bio-routes involved in the translocation of gut origin microbiota into the mammary glands. Macrophages can distinguish between pathogenic and commensal microflora in the gut, and they translocate probiotic strains from maternal gut into the mammary glands [10]. Despite their beneficial nature, the presence of any microbial cells or their antigens in the human blood would be considered as infections, and there must be a specific route for gut microbial translocation—more likely, we termed it the 'microbial bio-route'. In this context, the aim of this study was to discuss the composition of human milk microbiome and potential microbial translocation into the human milk. Furthermore, we also highlighted the factors affecting human milk microbial compositions and their likely effects on human health.

2. General Features of Human Milk Microbiota

Breast milk harbors a unique microbial population that varies across countries, ethnicities, or even among communities [11]. In the 1970s, there has been a quick rise in the number of HBM banks in the United States and other European countries for newborns in intensive care units (ICUs). The use of preserved milk raised concerns about the sterility of the milk and hygiene issues of the donors [12,13]. Various microbiological and biochemical analysis were performed on the HBM to check its safety before its availability for the newborns (0–2 months) and infants (0–1 year)/babies (0–4 years). These clinical tests eventually rejected the long-standing dogma that considered HBM as a sterile fluid [11]. Currently, powerful cutting-edge technologies in molecular biology research have dramatically outperformed the traditional techniques in the assessment of the HBM microbial quality. Currently used molecular techniques such as pyrosequencing of the DNA have been extensively used to identify the complex microbial population in the HBM. These techniques have proven that the HBM holds a unique microbial niche with outstanding benefits for the infants' growth and development [14]. However, HBM microbiome studies are not completed yet and are still inconclusive.

As culture-dependent methods have confirmed the presence of several bacteria in the HBM collected from healthy women, HBM sample collections were questioned [15]. Figure 1 shows the global distribution of microbial phyla at their different sites including maternal gut, breast milk, and the infant's oral cavity. The origin of HBM microbiota is still a matter of debate. However, growing evidence suggests that maternal gastrointestinal microbiota and maternal breast skin or the infants' oral microbiota might contribute to the microbial population in human milk [16,17].

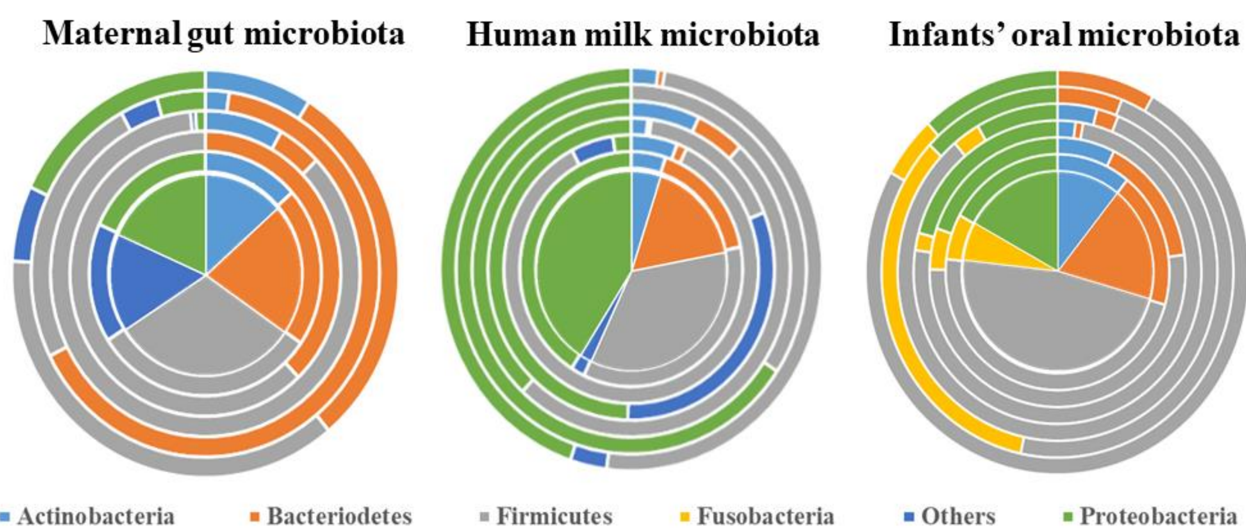

Figure 1. The microbiota composition of the maternal gut, human milk, and infants' oral cavity: a description of relative abundances of essential phyla in each microbiota composition. Concentric cake diagrams schematically represent inter-individual variability.

3. Microbial Partners of Human Milk 'Factory'

Every breastfed baby receives approximately 10^4–10^6 bacterial cells daily, consuming almost 800 mL of HBM [5,13,18,19]. Most of these microbiotas exist naturally within the HBM, and their diversity is unique. Culture-dependent and -independent techniques have both revealed the diversity of the microbial population present in the HBM [14,20]. Relatively, the HMB has a large variability at the intra-individual level, and it undergoes continuous changes over the lactation period [20]. Figure 2 illustrates the most isolated microbial species from the human milk samples.

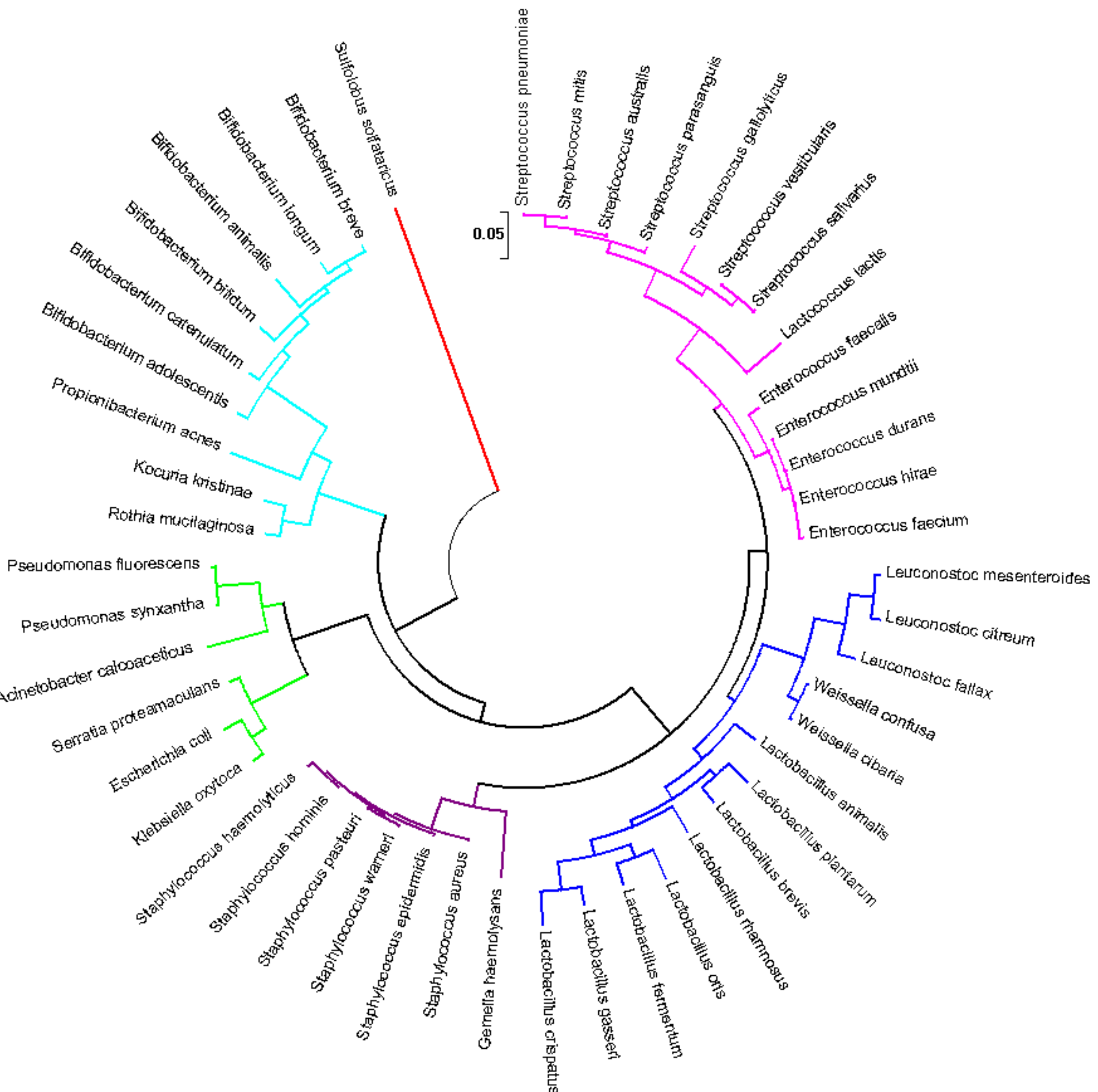

Figure 2. 16S rRNA gene-based phylogenetic tree of the human milk core microbiota. The tree was constructed based on the information provided by Patel et al. [21] and Sakwinska et al. [22] with the inclusion of additional species, of which the genome sequence data have been available since 2015. The analysis involved 49 nucleotide sequences. All the evolutionary analyses were performed by using MEGA (version 7.0). All positions comprising gaps and missing data were removed. The final dataset observed 1266 positions.

3.1. *Predominance of Skin Microflora*

Fitzstevens et al. [14] reported that *Staphylococcus* and *Streptococcus* strains were commonly found in almost all culture-independent quantification studies of HMB. The members of *Staphylococcus* and *Streptococcus* genera are dominant skin microflora including *Propionibacterium*, which was also reported to inhabit the infant gut ecosystem. Therefore, their predominance in human milk indicates that they may be derived from maternal skin or from gut microbes [11]. A study revealed that the microbial load in the milk samples of healthy mothers originates from maternal areolar skin. It was predicted that the skin bacteria from the breast surfaces of the nipple or areola could gain access through the ducts of the mammary glands during breastfeeding [23,24].

Still, the question arises here, why does breast milk contain an abundance of opportunistic pathogenic genera such as *Staphylococcus* and *Streptococcus*? Traditionally, greater amount of *Staphylococcus aureus* and *Staphylococcus epidermidis* in the HBM samples, along

with other genera including *Streptococci* and *Corynebacterium*, were considered as the main agents causing mastitis [25]. Mastitis is a condition of inflammation in lactating mammary glands caused by microbial dysbiosis in the human milk microbiome and leads to opportunistic pathogenic bacteria outgrowth and a decline in healthy and stable commensal bacteria [26]. Opportunistic pathogenic bacteria are the normal microflora that outgrow in numbers due to several conditions including the compromised host's immunity [15]. Patel et al. [21] reported that individuals with subacute mastitis and acute mastitis had higher abundance of *Proteobacteria* than *Firmicutes*.

In addition, there have been several studies that reported that commensal skin microflora including *Staphylococcus* and *Streptococcus* were also found to be predominant in the breast milk samples collected from healthy mothers [5,20,27]. O'Sullivan et al. [28] reported that skin originated coagulase-negative *Staphylococci* was able to produce bacteriocin, which inhibits the growth of pathogenic bacteria. In addition, HBM contains various antimicrobial proteins and peptides such as lactoferrin, beta-defensins, and alpha-defensin [1,29]. Antimicrobial proteins and peptides present in breast milk show broad inhibitory activity against a variety of pathogens like *Streptococcus epidermidis*, *S. aureus*, *E. coli*, and *Streptococcus agalactiae* [26].

Previously, Hunt et al. [30] described the survivability of these skin microflorae in human milk. The relative abundance of *Staphylococci*, especially *S. aureus* and *S. epidermidis* strains, was expected due to the influence of human milk oligosaccharides (HMO). The HMO are well-known for promoting the growth of various commensal bacteria in HBM including *Bifidobacterium* spp. [31,32]. However, these HMOs were also found to stimulate the growth and proliferation of *S. aureus* and *S. epidermidis* strains. Another interesting fact is that these two strains fail to metabolize HMOs available in breast milk [25]. Hence, conjectures can be made that this (above stated reason) could be the main factor for the abundance of skin microflora in the breast milk, especially *Staphylococci* and *Streptococci*. After breaching into the mammary glands from the areolar skin, these skin microflorae could be stimulated to multiply by breast milk components. However, the bacterial group survived by utilizing simple sugars rather than competing with the commensal bacteria of the breast milk for HMOs.

Microbiota present naturally in human skin and breast milk plays a crucial role in the immune system's control of a newly born baby. During the breastfeeding process, a significant microbial transmission potential occurs across the areolar skin barriers into the mammary glands [10]. Eventually, this transfer into the infants' gastrointestinal tract will build a neutral microbial ecosystem consisting of beneficial and commensal microorganisms. The introduction of these skin commensals into the infants' gut modulates the innate immunity [15,30,33–36].

3.2. Human Milk Is a Probiotic Consortium

Martin and colleagues [37] performed one of the earliest isolations on probiotics from HBM. The study successfully described 78 rod-shaped lactic acid bacterial isolates that grew on MRS medium [37]. The presence of *Lactobacillus* and *Bifidobacterium* strains in the human milk has also been confirmed by other studies [38–40]. Sinkiewicz and Ljunggren [38] described the occurrence of *Lactobacillus reuteri* strains in 220 breast milk samples by the culture-dependent method. Ozgun and Vural [41] characterized 100 presumptive *Lactobacillus* isolates from colostrum samples by using the API 50 CHL system from BioMeriéux. This study identified several *Lactobacillus* strains of *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, and *L. reuteri*. However, certain lactic acid bacterial isolates share common phenotypes [41]. Soto et al. [42] utilized qualitative PCR analysis, which revealed the presence of *Lactobacillus* and Bifidobacterial DNA in 160 samples. The most frequently detected *Lactobacillus* species were *L. salivarius*, *L. fermentum*, and *L. gasseri*. The study also reported the presence of *Bifidobacterium breve* as the most common Bifidobacterial species [42]. In addition, Biagi et al. [43] also found that the majority of probiotic bacterial OTUs, especially assigned to the Bifidobacterial

group, were shared between breast milk and stool samples of the same mother–infant pairs. This gives credence to the idea that the mother's milk acts as a source of pioneer probiotic bacteria for the infant's gut microbiota [33,43].

Currently, more *Lactobacillus* and *Bifidobacterium* strains have been isolated from human milk to be utilized as potential probiotics [44,45]. Rajoka et al. [46] identified three *Lactobacillus* strains recently isolated from human milk that exhibited probiotic properties. The study also reported anti-cancer properties in their cell-free culture supernatants. Damaceno et al. [5] characterized three probiotic potential strains, namely *L. gasseri*; *B. breve*, and *L. salivarius* from healthy human milk. These strains agglutinated with different pathogens instead of producing bacitracin. Besides, *L. fermentum* originating from human milk has been exploited extensively to treat mastitis problems [47]. Several orally administrated probiotic bacteria were also previously isolated from human milk samples [48,49].

Compared to many bacterial groups in mothers' milk, the presence of *Lactobacillus* and *Bifidobacterium* spp. has much more beneficial impact on the neonatal immune system [50,51]. In addition, the presence of probiotic strains including *Lactobacillus* and *Bifidobacteria* has a protective role where these bacterial strains suppress respiratory and diarrheal infections at the initial infantile age [52,53]. Björkstén et al. [54] reported that infants that have a slower colonization or a lower number of probiotic enterococci and Bifidobacteria may be more vulnerable to gastrointestinal or allergic problems. Other study by Johansson et al. [55] demonstrated that early colonization with Lactobacillus were shown to decrease the effect of allergy. Despite there being no clear protective effect on allergy response reported, breast milk is composed of key players to combat allergy [56,57]. Another recent systematic study demonstrated that the fecal microbiota of infants with colic found that Bifidobacteria and Lactobacilli were greatly decreased in infants with colic compared to the control infants [58]. Furthermore, the colic phenotype was found to be positively associated with various *Proteobacteria* groups, but negatively associated with bacteria belonging to the *Firmicutes* phyla, which includes certain *lactobacilli* and canonical groups that manufacture butyrate and lactate. Interestingly, some studies have found that administering a *Lactobacillus* strain believed to be from human milk to infants with infantile colic can be beneficial [58,59].

3.3. Presence of Other Microorganisms

Besides *Proteobacteria* and *Firmicutes*, another common genus found in most of the breast milk samples is *Bacteroides*. This bacterial genus is generally found in human colostrum. Thus, their presence in HBM might have a vital role in the early establishment of newborn gut microbiota [60]. *Bacteroides fragilis* can interact with intestinal dendritic cells to stimulate cytokine production. This leads to T-cell proliferation in the lamina propria and promotes physiological expansion of mucosal immunity in neonates [60,61]. Another of the most abundant genus detected in healthy breast milk is *Acinetobacter* [3,22,23]. However, its presence varies greatly in different studies. Sakwinska et al. [56] reported that abundance of *Acinetobacter* was related to poor aseptic technique during sampling. Patel et al. [21] reported a lower number of *Acinetobacter* in the breast milk samples collected after sterile cleaning and refusal of foremilk samples.

The presence of fungal strains, especially yeast species in the early development of the infant's gut, was confirmed in human milk [62]. Further information about the naturally existing fungal species in HBM is still lacking. Most of the previous detection has focused on breast candidiasis, the mammary infection by pathogenic *Candida* species [27,63]. Jiménez et al. [27] confirmed the presence of fungal sequences in a metagenomics analysis on HBM samples collected from mastitis-suffering mothers. Boix-Amorós et al. [64] reported the viable presence of *Malassezia*, *Candida*, and *Saccharomyces* species in the HBM samples from healthy mothers. This was the first evidence to support the natural existence of fungal species in the HBM. In addition, several studies have also described the presence of fungal strains in the milk samples of other mammals [15,40,65].

3.4. Predicting the Core of Human Milk Microbiota

Human milk core microbiota have been estimated by culture-independent techniques [21,27,30]. Hunt et al. reported that the core of human milk microbiota consisted of nine bacterial genera. Based on the pyrosequencing technique, various reports have indicated the presence of *Streptococcus*, *Staphylococcus*, *Serratia*, and *Corynebacteria* as the most abundant genera in human milk, besides *Pseudomonas*, *Ralstonia*, *Propionibacterium*, *Sphingomonas*, and *Bradyrhizobiaceae* [30]. In contrast, Jimenez et al. used metagenomics analysis on the milk samples from healthy mothers. Early reports suggest a microbial core composed of seven genera including *Staphylococcus*, *Streptococcus*, *Bacteroides*, *Faecalibacterium*, *Ruminococcus*, *Lactobacillus*, and *Propionibacterium* [15]. Recently, Williams et al. reported a microbial core of 10 bacterial groups that were abundantly found at each time point throughout six months of postpartum. *Streptococcus, Staphylococcus*, and *Propionibacterium* were found in all samples, whereas *Pseudomonas, Veillonella, Pilibacter, Gemella, Bacteroides, Prevotella*, and *Corynebacterium* were found in more than 90% of samples [66]. Meanwhile, Murphy et al. [20] reported a core microbiota consisting of 12 genera including *Pseudomonas, Staphylococcus, Streptococcus, Elizabethkingia, Variovorax, Bifidobacterium, Flavobacterium, Lactobacillus, Stenotrophomonas, Brevundimonas, Chryseobacterium*, and *Enterobacter*. This study characterized the bacterial population in breast milk and infant stool collected over the first three months of life, among 10 mother–infant pairs [20].

Therefore, determination of core microbiota in human milk is not easy to standardize due to multiple factors such as geographic locations, milk collection and storage, or analytical methods. Despite these variabilities, the core human milk microbiota is mostly dominated by four bacteria phyla, which are *Firmicutes* (such as *Staphylococcus, Clostridium, Lactobacillus*), *Actinobacteria* (such as *Propionibacterium*, *Corynebacterium*), *Proteobacteria* (such as *Pseudomonas, Ralstonia, Sphingomonas, Bradyrhizobiaccea*), and *Bacteroidetes* (such as *Prevotella*) [14,15,21,30,66]. Fitzstevens et al. suggest that this human milk microbial core might vary, but the reported additional genera in several studies are not consistently represented in all of the studies [14]. Furthermore, these milk microbial compositions are dynamic where it changes from highly diverse as lactation progresses [20,36,60].

Recently, it was confirmed that the microbial core in the human milk is not randomly distributed [60]. The microbial composition in the human milk might be due to specific localization. The study performed the first microbial network analysis in the breast milk samples using artificial neural networks (ANNs) to highlight the natural links among variables. Microbial networking enables a new understanding of the interactions existing within a bacterial ecosystem. Microbiome is dynamic and highly heterogeneous, where the role of each member is not often well-defined [67]. As a result, the bacterial network is proving to be a valuable method for deciphering microbial associations and assessing the effect of different interactions with the host. The study will identify the main “hubs”, which is represented by the utmost significant member in a bacterial population [60,67]. Drago et al. reported that a streptococci variant, *Abiotrophia* spp., is an important hub in the microbial network found in colostrum and mature milk from Italian women [68]. This bacterial genus is a common oral cavity microorganism that can also be found in the genitourinary tract and gastrointestinal tract [69]. Thus, the researchers hypothesized that its existence in human milk could be attributed to direct entrance through the mammary ducts while breastfeeding [30] or the entero-mammary pathway [52]. Similarly, the study also found lactic acid bacteria being main hubs for microbial networking in the colostrum from Italian mothers. Furthermore, this analysis showed that mature milk samples had a higher abundance of anaerobic intestinal bacteria than the colostrum samples [68].

4. Microbial Transmission: Solving the Labyrinth Path

Diverse hypotheses have been proposed for the common microorganisms between mother and infants. The suggested core microbiota in the human milk is similar to predominant bacterial phyla found in the human body [60,68].

4.1. From the Areolar Skin

Human body skin is a critical interface to protect internal cells from the excessive loss of moisture and entry of microorganisms [70,71]. Colonization of skin microbiota begins from the delivery process [72]. Then, immunotolerance against commensal microorganisms is established during childhood and allows for the sustainability of skin microbiota composition. During adulthood, the skin microbiota develops a balance that is unique to every individual [71,73]. Beyond this individual microbial stability, the human skin microbiota also vary across different bodies due to multiple factors including gender, climate, lifestyle, and maintenance habits [74,75]. The four major phyla that have been found in healthy skin were *Actinobacteria, Firmicutes, Proteobacteria*, and *Bacteroides*. Among them, *Staphylococcus, Propionibacterium*, and *Corynebacterium* were the three predominant genera found in human skin [70,76]. Female skin microbiota have been reported as being more diverse than male [73]. The gender differences impact the skin microbial communities due to behavioral characteristics such as the influence of hygiene practices and the use of cosmetics [76]. The greater bacterial diversity on female skin might also be due to the skin pH where men generally have a more acidic skin [73].

Chan and colleagues have published the first report on the quantification of microbial population on women's nipple and areolar skin [77]. Researchers used the 16S rRNA gene sequencing technique to classify the microbiota found on the nipple surface. Both nipple skin samples from healthy and breast cancer patients showed the presence of *Proteobacteria, Firmicutes*, and *Bacteroides* as the predominant genera [77]. However, the study did not correlate identified areolar microorganisms with breast milk or with the infants' gut. Pannaraj et al. demonstrated the first evidence that linked bacterial community in the HBM with areolar skin microbiota [23]. It was found that the newborn received the highest contribution of bacteria from mother's milk and areolar skin during the first month of life and the composition of bacteria received decreased as the infant grew up [23]. The Proteobacteria including *Moraxellaceae, Enterobacteriaceae*, and *Pseudomonadaceae* constituted the dominant phylum in the HBM whereas *Firmicutes*, especially *Staphylococcaceae* and *Streptococcaceae* dominated the areolar skin [23,28].

4.2. Cross-Contamination from Infants' Oral Cavity

The work done by Pannaraj et al. reported the involvement of areolar skin microbiota in the breast milk [23]. The study also found that about 60% of infant gut microbiota came from other sources than breast milk and areolar skin. However, the study did not further characterize the sources [23]. One of the possible causes could be translocation from thenewborn oral environment. When an infant is born by the natural method or C-sections it's oral cavity is exposed to the surrounding microbiota [43]. Li et al. reported that oral microbiota differs between infants delivered by natural and C-section [72]. The naturally delivered infants contained an abundance of *Lactobacillus, Prevotella* and *Gardnerella* genera than the C-section infants who were more likely to have skin microflora such as *Staphylococcus, Pseudomonas, Desulfovibrio*, and *Petrimonas* [68]. Naturally born infants could have placental or vaginal microflora as they might take up some of the amniotic fluid during delivery. This was confirmed with the discovery of various microorganisms found in the meconium (first stool of infants) [78]. Therefore, some of the bacteria from the infants' oral cavity could cross-contaminate and move into the mammary glands during suckling. Biagi et al. found that infants' oral microbiota were the least diverse as *Streptococcaceae* are the most common with an average relative abundance of 69.8 percent. The study also reported that the baby's mouth microbiota had similar identity between dominant *Streptococcus* detected in their mothers' milk. This finding suggests that infants' oral microbiota can produce a seeding consequence on the mammary gland microbial community during nursing [43]. However, this factor does not clarify the presence of some bacteria in the pre-colostrum, which are secreted in some women before delivery [3,37,43].

4.3. Entero-Mammary Pathway: The 'Silk Route' to Trade Microbiota from Maternal Gut to Infant

4.3.1. Link between Maternal Gut and Breast Milk

The human gastrointestinal environment contains the highest and most diverse microbial population compared to other body parts. Starting from the oral cavity to the rectum, the gastrointestinal tract includes more than 800 species of microflora [79,80]. The gut microbiota is generally dominated by *Firmicutes* and *Bacteroidetes* phyla where their presence and abundance are crucial in regulating body metabolism and energy harvesting [81,82]. However, the gut microbial population often changes over time with age, besides additional factors such as type of diet, ingestion of alive microorganisms, host genotype, and health status [80,81]. The gestation cycle is a structural transition in the female body that includes hormonal, immunological, and metabolic modifications to promote fetal development and growth. During this period, endocrine secretions (especially progesterone and estrogens) are higher and there is major alteration in the immune response [9,79,81]. In addition, there are also noticeable changes in the maternal gut microbiota during pregnancy [7,83].

A healthy gestation period is described with increased bacterial loading and changed gut microbial composition [9]. Koren et al. first reported the gradual change of gut microbiota during early pregnancy [83]. The feces of 91 pregnant women in their first to third trimesters were analyzed by amplifying the V1–V2 variable region of the 16S rRNA gene. Microbial composition of first trimester women was similar to healthy non-pregnant women. However, gut microbiota composition evolved dramatically during pregnancy with amplified loads of *Actinobacteria* and *Proteobacteria,* but their individual richness was reduced [83]. Similarly, Smid et al. also described that the abundance of maternal gut microbiota was reduced, and their variety and consistency were increased from early pregnancy ($\leq$20 weeks) to the end of the third trimester [7]. The changes in gut microbial population over time plays a critical role in normal pregnancy in promoting weight gain, raising maternal metabolic adaptations, and supporting the growth of the fetus [9,81,83]. Even after birth, the maternal microbiota does not appear to return to its previous state [83]. As the postpartum period is linked to significant hormonal shifts, it was expected that it could reveal more interesting facts of the hormonal effect on the maternal gut microbiome [9].

Despite the microbial transition before or after postpartum periods, it is an immensely popular fact that maternal gut has a great influence on the breast milk microbiota. Martin et al. performed the earliest comparison on the origin of milk microbiota using random amplification of the polymorphic DNA (RAPD) technique [37]. The RAPD profiles of lactic acid bacteria isolates from breast milk were compared with other isolates collected from various body sites. The study explained that none of the lactic acid bacteria isolates from the breast milk were identical to those from the breast areolar skin surface [37,84]. This is also supported by the research done by Soto et al. [42]. The study reported that the presence of skin microflora in the HBM samples seems to be widespread. However, the presence of *Lactobacillus* and *Bifidobacterium* must originate from the maternal gastrointestinal environment [42].

Albesharat et al. conducted a study for a large number of isolates by using RAPD and matrix assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) analyses [85]. The researchers found that the RAPD genotypes of *L. plantarum*, *L. fermentum*, *L. brevis*, *Enterococcus faecium*, *Enterococcus faecalis*, and *P. pentosaceus* recovered from breast milk, mothers' guts, and infants' feces were identical [85]. Jost et al. utilized the pyrosequencing technique to reveal vertical sharing of microbiota between maternal gut, breast milk and infant's gut [33]. Despite the predominance of skin microflora, the study also found that gastrointestinal anaerobic bacteria including *Bacteroides*, *Bifidobacterium*, *Blautia*, *Clostridium*, *Collinsella*, *Parabacteroides*, *Veillonella*, and facultative *Lactobacillus* spp. were detected in all three ecosystems analyzed. Jost et al. reported that *Bacteroides* and *Bifidobacterium* strains had the highest potential for vertical transfer from maternal gut into her breast milk samples [33]. The above-mentioned studies assist the statement that the

maternal gut bacteria reach the breast milk and colonize the gut of the breast-fed neonate through entero-mammary pathway trafficking [17,86].

However, only certain gut species have been identified from breast milk. Sampling and selective transfer of a specific strain or composition of maternal gut microbiota into the mammary glands remain to be completely explored. Milani et al. performed metagenomics investigation to highlight the transmission of probiotic Bifidobacterial communities as they are important representatives in the intestinal microbiota of infants [86]. With Bifidobacterial ITS profiling coupled with shotgun metagenomics analyses performed in the study, a common Bifidobacterial profile was identified to be similar among the mother–infant pairs. The targeted genome reconstruction from the microbiome also confirmed that these specific Bifidobacterial strains were persistent in the infant gut for six months after birth [69,86]. Later, the same group revealed that Bifidobacterial communities are widely spread among mammalian groups that include domesticated animals such as dogs, cats, cows, sheep, goats, horses, and pigs as well as 46 other non-primate mammals including humans and 13 non-human primates [8]. The study reported that the Bifidobacterial strains were found to be transferred from the mother through breast milk. These findings support the hypothesis that probiotics bacteria found in HBM, especially Bifidobacterial strains, are directly acquired from the maternal gut. However, they might be found at very low abundance, and this could be to favor an ultimate transfer to the following generation [8,17,69,86].

4.3.2. Mucosal Sampling and Migration of Dendritic Cells

The pathway of transmitting maternal microbiota from the gut to breast milk (entero-mammary pathway) can be linked to the improvement of the immunity in newborns [45,87]. The mother passes down some of her immunoglobulin (Ig) to the developing fetus to acquire passive immunity during the pregnancy period. IgG is the only antibody that can cross the placental barrier in humans through endosomes within the syncytiotrophoblasts of the placenta [88,89]. After giving birth, the maternal immunity is still being successfully transferred and passive immunity is achieved as the lactating mammary glands takes over the functionality of the placenta [89]. Breast-feeding maintains a strong interaction between the mother and her infant by continuing the transfer of maternal immunoglobulin molecules into the newborn.

SIgA is the predominant class of Ig found in HBM and is released by the antibody-secreting cells found in the mammary glands. However, the mammary gland does not seem to stand alone because it is highly influenced by the antibodies produced by the mother in her own gut [90]. During the late pregnancy and lactation stage, the maternal IgA antibody-secreting cells, found in the gut and in the respiratory system, are translocated into the mammary glands [91,92]. The gut mucosal epithelial chemokine CCL28 is the key regulator of the build-up of IgA antibody-secreting cells. The build-up of these cells occurs in the lactating mammary gland. A study using BALB/c mice as the animal model revealed that blocking of CCL28 can lead to significant accumulation of IgA plasma cells in lactating mammary glands [93]. Therefore, the data suggest an internal pathway to transport these antibody-secreting plasma cells from the maternal gastrointestinal tract to her mammary glands, without involving blood circulatory vessels. Furthermore, the translocation is regulated by specific carrier cells [51].

It has been recognized for many years that human lymphatic vessels exhibit a high capacity for accumulating particulate material. The human lymphatic system not only drains the lymph, but is also responsible for moving the immune cells of lymphocytes and antibodies throughout the body to help initiate and participate in an immune response. Several blind ended vessels and fine capillaries of the lymphatic system are found throughout the human body [94,95]. Gut associated lymphoid tissue (GALT) is the main lymphatic present around the gastrointestinal tract (Figure 3) comprising of Peyer's patches, isolated follicles, and mesenteric lymph nodes [94,96]. The GALT serves as antigen sampling and inductive sites of the mucosal immune system. SIgA cells from the gut can adhere selectively to specific M cells found in the intestinal Peyer's patches. This will mediate the translocation

of the SIgA cells from the intestinal lumen to GALT, and finally to mammary glands via the entero-mammary link [97]. The migration of SIgA cells from the gastrointestinal tract to mammary glands is believed to be under the regulation of hormones such as prolactin, estrogen, and progesterone [98].

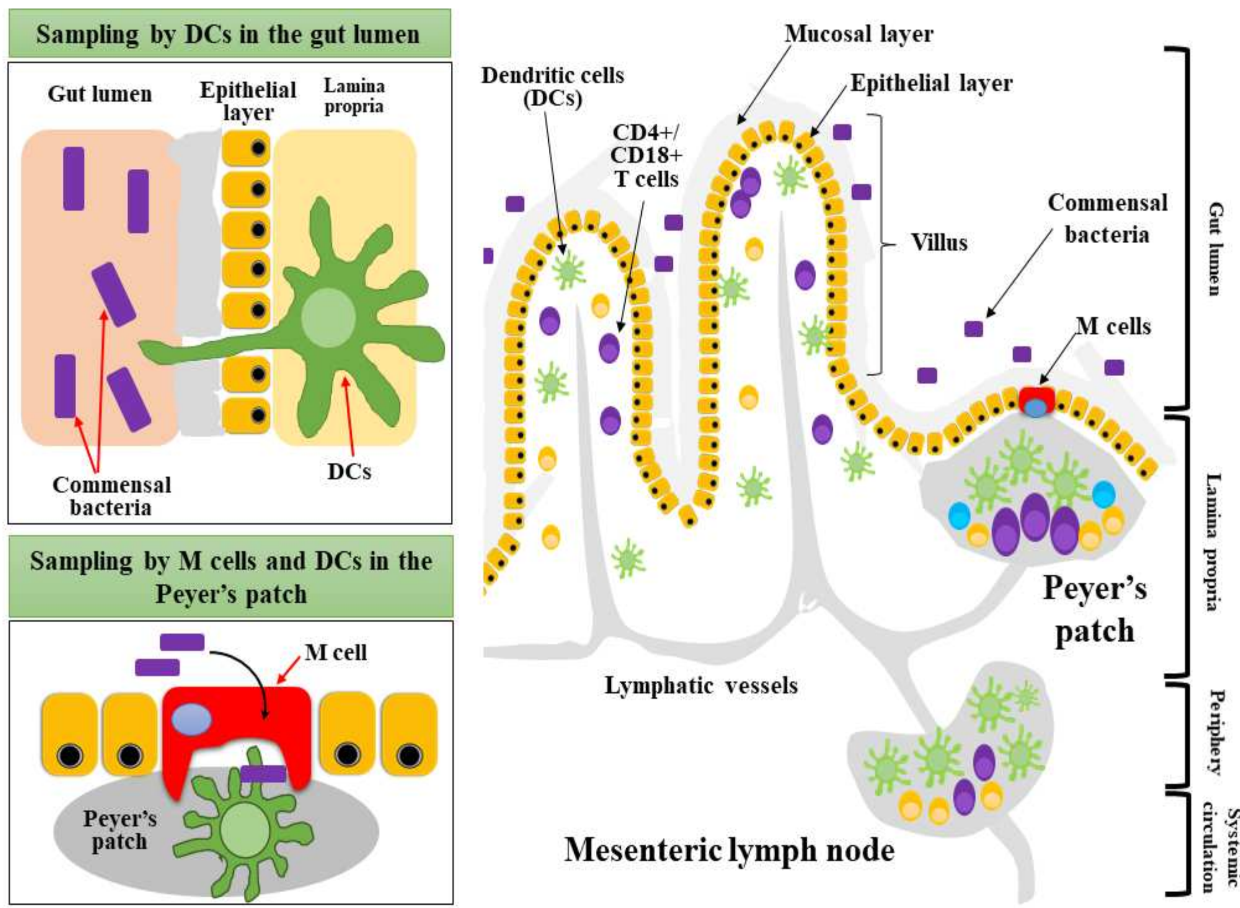

Figure 3. GALT containing lamina propria, Peyer's patches, and mesenteric lymph nodes (MALT). DCs are present in the lymphoid compartments.

Migration of antibody-secreting cells such as SIgA to HBM, originating from the maternal gut, could clearly explain the existence of lymphatic systems and the entero-mammary pathway [91]. However, the sampling and translocation of maternal gut microorganisms into breast milk is best explained by the dendritic cells (DCs). These cells are found throughout many sites including intestinal tissues. DCs actively migrate between the intestinal lamina propria, GALT, Peyer's patches, isolated lymphoid follicles, and in the mesenteric lymph nodes [97,99]. The main function of DCs is to sample antigens found in the intestinal lumen, then migrate into lymphatic nodes and present antigens into T cells to induce their proliferation and differentiation [99]. Several studies have suggested that DCs such as CD11c+ and CX3CR1+ macrophage cells can extend their trans-epithelial dendrites through the tight junction in the intestinal epithelium to capture luminal antigens or bacteria [33,99,100].

Farache et al. used 2-photon microscopy in live mice to evaluate the microbial sampling by CD103+ DCs in the mesentery lymph nodes [101]. These DCs engulfed *Salmonella* bacteria in the lamina propria and rapidly retracted toward the soma [101]. The study reported that CD103+ DCs translocated *Salmonella* bacteria in two steps [102–104]. First, some DCs were found to sense the presence of bacteria and started to secrete chemokines to stimulate more DCs to migrate to the epithelium. Then, the newly arrived DCs extended

their dendrites toward the lumen by penetrating the tight junction between intestinal cells. The dendrites were extended in response toward the chemokines and started to engulf the bacteria. The DCs will become mature plasma cells when returning to the lamina propria through lymphatic vessels (Figure 3). The presence of antigens on DCs stimulated T cells to initiate adaptive immunity [101,104].

The maternal microbiota are believed to be transferred to the mammary glands by DCs through the above explained lymphatic system and the entero-mammary pathway [105,106]. However, the precise selection of microbiota and how DCs differentiate commensal or pathogenic bacteria are yet to be explored. Human gastrointestinal microbiota include a large and diverse community including hundreds of commensal bacteria as well as pathogenic species. The pathogenic microflora induces specific inflammatory responses against commensal microbiota in the competitive environment. Bloom et al. demonstrated that germ-free mice injected with Gram-negative obligate anaerobes originating from the intestinal microbiota consortium would be sufficient to induce spontaneous colitis [107]. The activation of inflammatory responses by the intestinal microflora could mediate subsequent host pattern-recognition receptors [108]. This also leads to the recruitment of specific macrophages and DCs to activate adaptive immune responses [107]. These mononuclear phagocytic cells (macrophages and DCs), which normally reside in the intestinal lamina propria, also play a critical role in preventing inappropriate activation of inflammatory responses to the normal microflora [107,108]. But how does the immune system discriminate between commensal and pathogenic bacteria? Several commensal bacteria including *Bifidobacteria* strains have been found to induce tolerance in monocyte-derived DCs, which can recognize the specific pathogen-associated molecular patterns [99,108]. This step could help to precisely discriminate the intestinal microflora. This finding has been utilized to explain DCs translocation of non-pathogenic gastrointestinal bacterial strains like *Lactobacillus* and *Bifidobacterium* spp. [10].

We suggest that these translocation patterns could explain the entero-mammary pathway transfer of gut microbiota into mammary glands (Figure 4). DCs sample the commensal bacteria from maternal gut during pregnancy or after birth. After luminal sampling, DCs could travel in the lymphatic systems and reach axial lymphatic nodes. Then, DCs or microbes could migrate to the internal mammary lymphatic node [39,100,109]. Bioluminescence imaging was performed by de Andrés et al. to show that lactic acid bacteria could be transported in vivo during pregnancy in mice [6]. In this study, two strains of *L. lactis* MG1614 and *L. salivarius* PS2 were genetically modified to harbor luminescent response producing lux operon genes. Both strains could be isolated, and the lux genes were detected by PCR techniques from either milk or mammary gland biopsies after their oral administration to pregnant mice [6]. This clearly proves the existence of an entero-link between the gut and mammary glands involving lymphatic vessels and DCs (Figure 4). We believe that the human lymphatic system is the major path for the translocation of microorganisms in pregnant women. Perez et al. examined the presence of commensal microflora in breast milk and peripheral blood [87]. The study reported that microbial translocation occurred from the intestine to mesenteric lymph nodes and mammary glands during end stages of pregnancy and lactation period in mice [87].

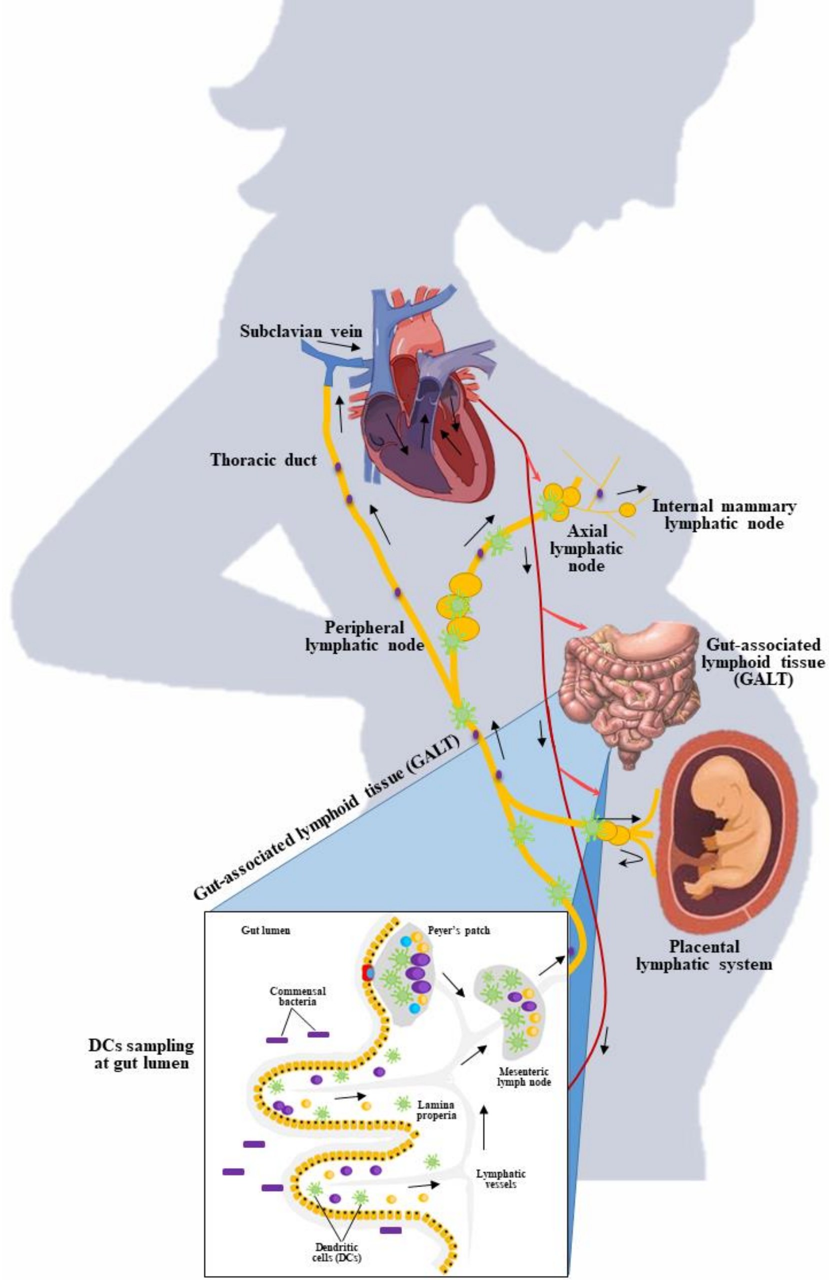

Figure 4. Entero-mammary pathway showing the movement of gut microbial groups into the mammary glands.

4.3.3. Evidence on Gut Transmission to Breast Milk

Recently, several reports have shown that orally administrated probiotic microorganisms to pregnant women could also be traced in the breast milk or in the infants' stool (Table 1). These studies have proven the homogeneity of bacterial strains with more precise molecular techniques [48,110–112]. Abrahamsson et al. utilized culture-based techniques and demonstrated that 12% of women in their probiotic group had viable *L. reuteri* strains in their colostrum samples compared with 2% in the placebo group [111]. Similarly, Arroyo et al. reported the presence of *L. fermentum* CECT5716 and *L. salivarius* CECT5713 in the milk of mothers who had undergone probiotic treatment for mastitis after 21 days of supplementation [112]. In another small trial, four of 10 women had viable cultures of *L. rhamnosus* strain LC705 isolated from breast milk samples after supplementation with *L. rhamnosus* strain LC705 [113]. Additionally, the results of subsequent trials also

suggested that maternal supplementation with *L. fermentum* CECT5716 may increase levels of microbial population in the breast milk samples compared to the control and under antibiotic treatments [48].

Table 1. Oral administration of probiotic strains and their respective re-detection methods.

References	Oral Administration	Method of Detection
Arroyo et al. [112]	*L. fermentum* CECT5716	16s rRNA sequence
Nasiraii et al. [113]	*L. rhamnosus* strain LC705	qPCR and 16S rRNA sequencing
Fernández et al. [52]	*L. salivarius* PS2	MALDI-TOF and PFGE
Hurtado et al. [48]	*L. fermentum* CECT5716	MALDI-TOF mass spectrometry
Jiménez et al. [27]	*L. salivarius* CECT5713 and *L. gasseri* CECT5714	species-specific PCR, 16S rRNA sequencing and PFGE

The orally administrated bacterial strains detected in breast milk such as *L. fermentum* CECT5716, *L. salivarius* CECT5713 [112], or *L. rhamnosus* strain LC705 [113] were initially isolated from breast milk samples. This could imply the presence of selective sampling and translocation of these bacteria, which have a natural affinity toward breast milk origin bacteria. In this context, translocated bacteria might have specific induction mechanisms to activate DCs [39]. Therefore, the natural gastrointestinal microorganisms or artificially administered probiotic strains are selectively translocated from the maternal gut into mammary glands through the entero-mammary pathway. Because of the small number of mother–infant pairs studied, the biological validity of such results is limited.

GALT lymphoid cells migrate to the mammary gland in humans and rodents, establishing an entero-mammary bond and leading to the so-called "normal mucosal mechanism," in which immune cells migrate between distant mucosal sites. In contrast to these observations, cattle studies have shown that lymphoid cell movement between the gut and the mammary gland is limited, suggesting that the entero-mammary relationship in ruminants is less functional [10]. While most mononuclear phagocytes in breast milk are produced from peripheral blood monocytes, it has recently been hypothesized that a proportion of these mononuclear phagocytes are dendritic cell-like cells that originate in GALT. These cells collect luminal microbiota, and then transport these microbial components to the mammary gland. This pathway teaches the neonatal immune system to recognize commensal-associated bacterial molecular patterns and to react properly to them [19,83].

4.4. Final Microbial Consortia of Human Milk

Microbial population in the human milk could be originated from different sources. Based on the core microbiota, it is suggested that the microbiota mainly originate from the maternal digestive tract, breast areolar skin, and from the infants' oral cavity during suckling (Figure 5). The maternal gut bacteria seem to be selectively sampled and these bacteria may reach the mammary glands through an endogenous route of the entero-mammary pathway [10]. The gut origin lactic acid bacteria and others were sampled by DCs and arrive to breast tissues and mesenteric lymphatic nodes. The lactic acid bacteria probably form biofilms on the lactiferous tubules of the mammary duct system [91]. Using several developmental stages, the mammary gland trains for lactation [114]. In late pregnancy, the alveolus system of the mammary gland will be maximally grown, and this will provide a favorable environment for the formation of a biofilm. In addition, during the suckling process, the microorganisms from the nipple and areola skin as well as the infants' oral cavity could be mixed in the breast milk produced [23]. However, in healthy women, these common skin microflorae might not breach deep inside the alveolar system. Biofilm formation by *Staphylococcus* or yeast are often related to the mastitis problem [15].

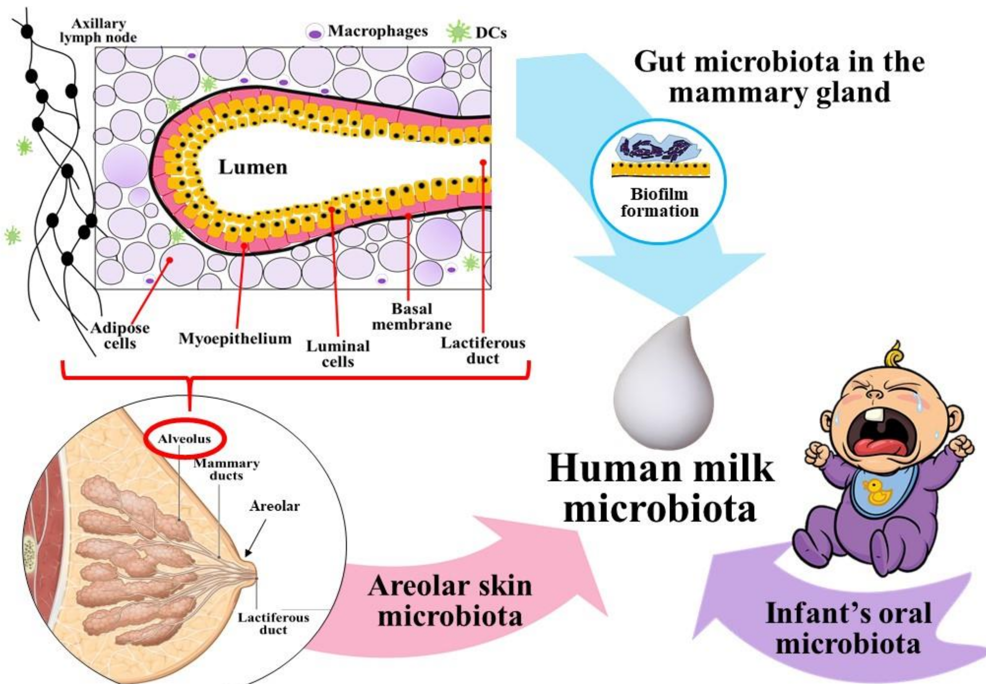

Figure 5. The three possible routes of microbial load in human breast milk.

5. Factors Affecting Microbial Load in the Breast Milk

5.1. Mode of Delivery

The method of delivery was one of the reasons that had a significant impact on the microbiota composition of breast milk. There are two types of methods of delivery: caesarean delivery and natural delivery. Caesarean is the delivery through the incisions made in the mother's abdomen and uterus. Toscano et al. [60] explained that the breast milk produced by a mother who delivers naturally has a greater bacterial richness such as commensal and pathogenic microorganisms (ex: *Streptococcus* and *Haemophillus*) compared to C-section breast milk microbiota. Thus, C-section and natural delivery have higher abundance of anaerobic bacteria compared to aerobic bacteria. As reported by Cabrera-Rubio et al. [115], mothers who give birth via caesarean delivery have a low amount of *Leuconostocaceae* and a high amount of *Carnobacteriaceae* compared with those who give birth by natural delivery. Nevertheless, this finding contradicts the data from a study published by Urbaniak et al. [116], which states that there is no effect on microbial profiles through the mode of delivery.

Another study reported that the differences in the milk produced by both delivery methods emerged from the oral colonization determined by the portal where the newborn exits [116]. Specifically, infants born naturally acquire maternal bacteria from the vaginal environment while maternal skin and breast milk transfer maternal bacteria to infants born through the caesarean method [117]. In addition, work done by Khodayar-Pardo et al. [118] reported that the total concentration of *Streptococcus* spp. was higher, and concentration of *Bifidobacterium* spp. was lower in C-section delivery compared to natural delivery. Meanwhile, natural delivery showed a higher correlation between *Gammaproteobacteria* and *Putrescine*, but maintained a positive correlation with *Pseudomonas fragi* [11].

5.2. Lactation Period

Breast milk is an infant's first intake of nutrition, and its structure varies from colostrum to mature milk depending on the infant's needs. Breast milk from healthy mother is considered a continuous source of bacteria [3,115,118,119]. There are three stages of lactation. Stage I involves secretory initiation, which appears during pregnancy and regulates the development of secretory cells from mammary alveolar cells. At this stage,

the mammary gland can produce the immunoglobulin-rich mammary secretion known as colostrum. Next, in stage II, the onset of milk secretion, which is known by transition milk. Lactose is the most common milk osmolyte, and an increase in intracellular lactose attracts water into the lactocytes, resulting in a significant increase in milk volume in stage II of lactogenesis. Finally, stage III is mature milk, which shows up one month after delivery and is affected by hormonal signaling, lifestyle, and diet [115,118,119].

Boix-Amorós et al. [3] reported that all three stages of lactation are dominated by *Staphylococcus* and *Acinetobacter* genera in colostrum, then, transition milk contained *Pseudomonas* and *Streptococcus*, and finally, *Acinetobacter* was abundant in mature milk. Besides, their bacterial patterns were also composed of other genera including *Finegoldia, Streptococcus, Corynebacterium*, and *Peptoniphlus*. The highest bacterial diversity was found in the transition milk with nine genera of bacteria compared to colostrum and mature milk. However, *Staphylococcus aureus* and *epidermis* were not detected in samples of healthy mothers [3]. In contrast, in a study published by Li et al., no substantial alteration in the abundance of 17 bacterial families among the samples that had been collected in the three different stages of lactation was detected [36]. The common genera reported in colostrum are *Weisella, Leuconostoc, Staphylococcus, Streptococcus* and *Lactococcus*, but the lactic acid genera showed the highest abundance in milk between one and six months after giving birth [115].

Khodayar-Pardo et al. reported that the total counts of *Bifidobacterium* and *Enterococcus* increased throughout the lactation period [118]. Total concentration of *Bifidobacterium* spp. and *Enterococcus* spp. were lower in colostrum compared to the transition and mature milk. There was a good relationship between colostrum and transition milk as well as transition milk and mature milk [118]. *Bifidobacterium* spp., *Lactobacillus* spp., and *Staphylococcus* spp. showed positive correlation between colostrum and transitional milk. Moreover, *Lactobacillus* is the most common genus in colostrum and the most abundant throughout the lactation stage. Additionally, *Bifidobacterium* and *Enterococcus* counts were greater in colostrum and showed differences in transitional milk and mature milk, which increased throughout the lactation period and depends on the infants' need in each period [10,120].

5.3. Maternal Nutrition

Breast milk is a complex biological fluid that contains many nutrients that come from the maternal diet [1,49]. Offspring microbiome as well as maternal immunoglobulins and macronutrients will be equally affected by the components of the breast milk [120]. The energy content of breast milk and fat concentration will be affected by the nutritional status of the mother. HBM not only contains diverse microbiota, but is also considered the greatest source of nutrients for the infants' stable growth and development [1,49]. Breast milk proteins also show antimicrobial activities. One of the immunoglobulins known as SIgA aids in fighting the pathogenic bacteria and yeast such as *E. coli, V. cholerae*, and *C. albicans*, which can be found in human milk [51]. Breast-fed infants have fewer pathogenic bacteria such as *E. coli* and *Streptococci*, but more *Lactobacilli* and *Bifidobacterium* whereas formula fed infants had a higher number of enterococci and clostridia [45]. Antimicrobial components in breast milk suppress the growth of potential pathogenic bacteria and activate the progress of beneficial bacteria. Studies showed that antimicrobial compounds such as bacteriocins and hydrogen peroxide from lactic acid bacteria can suppress the growth of various pathogenic bacteria [37,45,51]. However, the difference in the physicochemical conditions of the intestinal environment may favor some bacterial growth and suppress the others.

Probiotic, prebiotic, and symbiotic components in the body during prenatal and postnatal periods trigger changes in maternal microbiota [45]. Generally, breast milk generates an environment that aids the growth of *Lactobacilli* and *Bifidobacterium* and suppresses pathogenic bacteria [18]. Both bacteria play an important role in reducing the risk of spontaneous preterm delivery and in increasing the serum level erythrocyte glutathione reductase (antioxidant activity) [121]. Besides, *Lactobacillus* GG (LGG) supplementations raise the cord blood level. They also increase anti-inflammatory cytokines, IFNγ, and

TGFb1 in HBM. The perinatal administration of LGG reduces the clinical effects of dermatitis, itching frequency, plasma IgE levels, and elevated levels of IFNγ in skin biopsies. The combination of LGG and *B. lactis* Bb12 ameliorates glucose homeostasis in healthy young females during and after pregnancy. The findings from previous studies show that probiotics supplements play a prominent role in preventing allergy development in both the mother and infant [29,122,123].

Prebiotics such as oligosaccharides introduced into the small intestine of a mother for digestion purposes are responsible for changing the maternal microbiota. O'Sullivan et al. stated that the mass spectrometry-based tool revealed that HMOs support the competitive growth of *Bifidobacterium* species such as *Bifidobacterium longum* and *B. breve* [28]. The combination of prebiotics supplementation of galacto-oligosaccharide (GOS) and fructo-oligosaccharide also changed the maternal microbiota. Moreover, the increase in bacterial derived metabolites such as inulin and GOS increased the number of bacteria, which synthesize folate. Folates are present in the large intestine for digestion and in blood stream for fetal development. Short chain fatty acids (SCFAs) are another type of metabolite produced by the prebiotics. SCFAs such as acetate, propionate, and butyrate are metabolized in epithelial cells and serve as an energy supply for the mother as well as the developing fetus. SCFAs also regulate the human gamma- to beta-globin gene switching process to produce hemoglobin [1,121,124].

Symbiotic treatment combines probiotics and prebiotics that help combat allergic disorders in children by altering the microbiome of the mother after delivery. They improve the response to *Haemophilus influenza* type b (Hib) immunization, which increases antibody concentrations responding to diphtheria, tetanus, or Hib. The presence of symbiotic components inside a maternal body stimulates the growth of the delivered probiotic bacteria. It further causes the formation of SCFAs that are anti-pathogenic and immune-modulating agents. The changes occurring in the maternal microbiota by the existence of probiotics, prebiotics, and symbiotic contribute to the offspring's health during early development [51,125].

5.4. Maternal Health Status

Maternal health is an important factor contributing to the microbial changes during and after giving birth [126]. The changes in the levels of *Bifidobacterium* spp. and cytokines correlate with obesity. The increase in *Staphylococcus* spp., leptin, and proinflammatory fatty acid levels caused by obesity also reduces microbial diversity [127]. In the breast milk of mothers with celiac disease, the levels of cytokines, *Bacteroides* spp., and *Bifidobacterium* spp. were reduced [128]. Women diagnosed as HIV-positive had higher bacterial diversity and higher prevalence of *Lactobacillus* spp. compared to HIV-negative women [129,130]. Obviously, women consume different types of medications, treatments, or antibiotics. Antibiotics reduce the concentration of *Bifidobacterium*, *Staphylococcus*, and *Eubacterium* spp. in milk samples while increasing the incidence of *Lactobacillus*, *Bifidobacterium*, and *Staphylococcus* spp. [16]. Chemotherapy alters maternal microbiota by reducing bacterial diversity as the chemo-drugs kill both beneficial and harmful cells and microorganisms in the body [16,116].

5.5. Breast Feeding Practices

The World Health Organization (WHO) strongly recommends exclusive breastfeeding at least for the first six months of life and a continuous breastfeeding for two years or beyond is highly encouraged. However, some babies are unable to feed at the breast due to abnormalities, prematurity, or other illnesses [131]. These babies are fed with expressed milk or pumped milk. During recent years, expressed milk is common in many parts of the world [132]. However, the impact of these indirect breastfeeding practices on the infant's health outcomes is still unknown. A recent study demonstrated that indirect breastfeeding could cause a significant impact on microbial diversity and the composition of human milk [133]. The study reported that the microbial composition of breast milk was differ-

ent in indirect versus direct breastfeeding and also in manually expressed and pumped milk samples. Higher abundance of opportunistic pathogens such as *Stenotrophomonas* and *Pseudomonas* in the pumped milk could pose the risk of infants' respiratory infection asthma [133]. Another study conducted on 393 Canadian mother–infant pairs demonstrated that pumped human milks were enriched with pathogenic microflora and had a lower abundance of bifidobacterial compositions [134]. Both studies suggested that direct contact of the infant's mouth with maternal breast skin is important (Figure 5). The co-occurrence of other microflora is associated with many variables including hygiene, cleanliness of bottles and nipples, type of the pump, milk storage conditions, and duration of storage [133,134]. However, pump-feeding is not recognized as a cause of infant diseases and other health complications. Pump feeding must be acknowledged as this method is the only savior for some mothers, especially those suffering from mastitis. However, pump feeding of breast milk is not similar to direct breast feeding. More studies must be conducted to understand the impact of pumping on milk composition to optimize the process.

6. Functionality of Human Milk Microbiota

Because breast milk contains a variety of commensal bacteria, it is considered as a reservoir of bioactive ingredients useful for infants. The microbiota of breast milk contributes to infant wellbeing for its ability to prevent infections, inflammations, allergies, and Enterocolitis.

6.1. Anti-Infections

Weisella, Leuconostoc, Staphylococcus, Streptococcus, and *Lactococcus* are important organisms in colostrum samples. During newborn development, approximately from one to six months, the inhabitants of the oral cavity such as *Veillonella, Leptotrichia*, and *Prevotella* increased significantly in the breast milk [115]. These microorganisms also inhibited the growth of *E. coli, Salmonella* spp., and *Listeria monocytogenes* [4]. Next, reuterin secreted by *L. reuteri* is another antimicrobial compound for the colonization of infant gut. *L. gasseri* CECT5714, *L. salivarius* CECT5713 and *L. fermentum* CECT5714 have the capability to inhibit the adhesion of *Salmonella cholerasuis* to mucins [4,42]. Besides, the growth of pathogen microorganisms such as *S. aureus, S. typhimurium, Yersinia enterocolitica*, and *C. perfringens* are controlled by *Lactobacilli* and *Bifidobacteria*. The *Klebsiella cloacae* group have been reported to be frequent gastrointestinal colonizers of neonates [33].

6.2. Anti-Inflammation

The microbiota of human milk reduces the inflammatory process, which involves the response of TH2 lymphocytes. *L. fermentum* CECT5716 and *L. salivarius* CECT5713 activate the NK cells, activate the CD4and CD8 T cells, and regulatory T cells. Vaccination against diseases such as poliomyelitis, tetanus, and diphtheria improved the immunomodulatory effects of breast milk microbiota in humoral response in breast-fed babies more than formula-fed infants [4,135].

6.3. Metabolic Functions

Microbiota of human milk produces different types of metabolites. Lactate-utilizing bacteria in the human milk, namely *Eubacterium* and *Anaerostipes* species produce butyrate. Butyrate, being a functional metabolite that plays a crucial role in modulating intestinal function of infant by increasing fecal concentration, fecal moisture, volume, and stool frequency. Other functions of butyrate in early age development include gene expression, cell differentiation, gut tissue development, immune modulation, oxidative stress reduction, and diarrhea control [83,125].

6.4. Allergic Prevention

The intestinal microbiota of a breast-fed infant is predominated by *Bifidobacterium*. Data showed a significant correlation in *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* frequencies and counts in women suffering from allergies [136]. Besides, the amount of *Bifidobacterium* is lower in allergic mothers when compared to non-allergic mothers. Accordingly, the feces of infants from allergic mothers contain lower amount of *Bifidobacterium*. Thus, *Bifidobacterium* in breast milk contributes to allergic prevention in the mother and newborn [136]. Chiu et al. performed an experiment of different strains of *Bifidobacterium* to determine the potential immunomodulatory strains that can cause human peripheral blood monocular cells to produce cytokines [137]. Data showed that *B. adolescentis* DB-2458, *B. longum* subsp. *infantis* GB-1496, and *B. longum* HB-762 were the most potential species to induce a high level of IL-10 and TGF-b cytokines.

6.5. Enterocolitis Prevention

Some of the bacteria present in breast milk can increase mucin development and decrease intestinal permeability to improve the function of the intestinal barrier [115]. The maternal immune system consists of both secreted and diffused immunoglobulins such as SIgA and IgG. The breast milk contains IgG, SIgA, the most important bacterial pathogen of the neonate, *E. coli*, specific growth-enhancing *lactobacilli*, macrophages, and lymphocytes, which prevent the development of enterocolitis in infants [138,139].

6.6. Growth and Development of Immune System

The human gut microbiota, which consists of 500 to 1000 microbial species, is also responsible for the enhancement of infant immune system. Most of the fecal population is composed of *Bifidobacteria*, with smaller numbers of *E. coli*, *Bacteroides*, and *Clostridia* [140]. Infants undergoing caesarean delivery have lower numbers of *Bifidobacteria* and *Bacteroides*, whereas they are more often colonized with *C. difficile*. These infants have higher chances of developing atopic disease and prematurity. Many oligosaccharides and glycoconjugates produced by glycosyltransferases in the mammary gland are transferred to human milk and serve as receptors to interfere with the binding of pathogens to epithelial cells. This prevention is necessary to enhance the newborn's immune system through the breast milk [45,141].

7. Conclusions

The human milk consists of a dynamic microbial ecology with extraordinary properties and functions. These microorganisms are actively transferred from mother to infant via breast-feeding. The human milk microbiota have been proven to have great impact on the neonatal immune system, optimizing nutrient metabolism, the intestinal barrier function, and enhancing maturation of the digestive tract. These microorganisms enter human milk via several pathways including spreading through the mother's breast skin and movement from the infant's oral cavity and through a special route by the entero-mammary pathway. DCs offer unique microbial translocation mechanisms from the maternal gut into mammary glands. Among the trillions of strains, maternal gut commensal microorganisms are specifically selected and translocated. Several fundamental questions regarding the gut and breast mammary gland axis remain unanswered. The gut–mammary gland axis has a paramount role in the human body system and programming health for life. A precise understanding of the existence of the entero-mammary pathway requires more sophisticated experimental and clinical studies. The growing technology of -omic tools such as metagenomics, transcriptomics, and metabolomics has opened new avenues to identify and understand the existence of specific bacteria in the human milk and the gut. The strain-level identification of microbiome must also be performed in the bloodstream and within the immune cells involved in the entero-mammary transfer. In conclusion, a comprehensive understanding of the complete scenario of events of such mechanistic pathways will offer novel interventions to improve the health status of newborns. Emerging knowledge offers

novel opportunities to modulate the gut microbial composition to promote maternal-infant health.

Author Contributions: S.S. and H.A.E.E. conceived the review. S.S., H.C.K., K.H.N., R.A.M., D.S., S.H. and R.Z.S. wrote the manuscript. D.J.D., V.K.G., M.W., B.A., T.V. and H.A.E.E. wrote, edited, and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: S.S. and H.A.E.E. would like to thank RMC, Universiti Teknologi Malaysia (UTM), for financial support from grant no. R.J130000.7609.4C284 and R.J130000.7609.4C240.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: S.S. would like to extend this thanks to the School of Postgraduate Studies (SPS), Universiti Teknologi Malaysia (UTM) for the financial support of Zamalah's PhD Scholarship session 2017/2018. S.S. Thanks to Thevarajoo, S. for their technical advice on the construction of a 16S rRNA gene-based phylogenetic tree (Figure 2).

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

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Article

An In Vitro Study of Different Types of Greek Honey as Potential Natural Antimicrobials against Dental Caries and Other Oral Pathogenic Microorganisms. Case Study Simulation of Oral Cavity Conditions

Chrysoula (Chrysa) Voidarou [1,*], Maria Antoniadou [2], Georgios Rozos [1], Athanasios Alexopoulos [3], Elpida Giorgi [4], Athina Tzora [1], Ioannis Skoufos [1], Theodoros Varzakas [5] and Eugenia Bezirtzoglou [4]

[1] Laboratory of Animal Health, Food Hygiene and Quality, Department of Agriculture, University of Ioannina, 47100 Arta, Greece; clevervet@hotmail.com (G.R.); tzora@uoi.gr (A.T.); jskoufos@uoi.gr (I.S.)
[2] School of Dentistry, National and Kapodistrian University of Athens, Goudi, 11527 Athens, Greece; mantonia@dent.uoa.gr
[3] Laboratory of Microbiology, Biotechnology & Hygiene, Department of Agricultural Development, Democritus University of Thrace, 68200 Orestiada, Greece; alexopo@agro.duth.gr
[4] Laboratory of Hygiene and Environmental Protection, Medical School, Democritus University of Thrace, 67100 Alexandroupolis, Greece; elpidagiorgi94@gmail.com (E.G.); empezirt@yahoo.gr (E.B.)
[5] Department of Food Science and Technology, University of the Peloponnese, Antikalamos, 24100 Kalamata, Greece; theovarzakas@yahoo.gr
* Correspondence: xvoidarou@uoi.gr

Citation: Voidarou, C.; Antoniadou, M.; Rozos, G.; Alexopoulos, A.; Giorgi, E.; Tzora, A.; Skoufos, I.; Varzakas, T.; Bezirtzoglou, E. An In Vitro Study of Different Types of Greek Honey as Potential Natural Antimicrobials against Dental Caries and Other Oral Pathogenic Microorganisms. Case Study Simulation of Oral Cavity Conditions. *Appl. Sci.* **2021**, *11*, 6318. https://doi.org/10.3390/app11146318

Academic Editors: Monica Gallo and Wojciech Kolanowski

Received: 28 May 2021
Accepted: 6 July 2021
Published: 8 July 2021

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Abstract: To study the antibacterial effect of different Greek honeys, samples of citrus honey, *Saturja* spp. Honey, and oregano and sage honey were collected directly from producers. Manuka honey and artificial honey were used as controls. The honeys were diluted in various concentrations to determine the minimum inhibitory concentration (MIC) and were also placed in agar wells to determine the inhibitory zones of growth. The bacteria tested were two reference strains and five pathogens isolated from patients with various dental ailments. A series of samples were diluted with artificial saliva instead of distilled water to simulate the conditions in the oral cavity. The results show that in most cases the Greek honeys, and particularly the citrus honey and the oregano and sage honey, outperformed the antibacterial activity of manuka honey against all tested bacteria. This performance was due to the hydrogen peroxide as well as to other components of the honeys, that is, peptides and other substances such as phenolic compounds and flavonoids. Artificial saliva enhanced the antibacterial effect of the honeys in comparison to distilled water.

Keywords: honey; *Staphylococcus aureus*; *Streptococcus mutans*; *Fusobacterium nucleatum*; antibacterial activity; oral cavity; artificial saliva

1. Introduction

The oral cavity and its tissues form a complicated structure which consists of various anatomical elements of different fine structure and physiology. The main function of the oral cavity—apart from speech—is mastication of the food. It is the entrance of the digestive tract and the interaction of its anatomical elements with food may cause imbalance to the populations of 500 to 700 species of microorganisms which are estimated to inhabit the ecosystem of the oral cavity [1–3]. The ultimate result of this imbalance is the development of various lesions to the mucosal surfaces or to the teeth. Furthermore, at least 100 systemic diseases may induce lesions in the oral cavity, more than 500 medicines have oral manifestations, while 145 commonly prescribed drugs cause dry mouth [4–6]. Various pathogens, such as *Streptococcus mutans* exhibit their action causing serious health and oral health problems such as dental caries, while *Staphylococcus aureus* is found in periodontal pockets and other inflammations of the oral mucosa finally resulting in loss of

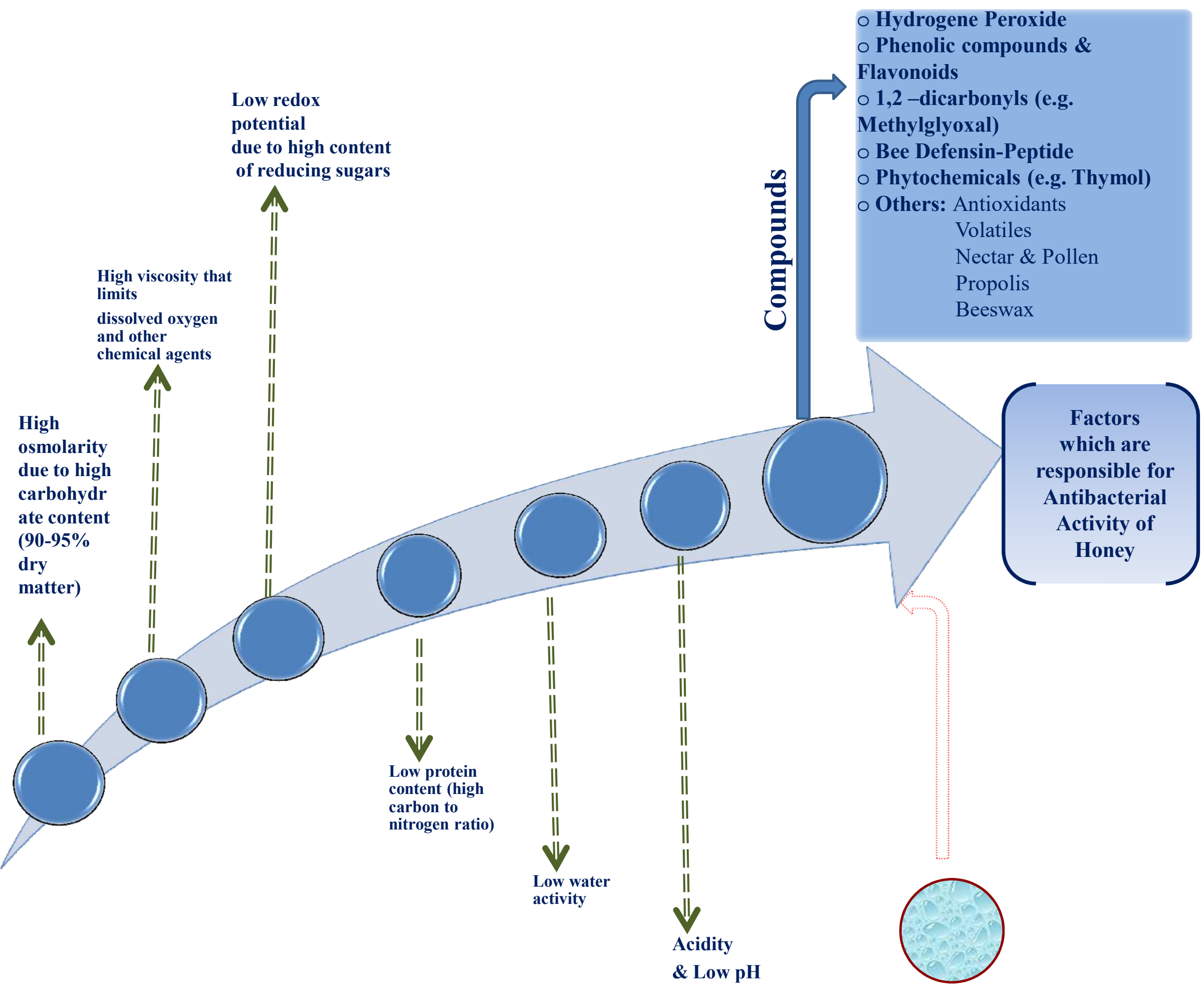
teeth [7,8]. In addition, it has been clinically demonstrated that bacteria from oral cavity are transported to other locations in the body causing infections such as inflammatory bowel disease (IBS) or participating in the pathogenesis of diseases such as the cirrhotic liver or Alzheimer's disease, diabetes melitus, adverse pregnancy outcomes, obesity, and polycystic ovary syndrome [1]. For all these reasons, the oral microbiome is of tremendous importance not only to oral health but also to the health of the whole organism. Antibiotics were and still are in the frontline of defense against infections, but their major disadvantage is that more and more resistant bacteria emerge. This resistance is not limited to commonly prescribed antibiotics but concerns even the so-called last resort antibiotics [9,10]. It is obvious that a new alternative strategy is needed and for that reason many researchers have focused their efforts on natural products of the pre-antibiotic era, to discover their salient or hidden antibiotic properties [11].

Honey is such a product with a very long history in human medicine. Since the dawn of humanity, honey has been used for its wound healing properties [12]. The fathers of medicine in classical antiquity mention honey as an excellent medicine for burns and trauma management [13,14]. Being a popular therapeutic in many cultures, honey is considered ideal for gastric and other ailments [15,16]. Chemically, honey is a supersaturated solution of carbohydrates (90–95% of its dry matter) containing no more than 20% of water (usually 17%) [17]. The remaining small portion consists of more than 180 other compounds which include proteins, vitamins, minerals, phenols, and flavonoids. Most researchers agree that the exact composition of the different types of honey depends on their botanical, seasonal, and geographical sources [7,18–22].

The aim of this study is to test the antibacterial activity of different types of honey (from different botanical sources but gathered at the same period and from the same area) against some pathogenic bacteria isolated from dental caries lesions. Furthermore, to determine their efficacy as well as their differences depending on their floral origins and hence stress their possible role for the prevention or the treatment of such lesions.

The Antibacterial Effectiveness of Honey

The antibacterial effectiveness of honey has been demonstrated in many studies [23–27]. The exact mechanism is not yet clear, although it is commonly attributed to (i) the high sugar content, which induces high osmolarity and inhibits bacterial growth, (ii) oxygen peroxide, which is produced by the activation of glucose oxidase and (iii) the low and acidic pH (usually 3.2–4.5) [6,7,22,26]. However, there is more to be said on the matter. Some studies have shown that «artificial» honey (a solution saturated in mono- and oligosaccharides) is not always able to inhibit bacterial growth effectively and that adding catalase does not remove all the antibacterial activity of honey [28,29]. These findings strongly suggest that there must be other factors which contribute to the total antibacterial effect of honey and that these factors act either separately or synergistically [26,27]. Such factors could be peptides such as defensin-1 or the phenolic compounds as well as the flavonoids, which are contained in honey (Scheme 1) [30,31].



Scheme 1. Schematic representation of the main factors involved in antibacterial activity of honey.

Manuka honey is a well-studied product famous for its antibacterial properties and serves as a paradigm and a reference material for similar studies of other honeys. The high phenol content of manuka honey is impressive and accounts for a large part of its antibacterial activity. However, it is the methylglycoxal, a compound derived from dihydroacetone, that completes its antibacterial activity [32–34]. Another substance (its structure not yet identified by the researchers) described in manuka honey induces cytokine production by interaction with TLR4 on macrophages [35]. This study has demonstrated that the activity of cytic cells intimately involved in the repair of wounded tissue is modulated by honey. The mechanisms by which honey affects the release of anti-inflammatory agents and growth factors from monocytic cells are so far unclear, and this represents an area for further study, e.g., whether honey affects other cell types, particularly endothelial cells and fibroblasts. Finally, manuka honey affects the proteins forming the septal ring and thus affecting the cell division [36]. Such findings fuel the research of the antibacterial properties of different honeys as alternatives to standard antibiotics.

2. Materials and Methods

2.1. Honey Samples

A total of 60 raw, freshly harvested, untreated, and unpasteurized honey (bee *Apis-mellifera*) samples were received from local producers, in Epirus province in Greece. The samples were originated from different botanical sources, as following: 20 of citrus origin, 20 of *Satureja* spp. origin, and finally 20 of oregano and sage origin. Technically, since a

botanical analysis of the pollen content has not been performed, all these samples should have been classified as multifloral honey. However, they were classified according to the dominant plant species in their geographical origin. Additionally, the given "botanical source" for every type of honey was identified by the beekeeper's information based on the major species flowering at the harvest season at the period of honey collection. Honeys were collected from beekeepers who transfer their hives each year during the blossom period at the mountain fields where these plant species are dominant. This is a traditionally tested practice to produce honey with flavor of a particular type (coniferous, thyme, oregano, etc.). A portion of 1000 g (from each one local producer) of each sample was collected in a sterile universal container and kept at 2–8 °C in the dark, until tested. The sampling and labelling process were performed by different associates than the ones who performed the analyses, to maintain a blind character of the study. The samples did not contain any additives or diluents and had not been heated. They were evaluated for their microbiological quality by being dissolved in cation-adjusted Mueller Hinton broth (CAMHB; Oxoid, Ltd., Basingstoke, Hampshire, England) and subsequently inoculated into sheep blood agar (Columbia Agar base with 5% Sheep blood, Becton Dickinson) and incubated aerobically at 37 °C for 48 h. Samples showing growth of bacteria or growth of more than 4–5 colonies of yeasts were excluded from the study; only 2 samples were excluded and were substituted by others suitable for the purposes of the study, so that the total number of suitable samples was 20 for every botanical source (geographical origin/ dominant plant species).

2.1.1. Control Indexes of the Experimental Design

Artificial honey was prepared by dissolving 3 g sucrose, 15 g maltose, 81 g D-fructose, and 67 g D-glucose in 34 mL sterile water and stored in sterile bottles (all sugars were supplied by Sigma-Aldrich) [20,37]. This dilution of sugars represents the proportions of the four predominant sugars in natural honey samples [38]. AM HEALTH Manuka Health MGO™550+ (25+) (Lower Hutt, New Zealand) was used as a positive control.

2.1.2. Determination of Physicochemical Parameters (All the Analyses Were Done in Triplicate)

pH: 10 g of each honey sample were diluted in 75 mL in CO_2 -free distilled water for measuring the pH value by the aid of a portable pH-meter (Sentron (1001) [39]. The pH-meter was calibrated by two standard recognition buffers before the analysis; pH 4 and pH 10 (as specified by the manufacturer) and each measurement was carried out in triplicate.

Determination of H_2O_2 content: H_2O_2 content in honey samples was determined by using the Megazyme GOX assay kit (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland), which is based on H_2O_2 release, after glucose oxidase catalysis of the oxidation of β-D-glucose to D-glucono-δ-lactone [40–42]. As a standard, H_2O_2 diluted to 9.8–312.5 μM was used. Other studies reported that the maximum levels of accumulated H_2O_2 that occurred in honey solutions were found in solutions diluted to concentrations between 30 and 50% [31], hence 40% (*w*/*w*) honey solutions in 0.1 M potassium phosphate buffer (pH 7.0) were prepared and immediately measured for their H_2O_2 content. Each honey sample as well as every H_2O_2 standard were tested in triplicate in a 96-well microplate. The absorbance of the reaction was then measured at 510 nm using a microplate reader [43].

Determination of free, lactonic, and total acidity: The free, lactonic, and total acidity were determined by equivalence point titration according to AOC, 1990 [44,45] and the results are expressed as meq/kg.

Determination of Total Phenolic Content (TPC): The TPC of the honey samples was measured in accordance with the Folin-Ciocalteu method, with a minor modification [20,46,47]. First, 20 μL volume of the sample was added to a tube containing 1 mL of ultrapure water. Subsequently, 100 μL of Folin-Ciocalteu reagent was added to the mixture, and the tube was stoppered and allowed to stand at room temperature for 3 min. Thereafter, 280 μL of 25% *w*/*v* sodium carbonate solution and 600 μL of ultrapure water were added to the

mixture. Following 1 h of incubation at room temperature in the dark, the absorbance was measured at 765 nm versus a blank containing Folin-Ciocalteu reagent and ultrapure water and the measurement of absorbance was conducted. The optical density of the sample (20 μL) in 25% *w*/*v* solution of sodium carbonate (280 μL) and ultrapure water (1.7 mL) at 765 nm was also measured. The TPC was determined using a standard curve of absorbance values correlated with standard concentrations (50–1500 μg/mL) of gallic acid. The results are expressed as gallic acid equivalents (GAEs) using the standard curve (absorbance versus concentration) prepared from authentic gallic acid and the TPC was expressed in mg of gallic acid equivalents (mg GAE/100 g of honey).

Determination of Flavonoids: The total flavonoid content (TFC) of honey samples was determined by the aluminum chloride method [48]. Subsequently, 1 mL of a honey solution (1 mg/mL) was mixed with 0.3 mL $NaNO_2$ (5%). After 5 min a solution of 0.3 mL $AlCl_3$ (10%) was added and six minutes later the tested honey samples were neutralized with a 2 mL of NaOH solution (1 M). The mixture was shaken, and the absorbance was measured for all samples at 510 nm using a spectrophotometer. Quercetin (Sigma–Aldrich, St. Louis, MO, USA), which is having a moderate absorbance, was used as the standard and a calibration curve was made using a standard solution of quercetin 20–100 mg/L. The results were expressed in mg for Quercetin Equivalents (CE)/100 g of honey, as the average of triplicate measurements [49].

2.2. Determination of the Antibacterial Activity

2.2.1. Tested Microbial Strains

The strains of the pathogenic bacteria that were tested as cell- targets to assess the antibacterial activity of honey were the following:

- *Staphylococcusaureus* subsp. *aureus*, methicillin, and vancomycin resistant (source: dental septicaemia)
- *Staphylococcus aureus* subsp. *aureus*, methicillin, and vancomycin resistant (source: tooth abscess)
- *Staphylococcus aureus* subsp. *anaerobius* (source: septicaemic gingivitis)
- *Streptococcus mutans* (source: oral cavity)
- *Fusobacterium nucleatum* (source: oral cavity)
- *Staphylococcus aureus* subsp. *aureus*, reference strain ATCC 12600
- *Staphylococcus aureus* subsp. *αnaerobius*, reference strain ATCC 35844

All the above strains were identified and classified by standard laboratory procedures, which are followed by the National and Kapodistrian University of Athens, School of Dentistry.

Antibiotic Susceptibility Assay

Antibiotic susceptibility for the used bacterial strains and the reference strains was detected using the disc diffusion method, according to the standards set by The National Committee for Clinical Laboratory Standards (later renamed The Clinical Laboratory Standard Institute-CLSI) [50,51]. An aliquot of 100 μL of an overnight culture was diluted in saline solution to about 1.5×10^8 CFU/mL (0.5 McFarland turbidity standard). Mueller–Hinton agar (Oxoid Ltd., Basingstoke, UK) plates were flooded with this suspension in order confluent colonies given. The inoculated plates could stand at room temperature for 15 min prior to dispensing the paper discs and were then placed at 37 °C for 24 h. The diameters of the clear zones around each disc were measured after incubation.

In the present study, 9 antibiotic discs were used to determine the antibiotic resistance of the wild pathogenic *Staphylococcus* tested strains: Vancomycin (VA, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Erythromycin (E, 15 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Ampicillin (AMP, 10 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Oxacillin (OX, 1 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Ciprofloxacin (CIP, 5 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Sulfamethoxazole with trimethoprim (SLT, 25 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Oxytetracycline (OXY, 30 μg, Oxoid Ltd., Basingstoke,

Hampshire, UK), Ceftriofur (CFT, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), and Amoxicillin with Clavulanic acid (AMC, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK).

For the *S. mutans* strain, the antibiotics employed in this study were: Ampicillin (AMP, 10μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Cefotaxime (CTX, 30 μg, HiMedia Labs, Einhausen, Germany), Erythromycin (E, 15 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Cephazolin (kZ, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Methicillin (ME, 5 μg Oxoid Ltd., Basingstoke, Hampshire, UK), Lincomycin (L, 2 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Clindamycin (CC, 2 μg, Abtek Biologicals Ltd-UK, Liverpool, UK), Vancomycin (VA, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK) Metronidazole (MTZ, 5 μg, Abtek Biologicals Ltd-UK, Liverpool, UK), Rifampicin (RIF, 5 μg, HiMedia Labs, Einhausen, Germany), Ciprofloxacin (CIP, 5 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Ofloxacin (OF, 5 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Gentamycin (GEN, 10 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Penicillin G (P10, 10 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Chlortetracycline (CTE, 30 μg, Oxoid Ltd.), Doxycycline (DO, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Chloramphenicol (CHL, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Tetracycline (TE, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK) and Amoxicillin (AMO, 30 μg, NEOSENSITABS™-Rosco Diagnostica, Taastrup, Denmark). Finally for the *F. nucleatum* strain the tested antibiotic discs were: Ampicillin (AMP, 10 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Clindamycin (CC, 2 μg, Abtek Biologicals Ltd-UK, Liverpool, UK), Erythromycin (E, 15 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Metronidazole (MTZ, 5 μg, Abtek Biologicals Ltd-UK, Liverpool, UK), Amoxicillin-clavulanic acid (AM, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Tetracycline (TE, 30 μg, Oxoid Ltd., Basingstoke, Hampshire, UK), Trovafloxacin (TROVAN, 30 μg, Pfizer Pharmaceuticals, Berlin, Germany) and Azithromycin (AZM, 15 μg, Oxoid Ltd., Basingstoke, Hampshire, UK).

The inhibition zone was measured after 24 h of aerobic and anaerobic incubation at 37 °C. The experiments of each antibiotic were performed in triplicate. The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) methodology.

2.2.2. Study Design

Used Solvents

In the present study, as a solvent to prepare various solutions and concentrations of honey, phosphate-buffered saline (PBS) was used. A simulation of in vivo (oral cavity) testing was attempted and all the tests for the determination of the minimum inhibitory concentration (MIC), with the difference that artificial saliva also was used as solvent of the honey samples.

The artificial saliva was prepared according to the composition used in dental studies, as shown in Table 1 [52,53]. The mucin used in this study was porcine gastric mucin, which comprises of both human mucins type MUC5AC and type MUC6 [54] and is said to simulate human saliva rymucins (mucin type MUC5B) [55].

Introduction on the Methods of Assessment of Antimicrobial Potency of the Different Honey Types

For the assessment of the antibacterial potency of the different types of honey, two different in vitro methods were used: (i) Agar wells diffusion method, and (ii) determination of the minimum inhibitory concentration by microtiter plates. The first method is based on the inhibition of bacterial growth in a circular zone around the well. The second method is based on the inhibition of bacterial growth in different dilutions of honey. In order to investigate the possible modes of antibacterial action involved, four different techniques of MIC determination by microtiter plates were used: (a1) addition of catalase, with PBS as solvent (a2) addition of catalase, with artificial saliva as solvent, (b1) addition of proteinase K, with PBS as solvent and (b2) addition of proteinase K, with artificial saliva as solvent.

Table 1. Chemical composition of artificial saliva. Reprinted with permission from Refs. [55,56] 2009 Elsevier.

Components in Artficial Saliva		Concetration (g/L)
Sodium chloride	NaCl	1.594
Ammonium nitrate	NH_4NO_3	0.328
Potassium phosphate	KH_2PO_4	0.636
Potassium chloride	KCl	0.202
Potassium citrate	$K_3C_6H_5O_7 \cdot H_2O$	0.308
Uric acid sodium salt	$C_5H_3N_4O_3Na$	0.021
Urea	H_2NCONH_2	0.198
Lactic acid sodium salt	$C_3H_5O_3Na$	0.146
Porcine gastric Mucin Type II		1.35
D- (+) glucose		0.1
α-amylase		100,000 U
Lysozyme		750 U
Water	Four times distilled H_2O and 0.1 M NaOH used to achieve pH 6.8	

Agar Well Diffusion Assay

It should be noted that the agar well diffusion assay was performed only for the 50% dilution of honey to simulate the dilution of honey in the oral cavity. It is estimated that a spoon of honey was screened for its antibacterial activity, according to the agar well diffusion method proposed by the Clinical and Laboratory Standards Institute (CLSI, former NCCLS) guidelines [20]. Briefly, overnight bacterial cultures grown in Mueller-Hinton broth were adjusted to 0.5 McFarland turbidity standard (~1.5×10^8 CFU/mL). Mueller-Hinton agar plates were inoculated with roughly 10^6 CFUs over the entire surface of the plate. Wells of 8 mm in diameter were cut into the surface of the agar using a sterile cork borer. Subsequently, 100 µL (50% *w*/*v* in phosphate-buffered saline (PBS)) of the tested honey types, manuka honey, and artificial honey were added separately to each well. The plates were incubated aerobically and anaerobically at 37 °C for 16–18 h. Antibacterial activity was assessed by measuring (with a calliper) the diameter of the inhibition zones surrounding the wells, including the diameter of the well. The diameter of the inhibition zone, if present in the negative control, was recorded and subtracted from the inhibition zones of the tested honey, as well as of manuka honey. The experiment was repeated three times.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the honey types was determined in sterile 96-well polystyrene microtiter plates (Kisker Biotech GmbH and Co. KG, Steinfurt, Germany) by using a spectrophotometric bioassay, as previously described [20,57]. Briefly, overnight bacterial cultures grown in Mueller-Hinton broth were adjusted to a 0.5 McFarland turbidity standard (~1.5×10^8 CFU/mL). Approximately 5×10^4 CFUs in 10 µL Mueller-Hinton broth were added to 190 µL of 2-fold diluted test honey (honey concentration ranged from 75 to 0.58% *w*/*v*) in Mueller-Hinton broth. Two-fold serial dilutions of the same range of manuka honey and artificial honey were included for comparison. The control wells contained only Mueller-Hinton broth-inoculated with bacteria. The optical density (OD) was determined at 630 nm using a microplate reader (Multi-detection reader, BioTek®), just prior to incubation (t = 0) and after 24 h of incubation (t = 24) at 37 °C. MIC was determined as the lowest honey concentration that results in 100% growth inhibition.

Determination of Minimum Inhibitory Concentration (MIC) Performed After Enzymatic Treatment of Honey Samples with Catalase and Proteinase K

This determination was done following two options:

(a) by using catalase, which degrades hydrogen peroxide, allowing evaluation of the contribution of hydrogen peroxide production to antibacterial activity [58].

(b) by adding proteinase K, with which the part of antibacterial activity that is due to proteins and peptides in honey can be assessed [59].

Catalase stock solution was prepared by dilution of 30 mg powder of catalase originating from bovine liver (SERNA, Heidelberg, Germany) to 10 mL phosphate buffer (pH 7.4. In 1.5 mL honey 50% *v*/*v* (750 µL honey and 750 µL Muller Hinton Broth), 28 µL of the stock solution were added so that the final concentration of catalase was 600 U/mL. The dilution of honey was put in the incubator shaker for 16 h at 37 °C in 210 rounds. Subsequently, all the different honey solutions were prepared to determine the MIC. For the proteinase K stock solution of 10 mg/mL concentration, 10 mg of proteinase powder (Ambion®, Inc., Huntingdon, Cambridgeshire, UK) were diluted in 1 mL distilled water. The same procedure was followed as described for catalase to obtain the different dilutions, so that the final concentration of proteinase Kwas 100 µg/mL. Catalase- and proteinase K-treated honey samples were then used in the antibacterial assay to determine the MIC values, as described in paragraph "Determination of Minimum Inhibitory Concentration (MIC)".

Controls containing no honey (positive growth control), and no honey with catalase or proteinase K (catalase only control/ proteinase K only control) were included to evaluate the effect of catalase/proteinase K alone on bacterial growth.

The elevated MIC values of the treated honey compared to the untreated honey revealed the presence of hydrogen peroxide and/or proteinaceous compounds, which contributed to the antibacterial activity of the tested honey types.

Three-fold samples for every honey and for every concentration were examined.

Statistical Analysis

The physicochemical characteristics of honeys were expressed as means (±SD) of triplicate analyses. Results from the well diffusion assays and minimum inhibitory concentration are also presented as means (±SD) of mm or honey concentration (%). Analysis of variance with Tukey's post-hoc comparison was used to compare either the physicochemical characteristics of the samples or the antibacterial effects. Spearman's rho correlation coefficient was used to indicate any correlation between the physicochemical characteristics and cluster analysis to distinguish groups of observations with similar characteristics. All statistical analyses were performed with SPSS v. 21 statistical package (IBM Corp. Armonk, NY) with a significance level at $p < 0.05$.

3. Results

In citrus honey samples ($n = 20$), pH values ranged from 3.5 to 4.5 with a mean value of 3.8 ± 0.3, hydrogen peroxide from 10.4 to 61.0 µg/g (mean 32.2 ± 15.1 µg/g), free acidity from 10.6 to 27.4 (15.6 ± 4.4), lactonic acidity from 3.1 to 10.3 (6.2 ± 2.3), TPC from 22.4 to 75.6 (41.4 ± 11.9 mg GAE/100 g), and TFC from 0 to 2.5 (1.1 ± 0.5 CE/100 g).

The average values of all variables varied considerably among the samples studied as indicated by the ANOVA procedure (Table 2). Cluster analysis (nearest neighbor method, squared Euclidean) revealed that one major group of observations contained 19 samples and another group consisting of the 3 observations of sample No.4 was distinguished by the increased value of free acidity and TPC, as indicated in Table 3. Correlation analysis revealed only one strong positive correlation between TPC and free acidity (Spearman rho = 0.73, $p < 0.05$).

Table 2. Physicochemical characteristics of the 20 citrus honey samples.

Honey	pH	H_2O_2 (µg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFCmg Quercetin Equivalents (CE)/100 g of Honey
1	3.9	11 ± 0.4 ^{a}	13.6 ± 0.2 ^{de}	9.1 ± 0.2 ^{f}	35.1 ± 0.4 ^{d}	0.65 ± 0.2 ^{ab}
2	3.5	37 ± 0.9 ^{e}	12.1 ± 0.4 ^{bc}	5.3 ± 0.4 ^{d}	29.5 ± 0.2 ^{b}	1.2 ± 0.2 ^{abc}
3	3.7	11 ± 0.56 ^{a}	22.1 ± 0.1 ^{gh}	7.8 ± 0.2 ^{e}	52.1 ± 0.7 ^{h}	0.8 ± 0.7 ^{a}
4	3.5	17 ± 0.8 ^{b}	27 ± 0.6 ^{i}	3.2 ± 0.2 ^{a}	75.1 ± 0.8 ^{j}	2.2 ± 0.3 ^{d}
5	3.5	47 ± 2.54 ^{g}	11.7 ± 0.1 ^{abc}	4.5 ± 0.2 ^{cd}	45.3 ± 0.7 ^{f}	1.9 ± 0.4 ^{cd}
6	3.6	32 ± 1.51 ^{d}	12.8 ± 0.3 ^{cd}	4.5 ± 0.4 ^{cd}	29.2 ± 0.7 ^{b}	0.55 ± 0.3 ^{a}
7	3.8	52 ± 2.74 ^{h}	15.2 ± 0.2 ^{e}	7.1 ± 0.2 ^{ce}	33.4 ± 0.2 ^{c}	0.7 ± 0.2 ^{ab}
8	3.9	41 ± 1.98 ^{ef}	22.2 ± 0.3 ^{h}	4.8 ± 0.1 ^{d}	45.3 ± 0.5 ^{f}	1.1 ± 0.3 ^{abc}
9	3.8	39 ± 1.09 ^{e}	17 ± 0.2 ^{f}	4.1 ± 0.5 ^{abc}	32.4 ± 0.7 ^{c}	0.8 ± 0.2 ^{ab}
10	3.9	40 ± 1.1 ^{ef}	18.1 ± 0.3 ^{f}	7.1 ± 0.3 ^{e}	45.2 ± 0.3 ^{f}	1.2 ± 0.2 ^{ac}
11	3.5	39 ± 0.4 ^{e}	11.2 ± 0.7 ^{ab}	9.4 ± 0.3 ^{fg}	33.7 ± 0.5 ^{cdf}	0.7 ± 0.2 ^{ab}
12	3.6	22 ± 1.04 ^{c}	10.8 ± 0.2 ^{a}	9.2 ± 0.1 ^{f}	44.7 ± 0.2 ^{f}	0.7 ± 0.3 ^{ab}
13	3.9	59 ± 1.77 ^{i}	14.2 ± 0.2 ^{e}	4.2 ± 0.4 ^{bc}	53.1 ± 0.7 ^{h}	0.9 ± 0.1 ^{ab}
14	4.1	55 ± 1.74 ^{hi}	11.8 ± 0.3 ^{abc}	3.5 ± 0.1 ^{ab}	22.7 ± 0.4 ^{a}	1.6 ± 0.2 ^{cd}
15	4.2	24 ± 0.8 ^{c}	14.2 ± 0.7 ^{e}	3.7 ± 0.1 ^{abc}	39.1 ± 0.1 ^{e}	1.5 ± 0.7 ^{abcd}
16	3.7	17 ± 0.8 ^{b}	14.1 ± 0.5 ^{e}	3.3 ± 0.2 ^{a}	44.2 ± 0.2 ^{f}	0.8 ± 0.2 ^{ab}
17	3.6	14 ± 0.8 ^{ab}	17.1 ± 0.4 ^{f}	7.1 ± 0.6 ^{be}	50.2 ± 0.4 ^{g}	0.9 ± 0.1 ^{ab}
18	4.1	29 ± 0.7 ^{d}	21 ± 0.1 ^{g}	8.9 ± 0.1 ^{f}	55.1 ± 0.1 ^{i}	1.2 ± 0.2 ^{bc}
19	4.5	14 ± 2.1 ^{ab}	14.3 ± 0.4 ^{e}	10.2 ± 0.1 ^{g}	29.7 ± 0.2 ^{b}	1.5 ± 0.2 ^{acd}
20	3.9	44 ± 1.11 ^{fg}	11.7 ± 0.2 ^{abc}	7.1 ± 0.7 ^{e}	33.4 ± 0.7 ^{c}	0.7 ± 0.2 ^{ab}
		F = 349.7, $p < 0.01$	F = 443.5, $p < 0.01$	F = 163.9, $p < 0.01$	F = 1800, $p < 0.01$	F = 6.53, $p < 0.01$

Different superscript letters in a column indicate statistically significant differences (ANOVA, Tukey's HSD post-hoc comparison with a 95% confidence level).

Table 3. Centroids of the 2 clusters formed by the physicochemical analysis of the 20 citrus honey samples.

Cluster	pH	H_2O_2 (µg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFCmg Quercetin Equivalents (CE)/100 g of Honey
1	3.8	33.0	15.0	6.36	39.65	1.0
2	3.5	17.0	26.96	3.2	75.1	2.2

In *Satureja* spp. honey samples ($n = 20$), pH values ranged from 3.0 to 4.1 with a mean value of 3.4 ± 0.3, hydrogen peroxide from 2.5 to 15.5 µg/g (mean 6.9 ± 3.9 µg/g), free acidity from 16.2 to 79.9 (33.8 ± 17.5), lactonic acidity from 0.3 to 33.3 (11.8 ± 6.2), TPC from 24.2 to 149.6 (73.4 ± 32.7 mg GAE/100 g), and TFC from 0.6 to 5.3 (2.5 ± 1.2 CE/100 g). The average values of all variables varied considerably among the samples studied as indicated by the ANOVA procedure (Table 4). Cluster analysis (nearest neighbor method, squared Euclidean) revealed that one major group of observations contained 19 samples and another group consisting of the 3 observations of sample 3 were distinguished by the increased value of TPC and the lower value of hydrogen peroxide as indicated in Table 5. There were no strong (Spearman rho > 0.70) and statistically significant ($p < 0.05$) correlations between the various physicochemical characteristics in those honey samples.

Table 4. Physicochemical characteristics of the 20 honey *Satureja* spp. samples.

Honey	pH	H_2O_2 (μg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFC mg Quercetin Equivalents (CE)/100 g of Honey
1	3.2	6 ± 0.7 ^{cd}	22 ± 0.5 ^{c}	16.8 ± 0.2 ^{cde}	78.5 ± 0.1 ^{g}	2.5 ± 0.4 ^{bcde}
2	3.4	14 ± 0.4 ^{g}	17.9 ± 0.2 ^{a}	7.1 ± 0.3 ^{abcd}	99.3 ± 2.1 ^{h}	3.2 ± 0.3 ^{e}
3	3.6	3 ± 0.6 ^{a}	28 ± 0.3 ^{d}	16.5 ^{bcde}	117.1 ± 1.4 ^{j}	1.7 ± 0.5 ^{abcd}
4	4.1	3 ± 0.2 ^{a}	17 ± 0.8 ^{a}	1.7 ± 0.2 ^{a}	65.2 ± 0.7 ^{e}	2.9 ± 0.2 ^{bcde}
5	3.9	5 ± 0.1 ^{bc}	17.8 ± 0.9 ^{a}	18.2 ± 0.3 ^{de}	55.7 ± 0.3 ^{d}	5.2 ± 0.1 ^{f}
6	3.6	7 ± 0.4 ^{de}	28 ± 0.4 ^{d}	6.8 ± 0.1 ^{abcd}	147.8 ± 1.7 ^{l}	3.4 ± 0.4 ^{de}
7	3.2	11 ± 0.3 ^{f}	28 ± 0.1 ^{d}	6.5 ± 0.3 ^{abc}	130.1 ± 2.1 ^{k}	1.4 ± 0.4 ^{ab}
8	3.5	8 ± 0.4 ^{e}	45.2 ± 0.4 ^{f}	17.1 ± 0.2 ^{cde}	47.8 ± 0.7 ^{c}	0.9 ± 0.2 ^{a}
9	3.1	8 ± 0.7 ^{e}	55 ± 0.2 ^{h}	8.5 ± 0.3 ^{abcd}	55.2 ± 0.7 ^{d}	3.6 ± 0.4 ^{e}
10	3.0	10 ± 0.4 ^{f}	28 ± 0.1 ^{d}	7.8 ± 0.1 ^{abcd}	65.7 ± 0.9 ^{e}	1.7 ± 0.4 ^{abc}
11	3.6	8 ± 0.1 ^{e}	17 ± 0.3 ^{a}	11.2 ± 0.3 ^{abcd}	40.3 ± 0.5 ^{b}	2.2 ± 0.4 ^{bcd}
12	3.7	6 ± 0.1 ^{cd}	72 ± 0.1 ^{i}	5.3 ± 0.6 ^{ab}	65.1 ± 0.8 ^{e}	2.3 ± 0.1 ^{bcd}
13	4.0	3 ± 0.4 ^{a}	32.2 ± 0.4 ^{e}	11.5 ± 0.3 ^{abcde}	110.1 ± 3.2 ^{i}	3.1 ± 0.4 ^{de}
14	3.1	3 ± 0.3 ^{a}	79.7 ± 0.2 ^{j}	10.9 ± 0.1 ^{abcd}	72.3 ± 1.1 ^{f}	1.4 ± 0.4 ^{ab}
15	3.3	15 ± 0.7 ^{g}	32 ± 0.2 ^{e}	16.2 ± 0.7 ^{bcde}	58.1 ± 2.3 ^{d}	5.2 ± 0.2 ^{f}
16	3.0	5 ± 0.5 ^{bc}	27.3 ± 0.1 ^{d}	16.8 ± 0.7 ^{bcde}	40.1 ± 3.1 ^{b}	0.9 ± 0.2 ^{a}
17	3.6	4 ± 0.1 ^{b}	31 ± 0.2 ^{e}	10.8 ± 0.4 ^{abcd}	24.8 ± 0.9 ^{a}	3.5 ± 0.9 ^{de}
18	3.1	3 ± 0.4 ^{a}	19.3 ± 0.4 ^{b}	22.7 ± 0.2 ^{e}	98.2 ± 3.1 ^{h}	1.7 ± 0.2 ^{abc}
19	3.2	14 ± 0.2 ^{g}	32 ± 0.2 ^{e}	17.5 ± 0.3 ^{cde}	59.5 ± 0.4 ^{d}	2.2 ± 0.4 ^{bcd}
20	3.1	3 ± 0.1 ^{a}	48.4 ± 0.3 ^{g}	6.9 ± 0.2 ^{abcd}	37.8 ± 2.1 ^{b}	1.7 ± 0.7 ^{abc}
		$F = 286.6, p < 0.01$	$F = 6444, p < 0.01$	$F = 6.72, p < 0.01$	$F = 1149, p < 0.01$	$F = 28.23, p < 0.01$

Different superscript letters in a column indicate statistically significant differences (ANOVA, Tukey's HSD post-hoc comparison with a 95% confidence level).

Table 5. Centroids of the 2 clusters formed by the physicochemical analysis of the 20 *Satureja* spp. honey samples.

Cluster	pH	H_2O_2 (μg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFC mg Quercetin Equivalents (CE)/100 g of Honey
1	3.4	6.9	33.9	11.4	72.66	2.5
2	3.6	3.6	28.3	33.3	115.9	1.5

In oregano and sage honey samples ($n = 20$), the pH ranged from 3.0 to 4.2 with a mean value of 3.4 ± 0.3, hydrogen peroxide from 4.6 to 17.3 μg/g (mean 9.6 ± 3.4 μg/g), free acidity from 27.4 to 99.3 (49.1 ± 20.8), lactonic acidity from 3.6 to 22.3 (11.2 ± 4.4), TPC from 22.6 to 89.1 (47.6 ± 17.4), and TFC from 0.6 to 7.0 (3.5 ± 1.3).

The average values of all variables varied considerably among the samples studied as indicated by the ANOVA procedure (Table 6). However, cluster analysis (nearest neighbor method, squared Euclidean) revealed some similarities among the samples based on the results of the physicochemical examination. As indicated in Table 7, all samples had similar physicochemical characteristics, except for sample 14, which had almost twice the concentration of hydrogen peroxide and TPCm but approximately half the concentration of free acidity and TPC. No strong correlations (Spearman rho > 0.7, $p < 0.05$) were observed between the various physicochemical parameters of oregano and sage honey samples.

Table 6. Physicochemical characteristics of the 20 oregano and sage honey samples.

Honey	pH	H_2O_2 (μg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFC mg Quercetin Equivalents (CE)/100 g of Honey
1	3.1	11 ± 0.2^{de}	52 ± 0.3^{i}	11.5 ± 0.1^{d}	38.2 ± 0.7^{d}	5.1 ± 0.7^{fgh}
2	3.6	9 ± 0.3^{c}	38.2 ± 0.7^{e}	15.2 ± 0.7^{ef}	44.2 ± 0.2^{e}	3.7 ± 0.5^{cdef}
3	3.5	6 ± 0.7^{ab}	99.1 ± 0.2^{o}	9.1 ± 0.2^{bc}	86.1 ± 3.1^{k}	4.4 ± 0.6^{defg}
4	3.2	5 ± 0.2^{a}	87 ± 0.2^{n}	4.5 ± 0.7^{a}	45.3 ± 1.3^{e}	4.1 ± 0.2^{def}
5	3.5	5 ± 0.3^{a}	65.1 ± 0.7^{k}	11.2 ± 0.7^{d}	55.7 ± 0.9^{g}	2.8 ± 0.4^{bcd}
6	4.1	11 ± 0.7^{de}	55 ± 0.1^{j}	14.2 ± 0.7^{e}	62.7 ± 2.1^{h}	2.9 ± 0.6^{bcd}
7	3.6	13 ± 0.3^{f}	47.8 ± 0.3^{h}	5.6 ± 0.2^{a}	33.9 ± 0.7^{c}	3.3 ± 0.4^{cd}
8	3.0	10 ± 0.4^{cd}	38.2 ± 0.9^{e}	7.8 ± 0.2^{b}	61.4 ± 0.4^{h}	4.7 ± 0.4^{defg}
9	3.1	12 ± 0.1^{ef}	71 ± 0.1^{l}	11.3 ± 0.5^{bd}	50.7 ± 2.7^{f}	3.7 ± 0.2^{cef}
10	3.2	5 ± 0.4^{a}	41.2 ± 0.2^{g}	11.1 ± 0.7^{d}	42.7 ± 0.2^{de}	3.1 ± 0.4^{bcde}
11	3.5	13 ± 0.2^{f}	32.7 ± 0.3^{bc}	22.3 ± 01^{h}	32.9 ± 3.5^{bc}	2.3 ± 0.2^{abc}
12	3.6	11 ± 0.2^{de}	27.9 ± 0.7^{a}	17.2 ± 0.7^{g}	40.8 ± 0.8^{de}	1.8 ± 0.3^{ab}
13	3.0	7 ± 0.2^{b}	32.1 ± 0.7^{b}	10.3 ± 0.1^{cd}	22.8 ± 0.2^{a}	6.2 ± 0.8^{h}
14	3.5	17 ± 0.4^{h}	29.2 ± 0.7^{a}	16.3 ± 0.4^{fg}	85.3 ± 0.2^{k}	3.1 ± 0.3^{bcde}
15	4.2	11 ± 0.2^{de}	33.2 ± 0.4^{bc}	10.2 ± 0.7^{cd}	37.8 ± 0.8	1.1 ± 0.5^{a}
16	3.9	15 ± 0.3^{g}	35.7 ± 0.2^{d}	4.2 ± 0.4^{a}	29.1 ± 0.7^{b}	2.4 ± 0.3^{abc}
17	3.1	7 ± 0.2^{b}	40.2 ± 0.4^{fg}	14.2 ± 0.1^{e}	33.8 ± 0.7^{c}	4.1 ± 0.8^{def}
18	3.2	6 ± 0.1^{ab}	33.7 ± 0.2^{c}	7.8 ± 0.2^{b}	41.5 ± 0.8^{de}	3.3 ± 0.4^{cd}
19	3.0	7 ± 0.3^{b}	85.1 ± 0.6^{m}	9.1 ± 0.7^{c}	71.2 ± 1.7^{j}	3.6 ± 0.2^{bcd}
20	3.6	12 ± 0.2^{ef}	39.7 ± 0.2^{f}	11.7 ± 0.1^{d}	37.5 ± 0.7^{i}	5.8 ± 0.6^{egh}
		$F = 315.03$, $p < 0.01$	$F = 6047$, $p < 0.01$	$F = 243.8$, $p < 0.01$	$F = 428.6$, $p < 0.01$	$F = 21.4$, $p < 0.01$

Different superscript letters in a column indicate statistically significant differences (ANOVA, Tukey's HSD post-hoc comparison with a 95% confidence level).

Table 7. Centroids of the 2 clusters formed by the physicochemical analysis of the 20 oregano and sage honey samples.

Cluster	pH	H_2O_2 (μg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFC mg Quercetin Equivalents (CE)/100 g of Honey
1	3.2	9.21	50.21	10.92	45.65	3.55
2	3.5	16.9	29.13	16.23	85.23	3.03

In Table 8, the mean (±standard deviation) of physicochemical characteristics of the three types of honey included in the study, i.e., citrus honey, honey from *Satureja* spp. and oregano and sage honey are presented.

Among the statistically significant differences observed between the characteristics of the various honey types, the most profound were the differences in free acidity and hydrogen peroxide concentration (Table 8). Honey samples produced from hives foraging citrus varieties of plants exhibit approximate half of the acidity of honeys produced from *Satureja* spp. and almost one third of the ones produced from oregano and sage plant species. However, as shown in Table 8, citrus honeys contained three to five times more the concentration of hydrogen peroxide compared to the other two types.

Table 8. Physicochemical characteristics of the honey types used in the study.

Honey samples (n)	pH	H_2O_2 (μg/g Unprocessed Honey)	Free Acidity	Lactonic Acidity	TPC (mg GAE/100 g of Honey)	TFC mg Quercetin Equivalents (CE)/100 g of Honey
Citrus (20)	3.81 ± 0.3 [a]	32.19 ± 15.0 [a]	15.6 ± 4.3 [a]	6.2 ± 2.3 [a]	41.6 ± 11.9 [a]	1.07 ± 0.5 [a]
Satureja spp. (20)	3.41 ± 0.3 [b]	6.89 ± 3.9 [b]	33.8 ± 17.4 [b]	11.8 ± 6.2 [b]	73.4 ± 44.6 [b]	2.48 ± 1.2 [b]
Oregano and sage	3.42 ± 0.3 [b]	9.59 ± 3.5 [b]	49.1 ± 20.8 [c]	11.2 ± 4.4 [b]	47.6 ± 17.4 [a]	3.52 ± 1.5 [c]
	F = 30.02, $p < 0.05$	F = 136.1, $p < 0.05$	F = 66.9, $p < 0.05$	F = 26.5, $p < 0.05$	F = 34.0, $p < 0.05$	F = 76.9, $p < 0.05$

Different superscript letters in a column indicated statistically significant differences among the honey types for each characteristic (ANOVA, Tukey's HSD post-hoc comparison).

3.1. Antimicrobial Activity

In well diffusion assays, all honey samples had an antibacterial effect against pathogens (and reference strains) when compared with artificial honey, where only the osmotic effect is considered (Table 9, Figure 1a–g). The average of inhibition zones from honeys of *Satureja* spp. were 16.7 ± 4.1 mm, oregano and sage 15.9 ± 3.5 mm, and of citrus 15.9 ± 3.9 mm indicating that samples of *Satureja* spp. were among the most effective, particularly against S. *aureus* A, *S. aureus* B, *S. aureus* ATCC 12600, *S. mutans,* and *F. nucleatum.* The most sensitive strain was proven to be *S. mutans,* with an average inhibition zone of 19.5 ± 4.7 mm and the least sensitive being *S. anaerobius* and *F. nucleatum,* with average inhibition zones of 14.0 ± 3.8 mm and 14.3 ± 3.1 mm, respectively, which indicates differences among the susceptibility of the strains (ANOVA F = 25.64, $p < 0.05$). Almost all honey samples were more effective against pathogens than the manuka honey. Overall, the manuka honey gave an average inhibition zone of 13.7 ± 2.2 mm in comparison to 16.0 ± 4.0 mm of the local samples. In these experiments, *F. nucleatum* was the least sensitive to manuka (mean zone 11.7 ± 0.9 mm) and *S. aureus* ATCC 12600 the most sensitive (mean zone 15.9 ± 2.5 mm).

The results from MIC experiments showed that local honey samples were effective enough against the seven bacteria tested alone or after treatment with proteinase K, catalase, or by using artificial saliva as diluent. A sample of commercially available manuka honey, equally diluted, was included in the study for comparison (Figures 2a–g and 3a–g). On average 9.3 ± 7.6% (*w/v*) of honey inhibited the growth of our pathogens. Again, samples from *Satureja* spp. floral source were the most effective (mean MIC 8.2 ± 6.2%) followed by citrus honey (mean MIC 9.6 ± 6.9%) and oregano and sage (mean MIC 10.2 ± 9.2%). In those experiments, *S. aureus* A was the most sensitive strain (mean MIC 8.1 ± 3.6%) and *S. aureus* spp. *anaerobius* the least sensitive (mean MIC 17.0 ± 13.0) with the rest of the pathogens being among the above. When the above experiments were repeated with manuka honey, an increase to the MIC (or a decrease in effectiveness) was observed from 9.3 ± 7.6 to 10.7 ± 6.4 for all strains and honey samples (ANOVA F = 20.9, $p < 0.05$). *S. mutans* (MIC 6.2 ± 0.0), *S. aureus* ATCC 12600 (MIC 6.2 ± 0.4), *S. anaerobius* (MIC 6.4 ± 1.1), and *S. anaerobius* ATCC 35844 (MIC 6.25 ± 0) were the most sensitive to manuka honey and *F. nucleatum* the least, with a mean MIC of 25.0 ± 0.

Table 9. Antimicrobial activity of honeys: Minimum inhibitory concentration (MIC) expressed in % *w*/*v* by broth dilution and inhibition diameter (mm) including well (8.0 mm) of honeys at 75% by well diffusion assays (WDA) against various pathogens.

Bacterial Species	Honey Origin (N)	Well Diffusion Assay (mm)		MIC 75% (*w*/*v*)				MIC % (*w*/*v*) Using Artificial Saliva			
		Honey	Manuka	Honey	Manuka	+ Catalase	+ Proteinase K	Honey	Manuka	+ Catalase	+ Proteinase K
S. aureus A	Citrus (20)	13.9 ± 3.6^{b1}	12.4 ± 0.9^{a2}	8.9 ± 3.7^{b1}	12.5 ± 0^{a2}	30.6 ± 13.5^{b3}	11.8 ± 6.5^{b2}	7.9 ± 3.9^{a1}	21.9 ± 5.4^{a2}	27.5 ± 16.7^{a3}	9.2 ± 8.2^{a1}
	Satureja spp. (20)	17.1 ± 4.4^{c1}	12.3 ± 1.5^{a2}	7.3 ± 3.6^{a1}	12.5 ± 0^{a2}	7.5 ± 3.5^{a1}	7.6 ± 3.3^{a1}	6.7 ± 3.6^{a1}	23.1 ± 4.5^{a2}	7.5 ± 3.5^{b1}	6.1 ± 5.4^{b1}
	Oregano and sage (20)	15.1 ± 2.3^{b1}	12.1 ± 1.2^{a2}	8.1 ± 3.4^{ab1}	12.5 ± 0^{a2}	8.1 ± 3.3^{a1}	16.5 ± 7.2^{c2}	7.2 ± 4.1^{a1}	25.0 ± 0^{b3}	8.1 ± 3.4^{b1}	12.8 ± 8.3^{c2}
	Artificial honey	8.0 ± 0^{a1}	13.0 ± 20^{a2}								
S. aureus B	Citrus (20)	13.4 ± 2.3^{b1}	12.1 ± 1.1^{a2}	10.3 ± 3.0^{b1}	12.2 ± 1.4^{b1}	31.6 ± 16.1^{b2}	10.5 ± 4.7^{a1}	9.4 ± 3.6^{c1}	14.1 ± 4.8^{b2}	29.4 ± 16.4^{a3}	7.8 ± 4.8^{a1}
	Satureja spp. (20)	17.5 ± 4.2^{c1}	13.0 ± 1.3^{b2}	6.4 ± 3.4^{a1}	11.8 ± 1.9^{b3}	8.6 ± 5.0^{a2}	8.7 ± 4.9^{a2}	6.4 ± 3.4^{a1}	11.9 ± 1.9^{a3}	8.8 ± 7.3^{b2}	7.3 ± 5.6^{a12}
	Oregano and sage (20)	16.1 ± 2.4^{c1}	12.5 ± 0.9^{ab2}	7.8 ± 3.6^{a1}	10.9 ± 2.7^{a2}	8.4 ± 3.4^{a1}	14.4 ± 7.5^{b3}	7.8 ± 3.7^{b1}	10.9 ± 2.7^{a2}	11.6 ± 6.6^{b2}	12.8 ± 6.9^{b2}
	Artificial honey	8.0 ± 0^{a1}	13.0 ± 1.2^{ab2}								
S. aureus ATCC 12600	Citrus (20)	17.0 ± 1.2^{b1}	16.3 ± 1.3^{a1}	5.8 ± 1.1^{b1}	6.25 ± 0^{a1}	25.6 ± 13.9^{c2}	6.9 ± 2.7^{a1}	7.8 ± 4.4^{c1}	6.25 ± 0^{1}	22.5 ± 7.6^{a2}	6.25 ± 0^{b1}
	Satureja spp. (20)	19.3 ± 1.8^{d1}	15.5 ± 1.1^{a2}	4.8 ± 1.9^{a1}	6.1 ± 0.7^{a2}	7.3 ± 3.6^{a3}	5.8 ± 2.9^{a12}	2.9 ± 0.6^{a1}	6.25 ± 0^{2}	7.8 ± 5^{b3}	1.2 ± 3.7^{a4}
	Oregano and sage (20)	18.3 ± 1.6^{c1}	16.0 ± 4.0^{a2}	5.2 ± 1.6^{ab1}	6.25 ± 0^{a2}	15.3 ± 8.3^{b4}	8.6 ± 5.8^{b3}	5.5 ± 1.4^{b1}	6.25 ± 0^{12}	7.8 ± 6.1^{b2}	8.4 ± 7.2^{c2}
	Artificial honey	8.0 ± 0^{a1}	16.0 ± 2.0^{a2}								
S. aureus anaerobius	Citrus (20)	18.7 ± 2.7^{c1}	14.9 ± 1.5^{a2}	5.54 ± 1.4^{a1}	6.6 ± 1.4^{a1}	24.1 ± 14.4^{b2}	7.0 ± 2.4^{a1}	4.7 ± 1.6^{a1}	12.5 ± 0^{a2}	7.2 ± 2.2^{a3}	5.5 ± 3.9^{a1}
	Satureja spp. (20)	11.4 ± 1.4^{b1}	15.1 ± 1.4^{a2}	16.1 ± 10.4^{b2}	6.25 ± 0^{a1}	15.1 ± 8.7^{a2}	14.4 ± 10.3^{b2}	10.5 ± 5.8^{b12}	11.9 ± 2.7^{a12}	12.9 ± 8.6^{b2}	9.8 ± 5.2^{b1}
	Oregano and sage (20)	12.2 ± 1.3^{b1}	15.3 ± 1.5^{a2}	29.4 ± 10.7^{c2}	6.6 ± 1.4^{a1}	29.4 ± 10.7^{c2}	26.5 ± 14.1^{c2}	25.0 ± 19.5^{c2}	11.9 ± 4.8^{a1}	24.7 ± 14.6^{c2}	10.0 ± 11.6^{b1}
	Artificial honey	8.0 ± 0^{a1}	15.0 ± 1.4^{a2}								
S. aureus anaerobius ATCC 35844	Citrus (20)	18.7 ± 1.8^{c1}	14.3 ± 0.9^{a2}	5.4 ± 1.5^{a1}	6.25 ± 0^{a1}	30.0 ± 12.8^{b2}	6.6 ± 2.9^{a1}	4.9 ± 1.8^{a1}	12.5 ± 0^{b2}	10.9 ± 3.2^{a3}	7.8 ± 4.4^{a4}
	Satureja spp. (20)	13.6 ± 1.2^{b1}	14.8 ± 1.2^{ab}	12.2 ± 5.0^{c2}	6.25 ± 0^{a1}	13.9 ± 9.5^{a23}	16.8 ± 6.5^{c3}	9.8 ± 3.7^{b1}	11.25 ± 3.8^{a1}	10.1 ± 3.8^{a1}	12.5 ± 9.8^{b1}
	Oregano and sage (20)	14.0 ± 1.2^{b1}	15.1 ± 1.0^{b2}	8.3 ± 3.6^{b1}	6.25 ± 0^{a1}	28.4 ± 13.5^{b2}	8.7 ± 4.9^{b1}	16.6 ± 15^{c2}	11.9 ± 2.7^{ab12}	9.7 ± 5.8^{a1}	22.5 ± 15.7^{c3}
	Artificial honey	8.0 ± 0^{a1}	15.0 ± 2.0^{ab1}								
Streptococcus mutans	Citrus (20)	18.3 ± 4.7^{b1}	14.1 ± 2.7^{a2}	6.2 ± 3.4^{b1}	6.25 ± 0^{a1}	22.5 ± 17.4^{b2}	8.3 ± 5.3^{b1}	6.1 ± 0.7^{a1}	6.25 ± 0^{a1}	6.25 ± 3.4^{a1}	5.6 ± 1.9^{a1}
	Satureja spp. (20)	20.7 ± 3.7^{c1}	13.7 ± 2.5^{a2}	4.5 ± 1.8^{a1}	6.25 ± 0^{a2}	6.1 ± 2.5^{a2}	5.1 ± 2.4^{a1}	6.1 ± 0.7^{a1}	6.25 ± 0^{a1}	6.25 ± 0^{a1}	4.0 ± 7.7^{a2}
	Oregano and sage (20)	20.1 ± 4.9^{bc1}	13.8 ± 2.5^{a2}	6.25 ± 0^{b1}	6.25 ± 0^{a1}	9.4 ± 6.0^{a2}	8.1 ± 4.5^{b2}	6.25 ± 0^{a1}	6.25 ± 0^{a1}	6.9 ± 1.9^{a1}	8.1 ± 4.0^{b2}
	Artificial honey	8.0 ± 0^{a1}	14.0 ± 2.0^{a2}								
Fusobacteriumnucleatum	Citrus (20)	11.3 ± 1.3^{b1}	12.1 ± 1.0^{b2}	25.0 ± 0^{c1}	25.0 ± 0^{a1}	18.7 ± 20.9^{b2}	14.1 ± 12.2^{b2}	11.6 ± 2.2^{a1}	12.5 ± 0^{a1}	13.7 ± 21.8^{b1}	22.5 ± 7.6^{c2}
	Satureja spp. (20)	16.9 ± 2.2^{d1}	11.5 ± 0.9^{a2}	5.9 ± 0.9^{a1}	25.0 ± 0^{a3}	8.4 ± 5.7^{a2}	7.5 ± 4.7^{a12}	5.6 ± 1.2^{b1}	12.5 ± 0^{a2}	5.3 ± 2.2^{a1}	1.6 ± 3.9^{a3}
	Oregano and sage (20)	15.1 ± 2.1^{c1}	11.5 ± 0.8^{a2}	6.25 ± 0^{b1}	25.0 ± 0^{a3}	13.1 ± 9.3^{ab2}	5.6 ± 2.7^{a1}	5.3 ± 1.7^{b1}	12.5 ± 0^{a3}	13.7 ± 7^{b3}	9.7 ± 6.7^{b2}
	Artificial honey	8.0 ± 0^{a1}	10.9 ± 1.0^{ab2}								

Different superscript lower letters in columns indicate statistically significant differences (ANOVA with Tukey's HSD post-hoc comparison) between the honey types of each experiment and for each strain. Different superscript numbers in rows indicate statistically significant differences (ANOVA with Tukey's HSD post-hoc comparison) for each honey within the various experiments (well diffusion assay, MIC with diluted honey, MIC with artificial saliva).

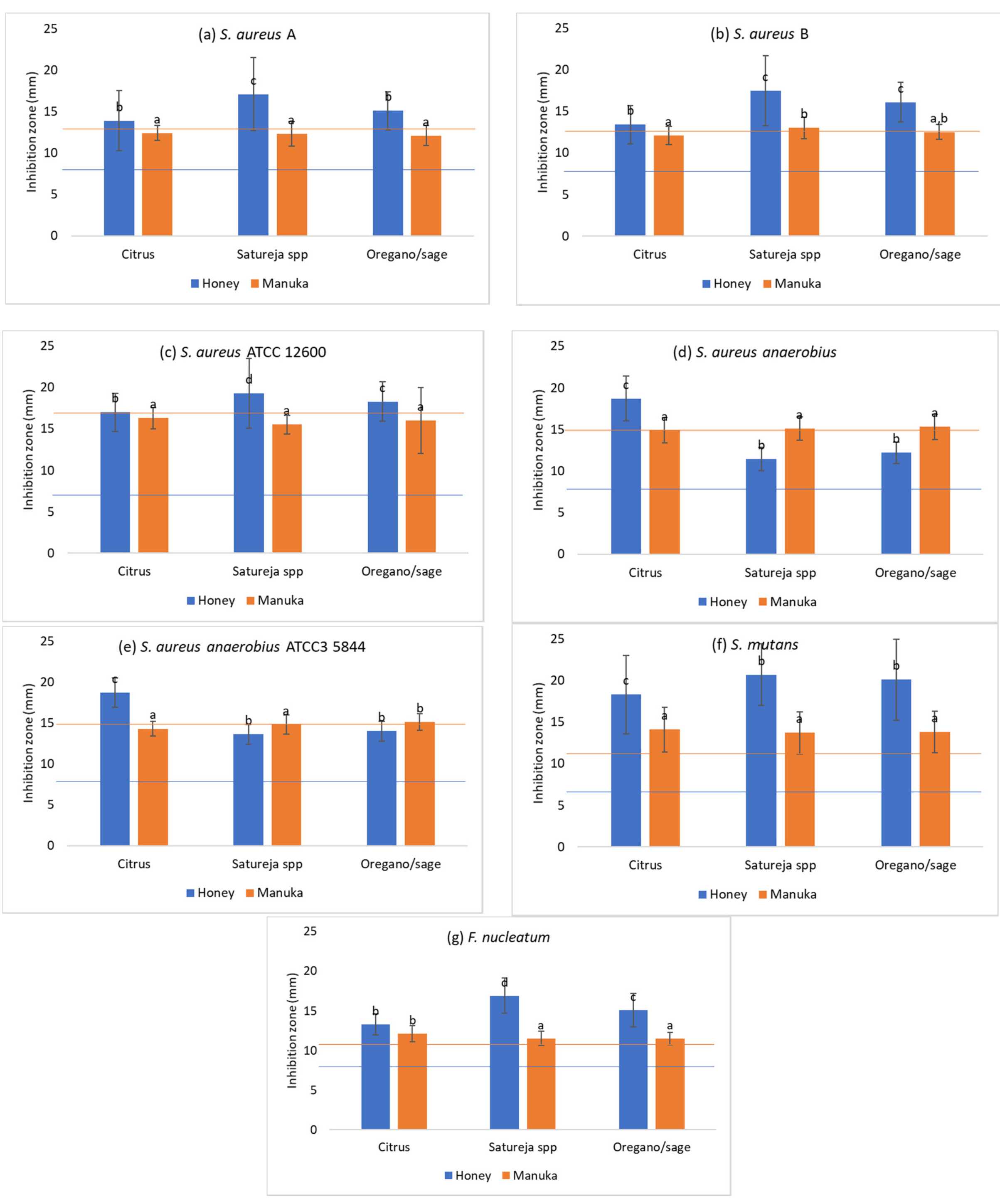


Figure 1. (**a**–**g**): Antimicrobial effect of local and manuka honey samples estimated by the well diffusion assay against seven pathogens. Lines indicate the inhibition zone of artificial honey in local honey experiments (blue) and with manuka (yellow).

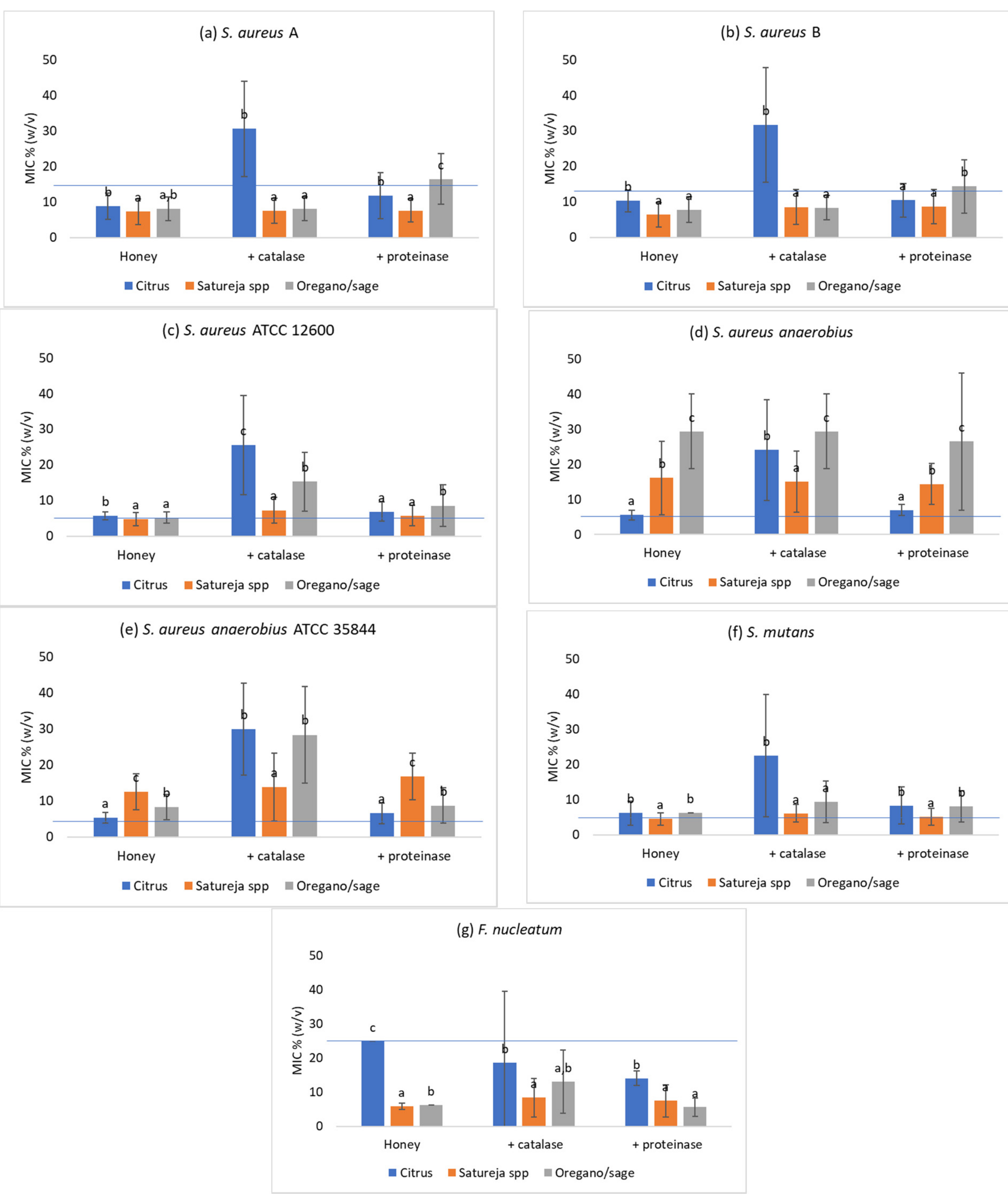


Figure 2. (**a**–**g**): Minimum inhibitory concentration of local (n = 60) honey samples treated with and without catalase or proteinase, as well as manuka honey (n = 1) against seven oral pathogens. Different letters indicate differences among the types of honey within each treatment. Line (blue) indicates the MIC value of manuka.

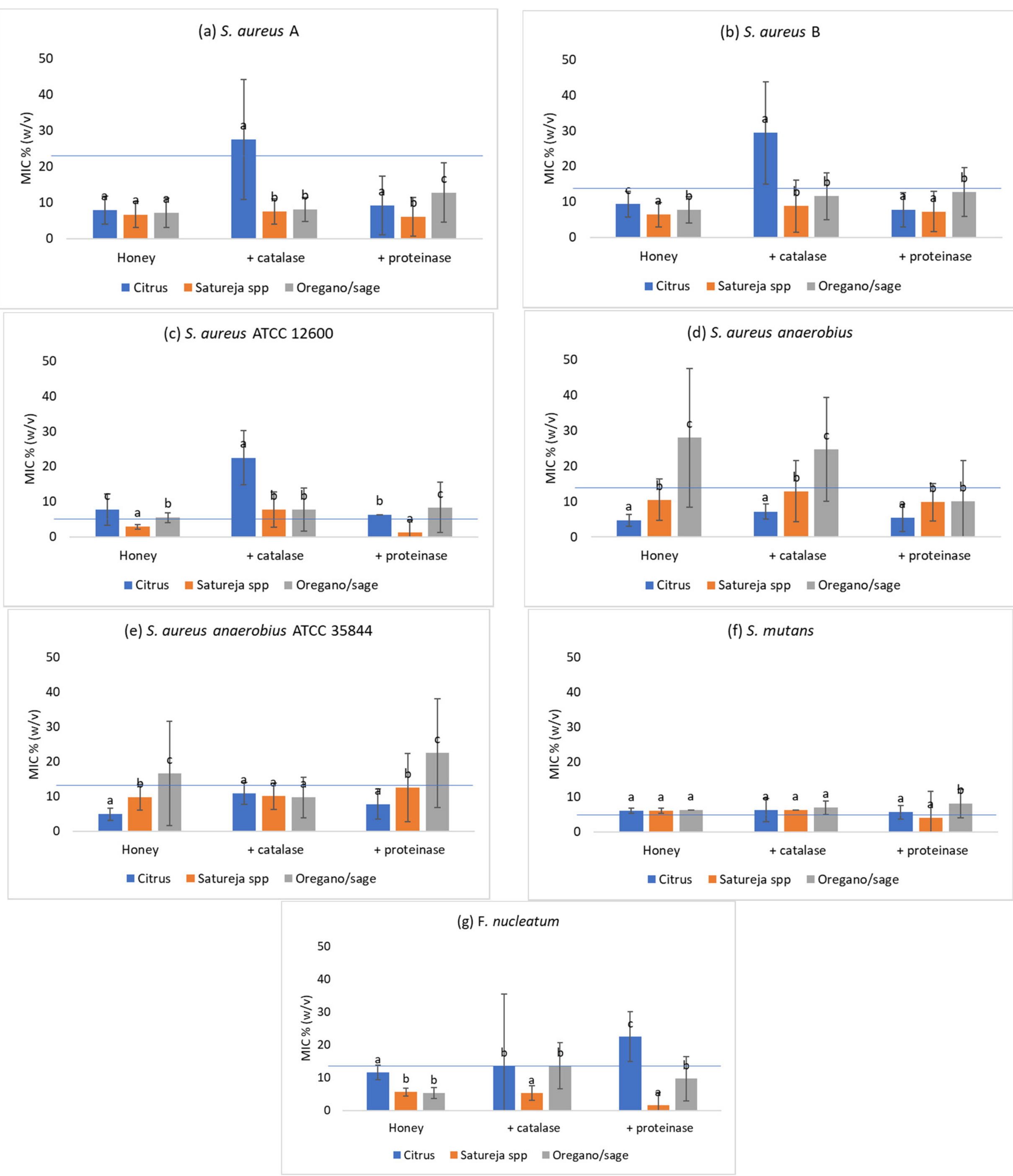


Figure 3. (**a**–**g**): Minimum inhibitory concentration of local (n = 60) honey samples treated with and without catalase or proteinase as well as manuka honey (n = 1) against seven oral pathogens with artificial saliva as diluent. Different letters indicate differences among the types of honey within each treatment. Line (blue) indicates the MIC value of manuka.

As already mentioned, the estimation of the minimum inhibitory concentration was accomplished first by using multiple dilutions of honey in deionized water and again with artificial saliva as dilutant. In those later experiments there was a decrease in MIC values below the values presented in MIC experiments with ordinary dilutions, or in other words, an increase in effectiveness of the local honeys. The reduction in MIC values was statistically significant (ANOVA F = 11.6, $p < 0.05$) from an average of 9.3 ± 7.6 to 8.3 ± 7.6, which is proof of the active involvement of (artificial) saliva in the antimicrobial action of honey. However, the use of artificial saliva with manuka honey did not result in a similar

increase of effectiveness but on the contrary, there was an average increase in MIC by 1.5% from 10.6 ± 6.4 to 12.1 ± 5.9%.

Finally, as expected the results from the well diffusion estimations were negatively correlated with those of the MIC (Spearman Rho = −0.65, $p < 0.05$), indicating an overall consistency of our data.

3.2. Antibiotic Susceptibility of Used Strains

All strains were tested for their susceptibility in common antibiotics. *S. aureus* (A) and (B) (both methicillin and vancomycin resistant) were proven to be multi-resistant in 66.7% of the drugs (Figure 4). *S. mutans*. was also multi-resistant in 7 out of 19 antibiotics. The two *S. aureus* reference strains were sensitive in all tested antibiotics, while *F. nucleatum* was resistant only in ampicillin (10 µg) and erythromycin (15 µg).

Strain	AMC	AMO	AMP	AZM	CC	CFT	CHL	CIP	CTE	CTX	DO	E	GEN	KZ	L	ME	MTZ	OX	OF	OXY	P10	RIF	SLT	TE	TROVAN	VA
S. aureus A	R		R			S		R				S						R		S			R			R
S. aureus B	R		R			S		R				S						R		S			R			R
S. aureus anaerobius	R		R			S		S				S						S		S			R			S
S. aureus ATCC 12600	S		S			S		S				S						S		S						S
S. aureus anaerobius ATCC35844	S		S			S		S				S						S		S						S
S. mutans		R	R		S		S	S	S	R	S	R	S	R	S	S	S		S		R	S		S		R
F. nucleatum	S		R	S	S							R					S							S	S	

Figure 4. Antibiotic susceptibility profile of the strains used in the study (see in the text for antibiotic abbreviations).

3.3. Effect of Physicochemical Characteristics in Antimicrobial Action

Multiple regression analysis with the results of well diffusion assays or MICs (with and without artificial saliva) as the dependent variable and the physicochemical characteristics of the honey samples as independent variables, showed that (a) no significant contribution of these characteristics was observed in well diffusion assays for the three types of honey studied (ANOVA F = 2.06, $p > 0.05$); (b) similarly, no critical factors were identified in the manuka well diffusion experiments (ANOVA F = 0.06, $p > 0.05$), (c) only TPC was a critical factor (ANOVA F = 10.6, $p < 0.05$) for MIC with no artificial saliva results, (c) all physicochemical characteristics (except pH) were contributing factors in the results of MIC experiments with the addition of catalase (ANOVA F = 63.7, $p < 0.05$) and all factors (except pH and TPC) contributed in MIC values in experiments with the addition of proteinase (ANOVA | F = 5.44, $p < 0.05$). When artificial saliva was used as a diluent in MIC experiments, multiple regression analysis revealed that the contributing factors were free acidity and TPC (ANOVA F = 8.19, $p < 0.05$) when the three types of honey were tested. In MIC experiments with artificial saliva and catalase addition, the concentration of hydrogen peroxide, lactone acidity and TPC were indicated as contributors (ANOVA F = 20.17, $p < 0.05$). When proteinase was added, then all physicochemical characteristics (except pH and TFC) were identified as critical factors (ANOVA F13.26, $p < 0.05$) while no characteristics appeared to contribute in manuka MIC experiments (ANOVA F = 1.07, $p > 0.05$).

3.4. Effect of Honey pH in Antimicrobial Action

The pH of the various honey samples ranged from 3.0 to 4.5 with a mean of 3.55 ± 0.36. Although there were statistically significant differences among the pH values from different floral sources (citrus, *Satureja* spp., oregano and sage) as shown in Table 9, it was not statistically possible to correlate (positively or negatively) these values to the antimicrobial action observed both in well diffusion experiments and in MIC estimations.

3.5. Effect of the Addition of Catalase

Except for *F. nucleatum*, all other pathogens including the reference ones exhibited a significant increase in MIC (Table 9) after the treatment with catalase. This is an indication

of decrease in the antimicrobial action of the honey samples and particularly in those of citrus origin. A profound decrease of antimicrobial action was the most noticeable among the reference strains, with the new MIC values being 4 to 6 times higher than those without catalase treatment. As already stated, *F. nucleatum* was the only strain in which the addition of catalase increases the susceptibility.

In most of the MIC experiments with artificial saliva as diluent, no statistically significant differences were observed. A significant increase of MIC was observed in citrus and oregano and sage honeys against *F. nucleatum* and in all honeys against *S. aureus* A, *S. aureus* B, and *S. aureus* ATCC 12600.

3.6. Effect of Proteinase K

In general, and regardless of the pathogen or the floral source, almost all honey samples showed a reduced activity after the proteinase K treatment from a total MIC average of 9.3 $\pm$ 7.6 (% w/v) to 10.4 $\pm$ 8.1 (% w/v). In one third of the experiments, those differences were statistically significant, as indicated in Table 9, while the rest showed no differences. Increased antimicrobial activity after proteinase K treatment was noted from citrus samples against *F. nucleatum*. Similar results were also obtained in MIC experiments where artificial saliva was used as diluent.

4. Discussion

Dental decay has been known since recorded history, but was not an important health problem until sucrose became a major component of the human diet. When sucrose is consumed frequently, an organism known as *S.mutans* emerges as the predominant organism, and it is this organism that has been uniquely associated with dental decay [60]. The tooth surface initially represents a carrier state relative to harboring this primary cariogen in the dental plaque on a smooth surface. The proportion of the cariogen in the flora is similar in both cases, but the location of *S. mutans* differs within the plaques. In the tooth destined to develop decay, *S. mutans* is located on the enamel surface, whereas in the tooth destined to remain caries free, *S. mutans* is confined to the saliva-plaque interface [61]. Debriding procedures, such as toothbrushing and flossing, might remove most plaque organisms except of *S.mutans*, including *S. aureus* and *F. nucleatum*, tested in this study, but could leave untouched those bacteria either firmly attached to the enamel surface or sequestered in defects and cavities in the enamel or dentin surface [60,62,63]. In such cases, alternative natural diet sources such as honey tested here could inhibit the proliferation of the previous mentioned pathogen's population, resulting in controlling or even better, preventing dental caries. Other pathogens can also proliferate in the oral cavity inducing periodontitis and other soft tissue diseases resulting in the loss of teeth and low quality of life [60,62,63]. Within the limitations of this in vitro study, different honey samples, originating from the region of Epirus in Greece, were found to be effective against dental pathogens when treated in artificial saliva means. Artificial saliva has rheological, lubrication, and antibacterial properties similar to the ones of natural saliva and is used with good clinical results in patients suffering from xerostomia after radiation and other similar ailments, which reduces the production of natural saliva [64].

In our study, despite the strain and botanical dependent variation of our results, the majority of our samples performed better than manuka honey. This is an important finding, because manuka honey is considered by most researchers as the "front-runner of honeys for non peroxide antimicrobial activity" [65]. Hydrogen peroxide is not accumulated in manuka honey due to the destruction of glucose oxidase by methylglycoxal [66] and this is perhaps one reason for the outperformance of antibacterial activity of the Greek honeys. These honeys can act as both bacteriostatic and bactericidal depending on the concentration used. Pasture honey (4–8%) and 5–11% manuka honey were found to be bacteriostatic, whereas bactericidal activity was achieved at 5–10% and 8–15% concentrations, respectively [15,27,67]. In contrast, artificial honey (sugar solution which mimics the composition of honey) showed only bacteriostatic activity (at 20–30% v/v) and not bactericidal in the

study of Bansal et al. (2005) [68]. In the present study, the bacteriostatic result was not differentiated from the bactericidal effect, mostly because (a) the aim of the study was to assess the total antibacterial activity and (b)—to a lesser degree—from a therapeutic point of view it is the inhibition of bacterial growth that matters most.

Mechanisms of antimicrobial activity of honey are different from antibiotics, which destroy the bacteria's cell wall or inhibit intracellular metabolic pathways. The antibacterial activity is related to four properties of honey [11,17,22,26,33,69]. First, honey draws moisture out of the environment and thus dehydrates bacteria. The sugar content of honey is also high enough to hinder the growth of microbes, but the sugar content alone is not the sole reason for honey's antibacterial properties [68]. The second antimicrobial reason of honey is the pH values. The pH of honey is between 3.2 and 4.5 [26,27,38,70]. This acidity is low enough to inhibit the growth of most microorganisms of the ones tested here [26,27,38,70–72]. Although statistically significant differences were observed among the pH values off the different honey types, it was not possible to conclude any positive (or negative) statistically significant effect of these differences on the antimicrobial action either in well diffusion experiments or in MIC estimates. Furthermore, hydrogen peroxide produced by the glucose oxidase is one of the most important antibacterial components of honey, although some authors believe the non-peroxide activity is equally or even more important. Glucose oxidase is incorporated into honey during the foraging of the bees and oxidizes glucose to glucolactone [40,41]. This oxidation results in the production of gluconic acid and in the reduction of molecular oxygen to hydrogen peroxide [73]. The latter substance however can also be produced by polyphenol autoxidation [74]. In the present study, the hydrogen peroxide's antibacterial activity was assessed by the addition of catalase (Figures 2 and 3). Catalase can be found naturally in honey and is of pollen origin [75]. The results show (Figures 2 and 3) that for all types of honey and for all pathogens, MIC increases after the addition of catalase. This increase in MIC implies an increased susceptibility of the dental pathogens due to the hydrogen peroxide content of honey. However, hydrogen peroxide, although a significant bactericidal ingredient, is not the sole or the most potent cause of the antibacterial activity of unprocessed honey. In our study, citrus honey contains 3.35 and 4.67 more hydrogen peroxide than oregano honey and *Satureja* spp. honey, respectively (Table 8), yet 5 out of 7 pathogens and reference strains showed a smaller inhibition zone in comparison to the other honeys (Table 9). On the other hand, the bacterial susceptibility can perhaps be attributed to the strain or even species-specific factors. Furthermore, several phytochemical factors with antibacterial activity have been identified in honey [76,77]. All these substances have been described as non-peroxide antibacterial factors [78].

In most of the MIC experiments with artificial saliva as the diluent, no statistically significant differences were observed. A significant increase of MIC was observed in citrus and oregano and sage honeys against *F. nucleatum* and in all honeys against *S. aureus* A, *S. aureus* B, and *S. aureus* ATCC 12600. Artificial saliva alters the viscosity of honey as well as its colloid structure. These physicochemical changes reduce the susceptibility of certain bacteria and thus the increase in MIC values in our study. However, this effect must have a species-specific element since only the *S. aureus* strains were found most susceptible to all honeys. In addition to the previous information, volatiles, organic acids, lysozyme, beeswax, nectar, pollen and propolis are important chemical factors that provide antibacterial properties to honey [78–80]. Propolis, a natural resinous mixture produced by honeybees, which exhibits anti-microbial, anti-inflammatory, cytostatic, and cariostatic properties. has been used already for cosmetic crèmes for the skin and oral hygiene sources such as dentifrices. Propolis, as it is known, influences the cytoplasmic membrane and has an inhibitory effect on the bacterial motility and enzymatic activity. It has bacteriostatic activity at low concentrations and can be bactericidal at high concentrations [81]. It breaks down bacterial cell wall, cytoplasm and prevents bacterial cell division. There are reports on the effectiveness of propolis-containing dentifrices for the control of caries in young adults [82]. In addition, in the study of Ophori et al. (2010) [83], it was also established

that propolis and especially the ethanol extract of propolis (EEP) exerted bacteriostatic and bactericidal effects against *S. mutans*, respectively, at concentrations of 1.875 and 3.75 μg/mL or more. It was stated that organisms were the most susceptible to EEP at acidic pH followed by neutral and alkaline pH [84]. In another study, propolis mouth rinse was found to have an effective antimicrobial action against *S. mutans* [84]. In this sense, Greek honeys, which are already more effective than the manuka control source, as tested in this study, are estimated to better fulfill the pathogens inhibition status if specific extracted derivatives were also used in oral hygiene sources such as gels, mouth rinses, or dentifrices. Honey also contains oligosaccharides in large quantities. The sugar composition of honey from different floral sources was related to the growth inhibition of various intestinal bacteria [85]. Sugars do not possess antibacterial properties per se, but their concentration regulates the osmolarity and the osmolality of honey [86,87]. From the findings of our study, it is not clear however, if these differences can significantly affect the colloidal and rheological properties of different honeys and thus alter their antibacterial effect.

Despite their antibacterial effect, honey's carbohydrates could be a factor of cariogenesis in high-risk dental patients [62,63]. Of course, in this risk category, patient's use of honey is better than the sugar itself [62]. As shown in the study of Sela et al. (1998) [88], the initial microhardness of the surface of the enamel decreased significantly after consumption of a teaspoonful of honey in the subjects with a regular saliva flow, whereas in the irradiated dry-mouth patients, no enamel microhardness decrease occurred. The supposed solubility-reducing factor present in honey, which remains active in the absence of saliva but is inactivated by salivary enzymes, gives some support to the hypothesis that honey is less cariogenic or erosive in dry-mouth subjects [63,89]. These findings could most likely suggest that honey as a natural diet source could work better as an anticariogenic, anti-erosive, or wound healing factor in patients with hyposalivation [89–91]. However, diagnosed hyposalivation often comprises a sequela of severe systemic diseases, such as Sjögren's syndrome [91,92], diabetes mellitus [93], or cancer during the phase of chemotherapy and radiotherapy [94,95]. It can also be derived by age or certain drugs (i.e., intake of angiotensin-converting enzyme inhibitors, anorexiants, anticholinergic/antispasmodic agents, sedatives, anti-parkinsonism agents, antipsychotics, etc.) [96,97]. Possibly, in these patients, honey should be used through natural sources while propolis should be used in younger and healthy patients as extracts in oral hygiene products.

Moreover, it is reported that a part of the antibacterial activity might be attributed to the components of plant origin [97,98]. In our study, honeys of different plant origins were used, and the results show differences in the diameter of the inhibition zones as well as in MIC according to the plant origin. After the addition of catalase, which eliminates the activity of hydrogen peroxide, the still existing antibacterial activity of honey is entirely due to various phytochemicals. A portion of these substances is of protein or peptide origin, as the addition of proteinase K suggests, and the rest are phenolic, flavonoid, and other compounds with antibacterial activity, all of them derived from the plants that bees forage. Honey contains proteins and peptides with antibacterial activity. Brudzynski and Sjaard (2015) [99] identified fragments of glycoproteins, which exerted non-specific membrane permeabilization of the bacterial cells, resulting in a strong inhibition of growth. The same researchers (2014) [100] argued that compounds in Canadian honey act against bacterial cells in a mode similar to β-lactams. Four families of antibacterial peptides and proteins have been identified so far, in bees: apideacins, abaecins, hymenoptaecin, and defensin. These compounds represent the humoral defense of bees against pathogens and some of them, such as defensin 1, are incorporated into honey [100,101].

An interesting find is that in the case of citrus honey, most of the tested pathogens showed decreased susceptibility after the addition of protease K (Figure 3). The remaining antibacterial activity is due to phenolic and other non-protein compounds, and since the susceptibility of the pathogens was reduced, it follows that possibly the action of some of these compounds must have been inhibited by some peptides. Combarros-Fuertes et al. (2019) [102] demonstrated that the antibacterial activity of the phenolic and flavonoid

compounds of honey in MIC range from 0.05 g/mL to 0,40 g/mL. The use of artificial saliva in the present study served the purpose of simulating the conditions in the oral cavity. The results show that the antibacterial effect was enhanced in most cases. To our knowledge, this is the first study researching the effect of artificial saliva as a solvent to the antibacterial effect of honey. Having a greater viscosity than distilled water (the usual solvent), artificial saliva reduces to a lesser extent the viscosity and the colloidal structure of honey, which retains due to these properties more of its initial antibacterial activity.

A limitation of the present study is the lack of pollen analysis of the different types of honey. This analysis would verify the botanical source of each sample. We relied on the information about the geographical origin of the samples, which included the dominant plant species.

Finally, as a probable limitation in this study, we could point the use of a post-hoc multiple comparison methodology but without a Bonferroni reduction, since it works by reducing the *p*-values, making it possible to reject the valid conclusions (Type II error). We chose to follow the classical approach (without Bonferroni reduction) which, despite its flaws, remains a standard and convenient approach.

5. Conclusions

1. The Greek honeys and particularly the citrus and the oregano and sage honey, showed an impressive antibacterial activity against all oral pathogens tested in this study as well as the reference strains.
2. This antibacterial activity outperformed in most cases the one of manuka honey, which was used as control due to its well-studied and fully documented antibacterial activity.
3. A significant part of the antibacterial activity was due to hydrogen peroxide. Further studies are needed for evaluating the effect of other compounds such as peptides and non-peptides (phenolic compounds, flavonoids, and others) in the antibacterial action.
4. In in-vitro conditions, the antibacterial activity of honey is found to be enhanced in most cases when artificial saliva is used for its dilution.
5. There is an indication that in a clinical environment, Greek honeys can be used as anti-cariogenic, anti-erosive and/or oral wound healing factor in patients with hyposalivation.
6. Although further clinical research is needed, there is a strong indication that honey should be used in elderly patients through natural sources while propolis or other honey derivatives should be used in younger and healthy patients as extracts in oral hygiene products.
7. Our results are promising, and a future project must include not «artificial saliva» but saliva from volunteers, and perhaps not only healthy volunteers but also volunteers with specific dental lesions. In this case, the interaction between the natural microflora of the oral cavity and the pathogenic bacteria in the presence of various types of honey should also be studied.
8. The exact botanological profile of the various types of honeys should be investigated in order to classify them accurately and derive more specific clinical suggestions.

Author Contributions: C.V., G.R., A.T., M.A. and E.B.; methodology, G.R. and A.A.; software, C.V., M.A., I.S., A.T. and E.B.; validation, A.A., M.A., G.R., E.G. and T.V.; formal analysis, C.V. and A.A.; investigation, C.V., E.G., A.T., I.S. and E.B.; resources, C.V., A.A., G.R. and T.V.; data curation, C.V. and G.R.; writing—original draft preparation, C.V., M.A., A.T., T.V. and E.B.; writing—review and editing, C.V., T.V. and E.B.; visualization, C.V., A.T. and E.B.; supervision, C.V., A.T. and E.B.; project administration. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

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Review

Probiotics, Prebiotics, Synbiotics and Dental Caries. New Perspectives, Suggestions, and Patient Coaching Approach for a Cavity-Free Mouth

Markos Amargianitakis [1], Maria Antoniadou [1], Christos Rahiotis [1] and Theodoros Varzakas [2,*]

[1] Dental School, National and Kapodistrian University of Athens, 11527 Athens, Greece; markosamargian@gmail.com (M.A.); mantonia@dent.uoa.gr (M.A.); craxioti@dent.uoa.gr (C.R.)
[2] Department of Food Science and Technology, University of Peloponnese, 24100 Kalamata, Greece
* Correspondence: t.varzakas@uop.gr

Abstract: Probiotic therapy forms a new strategy for dental caries prevention. Probiotic microorganisms possess the ability to displace cariogenic microorganisms and colonize the oral cavity. They can produce various antimicrobial substances such as bacteriocins, bacteriocin-like peptides, lactic acid, and hydrogen peroxide. Dairy products may be ideal for probiotic administration in dental patients. Many other means have been proposed, primarily for those allergic to dairy components, such as capsules, liquid form, tablets, drops, lozenges, sweetened cakes, and ice creams. The last two forms can be used in a coaching approach for children and elderly patients who find it difficult to avoid sugary beverages in their daily routine and benefit from the suggestion of easy, cheap, and common forms of delicacies. In caries prevention, the concept of the effector strain is already considered an integral part of the contemporary caries cure or prevention strategy in adults. Adults, though, seem not to be favored as much as children at early ages by using probiotics primarily due to their oral microbiome's stability. In this non-systematic review we describe the modes of action of probiotics, their use in the cariology field, their clinical potential, and propose options to prevent caries through a patient coaching approach for the daily dental practice.

Keywords: probiotics; prebiotics; synbiotics; dental caries; effector strain; prevention; oral health

Citation: Amargianitakis, M.; Antoniadou, M.; Rahiotis, C.; Varzakas, T. Probiotics, Prebiotics, Synbiotics and Dental Caries. New Perspectives, Suggestions, and Patient Coaching Approach for a Cavity-Free Mouth. *Appl. Sci.* **2021**, *11*, 5472. https://doi.org/10.3390/app11125472

Academic Editor: Mary Anne Melo

Received: 26 April 2021
Accepted: 9 June 2021
Published: 12 June 2021

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1. Introduction

Dental caries is a multifactorial disease that occurs because of the ecological imbalance between the inorganic components of the hard dental tissues and biofilms [1]. It is the most widespread disease worldwide, with a prevalence approaching 91% of the adult population [2–5]. This trend is depicted particularly in USA's national expenditures on oral biofilm-associated diseases, which have surpassed the corresponding expenditures for heart conditions since 2006 [3]. In general, the human microbiome is in balance—symbiosis—with its host, the human body. However, the use of antibiotics seems to cause serious adverse effects, such as damage to the desired oral microbiome, pathogen resistance, and oral cavities more prone to dental caries, among other things [6,7]. For this reason, a newly derived and preventively oriented method, probiotic therapy (i.e., the use of desired and harmless microorganisms) has been gaining ground for the past few years. These facts requisite to form new strategies for dental caries prevention, especially in the post-COVID-19 pandemic recession-era worldwide.

In dentistry, probiotics utilization is being focused on advancing oral health by forestalling caries' and periodontal diseases' establishment [8]. In caries management, probiotics' rationale is that probiotic microorganisms possess the ability to displace cariogenic microorganisms and colonize the oral cavity [9,10]. In this review study, we focus our interest on the modes of action of probiotics as much as on the scientific effort from the advent of probiotics in cariology, until today. We also highlight some considerations regarding

their clinical potential in daily use and propose simple options to prevent caries experience or aggravation through a patient coaching approach for the daily practice.

2. Nomenclature

Probiotics were discovered in 1907, from the observation of the Nobel laureate in Immunology and Russian bacteriologist Ilya Ilyich Metchnikoff, that certain bacteria promote human intestinal health [11]. Since then, much has changed in probiotic nomenclature and its perspectives [12–16]. Today, probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" by the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) (2002) [17]. The term 'prebiotics', on the contrary, is used to describe "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the resident microflora, that confers benefits upon host wellbeing and health" [18]. The term 'synbiotic' is applied to products containing probiotics and prebiotics (Figure 1) [19].

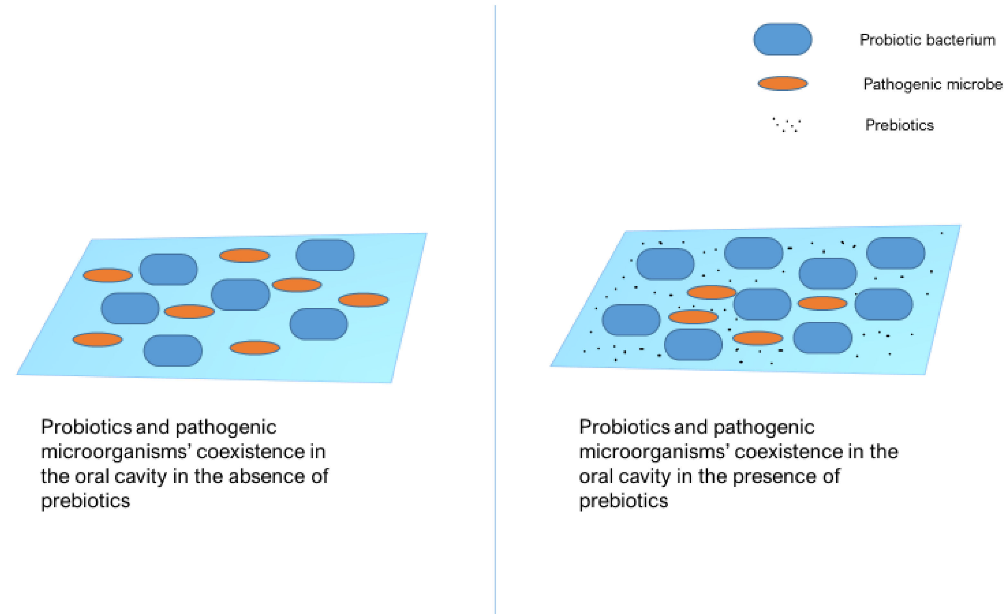

Figure 1. Synbiotics are products containing both probiotics and prebiotics.

It should be noted that fermented foods, although consisting of many microorganisms serving as probiotics, do not follow the cause explained for probiotics [10]. Fermented foods comprise edible products in which microbial activity is necessary to acquire stability, safety, and sensory properties. This is accomplished due to the ability of certain microorganisms (i.e., fermentation microorganisms/microbiomes) to decompose carbohydrates, thereby producing metabolites, such as lactic acid (lactic acid bacteria and *Enterobacteriaceae*), acetic acid (*Acetobacter* spp., *Gluconobacter* spp., *Bacillus subtilis* and yeasts), ethanol (heterofermentative lactic acid bacteria, *Enterobacteriaceae*, yeasts etc.), carbon dioxide, hydrogen peroxide, bacteriocins and antimicrobial peptides, which act alone or collectively to inhibit spoilage and the growth of several pathogens. Therefore, the microbiomes used for fermentation do not aim primarily to alter a human's microflora, even though many probiotic strains (e.g., *Lactobacilli* spp.) utilized in general medicine and dentistry have been derived from the fermentation industry.

3. Modes of Action of Oral Probiotics

Several microorganisms serve as oral probiotics. Probiotics that have been used in clinical trials are classified regarding the genus, the species, and the strain (Table 1).

Table 1. Microorganisms are serving as oral probiotics.

Genus	Species	Strain
Lactobacillus	*rhamnosus*	GG (ATCC 53103) [20–23], hct 70 [24], LB21 [25], LC 705 [22]
	reuteri	ATCC 55,730 (SD2112) [26–28], ATCC PTA 5289 [29–32], DSM 17,938 [29–32]
	casei	Shirota [33]
	paracasei	F19 [34], GMNL-33 [35], SD1 [36–38]
	achidophilus	ATCC 4356 [39], La-5 [40]
	salivarius	TI 2711 [41], WB21 [41]
	brevis	CD2 [42]
	bifidum [43]	
	bulgaricus [43]	
	sporogens [43]	
	thermophilus [43]	
Bifidobacterium	*animalis lactis*	BB-12 (ATCC 27536) [40,44–46], DN-173010 [47,48]
	bifidum	ATCC 29,521 [39]
	longum [49]	
Streptococci	*mutans*	A2JM [50]
	rattus	JH145^{TM} [51,52]
	oralis	KJ3^{TM} [52]
	uberis	KJ2^{TM} [52]
	dentisani	CECT7746 [53]
	salivarius	M18 [54]
Bacillus	*coagulans* [55]	

The probiotics' mechanisms of action generally have not been precisely determined [56]. Nevertheless, in general, the three main modes through which probiotics exert their action are (a) modulation of host's defense (b) direct destruction of pathogens, and (c) indirect removal of pathogens.

Probiotics and their extracellular products are found to interact with the host's mucous cells, determining in a strain-specific manner the cytokines' and chemokine's production, which leads to the enhanced phagocytic activity of macrophages, neutrophils, and Natural Killer (NK) cells [57]. For example, *B. lactis* Bb-12, *L. rhamnosus* GG, and *L. acidophilus* La1 increase the phagocytic capacity of leukocytes [58–61]. Probiotics, however, manipulate not only innate immunity but also stimulate adaptive immunity by increasing IgA levels in the serum and regulating the development of T helper cells and the proportion of Th1/Th2 cells [57,62–64]. In the oral environment, much less has been elucidated [56]. Indeed, specific probiotics inhibit the interleukin-8 (IL-8) response of the oral mucous cells caused by some periodontal pathogens as much as other inflammatory biomarkers, such as prostaglandin E2 (PGE2) [65–67]. Still, no alteration in salivary IgA levels has been observed [68]. Additionally, *L. paracasei* has been proved to augment the detectable counts of a defensin [69], salivary human neutrophil peptide 1–3 [70].

Probiotic bacteria can produce various antimicrobial substances, such as bacteriocins, bacteriocin-like peptides, lactic acid, and hydrogen peroxide. All of the above have an immediate effect on the host's microbiome, as they preconceive the death of specific pathogens, while the producer strains survive [71–73]. For example, *L. rhamnosus* GG secretes a broad-spectrum antimicrobial substance affecting many Gram-positive (*Streptococci*, *Lactobacilli*, *Clostridium* spp.) and Gram-negative bacteria (*E. coli*, *Bacillus fragilis*) [74]. *L. reuteri* produces

reuterin and reutericyclin [75,76], which exert their antimicrobial properties by inducing oxidative stress and altering the transmembrane ΔpH in target cells [77,78], respectively.

The indirect effects of probiotic microorganisms on the host's microbiome have to do with the phenomenon of competing with the pathogenic bacteria either for an adhesion niche or for essential nutrients [79–82]. Whenever salutary strains preoccupy potential sites of pathogens' adhesion, disease establishment is subverted [56]. The same happens when probiotic organisms excrete certain bio-surfactants that impede pathogenic bacteria's adhesion or when they modify the salivary pellicle per se [82,83]. The previous adhesion sites are altered in a direction rendering them not probable for pathogens to establish.

4. Caries Pathogenesis

For a thorough perception of the role of probiotics in caries prevention and therapy, an analysis of the mechanism via which caries lesions develop is imperative. The oral cavity constitutes a habitat for a wide variety of microorganisms [84]. The latter are found to colonize both the oral mucosa and stable surfaces, such as teeth, fixed and removable prosthodontic appliances, etc. It is those microorganisms that colonize tooth surfaces to which dental caries is attributed. These microorganisms conglomerate, thereby constituting a complex, tolerant antimicrobial agent mass called 'oral biofilm' or 'dental plaque'.

Oral biofilms are made up of a plethora of microorganisms. Some of them are harmless when present in the oral cavity, and some others possess a facultative pathogenic potential, which is called 'opportunistic pathogens' [85]. According to the 'Ecological Plaque Hypothesis', in the presence of health, all of them are in a state of symbiosis with each other as much as with the host. In fact, they play a crucial role in the host's health. Caries disease results from this symbiotic relationship's subversion, where a shift toward pathogens occurs, a state characterized as dysbiosis. In that case, sugars consumed through diet are taken up by pathogenic bacteria and are metabolized to lactic acid. Acids produced by dental plaque solubilize apatite crystals of the hard dental tissues (i.e., demineralization). Once acidic residues are removed, remineralization occurs (Figure 2).

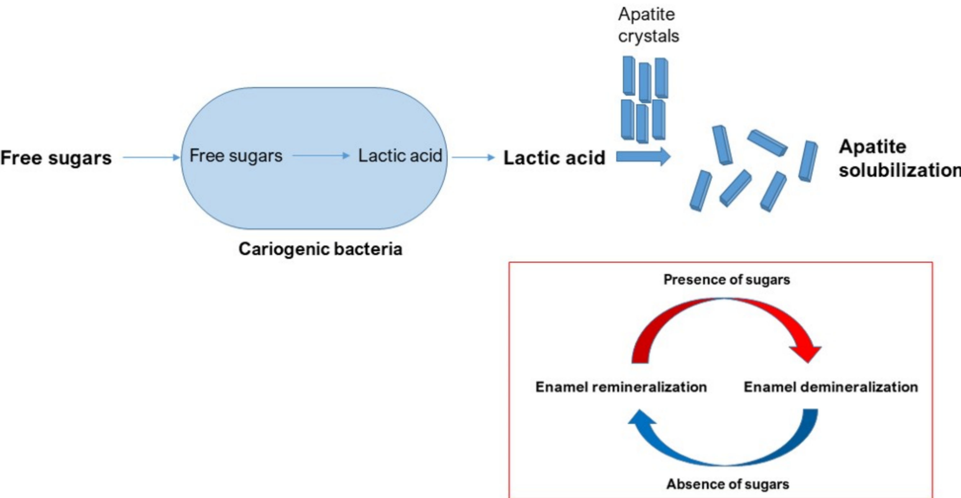

Figure 2. The process of caries development.

However, caries onset is not as simple as presented above. Many factors interplay among the host (salivary flow, apatite solubility), its oral microbiome synthesis, and the type of nutrients being taken up (high/low-sugar diet) [86]. Thus, the effects of acids produced during a sugar-rich diet of low frequency can be neutralized by saliva or alkali produced in the dental plaque, meaning that demineralization and remineralization phenomena are in equilibrium. In the case that a sugar-abundant diet is consumed more frequently, the constant low-pH conditions exert evolutionary pressure toward acid-adapted and acidogenic bacteria, such as *Streptococci* and *Actinomycetes*. If this frequency is even higher,

the most efficient acid-tolerating and acid-producing bacteria dominate. In the last two cases, demineralization of the tooth surfaces prevails at the expense of remineralization [85].

Several microorganisms have been correlated with different types, stages, and sites of cariogenesis [84]. The complex microbial composition of cavities at various stages is not consistent with the specific plaque hypothesis and supports a polymicrobial origin. Nowadays, dental caries cannot be considered a classical infectious disease that follows the conventional Koch's model. The microbial 'players' involved change through time depending on the tissue affected, and multiple species are responsible for the progressing lesion at different stages of the caries process [87].

5. Caries Management with Probiotics

Today, probiotics seem to be a salutary, newly derived method to control dental caries [9,10]. The rationale of probiotics use in caries management is that probiotic microorganisms can expel cariogenic microorganisms and colonize the oral cavity.

A wide variety of clinical trials have been conducted to examine probiotics' effect on dental caries and oral microflora. These studies are summarized in Table 2.

Among most of these trials, dairy products remained a common denominator as an administration milieu [46–48]. Milk's colloidal nature seems to be enamel protective [88], as it contains organic and inorganic compounds assisting in compensating cariogenic challenges [89]. Another substrate of dairy formulations, calcium lactate, also possesses anti-cariogenic properties [90]. Therefore, dairy products may be ideal for probiotic administration. However, many other means have been proposed to serve as probiotic administration agents, especially for those allergic to dairy components. Among those means stand capsules or liquid form [43], specially prepared straws or tablets [27,35,41], drops [29], lozenges [54], and even sweetened cakes [55] and ice creams [44].

The first-ever study introducing probiotics to dental clinical practice was that of Meurman and his colleagues [20]. They suggested that *Lactobacillus Gorbach-Goldin* (GG) – LGG, a strain earlier isolated by Gorbach and Goldin [91], can colonize the human oral cavity. Nonetheless, its long-term beneficial effect on oral health and the resulting alterations in oral microflora remained unilluminated. Näse et al. investigated *L. rhamnosus* GG for its in vivo long-term effect (7-month period) on dental caries [21,92]. They reported no significant changes in *S. mutans* salivary counts and caries prevalence occurred, but a significantly lower caries-risk than in the control group.

Following these results, Ahola et al. investigated the short-term anticaries effect of a cheese containing the same *Lactobacillus rhamnosus* strain plus *L. rhamnosus* LC 705 [22]. After a 3-week period, no statistically significant difference in *S. mutans* counts between the intervention and the control group occurred. However, the probiotic-containing cheese was found to exert its enhanced anticaries potential during the post-treatment period, as the *S. mutans* levels in saliva were significantly reduced.

In 2003, Montalto et al. examined two extra facets of probiotics intended for oral administration [43]. Probiotic species tested in this study were *L. sporogens* 16%, *L. bifidum* 12%, *L. bulgaricus* 12%, *L. thermophilus* 18%, *L. acidophilus* 20%, *L. casei* 10%, and *L. rhamnosus* 12%. The probiotics used, independently of the administration's milieu, were found to increase *Lactobacilli* salivary counts in contrast to *S. mutans*. In fact, capsule and liquid forms were found to lead to equivalent results. The only practical difference between these two types of administration is that Lactobacilli strains diffused into the oral cavity in the liquid state. Thus, a systemic effect of oral probiotics was suggested.

In 2004, Nikawa et al. highlighted the selective bactericidal influence of *L. reuteri* SD2112 (ATCC55730) on *S. mutans* in vitro and in vivo [26]. The individuals consumed a cup (95 g) of placebo yoghurt (*S. thermophilus* and *L. bulgaris*) once a day for two weeks during lunchtime in the first group. The following 2 weeks, a probiotic yoghurt containing *L. reuteri* and *S. thermophilus* was administrated in the same terms. In the second group, the opposite regimen was followed. Ultimately, both types of treatment significantly reduced the *S. mutans* salivary carriage, compared to its baseline values for each group.

Table 2. Clinical trials around oral probiotics utilized without aiming to preemptively colonize the oral cavity of subject.

Baseline Condition	Type of Study	Patient Type	Baseline Condition	Study Groups	Treatment	Probiotic Strains	Strain Concentration	Results
Meurman et al. (1994) [20]	Cohort study	Dental students (mean age 25 years)	Healthy	1 test group (n = 9)	2 × 250 g of probiotic yoghurt/day	*Lactobacillus rhamnosus* GG ATCC 53103	1×10^8 CFU/mL	LGG showed distinct growth in 8 of 9 subjects 2 weeks after treatment discontinuation.
Näse et al. (2001) [21]	Randomized, double-blind, placebo-controlled	Daycare children (1–6 years old)	Healthy Caries	Test (n = 231) Control (n = 220)	5 × (±250 mL) of probiotic milk/week during a 7-month period 5 × (±250 mL) of placebo milk/week for 7 months	*Lactobacillus rhamnosus* GG ATCC 53103	$5\text{–}10 \times 10^5$ CFU/mL	No significant differences in caries and MS scores. Significantly reduced caries-risk in the probiotic group, especially in the 3- to 4-year-old children.
Ahola et al. (2002) [22]	Randomized, double-blind, placebo-controlled	Young adults (18–35 years old)	Healthy	Test (n = 38) Control (n = 36)	5 × 15 g of probiotic cheese/day for 3 weeks 5 × 15 g of placebo cheese/day for 3 weeks	*Lactobacillus rhamnosus* GG ATC 53103 *Lactobacillus rhamnosus* LC 705	1.9×10^7 CFU/g 1.2×10^7 CFU/g	No significant difference in MS and yeast counts during intervention. Significantly reduced MS scores and a tendency toward fewer patients with high Lactobacilli counts in the probiotic group during the post-treatment period.
Montalto et al. (2004) [43]	Randomized, double-blind, placebo-controlled	Young adults (23–37 years old)	Healthy	Group A (n = 14) Group B (n = 16) Group C (n = 5)	(Capsuled probiotics + Liquid placebo)/day for 45 days (Liquid probiotics + capsuled placebo)/day for 45 days (Liquid and capsuled placebo)/day for 45 days	*L. sporogens* *L. bifidum* *L. bulgaricus* *L. thermophilus* *L. acidophilus* *L. casei* *L. rhamnosus*	16% 12% 12% 18% 20% 10% 12% (1.88×10^9 total CFU/day)	Significant increase in Lactobacilli groups in both probiotic groups. No change in MS counts in all groups.
Nikawa et al. (2004) [26]	Double-blind, placebo-controlled	Female dental hygienist students (20 years old)	Healthy	40 subjects in total	95 g of placebo yoghurt/day for 2 weeks + 95 g of probiotic yoghurt/day for 2 weeks 95 g of probiotic yoghurt/day for 2 weeks + 95 g of placebo yoghurt/day for 2 weeks	*L. reuteri* SD2112 (ATCC55730)	Data not provided	Probiotic yoghurt compared to the placebo yoghurt significantly reduced MS counts.
Caglar et al. (2005) [47]	Randomized, double-blind crossover	Young adults (21–24 years old)	Healthy	Test (n = 21) Control (n = 21)	Periods 1,3: run-in and wash-out, respectively Periods 2,4 (2 weeks each): 1 × 200 g of probiotic or placebo yoghurt/day	*Bifidobacterium animalis lactis* DN-173 010	7×10^7 CFU/g	Significant decrease in MS counts and a tendency toward Lactobacilli reduction due to probiotic yoghurt consumption.
Caglar et al. (2006) [27]	Randomized, placebo-controlled with 4 parallel arms	Young adults (21–24 years old)	Healthy	Group A (n = 30) Group B (n = 30) Group C (n = 30) Group D (n = 30)	200 mL of water/day through probiotic straw for 3 weeks 200 mL of water/day through placebo straw for 3 weeks 1 probiotic tablet/day for 3 weeks 1 placebo tablet/day for 3 weeks	*L. reuteri* ATCC 55730	10^8 CFU/straw 10^8 CFU/tablet	Significant decrease in MS counts and tendency toward reduction of Lactobacilli scores in both probiotic groups.
Caglar et al. (2008) [44]	Randomized, double-blind, placebo-controlled crossover	Young individuals (mean age 20 years old)	Healthy	Test (n = 23) Control (n = 24)	Periods 1,3: run-in and wash-out, respectively Periods 2,4 (10 days each): 1 × 53 g of probiotic or placebo ice-cream/day	*B. animalis lactis* Bb-12	1×10^7 CFU/g	Significant decrease in MS counts after probiotic ice-cream consumption. No change in Lactobacilli counts after both ice-creams intake.

Table 2. *Cont.*

Baseline Condition	Type of Study	Patient Type	Baseline Condition	Study Groups	Treatment	Probiotic Strains	Strain Concentration	Results
Stecksen-Blicks et al. (2009) [25]	Clustered, double-blind, placebo-controlled	Preschool children (1–5 years old)	Healthy Caries	Probiotic (n = 110) Placebo (n = 76)	1 × 150 mL probiotic milk (supplemented with 2.5 mg fluoride/L)/day for 21 months 1 × 150 mL standard milk fluoride)/day for 21 months	*L. rhamnosus* LB21	1×10^7 CFU/mL	Significant reduction in caries development after intervention milk intake. Lower, but not significant, MS proportion and no increase in total Lactobacilli counts in the probiotic group.
Singh et al. (2011) [40]	Randomized, double-blind, placebo-controlled, crossover	Children (12–14 years old)	Healthy	Group I (n = 20) Group II (n = 20)	1-week run-in, 54 g of placebo ice-cream/day for 10 days, 2-week wash out, 54 g of probiotic ice-cream/day for 10 days 1-week run-in, 54 g of probiotic ice-cream/day for 10 days, 2-week wash out, 54 g of placebo ice-cream/day for 10 days	*B. animalis lactis* BB-12 ATCC27536 *L. acidophilus* La-5	1×10^6 CFU/g 1×10^6 CFU/g	Significant decrease in MS counts after probiotic ice-cream consumption. No change in Lactobacilli counts after both ice-creams intake.
Chuang et al. (2011) [35]	Randomized, double-blind, placebo-controlled	Young adults (20–26 years old)	Healthy	Test (n = 42) Control (n = 36)	3 probiotic (+11% xylitol) tablets/day for 2 weeks 3 placebo (11% xylitol) tablets/day for 2 weeks	*L. paracasei* GMNL-33	3×10^8 cells/tablet	No change in MS and Lactobacilli levels in both groups during the experiment. Significant reduction in MS counts in the post-treatment period compared to the respective levels recorded immediately after treatment cessation.
Kavaloglu-Cildir et al. (2011) [29]	Randomized, double-blind, placebo-controlled, crossover	Children (4–12 years old)	Healthy Cleft Lip/Palate	Test (n = 19) Control (n = 19)	Periods 1,3: run-in and wash-out, respectively Periods 2,4 (25 days each): 5 probiotics or placebo drops/day	*L. reuteri* DSM 17938 *L. reuteri* ATCC PTA 5289	$\geq 1 \times 10^8$ CFU/5 drops $\geq 1 \times 10^8$ CFU/5 drops	No change in MS and Lactobacilli counts after the consumption of both drops.
Juneja et al. (2012) [24]	Randomized, double-blind, placebo-controlled	Children (12–15 years old)	Healthy Caries	Group I (n = 18) Group II (n = 18)	2 × 150 mL of standard milk/day for 3 weeks 2 × 150 mL of probiotic milk/day for 3 weeks	*L. rhamnosus* hct 70	2.34×10^9 CFU/day	Significant reduction of MS levels immediately after the intake of probiotic milk.
Burton et al. (2013) [54]	Randomized, double-blind, placebo-controlled	Schoolchildren (5–10 years old)	Healthy Caries	Test (n = 40) Control (n = 43)	2 probiotic lozenges/day for 3 months 2 placebo lozenges/day for 3 months	*S. salivarius* M18	3.6×10^9 CFU/lozenge	Significant reduction of plaque scores in the probiotic group. Children who presented a distinct oral colonization by M18 tended to possess lower counts of MS.
Teanpaisan et al. (2013) [36]	Randomized, double-blind, placebo-controlled	Young adults (18–25 years old)	Healthy Caries	Group A (n = 20) Group B (n = 17)	1 × 10 g reconstituted probiotic milk powder in 50 mL of water/day for 4 weeks 1 × 10 g reconstituted placebo milk powder in 50 mL of water/day for 4 weeks	*L. paracasei* SD1	$\geq 10^7$ CFU/g or mL	Significant decrease of MS levels and increase of Lactobacilli levels after probiotic milk powder consumption. The probiotic could be detected up to 4 weeks after the discontinuation of the intervention.
Yadav et al. (2014) [33]	Randomized, double-blind, placebo-controlled, crossover	Children (6–8 years old)	Healthy Caries	Test (n = 31) Control (n = 31)	Periods 1,3: run-in (7 days) and wash-out (30 days), respectively Periods 2,4 (10 days each): 1 × 10 mL of probiotic or placebo milk/day	*L. casei Shirota*	Data not provided	Significant reduction of MS counts after the intake of probiotic milk.

Table 2. *Cont.*

Baseline Condition	Type of Study	Patient Type	Baseline Condition	Study Groups	Treatment	Probiotic Strains	Strain Concentration	Results
Pinto et al. (2014) [48]	Randomized, double-blind, placebo-controlled, crossover	Orthodontic patients (median age 15 years)	Healthy	Group 1 (n = 15) Group 2 (n = 15)	1-week run-in, 200 g of probiotic yoghurt/day for 2 weeks, 4-week wash out, 1 × 200 g of placebo yoghurt/day for 2 weeks 1-week run-in, 200 g of placebo yoghurt/day for 2 weeks, 4-week wash out, 200 g of placebo ice-cream/day for 2 weeks	*B. animalis lactis* DN-173010	Data not provided	No significant difference in MS, Lactobacilli and total cultivable microorganisms counts after both yoghurts. Both yoghurts were equally efficient at reducing total cultivable microorganisms isolated from dental plaque.
Nishihara et al. (2014) [41]	Randomized, double-blind, placebo-controlled with 4 parallel arms +Cohort study	Sixth-year dental students (mean age 24.8 years) Dentists (mean age 30.0 years)	Healthy Healthy	Group 1 (n = 17) Group 2 (n = 16) Group 3 (n = 13) Group 4 (n = 18) 1 test group (n = 8)	1 probiotic (+280 mg xylitol) tablet for 1 month 1 probiotic (+450 mg xylitol) tablet for 1 month 1 Ovalgen (+100 mg xylitol) tablet for 1 month 1 xylitol (280 mg) tablet for 1 month 3 × 1 tablet/day for 2 weeks	*L. salivarius* WB21 *L. salivarius* TI 2711 *L. salivarius* WB21	6.7×10^8 CFU/tablet 2.8×10^8 CFU/tablet 2.0×10^9 CFU/tablet	No significant change in MS levels. Significant increase in Lactobacilli counts in the two probiotic groups and enhanced buffering capacity in *L. salivarius* TI 2711 and Ovalgen group. Significant decrease in salivary MS levels.
Keller et al. (2014) [30]	Randomized, double-blind, placebo-controlled	Adolescents (12–17 years old)	Healthy Caries	Test (n = 19) Control (n = 17)	2 probiotic tablets/day for 12 weeks 2 placebo tablets/day for 12 weeks	*L. reuteri* DSM 17938 *L. reuteri* ATCC PTA 5289	1×10^8 CFU/tablet 1×10^8 CFU/tablet	Significant decrease of fluorescence in decayed teeth over time in the probiotic group. No significant differences in fluorescence between the two groups.
Gizani et al. (2016) [31]	Randomized, double-blind, placebo-controlled	Adolescents and young adults (mean age 15.9 years)	Healthy Orthodontic treatment Caries	Test (n = 42) Control (n = 43)	1 probiotic lozenge/day for 17 months 1 placebo lozenge/day for 17 months	*L. reuteri* DSM 17938 *L. reuteri* ATCC PTA 5289	$\geq 10^8$ CFU/lozenge $\geq 10^8$ CFU/lozenge	Significant reduction of Lactobacilli counts and no alteration of MS levels in both groups. No difference in the incidence of white spot lesions between the two groups.
Ghasemi et al. (2017) [39]	Randomized, double-blind, placebo-controlled	Female students (19–27 years old)	Healthy	Group 1 (n = 25) Group 2 (n = 25)	200 g of probiotic yoghurt/day for 3 weeks 3 × 2 xylitol gums/day for 3 weeks	*L. acidophilus* ATCC 4356 *B. bifidum* ATCC 29521	1.5×10^8 total CFU/g	Significant reduction of MS counts in both groups with no significant difference between them.
Koopaie et al. (2019) [55]	Randomized, double-blind, placebo-controlled, crossover	Adolescents and adults (mean age 41.67 years)	Healthy	Group 1 (n = 20) Group 2 (n = 20)	70 g of probiotic cake/day for 1 week, 4-week wash-out period, 70 g of regular cake/day for 1 week 70 g of regular cake/day for 1 week, 4-week wash-out period, 70 g of probiotic cake/day for 1 week	*B. coagulans*	Data not provided	No statistical difference in MS levels after probiotic cake intake. Significant increase of MS counts after regular cake consumption. No significant alteration in salivary pH after the consumption of both cakes.
Javid et al. (2020) [46]	Randomized, double-blind, placebo-controlled	Students (18–30 years old)	Healthy Caries	Test (n = 33) Control (n = 33)	300 g of probiotic yoghurt/day for 2 weeks 300 g of placebo yoghurt/day for 2 weeks	*B. lactis* Bb-12	10^6 CFU/ml	Significant reduction in MS and Lactobacilli levels in the probiotic group.
Ferrer et al. (2020) [53]	Prospective, mechanistic pilot with two parallel follow-up groups	Adults (25–35 years old)	Healthy	Group 1 (n = 6) Group 2 (n = 5)	7 vials (multidose) containing the probiotic strain 2 vial (monodose) containing the probiotic strain	*S. dentisani* CECT7746	5.5×10^9 CFU/vial 4×10^{10} CFU/vial	Significant decrease of MS and significant increase in *S. dentisani* levels and salivary pH. The latter was stronger in the multi-dose schedule.

To cover the lack of knowledge about *L. reuteri's* effect on Lactobacilli salivary counts in humans, after the introduction of the strain *Bifidobacterium* DN-173 010 to the oral probiotic armamentarium [47], Caglar et al. selected *L. reuteri* ATCC 55,730 as intervention strain to illuminate this unknown aspect [27]. As concluded, probiotic-containing straws and tablets could be beneficial to *S. mutans* confinement. Two years later, Caglar and his affiliates presented the strain *Bifidobacterium lactis* Bb-12 as an anticaries-competent probiotic strain [44]. This study introduced a novel probiotic strain into the race against caries and suggested ice-cream as a possible means for probiotics administration.

Given the previous studies about probiotics' role in caries management, Stecksen-Blicks et al., came up with the highly promising idea of combining probiotic bacteria with fluoride [25]. This idea was based on the hypothesis that these two agents would act synergistically. Children in both the probiotic and placebo group consumed 150 mL of medium-fat milk at lunch for 21 months. As the results indicated, caries incidence increment was statistically significantly lower in the intervention group than in the control children. In contrast, salivary counts of caries-associated *S. mutans* and Lactobacilli were not affected.

With time passing by, innovative probiotic strains have been proposed for the fight against dental caries. For instance, *L. acidophilus* La5 combined with *B. lactis* Bb-12 [40], *L. paracasei* GMNL-33 [35], *L. rhamnosus* hct 70 [24], *S. salivarius* M18 [54], *L. paracasei* SD1 [36], *L. casei Shirota* [33], *L. acidophilus* ATCC 4356 combined with *B. bifidum* ATCC 29,521 [39] dismiss *S. mutans* from the oral cavity. Moreover, *L. paracasei* SD1, when being received once a day for 4 weeks can be retained in the oral cavity of healthy young adults for 4 additional weeks by the time the regimen has been interrupted [36]. Lately, a shift towards the study of the host-specific alterations caused by probiotics has been recorded. Remarkably, the potential of specific probiotics to decrease caries risk has been correlated with their property to increase saliva's buffering capacity [41,53], although no probiotic tested for an immediate anticaries ability has been shown to invert early caries development per se [30,31].

It is worth mentioning that in 2019, a highly promising study was conducted [55]. This trial investigated the effect of a *Bacillus coagulans*-abundant cake on *S. mutans* levels and salivary pH. It pointed out that the sweetened probiotic cake can keep *S. mutans* amounts low and comparable to those surveyed before cake consumption. Hence, it was proposed that cakes carrying probiotic flora may comprise a novel strategy against *S. mutans* [92]. This proposal is the modern trend in food policy [93].

6. Clinical Considerations on Probiotics' Effectiveness

In general, for a strain to serve as a probiotic, it should be capable of firmly attaching to the oral surfaces [94]. However, *Lactobacilli*, present weak adhesiveness on the tooth structure [95]. The latter raises a variety of speculations around their long-term restraint in retention sites. Data from research studying the effect of probiotics on their saliva concentration and their tooth structure content are limited. However, according to Meurman et al., during the second week after the discontinuation of probiotic treatment with a yoghurt supplemented with LGG, LGG's salivary counts show a decrease in subjects who were following the treatment [20].

Likewise, Busscher et al. investigated LGG, *L. acidophilus* and *B. bifidum*'s ability of adhesiveness on the tooth structure in vitro and in vivo [96]. In vitro data suggested that LGG possesses a by far inferior ability of adhesion to the clear enamel (without salivary pellicle) and the pellicle-coated enamel compared to the corresponding ability *L. acidophilus*. This difference was attributed to the hydrophilic character of LGG. Salivary samples collected from individuals who were subjected to the daily intake of these bacteria through a bio-yoghurt were free of Lactobacilli. It was concluded that the ecological conditions in the oral cavity of test persons were unfavorable for Lactobacilli to grow, as temporary colonization could not be achieved even in individuals without any evincible amounts of Lactobacilli.

Another study demonstrating the temporary colonization of the oral cavity by probiotic Lactobacilli is Petti et al. [97]. The authors investigated if *S. thermophilus* and *L. bulgaricus*-containing yoghurt presented any activity against the oral microbiome regarding whether these probiotics could colonize the human oral cavity. Some activity against oral Streptococci was detected, but this has not resulted from the probiotics' colonization because it elapsed once the treatment was discontinued.

Yli-Knuuttila et al., except for demonstrating LGG's inability to colonize young adults' oral cavity, signalized that permanent LGG colonization is possible, providing that early administration in childhood has taken place [98]. Devine and Marsh [99] explained that the latter finding was correlated with the instability of the resident microbiota in children [100]. After that, many studies were conducted to examine the possible long-term effect of probiotics in the child population. These trials are synopsized in Table 3.

One study mentioned above evaluated the possible correlation between probiotic use in combination with an agent controlling the oral microbiota [23]. Aminabadi et al. tested this eventuality by combining the salutary advantages of chlorhexidine (CHX) in oral microbiome control using LGG. They concluded that CHX increases—at least 5 weeks after ceasing the regimen—the stability of LGG oral colonization.

Taipale et al. evaluated the influence of *B. animalis lactis* Bb-12 (Bb-12) early administration on *S. mutans* and Bb-12 oral colonization [45]. Subjects 1–2 months old in the test group received tablets with the probiotic strain, whereas control groups consumed xylitol (X) and sorbitol (S) in the same manner. The whole regimen lasted until the infants became 2 years old. Qualitative PCR showed bare and no Bb-12 oral colonization at 8-month-old and 2-year-old children, respectively, significantly reduced *S. mutans* levels, and had no effect on Lactobacilli. Such results were extracted in a study carried out one year later for the strain *L. paracasei* F19 [34].

In 2014, Stensson and his team studied whether caries prevalence could be subverted through oral probiotic administration before establishing the oral microflora [28]. It is worth mentioning that although a significant reduction in caries incidence was reported, no caries-associated microbiological alterations were observed. Other strains of *L. reuteri* (DSM 17,938 and ATCC PTA 5289), when administered through lozenges twice a day, prevail against MS and Lactobacilli and benefit the salivary buffer capacity [32].

Hedayati-Hajikand et al., in turn, evaluated the effect of a commercially known probiotic product (ProBiora3 TM) filled with the strains *S. uberis* KJ2, *S. oralis* KJ3, *S. rattus* JH145 as an adjunct to the everyday oral hygiene of 2/3-year-old children [52]. Thus, ProBiora3 TM chewing tablets benefit early childhood caries increment when used in children's daily oral care.

More recent studies suggest that *L. rhamnosus* combined with *B. longum*, *L. paracasei* SD1, and L. *brevis* CD2 could also assist in attenuating the range of dental caries from early ages [37,38,42,49]. More specifically, *L. rhamnosus* combined with *B. longum*, despite being incapable of reducing *S. mutans* salivary levels, they do so as far as *Lactobacilli* are concerned and enhance the buffering capacity of saliva [49]. On the other hand, *L. paracasei* SD1 affects MS levels and inhibits caries development [37,38]. Finally, the latest research in the field suggests that *L. brevis* CD2 is competent for diabetic children because it improves some caries risk factors (e.g., reduction in salivary MS and maximum plaque pH fall, increase in lowest plaque pH) and gingival health [42].

Table 3. Clinical trials investigating probiotics ability to colonize the oral cavity of children preemptively.

Baseline Condition	Type of Study	Patient Type	Baseline Condition	Study Groups	Treatment	Probiotic Strains	Strain Concentration	Results
Aminabadi et al. (2011) [23]	Randomized, double-blind with 4 parallel arms	Children (6–12 years old)	Healthy	Group A (n = 35) Group B (n = 35) Group C (n = 35)	2 × 5 mL of 0.12% chlorhexidine/day for 2 weeks 15–20 mL of probiotic yoghurt for 3 weeks 2 × 5 mL of 0.12% chlorhexidine/day for 2 weeks + 15–20 mL of probiotic yoghurt for 3 weeks	*L. rhamnosus* GG	2 × 10^8 CFU/g	Significant decrease in MS counts in all groups; only in groups A and C it was persisted for 5 weeks after the end of treatment. In group C LGG levels were more prominent than in group B.
Taipale et al. (2012) [45]	Randomized, double-blind, placebo-controlled with 3 parallel arms	Infants (1–2 months old)	Healthy	Test (n = 32) Control 1 (n = 35) Control 2 (n = 29)	2 probiotic-tablets/day 2 xylitol-tablets/day 2 sorbitol-tablets/day Until the age of 2 years old	*B. animalis lactis* BB-12	5 × 10^9 CFU/tablet	Significant decrease in MS counts in the probiotic and the sorbitol groups at the age of 2 years. No observed permanent oral colonization of BB-12. Lactobacilli were unaffected.
Hasslöf et al. (2013) [34]	Randomized, double-blind, placebo-controlled	Infants (4 months old)	Healthy	Test (n = 56) Control (n = 62)	At least 1 serving of probiotic-cereals/day At least 1 serving of placebo-cereals/day	*L. paracasei* F19	1 × 10^8 CFU/serving	No significant difference in MS counts and caries experience between the two groups. No permanent establishment of LF19.
Stensson et al. (2014) [28]	Randomized, single-blind, placebo-controlled	Mothers (during the last month of gestation) + Infants (through the 1st year of life)	Healthy	Test (n = 60) Control (n = 53)	5 drop of probiotic-oil/day (last month of gestation and 1st year of life) 5 drops of placebo-oil/day (last month of gestation and 1st year of life)	*L. reuteri* ATCC 55730	10^8 CFU/5 drops	Significant decrease in caries prevalence in the probiotic group. No significant intergroup differences in *L. reuteri*, MS, Lactobacilli and sIgA counts.
Hedayati-Hajikand et al. (2015) [52]	Randomized, double-blind, placebo-controlled	Preschool children (2–3 years old)	Healthy Caries	Test (n = 54) Control (n = 56)	1 chewing probiotic-tablet/day 1 chewing placebo-tablet/day	*S. uberis* KJ2™ *S. oralis* KJ3™ *S. rattus* JH145™	≥1 × 10^8 total CFU/tablet	Significantly lower caries increment in the probiotic group.
Villavicencio et al. (2017) [49]	Randomized, triple-blind, placebo-controlled	Preschool children (3–4 months old)	Healthy Caries	Test (n = 136) Control (n = 227)	200 mL of reconstituted probiotic milk/day for 5 days a week during a 9-month period 200 mL of reconstituted standard reconstituted milk/day for 5 days a week during a 9-month period	*L. rhamnosus* *B. longum*	5 × 10^6 CFU/g of powdered milk 3 × 10^6 CFU/g of powdered milk	Significantly lower counts of Lactobacilli count and higher buffering capacity in the test group. No significant difference in caries prevalence, MS counts, salivary pH and dental plaque between the groups.
Pahum-unto et al. (2018) [37]	Randomized, double-blind, placebo-controlled	Preschool children (1.5–5 years old)	Healthy	Test (n = 62) Control (n = 62)	5 g of probiotic milk powder in 50 mL of water/day for 3 months 5 g of standard milk powder in 50 mL of water/day for 3 months	*L. paracasei* SD1	1 × 10^7 CFU/g	Significantly lower risk of MS levels increases and of caries development in the test group.
Alamoudi et al. (2018) [32]	Randomized, double-blind, placebo-controlled	Children (3–6 years old)	Healthy	Test (n = 90) Control (n = 88)	2 probiotic lozenges/day for 28 days 2 placebo lozenges/day for 28 days	*L. reuteri* DSM 17938 *L. reuteri* ATCC PTA 5289	≥2 × 10^8 total CFU/lozenge	Significant decrease in MS and Lactobacilli counts in the probiotic group. No statistical difference in plaque accumulation and buffer capacity between the groups.

Table 3. *Cont.*

Baseline Condition	Type of Study	Patient Type	Baseline Condition	Study Groups	Treatment	Probiotic Strains	Strain Concentration	Results
Manmontri et al. (2020) [38]	Randomized, double-blind, placebo-controlled with 3 parallel arms	Preschool children (1–5 years old)	Healthy Caries	Group I (n = 86) Group II (n = 89) Group III (n = 93)	1 × 3 g of placebo milk powder in 50 mL of milk for 7 days/week for 6 months 1 × 3 g of probiotic milk powder in 50 mL of milk for 7 days/week for 6 months 1 × 3 g of probiotic milk powder in 50 mL of milk for 3 days/week + 3 g of placebo milk powder in 50 mL of milk for 4 days/week for 6 months	*L. paracasei* SD1	1.8 × 10^7 total CFU/mL	Significantly lower counts of MS and higher levels of Lactobacilli in saliva in both probiotic groups than in the placebo group. No difference regarding these alterations between the probiotic groups
Lai et al. (2021) [42]	Randomized, double-blind, placebo-controlled	Children (4–14 years old)	Type 1 diabetes Caries	Test (n = 34) Control (n = 34)	2 probiotic lozenges/day for 60 days 2 placebo lozenges/day for 60 days	*L. brevis* CD2	2 × 10^9 CFU/lozenge	Significant decrease in salivary MS and in maximum plaque pH fall and significant increase in lowest plaque pH.

7. The Concept of the 'Effector Strain'

Generally, the 'effector strain' is a microorganism with zero pathogenic potential that can persistently colonize infection-susceptible host tissues and prevent tissues conquest by pathogens. This mechanism has been described as 'replacement therapy'. This method could be used for prevention, as much as for the cure of disease, and potentially could lead to 'herd protection' through effector strain's transmission from one individual to another [101]. In the case of dental caries, the use of certain *S. mutans* strains as 'effector strains' has been proposed by Hillman [102]. Specific *S. mutans* strains with low acidogenic potential due to a lactate dehydrogenase (LDH) deficiency and the ability to produce particular bacteriocins could be mobilized to serve as 'effector strains' [102–104]. LDH is an enzyme that plays a pivotal role in pyruvate to lactic acid conversion during the catabolism process of glucose by cariogenic bacteria [105]. On the other hand, bacteriocins possess antimicrobial properties against strains or species in close relativity with the producer one [106].

A series of *S. mutans* strains, JH1000, JH1001, JH1005, JH1010 have been found to produce a specific bacteriocin ('mutacin 1140' or 'MU1140'), a lantibiotic, in particular, with close relativity to nisin's structure [104,107], as the latter has been determined by Hurst [108]. Mutacin 1140 is highly bactericidal against a wide range of microorganisms, primarily Gram-positive (e.g., *Streptococcus sanguis*, *Streptococcus salivarius*, *Streptococcus pyogenes*, *Streptococcus mitis*, *Lactobacillus salivarius*, oxacillin—and vancomycin-resistant *Staphylococcus aureus* and *Actinomyces* species), and Gram-negative bacteria as well [104,109,110]. This is attributed to its ability to powerfully connect with lipid II [111,112], which is a crucial element in bacterial wall synthesis [113] and is targeted by a variety of antibiotics [114]. No adaptive resistance to MU1140 has been presented [109]. JH1000 and its close relatives JH1001, JH1005, and JH1010 were also tested for their ability to colonize rats' oral cavity [104]. JH1001 and JH1005 strains were significantly more competent to displace indigenous *S.mutans* strains and, conversely, to a lesser extent, displaced by extrinsic bacteria (e.g., *S. mutans* Ingbritt) than JH1010 strains. The strong correlation between the bacteriocin production and the producer strain's ability to preemptively infect the rodents' oral cavity indicated that JH1001 and its successor, JH1005, could be used as human probiotic strains in the future. The finding reinforced this thesis that JH1001 strain was indeed superinfecting and could displace indigenous *S. mutans* to a great extent in humans, but a minimal infection dose (MID) was not determined [115]. Two years later,

JH1005's ability to superinfect the human oral cavity was examined [116]. The results were more than encouraging, as *S. mutans* levels significantly decreased 7- and 38-fold in the post-treatment period. In contrast, the rest of the subjects' oral ecological flora remained unaffected, thereby satisfying an excellent precondition for a successful replacement therapy that in no way should effector strains unsettle human oral ecological balance to the extent that predisposition to other diseases is possible.

The key to achieving a fabricated probiotic combining low acidogenicity and high colonizing capacity was introducing the mutant LDH gene into JH1000 strains [117]. Initially, a natural selection favored the wild-type S.mutans JH1000 occurred by eliminating the mutant gene, suggesting that the LDH gene's mutation was lethal in JH1000 [117,118]. This problem could be overcome by limiting the glucose supply and by augmenting alcohol dehydrogenase (ADH) activity, which, as found, compensates for LDH deficiency in high sugar concentrations [119].

With the advent of the new millennium, Hillman et al. announced the construction of an effector strain, BCS3-L1, thanks to the insertion of the adh B gene of ADH (derived from *Zymomonas mobilis*) to the JH1140 strain (a mutant strain that produces two- to three-fold mutacin 1140 than JH1001) [120]. Animal studies highlighted that strain as ideal for replacement therapy inception, as it fulfilled all the prerequisites for an effector strain, which are the significantly reduced pathogenic potential, the selectivity in colonizing the tissues at risk of disease (i.e., the *S.mutans* niche), genetic stability, superinfecting competency, and prevention of pathogen outgrowth. Targeted mutations were introduced to BCS3-L1 through DNA recombination [50]. These mutations affected the genes dal and come. The dal participates in formation of the bacterial cell wall and the comE gene has a regulatory role in the uptake of exogenous DNA. To date, tests in rats foresee no harmful side effects of A2JM. Collectively, the A2JM strain has low acidogenicity, can colonize the oral cavity, produces high levels of MU1140, and is genetically stable.

Other strains are also under investigation. These include the LDH-deficient *S. rattus* JH145, which can displace *S. mutans* from the oral cavity of rats [121] and is included in the commercial ProBiora3 TM (*Streptococcus uberis* KJ2, *Streptococcus oralis* KJ3, *Streptococcus rattus* JH145) mouthwash, which is considered a safe and effective adjunct in maintaining dental health [51,122]. A new strategy for replacing dental caries places LDH- and gcrR-deficient and *S. mutans* in the foreground [123]. Hence, the deletion of this gene allows the LDH-deficient *S. mutans* to better adhere to tooth surfaces. This hypothesis has been confirmed both in vitro and in vivo [124].

At first, the concept of the effector strain constituted a radical notion in probiotic therapy. Now, it is considered an integral part of the contemporary caries cure or prevention strategy in adults. Moreover, the thorough study of the effector strain's capacities and its safety guarantees its clinical effectiveness.

8. Synbiotics: A New Perspective in Caries Management

As previously mentioned, the term 'synbiotic' regards products that consist of both probiotics and prebiotics. Gibson and Roberfroid first proposed prebiotics in 1995 to promote symbiosis in gut microbiota [19]. Today, there is clear evidence that prebiotics enhances host's immune function [125–128], selectively favoring health-promoting bacteria, such as *Lactobacilli* and *Bifidobacteria* [129–133], employing potential adhesion sites of pathogenic strains, thereby exerting anti-adhesive properties and repressing the virulence of human pathogens per se [130,132,134].

In dentistry, as in general medicine and the food industry [93], prebiotics are combined with probiotics to enhance the latter's ability to "outgrow" pathogens. Until today, only five combinations have been tested; all of them are at pre-clinical stages [135–139]. Glucomannan hydrolysates (GMH) or 3% galactooligosaccharides (GOS) and 1% fructooligosaccharides (FOS), when combined with *L. acidophilus*, suppress *S. mutans* growth [135–138]. In 2015, Kojima et al. proposed specific probiotic and prebiotic candidates that could be combined to serve as synbiotics [136]. The corresponding probiotics were specific strains

from the species *L. fermentum*, *L. plantarum*, and *L. paracasei*. The potential prebiotics were xylose, xylitol, and arabinose, which were the only saccharides tested to simultaneously inhibit *S. mutans* growth and promote the survival of *Lactobacilli*.

Another strategy of synbiotics evolving in the past few years is incorporating probiotics-specific prebiotics known for their ability to maintain the oral environment's pH at high levels when a cariogenic challenge occurs. These prebiotics are mainly urea and arginine [140]. Although urea benefits specific oral microorganisms [141–143], its anti-cariogenic effect is notable [144,145], no study about its potential use in synbiotics has been conducted. Arginine, an amino acid strongly positively correlated with caries absence in adults [146], is also well-documented [147]. In contrast to urea, arginine has found one application in synbiotics. This was accomplished by Bijle and his partners [139]. According to this study, arginine concentration is directly correlated with LGG's viability, inhibition of *S. mutans* per se, biofilm, in general, and lactic acid production, thereby preserving plaque pH after the treatment application. Nonetheless, clinical trials must be carried out soon to verify their application in the complex oral environment.

Undoubtedly, the concept of synbiotics in caries management has high promise. For the moment, it is still in its infancy. Further clinical trials, which will investigate the in vivo effect of these formulations on the oral microenvironment, especially on dental plaque and its pH, are necessary to clarify whether synbiotics can facilitate our attempts to decrease caries incidence.

9. Patient Coaching Approach on the Use of Probiotics for Caries Prevention

Research around probiotics indicates that these may be a good tool in healthcare delivery. It is expected that they will be a cooperative agreement to the patient's adjunct in oral health promotion, as probiotics are simple to consume and do not require any effort from the patients. The latter's compliance to the treatment strongly depends on their attitude towards it and how the dentist could show the short and long-term benefits of following diet instructions. Inevitably, we should persuade dental patients that probiotics are meaningful. This will not be achieved through traditional standardized health advice. Current healthcare advances dictate that the patient should be put in the epicenter and gain an active role in the doctor-patient relationship [148,149]. The motivation provided should be based on patients' customized needs and skills. A thorough understanding of the necessity of probiotics in the daily diet and the easy way of consumption they possess in contrast to more demanding oral hygiene practices will ensure their position in all dental patients' diet.

Many alterations are observed from infancy to adolescence as far as oral microbiota and dietary habits are concerned. While the microbiota of children's mouths are unstable, during puberty, they become consistent [100]. Infants and toddlers may be bottle-fed, whereas children and teenagers likely consume high-sugar- or high-starch-containing snacks and beverages [150,151]. Both habits favor caries establishment. In the case of infants, toddlers, and children, parents should be informed about the microbiological 'open window'. In this context, milk would be the probiotic carrier of choice, given the fact that they daily consume milk at breakfast time. Teenagers and children also, considering that they may not manage to refrain from a high-sugar diet, should be suggested to prefer probiotic sweetened foods instead of regular ones, as the former are considered to confer health benefits on the consumers [40,44,55].

Adults also have unique dietary patterns which need to be considered [152]. Those between the ages of 18 to 30 years old exhibit no specific nutritional pattern associated with caries disease. Those older than 30 years of age seem to consume high portions of sweetened beverages, sandwiches, and bread, indicating caries prevalence and severity in this group. In high caries-risk adults, the application of the effector strain may be unavoidable.

Older adults often confront serious health and socio-psychological problems, such as obesity, malnutrition, memory lapses, low mood, reduced resilience, etc. [153,154]. Their

oral health is also compromised [154]. Due to these problems and their advanced age, they subconsciously resist changing their attitude and quit caring about themselves, thereby facing a vicious circle of constant health impairment. This obstacle could be overcome if the dentist learns to encourage such patients and maximize the potential of collaboration and, by extension, of treatment. Considering that probiotics' daily intake does not require excellent skills or daily lives, they may consist of a minor, high profitable diet change for older patients. Again, in these people, the effector strain may have to be chosen for utilization.

People suffering from hyposalivation are vulnerable to oral diseases, including dental caries [155]. Diagnosed hyposalivation often comprises a sequela of severe systemic diseases, such as diabetes mellitus, Sjögren's syndrome, or cancer during the phase of chemotherapy and radiotherapy or may be derived by age or certain drugs [156–162]. In this context, these patients need to be diet coached to admit probiotics into their daily routine. Probiotic lozenges in the daily diet may be the best choice, as lozenges confirmedly increase salivary flow [163]. The inclusion of probiotics will help them surpass the potential jeopardy of caries development or other oral infections. Notably, cancer patients are expected to strictly follow probiotic treatment because they are more receptive to new therapies [164]. During anticancer therapy, the instability of their physical body should need diet highlights to surpass the treatment's stress. Nevertheless, cancer patients are surprisingly unwilling to follow diet recommendations for long; thus, probiotics could be an effective, cheap, and easy solution for positively affecting their oral condition at the first stages of treatment and the phase of maintenance [154].

10. Discussion

Dental caries is still a significant public health problem across the world. It has a multifactorial etiology. Health inequality influences general and oral health. In the early 1970s, Swedish children had some of the worst caries statistics in Europe. Accordingly, these inequalities were manifest between groups with lower and higher educational levels. Then, the Swedish government developed a national dental insurance system and proposed that all citizens be entitled to dental care on equal terms. At the same time, they organize public dental care, free of charge, for all children and adolescents up to and including 19 years of age. The result of this politics is the detrimental decline of the caries index. A decline in the incidence of dental caries is also observed in countries having established public health programs using fluoride for dental caries prevention, coupled with changing living conditions, healthier lifestyles, and improved self-care practices. Indeed, the use of fluoride is considered a public health benefit.

Diet also possesses a prominent place in caries establishment and prevention. The frequent consumption of sugars and starches in foods and beverages is the primary causative factor of cariogenesis [165–168]. This etiologic relationship can be mirrored through studies investigating dental caries incidence whenever sugar availability changes occurred [169,170]. A correlation of the types of sweetened foods with their cariogenicity does not express the precise action of sugars in the oral cavity in real-time, due to the interplay among sugars with the salivary flow and the preventive measures implemented [171]. Since sugars' role in caries development is evident today, it is sensible to orientate toward their intake confinement. This could be achieved either by reducing sucrose intake (or intake frequency) or replacing sucrose in the diet with sugar substitutes, such as sorbitol and xylitol, or by adding to the diet various food factors, including phosphates [172]. It is not clear yet whether these measures are efficient.

Therefore, as we approach the new era of preventive dentistry, and given that fluoride intake is not a nostrum [173], we should seek contemporary methods that are highly efficient, cost-effective, safe, and necessitate the least involvement of our patients. In general, probiotic strains are considered safe, as most of them have been informally consumed in fermented foods and utilized in general medicine for several years [10,174]. Short-term

and long-term clinical trials around oral probiotics confirm this, as they do not alter the oral microbial ecology in a direction prone to disease [22,27,28,45].

The effectiveness of the permanence of probiotics' impact on the mouth's microbial flora—and by extension, on oral health—strongly depends on the pre-existent microbial conditions prevailing in the oral cavity [99]. Adults seem not to be favored as much as children at early ages by using probiotics, primarily due to their oral microbiome's stability. This is why we need to consider probiotics better as a preventive method rather than as a therapy per se, meaning that we can implant certain microbial strains in our patients' mouth from an early age to augment the potential benefit to people's oral health in the long-run. Yet, this is not always the case. As it is known, public health measures and pharmaceutical regimens have played a vital role in overall in the dental health improvement and the prolongation of life [175–178]. This, in conjunction with falling birth rates, leads to an aging population [175,179]. Given that more people are getting older and preserving their natural teeth than in the past and are more likely to develop caries lesions [180–182], we need to develop methods that are addressing such a population [183]. In these people, the effector strain concept seems to be critical, as it allows us to displace established pathogens at an advanced age, at which naturally significant alterations in oral microecology have not been feasible previously. Perhaps another adult-oriented method to prevent caries lesion in the future may be the autonomous use of mutacin 1140 (MU1140) or its analogues, to confine *S. mutans* carriage, followed by the treatment of simple probiotic strains, such as *L. reuteri* strains, to ensure that a desirable integration of theirs in the mouth is competent and durable. The last suggestion is since mutacin 1140 is highly bactericidal for *S. mutans* [104], this is an ability that can be technically improved [184]. Also, MU1140 is not connected with acquired resistance by pathogens [109], its pharmaco-kinetic and -dynamic properties are well-known [110,185,186], and its analogues can now be produced through laboratory biochemical processes [187].

The most significant advantage of probiotics is that they confer benefits to patients' health through a minimal involvement of the latter. This will make them more acceptable as a new method. Furthermore, probiotics can now be contained in formulations, such as ice-creams and cakes [40,44,55], which contain high proportions of sugars, thereby promoting cariogenesis [171], and make those easily consumed, happy diet delicacies to work in favor of the good oral condition and not against it. The choice to entirely refrain from such products is not feasible, especially for children, who frequently consume a sugar-enriched diet [188]. Thus, the inclusion of probiotic strains in these products would be positive for preventing dental caries as they reduce *S. mutans* salivary levels. This will change in the future; the ways in which diet can benefit oral health, diminishing dental caries, through comfort in use and well-known products, will be accepted by everyone. It seems imperative that different probiotic formulas must be designed in the food industry in collaboration with dental professionals to make oral prevention and human sustainability a fact for future generations.

11. Conclusions

The introduction of probiotics to the field of cariology is auspicious for the decrease of caries prevalence. The most important fact of all is that they are addressing a broad spectrum of our patients' ages and health status through multiple manners and they can be readily and safely incorporated into daily use with the application of general coaching models, without necessitating particular toil from the side of the patients. The latter is of great significance for older people. The knowledge around oral probiotics' mechanisms of action, nonetheless, is still lacking. Further studies need to be conducted to understand their interaction with the host's cells and microbiome. In the meantime, they should be used as a preventive method rather than as a caries therapy per se tool.

Author Contributions: M.A. (Markos Amargianitakis); writing—original draft preparation, M.A. (Maria Antoniadou); writing—review and editing, C.R.; supervision, writing—review and editing, T.V.; project administration, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

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

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Review

Mushroom Nutrition as Preventative Healthcare in Sub-Saharan Africa

Tito Fernandes [1,*], Carmen Garrine [1,2], Jorge Ferrão [3], Victoria Bell [4] and Theodoros Varzakas [5]

[1] CIISA, Faculty of Veterinary Medicine, University of Lisbon, 1300-477 Lisbon, Portugal; carmen.garrine@fmv.ulisboa.pt
[2] Faculdade de Veterinária, Universidade Eduardo Mondlane, 3453 Maputo, Mozambique
[3] Vice-Chancellor's Office, Universidade Pedagógica, Rua Comandante Augusto Cardoso, 1260 Maputo, Mozambique; reitoria@up.ac.mz
[4] Faculdade de Farmácia, Universidade de Coimbra, 3000-548 Coimbra, Portugal; victoriabell@ff.uc.pt
[5] Department Food Science and Technology, University of Peloponnese, 24100 Antikalamos, Greece; t.varzakas@uop.gr
* Correspondence: profcattitofernandes@gmail.com; Tel.: +351-91-992-7930

Abstract: The defining characteristics of the traditional Sub-Saharan Africa (SSA) cuisine have been the richness in indigenous foods and ingredients, herbs and spices, fermented foods and beverages, and healthy and whole ingredients used. It is crucial to safeguard the recognized benefits of mainstream traditional foods and ingredients, which gradually eroded in the last decades. Notwithstanding poverty, chronic hunger, malnutrition, and undernourishment in the region, traditional eating habits have been related to positive health outcomes and sustainability. The research prevailed dealing with food availability and access rather than the health, nutrition, and diet quality dimensions of food security based on what people consume per country and on the missing data related to nutrient composition of indigenous foods. As countries become more economically developed, they shift to "modern" occidental foods rich in saturated fats, salt, sugar, fizzy beverages, and sweeteners. As a result, there are increased incidences of previously unreported ailments due to an unbalanced diet. Protein-rich foods in dietary guidelines enhance only those of animal or plant sources, while rich protein sources such as mushrooms have been absent in these charts, even in developed countries. This article considers the valorization of traditional African foodstuffs and ingredients, enhancing the importance of establishing food-based dietary guidelines per country. The crux of this review highlights the potential of mushrooms, namely some underutilized in the SSA, which is the continent's little exploited gold mine as one of the greatest untapped resources for feeding and providing income for Africa's growing population, which could play a role in shielding Sub-Saharan Africans against the side effects of an unhealthy stylish diet.

Keywords: food insecurity; mushroom nutrition; poverty; health promotion; health foods

Citation: Fernandes, T.; Garrine, C.; Ferrão, J.; Bell, V.; Varzakas, T. Mushroom Nutrition as Preventative Healthcare in Sub-Saharan Africa. *Appl. Sci.* **2021**, *11*, 4221. https://doi.org/10.3390/app11094221

Academic Editor: Wojciech Kolanowski

Received: 31 March 2021
Accepted: 30 April 2021
Published: 6 May 2021

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1. Introduction

The basic human Right to Food, a 73-year-old commitment of all countries, should have guaranteed each living person to be exempt from hunger, which is mainly generated and perpetuated by human decisions and the dependency on international trade agreements. Universally, the concept has evolved to Right of Adequate Food interlinking policies on agriculture and nutrient requirements with fields such as environment, climate, energy, education, social–economy, and marketing.

Despite regular and record numbers of national and international campaigns, programmes, initiatives, global development goals, universal declarations, technical and scientific articles, and books, Sub-Saharan Africa stands as the world's most food-insecure region. Albeit advances, hunger, food insecurity, and under nutrition still prevail as a serious hazard and the United Nations Zero Hunger Challenge by 2030 is in doubt and probably unachievable.

Agriculture and Fisheries, and associated sectors, are the main sectors of occupation for the majority of African people. Agriculture, sea, and river resources are the impelling cause of economic reform in Africa, since it bears the world's largest unfarmed arable land and marine assets, employing a large fraction of the population [1]. Nevertheless, SSA is a net food importer that is dependent on most agricultural and agro-food sectors, namely maize, rice, and wheat. This situation was aggravated in the last four decades, mainly due to rapid population growth, persistent economic inequality, climate change threats, and even claims of the legacy of colonialism.

In order to obtain a SSA food sovereignty, further to the general need to increase the production and productivity of cereals, untapped traditional and native food crops with expected nutritional attributes remain to be extensively researched and considered, having massive potential to improve the agri-food and fisheries' value chains [2].

Smallholder farmers and fisherman, with a valuable central role to play, have been struggling on a subsistence level often with no community-control and biodiversity-based food systems. There is no panacea for these issues, and the first movement must be on investments in agricultural and fisheries infrastructures and extension services, as smallholders are key actors in food security and in poverty reduction [3].

The leverage of agriculture for food and nutrition security is a means to improve human health and dietary patterns toward increasing agricultural diversity and ensure a balanced diet. However, there is little health research on diet quality based on what African people consume, and a rigorous evaluation of for example universal micronutrient supplementation effects from international aid programmes is extremely rare.

The practice for the past decades of vast and well-intended international aid, even from the United Nations WFP, to the region to curb food insecurity has been unsustainable, ineffective, with unintended consequences and may even ultimately cause harm. It is essential to link aid effectiveness to catalyse development strategies with a longer-term focus.

In SSA, comprising some 44 countries, despite poverty, chronic hunger, food insecurity, movement to renewal, and the arrival of new foods and eating habits, the traditional food choices have luckily prevailed and been considered beneficial in relation to health outcomes and sustainability [4].

However, it is changing, since with no trade agreements, many international organizations and food companies dump their products to gain market share in SSA. Since the 1960s, the African people have consumed increasing amounts of processed food.

The global food system is very complex and influenced by many different inputs, including farming, economics, politics, environment, transport, storage, and consumers; it must entail long-term dimensions on sustainability. These factors are aggravated in SSA, the second world region with the highest prevalence of under-nutrition as well as inadequate incomes or other resources.

Malnutrition is still one of SSA's primary concerns for enhanced human development. Due to inadequate dietary intake and lack of nutritional knowledge, there is a frequent concurrence of both under-nutrition and over-nutrition in the same population across the life course probably due to unbalanced diets or diseases [5]. There are multiple reasons for malnutrition and promoting actions must be multi-sectorial, although quite complex to coordinate.

In general, despite indications that Africans are smoking less and having more physical exercise than in developed countries, when food is available, the African diet rivals the healthy Mediterranean diet. African cuisine is a healthy way of cooking and may become an example if food diversity is enhanced. However, in African urban areas, with the growing acceptance of "Western" eating habits, one can expect more non-communicable diseases or chronic diseases (e.g., diabetes, cardiopulmonary diseases, cancer) for which African healthcare systems are unprepared [6].

Among many possible initiatives to improve food and nutrition security in SSA, it is important to identify consumer habits in each region. Ideally, a guideline needs to be specifically designed for each of the main six African regions or even per country.

The incorporation of African indigenous foods into the existing diet must be incentivized. African "superfoods" and other functional foods and beverages, with traditionally proven major health benefits, should be encouraged.

Knowledge about what is eaten is essential to dietetics and food science as well as for biodiversity, agriculture production, and the food industry. Food pattern recommendations usually limit the intake of salt, sugar, and saturated fats and are normally derived from established dietary guidelines. While these have been well structured in developed countries [7], only very few SSA countries have achieved this stage.

An overview of the African foods and ingredients and the importance of establishing national dietary guidelines that apply to each country or region are discussed. Contrary to what is known from ancient Asian civilizations, the ethnomycological knowledge of useful African mushrooms is scant. Furthermore, gut microbiota from SSA people have different and specific profiles, which need to be studied in order to match and determine their nutrient requirements. Since most rural SSA small farmers operate traditional subsistence lifestyles, it is important to evaluate their microbiota profile and role as well as the widespread antibiotic use [8].

This article is a contribution to the debate about the effectiveness of dietary interventions for African rural development, integrating agricultural interventions for food security with those for poverty reduction while shedding light on mushrooms as novel food source with health-promoting foods and a possible contributor to the African diet, income, health, and livelihood. Our review has several clear limitations, most importantly the lack of accurate data on food production and of epidemiological studies in SSA.

2. The African Diets

The diverse nature of African cooking has fantastic elements of different cultures: Arab, Black African, European, and Asian. African eating and drinking habits are significantly different in each African region. Presently, there are some five to six main African regions (Figure 1) and there are not many studies on food consumption patterns of the African people per country or among the 54 sovereign countries [9].

Figure 1. Main African regions.

Africans, namely rural communities, perform more physical exercise and do not eat many ultra-processed foods such as salty fatty packaged snacks, soft fizzy drinks, sweetened breakfast cereals, and instant convenience foods, which are ultra-processed and nutritionally unbalanced [10].

The traditional African diet comprises more wholesome and healthful foods rather than pre-treated food (Figure 2). In general, the defining characteristics of the traditional African cuisine are rich herbs and spices, fermented foods and beverages, and healthy and whole ingredients [11]. In comparison with other continents, very little meat, fish, and poultry is generally consumed in Africa [12].

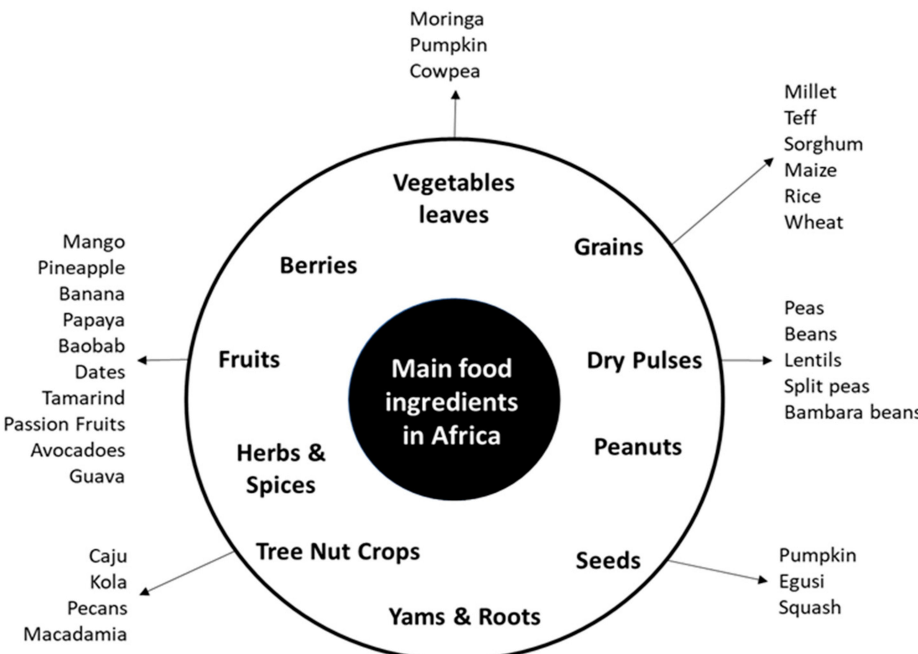

Figure 2. Main food ingredients in Africa.

It is considered that people in West Africa (Mali, Chad, Senegal, and Sierra Leone) enjoy healthier diets than their counterparts in the United States, the United Kingdom, Japan, or Canada [13].

Some select African high-nutrition foods, "superfoods" worthy of this title, have been identified, with high concentrations of essential nutrients such as phytonutrients, vitamins, minerals, enzymes, and antioxidants, although no single food holds the key to good health or disease prevention. They include moringa, baobab, yams, teff grain, leaves of kenkiliba, sesame, fonio grain, artemisia, tamarind, hibiscus, coconut, pumpkin, and amaranth leaves (Figure 3).

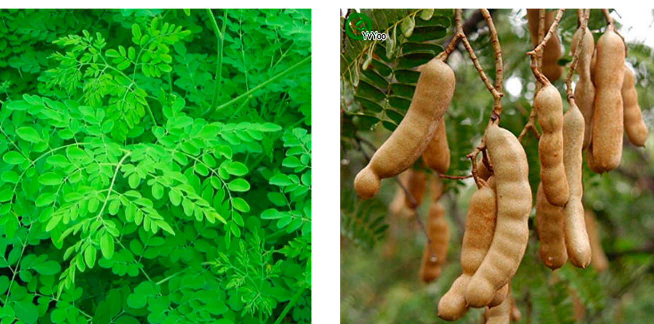

Figure 3. Moringa leaves and tamarind fruit, widely used as food and for medicinal purpose.

Other foodstuffs with good nutrient counts and health properties comprise local darky leafy greens, green tea, legumes/pulses, nuts and seeds, yoghurt, garlic, ginger, curcumin, avocado, sweet potatoes, seaweeds, and mushrooms. Cooking oils from fruits (e.g., palm), seeds (e.g., sunflower), and nuts (e.g., peanut) are widely used, while olive oil is also important but in northern Africa where Tunisia and Morocco are considered the largest producers in the world.

Meat, fish, seafood, and traditionally produced fermented dairy products (e.g., yogurt, cromwo, boeber, alouda, amasi, and leite azedo) are often used as a garnish, prepared with cooking oil, tomatoes, onions, salt and spices, poured into a mash or porridge made from cereal or cassava flour. Beef, goat, chicken, eggs, and mutton are quite expensive in SSA,

so these foods are reserved for special days. However, fish and seafood are abundant in coastal regions, rivers, and lakes.

Most countries should periodically review their multi-sectorial nutrition strategies and consider both more effective nutrition-specific intervention approaches (e.g., supplementation, fortification) and/or a shift to actions at the level of underlying causes (e.g., promoting optimal breastfeeding and complementary feeding, diet diversification). We have previously considered the issue of monotonous diet in SSA, lack of diversification, and fortification as a matter of business as much as science [14].

3. African Dietary Guidelines

Modern nutritional science is surprisingly young, and "Food-Based Dietary Guidelines" are suggestions based on science knowledge in the form of instructions for healthy eating. Designed for information to consumers, customers, and technical advisors, they must be suitable and relevant for each country, culturally appropriate, and easy to implement. Moreover, they should be harmonious, comprehensible, and memorable [15].

Authorities use a spectrum of schemes from optional to compulsory. The present global food regulatory framework is confusing and limiting, and specific measures exist for certain claims [16]. Many challenges still remain regarding the establishment of dietary guidelines integrating education, agriculture, health, environment, and industry.

Many developed countries have established dietary guidelines, but implementation plans are often not comprehensive enough for consumers. Dietary guidelines around the world are presented in different configurations, exhibited as pyramids, plates, baskets, texts, circle graphs, diagrams, and tables, yet they are similar in terms of content, giving consumers a number of advised food groups and daily servings to maintain optimum health [17].

In Africa, food-based dietary guidelines have been established in Benin, Kenya, Namibia, Nigeria, Seychelles, Sierra Leone, and South Africa, the latter in 2003. Food is a particularly sensitive commodity, but the majority of SSA countries did not develop their own food guidelines [18], and in general, the approach of the legal systems has been broadly consistent with the benchmark in international trade agreement channelled through the *Codex Alimentarius* Commission since 1963 [19].

In African history, most rural life has been devoted for household production and the procurement and preparation of foodstuffs, often low in nutrients, while food scarcity has constituted a major threat to survival. Unless the food quality (i.e., safety and nutrient composition) that Africans eat is addressed, the continent will not be able to address under-nutrition, obesity-related diseases, and even mental health.

We have enhanced the fact that some major nutrient contents of foods are reasonably well characterized, and their required levels of intake calculated. However, the subject becomes quite complex when accounting for the active bioavailability of the dietary compound rather than the dose ingested [20]. Furthermore, there are naturally occurring ca. 100,000 phytonutrients in plants, which are considered non-essential for growth and development but essential for lifetime good health [21].

We have previously reviewed several global dietary guidelines and noticed that with a few exceptions, fermented foods and mushrooms are generally absent as a recommended category of food for daily intake in Food Guides, reflecting a failure to appreciate the benefits resulting from these foods [17].

Current food systems in African agriculture, fisheries, and animal production, an outcome of a historic development pathway, are unsustainable with no diversification and no adequate integration of indigenous products [22,23]. Nevertheless, Africans eat starchy foods in the form of minimally processed or whole grains, legumes, beans, roots, and yams, rather than refined starches and sugary products with the benefit of a high carbohydrate intake supplying 55–75% of total dietary energy [24].

The majority of African cuisines have a different starch base (sorghum, millet, maize, teff, rice, sweet potatoes, cassava, and yams) because they supply plenty of calories. Starches are more filling, as they mislead the body and brain into feeling satiated [25]. Usually, it is

complemented with margarine or oil, supplying an extra source of energy but few essential nutrients where fried onions, garlic, and tomatoes make a basic curried sauce [26].

Inadequate nutrition, whether associated with deficiency disorders or chronic diseases, is embedded in impoverishment and neediness [27]. In the poorer regions of SSA, micronutrient malnutrition exists wherever there is undernourishment due to food shortages, and it is likely to become common where diets lack diversity, even in conjunction with sufficient energy intake [28].

Role of Mushrooms in the Dietary Guidelines

Foods supplying proteins in dietary guidelines have been organized under the concept of being either animal or plant based while other rich protein sources, i.e., mushroom-derived, has been relatively neglected [29]. The intake of mushrooms per day has been very low worldwide and regarded as a lavish delicacy; however, their inclusion adds essential shortfall micronutrients and bioactive components.

Mushrooms have a unique and key nutrient profile supporting the recommendation of lower energy intake and sodium, and they are uniquely high in vitamin D and protein as well as low in fat. Mushrooms can be supplied fresh, as biomass dietary supplements, or as extracts. However, the extracts are considered medicinal nutraceuticals and not as foods or dietary supplements, and the legislation regulating these dietary supplements remains unclear due to the fact that they can be considered as foodstuffs and/or medicinal products depending on various factors.

Below, we propose a graphic design of a general Food Guideline for Sub-Saharan Africa with the inclusion of indigenous products (Figure 4). It is an attempt since a sound or comprehensive Sub-Saharan African Food Guide Pyramid should be broader and include other information related to food safety, number of meals, amount of (un)refined foodstuffs, processing stages, access to potable water, food traditions, fermented food and beverages, salt and sugar consumption levels, methods of cooking, sociocultural habits, and even creed and religious faiths.

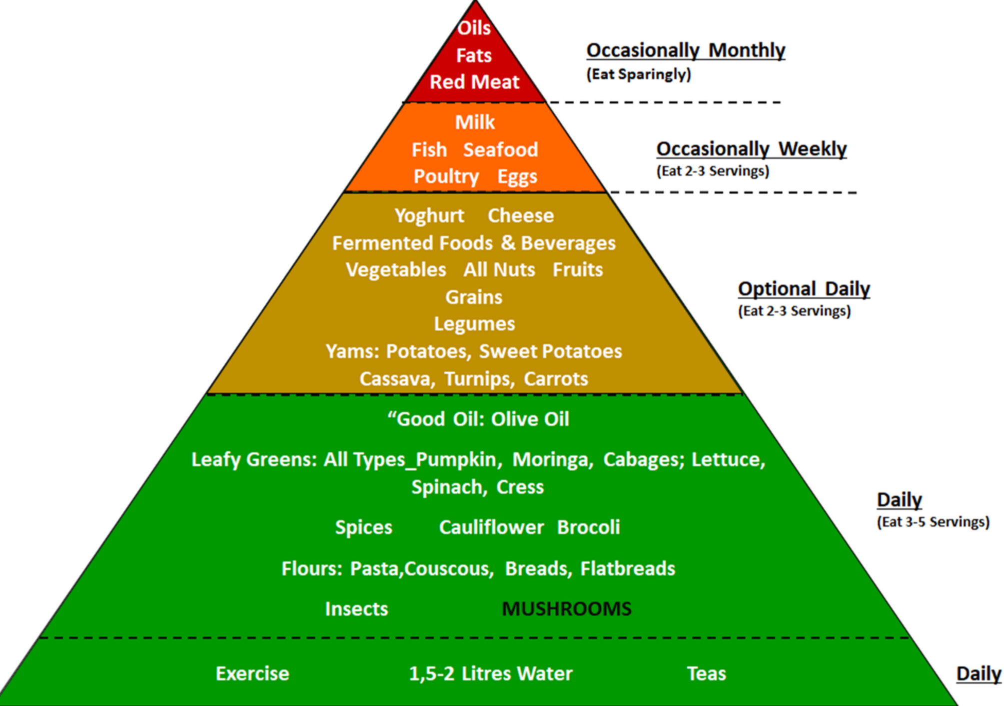

Figure 4. The Sub-Saharan Africa Food Guide Pyramid. There are variations in amounts recommended from different food groups. A "serving size" is a standard amount of a food, such as a cup or a spoon, but they are not fixed recommendations. It is recommended to drink a lot of water and teas, exercise physically daily, and spare on salt, sweets, candies, processed foods, squash drinks, and fizzy beverages.

We note the specific inclusion of mushrooms as a valuable food source of essential nutrients and bioactive components, namely protein and medicinal bioactive elements that have been in the past overlooked in global dietary guidelines.

The term "portion" means the amount of a food selected for a refection or snack. A portion size can vary from meal to meal, depending on energy concentration, whereas "serving size" is a measured amount of food usually recommended by the food manufacturer or an external agent. There is room for all foods in a well-balanced, healthy diet, but some should be eaten less often and in smaller portions than others. Appropriate amounts eaten takes practice and depends on the individual.

Even considering developed countries, only very recently there has been a pioneer recommendation of adding a serving (84 g/day) of mushroom mixtures to USDA Food Patterns [30].

In SSA, it is imperative to promote foods with a high ratio of micronutrients to energy content, diversifying food sources, moving away from the concept of food groups and adopting the use of local names for common foods and beverages. Each country, and probably each individual region, must design specific healthy eating patterns [31,32].

4. African Mushrooms

Mushrooms that have existed for centuries have spread all over the world, and their distribution in SSA has not been completely surveyed yet. Mushrooms are vitally important to human life and of primary importance for the environment as global decomposers of nature organic matter and recyclers in most ecosystems, and they have been used in SSA since the Palaeolithic period (5000–9000 BC), where their application has been historically related to spiritualism [33].

Mushrooms (aboveground macrofungi) engage central roles in SSA tropical forest ecosystems feeding on non-living organic matter and interacting with trees, and they can influence carbon and nutrient cycling and the maintenance of biodiversity. Truffles (subterranean macrofungi) from arid-land ecosystems enhance the capacity of their host plants to resist dry spells.

Fungi also help in the growth of most plants by developing mycorrhizal symbiotic associations, enhancing crop productivity and aiding to increase access to water and minerals for plants, and tolerance to stressful conditions. Zimbabwe, Swaziland, Namibia, South Africa, Malawi, Benin, and Ghana are the leading mushroom-producing °countries. The production of mushrooms on a small or large scale is quite simple, and good examples can already be seen in Cameroon, Congo, Ivory Coast, Kenya, and Namibia, where 10 kg of fresh mushrooms per square meter and more is achieved.

If fungi are to be the subject of a thriving application in industry and biotechnology in Africa, much more research and development is needed, and consequently mycological education. Some countries (e.g., Zimbabwe) have increased prominence of fungi in the primary school curriculum and awareness of edible and toxic mushrooms. Malawi has a Fungi Farm where children have the opportunity to learn about how the conservation of nature and ecology is key to how we can all live in harmony.

However, most SSA countries did not release guidelines or enact legislation in order to ensure the safe commerce of wild mushrooms due to food safety concerns, and present legislations do not yet mention macro fungi let alone their conservation.

Nutritional information on cultivated species of fungi is extremely vast; however, data on wild edible fungi remain scarce, these being collected for food and as an income [34].

Here, it is highlighted the potential and the current knowledge available on the nutrient, antioxidants, and bioactive components values of some African edible macrofungi underlining the applications of mushrooms as dietary foodstuffs in some major health concerns, and they are also often used for innovative biotechnological, medicinal, and ecological applications [35]. It does not underline their application in complementary folk medicine in this part of the world, but the need to consult indigenous people with this knowledge must be stressed.

The tropical and subtropical regions of SSA are characterized by higher mushroom diversity compared to North Africa. Mushrooms and truffles are considered valuable foods in many cultures being rich source of different types of essential nutrients, and they have been widely studied and reviewed [36,37]. White truffles are considered the most expensive food in the world; however, this bonus income has not been explored in SSA, where less expensive black desert truffles predominate, growing mainly in the rainy season.

No single sector or actor can establish food and nutrition security, and there is the need for a well-coordinated effort among them on cross-sectorial approaches and multi-partner platforms [38]. Cooperative endeavours under a multi-institutional program are required to seize the representative macro fungi species of SSA with a view to update their nutritional and health value [39].

Globally, there are up to 5 million species of mushrooms, of which around 1000 of these species can be found in SSA, and as there is no single tool for their identification, only some 7% have been accurately classified [40]. The poor discovery, identification, and certification of edible and medicinal species of mushrooms in SSA retarded its potential use in nutrition, as tonic, and as medicine [41,42].

Foraging for wild mushrooms in SSA Africa does play a significant role in sustaining their livelihood, but there are very few ethnomycological reports and research on mushroom genetic resources, the cultivation of undomesticated wild mushrooms, protection, and lineage improvement [43].

With the application of molecular methods, it is now possible to identify mushrooms previously considered non-existent in SSA. *Hericium erinaceus* was first reported in Tunisia, but it is common in SSA tropical forests in Ghana, Cameroon, Congo, Madagascar, and currently, it is also successfully cultivated and South Africa, exporting annually over 440 tons [44].

Mushrooms produce a vast set of extracellular carbohydrate-active enzymes and biological active molecules that degrade very complex compounds such as hemicellulose and lignin. The variety of enzymes is dependent on the habitat and specific substrates, so it differs among mushroom species and home ground [45].

Desert mushroom truffles, used for thousands of years in Africa, include genera such as *Phaeangium*, *Terfezia*, *Delastreopsis*, *Balstonia*, *Delastria*, *Leucangium*, *Mattirolomyces*, *Tirmania*, and *Tuber* [46], and they are of considerable interest for ecological reasons because of the low water input or organic matter required for sprouting.

Various types of mushrooms and truffles (Figure 5) are considered as natural biota in the North Africa deserts [47,48] and in South Africa, Namibia, and Botswana [49].

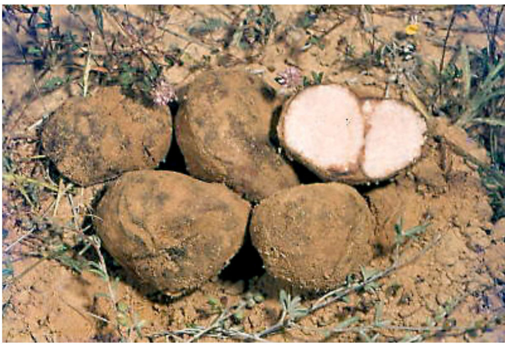

Figure 5. African desert truffles genus Terfezia.

Mushrooms belonging to species of *Termitomyces*, *Pleurotus*, *Lentinus*, *Lenzites*, *Trametes*, *Ganoderma*, *Pycnoporus*, *Coriolopsis*, and *Calvatia* have been reported to be used in folk medicine in West Africa. Some popular wild edible and medicinal mushrooms in West Africa include *Schizophyllum commune*, *Lactarius* spp., *Chantarellus platyphyllus*, *Volvariella volvacea*, and *Auricularia auricular-judae* [50,51]. In the Namibe desert, *Agari-*

cus campestris, *Calvatia lilacina*, *Coprinus comatus*, *Ganoderma* spp., *Schizophyllum commune*, *Volvariella volvacea*, and *Termitomyces*.

Specific to Africa, there are more than 1000 species from the family Termitidae, constituting 95% of soil insect biomass [52]. Although they are wild, the African mushrooms, and particularly those species associated with termites (*Termytomyces*), are considered "superior" to all other mushrooms [53]. There are about 30–40 mushroom species of *Termytomices* whose cap can reach 1 m in diameter, and most are highly valued as food (Figure 6) [54]. On the other hand, mushrooms of the species *Termitomyces*, *Agaricus*, *Boletus*, *Pleurotus*, *Cantharellus*, *Macrolepiota*, *Ganoderma*, and *Geastrum* have been reported in East and South Africa [37].

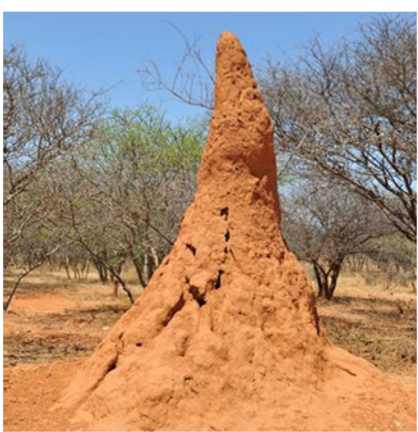

Figure 6. A termite and roadside vending of edible mushrooms, Termitomyces mushroom, a traditional source of seasonal income in miombo areas in Mozambique.

The indigenous population on miombo, a savanna type of ecosystem woodlands, relies directly on the timberland for their resources for wood fuel, food and wild fungi. This region extends from East Africa (Burundi, Kenya, Tanzania, Uganda) to the Zambezian region (Angola, DR Congo, Malawi, Mozambique, Zambia, Zimbabwe), where the most predominant mushrooms occurring include *Cantharellus* (15 spp.), *Lactarius* (incl. *Lactifluus*) (14 spp.), *Russula* (10 spp.), and *Amanita* (8 spp.) [55].

Despite its millennial existence and its empirical knowledge, harvesting wild mushrooms is not a well-known concept in Africa due to the threat of being poisonous and sociological impacts (myth, culture, and spirituality) [56,57], while commercial production exists but is still in its early stages.

Mushroom cultivation is a lucrative agricultural process to produce various essential nutrients from plant wastes, requiring a correct combination of compact space, temperate climate, high humidity, and organic substrates residues [58,59].

Bioactive Compounds of Mushrooms

Unlike other foods, macro-fungi mushrooms act through major categories of specific bioactive molecules: (1) polysaccharide β-glucans or polysaccharide–protein complexes; (2) triterpenes; (3) polyphenols; (4) alkaloids; (5) metalloids; (6) short-chain fatty acids; (7) enzymes; (8) lectins; (9) nucleotides.

Mushrooms feed on dead plant material, fulfilling an essential role in the carbon cycle while harbouring numerous species with diversity of metabolites of nutraceutical and therapeutic significance [60].

Mushrooms are heterotrophic (do not perform photosynthesis) and reproduce through spores absorbing complex organic compounds from the environment, as they are unable to synthesize their own organic matter [61]. The mycelia, which play important roles in the support and absorption of nutrients, rely solely on carbon obtained from other living organisms, i.e., plants, insects, and even other mushrooms, for growth.

Considerable debate is ongoing on the definitions of nutraceuticals (first coined in 1989 by Stephen L. DeFelice), functional foods (coined in Japan in the early 1980s), innovative food products, with extensive disagreements, and no existing international agreements, which compounds the confusion. We regard nutraceuticals as products, which other than nutrition are also used as alternative for pharmaceuticals.

There is abundant literature, and we have described the value of some edible mushrooms and culinary–medicinal mushrooms, their biomass and extracts, which contain many low molecular bioactive components termed secondary metabolites, since they are formed due to the enzymatic resections of primary substances (amino acids, sugars, vitamins) [62].

The most common secondary metabolites in mushrooms include polyphenols, phenolic acids, quinones, coumarins, groups of flavonoids, stilbenes, hydrolysable and condensed tannins, terpenes and terpenoids, alkaloids, lectins, sterols, lactones, antibiotics, and metal-chelating agents, all of which may activate the cell and humoral immunity, hence increasing resistance to disease [63–66].

The different bioactive polyphenolic compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents [67]. These large numbers of biological cell components and secondary metabolites have been shown to affect the immune system of the human consumer [68,69].

Lignocellulose is the most abundant natural biopolymer on earth, and its structure being so complex affects its biodegradation and rate-limiting steps in the global carbon cycle [70]. Most species of mushrooms synthesize enzymes that may play important functions in the human organism. The vast list of enzymes in mushrooms include hydrolases, glucoamylase, pectinase, acid protease, endo-1,4-β-glucanases and 1,3-β-glucosidase, esterases, phenol oxidases, polyketide synthase, hemicellulases (glucuronoxylanase, arabinoglucuronoxylanase, and glucomannanase), the ligninolytic system, cell wall lytic enzymes (laminarinase, 1,4-β-D-glucosidase, β-N-acetyl-D-glucosaminidase, α-D-galactosidase, xylanases, β-D-mannosidase, acid phosphatase, laccase, lignin peroxidase, manganese peroxidase, polygalacturonase-pectinase, ribonuclease, and many others [71,72]. Thus, mushroom enzymes can break down polyssacharides and the ligninolytic system, releasing compounds or providing a remarkable nutritive value [73].

The *Hericium* fruiting body contains polyphenol oxidases (PPOs), including tyrosinase and laccase, which are strong antioxidant substances [74]. Forest dweller *Hericium erinaceus* strains were first reported in temperate forests of North Africa and Ghana and known to have anti-peptic ulcer activity [44,75–77].

Several studies showed that *Pleurotus eryngii* and *Ganoderma lucidum* can produce laccases, which is a group of enzymes that can confer activity against HIV by inhibiting the reverse transcriptase [78,79].

Superoxide dismutase (SOD) is also present in some mushrooms, and its important physiological role is in the primary cellular antioxidant defense and its potential therapeutic use [80]. Proteolysis is an essential part of many physiological and metabolic processes in all biota, and basidiomycetes mushrooms are valuable sources of proteases used in defense mechanisms of living organisms and in biotechnological processes [81].

Multiple lectins produced by *Flammulina velutipes*, *Pleurotus ostreatus*, and *Ganoderma carpense* were shown to have potent inhibitory activity in vitro toward cancer cells through fungal ribotoxin-based immunotoxins, which are characterized by the ability to irreversibly block protein synthesis in neoplastic cells [82,83].

In addition to the presence of enzymes, we have previously discussed [84] the significance of mushroom low-molecular-weight secondary metabolites (e.g., terpenes, steroids, anthraquinones, and benzoic acid), which can regulate processes such as cell cycle regulation, apoptosis, autophagy, angiogenesis, metastasis, and signal transduction cascades, which are associated with the development of cancer [66,85,86].

Nucleosides and nucleotides, components of nucleic acids, participate in the genesis and retention of energy for basal metabolism, the synthesis of macromolecules, and cell–cell signalling interplay with cell surface protein receptors; this transmits signals and changes

inside the cell and hence the regulation of physiological processes in the human body via the vast array of purinergic and/or pyrimidine receptors [87].

Oxidative damage causes the production of over one hundred known modified nucleotides formed by multi-enzymatic reactions and by spontaneous chemical reactions. Mushroom constituents include these nucleic derivatives (nucleosides, nucleobases, and nucleotides), flavour determinants, and all are involved in several enzymatic reactions as target or as cofactors [88]. They are also used as fingerprint biomarkers to authenticate the mushroom species, and the pyrimidine ring structure, the backbone of the genetic material of DNA, has revealed therapeutic potentials and valuable medical applications, as shown below (Figure 7).

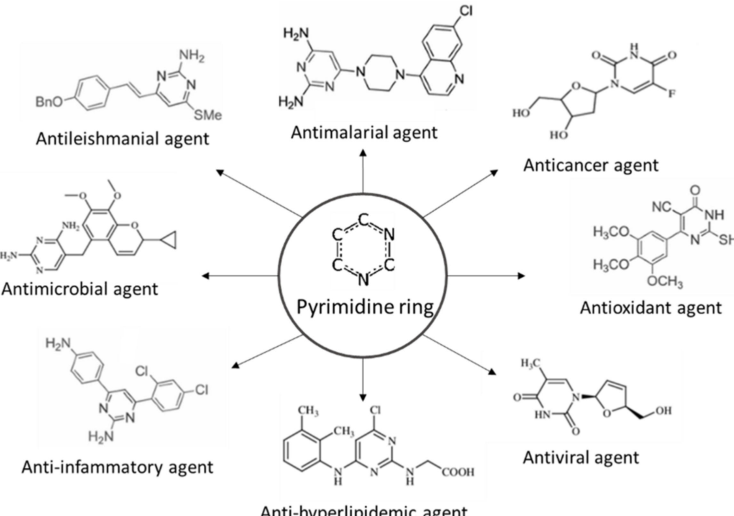

Figure 7. Antimicrobial, antioxidant, antimalarial, anticancer, and anti-inflammatory potential functions of pyrimidine derivatives (adapted from [89]).

From shiitake mushroom *Lentinus edodes*, the most studied species of mushroom and the second most consumed in the world, we can obtain lentinacin (eritadenine), a nucleoside compound well known to reduce total blood sugar and cholesterol [90]. DNA are composed of the same four nucleotides, and consumption is considered safe due to the likelihood of transfer and functional integration of DNA from ingested food, even modified, while gut microflora and/or human have developed sophisticated methods to suppress and annihilate exogenous DNA [91]. Nevertheless, there is a practical daily safe limit of nucleic acid intake (ca. 2–4 g) in human adults [92,93].

5. Anti-Inflammatory Role of Mushrooms

Inflammation, the cornerstone of pathology, is a complex protective mechanism where the blood flow raises to the area of tissue lesion or infection, which is a necessary part for recovery [94]. Inflammation a healing restorative process; however, it may be adverse also, because it destroys a lot of the fine cells in the process [95].

Based on the need to develop novel therapies, researchers have sought evidence supporting the impact of specific foods on inflammation in the body. Foods that may originate inflammation comprise processed carbohydrates such as white bread and pastries, fried chips, fizzy drinks, red meat, processed sausages, biscuits, desserts, and margarine.

Some foods, mushrooms included, have the capacity to suppress inflammation, but it is unclear how often and how much is needed for this benefit. Following an anti-inflammatory diet, one can fight off inflammation; however, although there is promising research for the impact of some foods, there is no anti-inflammatory miracle food, and although diet is crucial, it is not the single factor [96].

Consuming mushrooms does not necessarily show significant changes on induced inflammatory responses. The result is not surprising, since it would certainly be harmful

to strongly induce or suppress immune function following the ingestion of a commonly consumed food. Mushrooms also have an effect on immune function, but that effect is evident only when the immune system is challenged [97].

Common African mushrooms such as *Pleurotus tuber-regium*, *Termitomyces* spp., *Pleurotus* spp., and *Agaricus* spp. are rich in chitin, which can be hydrolyzed into glucosamine, which is involved in the creation of molecules that protect joints from inflammation [98,99].

Some mushrooms act directly on inflammation. *Cordyceps synensis*, a mushroom that is abundant and diverse in humid temperate and tropical forests at high altitude, not yet reported in SSA, contains a nucleoside compound, cordycepin, that stimulates the production of interleukin 10, an anti-inflammatory cytokine [100].

Wild or cultivated mushrooms, fresh or as dietary supplements, have anti-inflammatory activity occurring through inhibition of the NF-κB signalling pathway, which is a protein complex that controls cytokine production and cell survival, and it is a major transcription factor that regulates genes responsible for both the innate and adaptive immune response [101].

Poria cocos mushrooms also contain triterpenes, which have been shown to improve inflammation and treat tumors [102]. Other mushrooms exert an anti-inflammatory effect less directly by quenching damaging free radicals and counteracting oxidation. For instance, Chaga mushrooms (*Inonotus obliquus*) have antioxidant activity, protecting cells against oxidative damage [103,104]. Oyster mushrooms (*Pleurotus ostreatus*) have an antioxidant effect as well [105].

Much of the active polysaccharides, water soluble or insoluble, isolated from mushrooms, can be classified as dietary fibres (i.e., β-glucan, xyloglucan, heteroglycan, chitinous substance) and their glycoprotein complexes [106].

The chemical nature of extracted β-glucan varies from different sources. Cereals and other food contain 2.5–4.5% β-glucans, but these are not capable of controlling immune functions. However, mushroom β-glucans, which consist essentially of a (1,3)-β-linked with small numbers of (1,6)-β-linked side chains, can modulate the autoimmune mechanisms [107].

These biological response modifiers (1,3)-β-glucans interact with the intestinal cell wall and are absorbed into the lymph fluid, where they recruit neutrophils and macrophages and trigger the production of cytokines and stimulate immune function (Figure 8) [108].

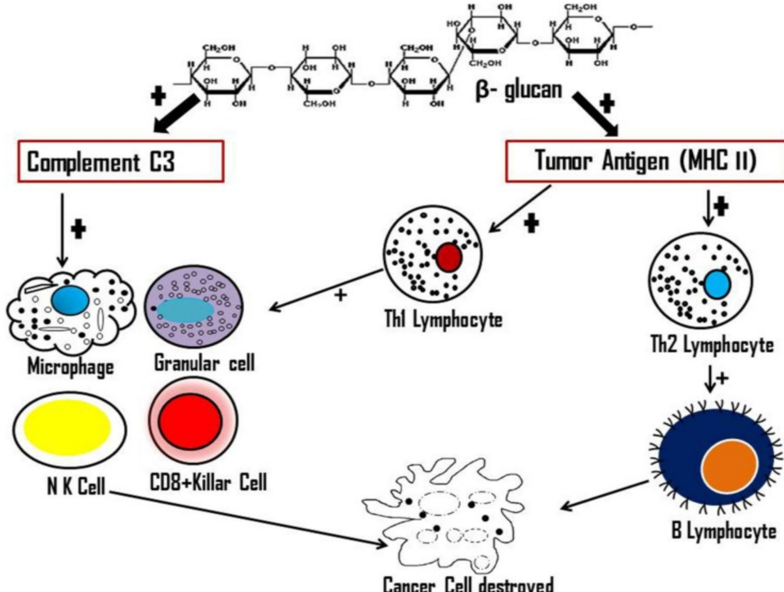

Figure 8. Mechanism of antitumor activity of β-glucan bioactive compound [109]. Normally, tumor cells do not express major histocompatibility complex (MHC-II) genes. NK = natural killer. Complement C3 = C3 (C3 deficiency are susceptible to bacterial infection).

Dietary supplements as biomass or extracts derived from the mushroom *Coriolus versicolor* are not foods; they have potential immunomodulating and antineoplastic activities, and they were shown to stimulate the production of lymphocytes and cytokines, such as interferons and interleukins, and they may exhibit antioxidant activities [110,111].

Neuroinflammation is a specialized immune response that occurs in the central nervous system, and it is linked to chronic neurodegenerative disorders (e.g., amyotrophic lateral sclerosis, multiple sclerosis, Huntington's disease, Parkinson's disease, and particularly Alzheimer's), negatively affecting mental and physical functioning being characterized by synaptic dysfunction and a gradual loss of neurons from specific regions [112,113].

Mushrooms incorporate ergothioneine, which humans are unable to synthesize, a unique antioxidant, cytoprotective, and anti-inflammatory derived from food histidine, but which accumulates to high levels in red blood cells and in many other tissues, functioning both as a therapeutic and possibly as a preventative agent of several diseases [114,115].

6. The Antiviral Role of Mushrooms

New viruses emerge all the time and can be serious threats to public health. Recently, it was reviewed how mushrooms represent a vast source of bioactive molecules, which could potentially be used as antivirals [116].

A virus is an infectious agent metabolically inert made up of a core of genetic material, either DNA or RNA, and an outer protein and lipid shell, which can only replicate using the host cell mechanisms [116].

Many of the common edible mushrooms and several non-edible mushroom dietary supplements are sources of natural bioactive compounds responsible for the prevention and treatment of viral diseases through their improvement of human immunomodulation (Figure 9) [117]. Numerous previous studies have demonstrated mushrooms as exhibitors of potential antiviral efficacy [118–120].

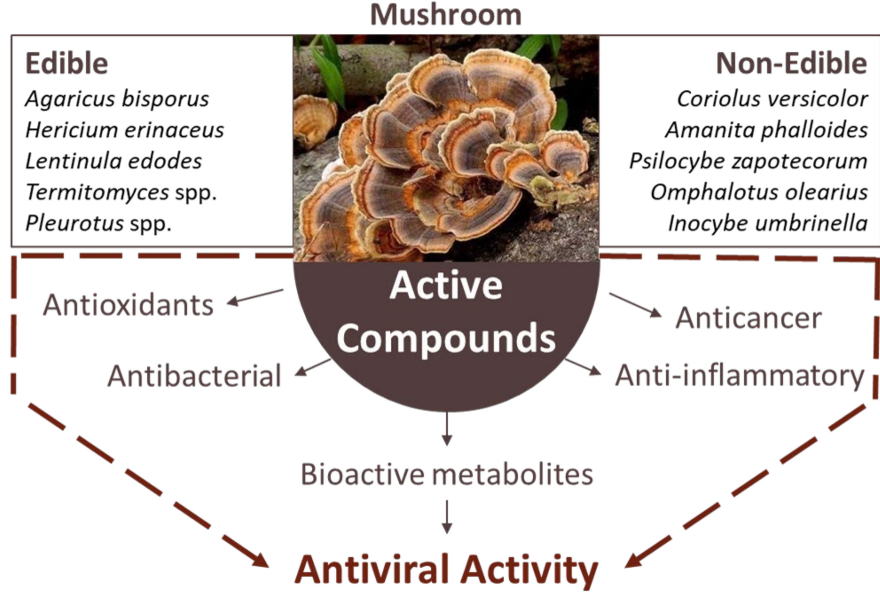

Figure 9. With over 400 bioactive compounds, mushrooms have shown a number of antiviral effects, and used as dietary supplements, functional food or medicinal products. They act by blocking virus entry into the cell, by inducing lysis of virus infected cells through activation of NK, CD8+ and T cells, by anti-neuroaminidase increased activity, and by innate immune support.

There are four mushrooms subjected to several clinical studies specifically for fighting viruses [121,122], but the following claims are still considered unsubstantiated at least for COVID-19 prevention and treatment: (1) Ganoderma: shown to kill the Influenza A virus, herpes, hepatitis, and H1N1 strain of the flu; (2) Cordyceps: fighting the Influenza virus by boosting the body NK cell activity as well as other virus-killing cytokines; additionally,

it has been shown to decrease inflammation in chronic asthma and other lung diseases; (3) Maitake: it has shown to actually stop the replication of the virus, which would be very helpful in allowing the body to fight it off without getting too overwhelmed and preventing a lot of excessive damage; additionally, it is also shown to boost the body supply of antiviral cytokines; (4) Shiitake: ability to stop the growth of the virus by preventing the entry into the cell; this mushroom has shown to be effective in fighting the herpes simplex virus, hepatitis C virus, HIV, and the influenza.

Mushrooms fight viral infections, and there are many studies on antiviral activities of several mushrooms against herpes (HHV-causing skin infections) [123], West Nile (mosquito-borne disease) [124], Orthpoxviruses (variola agent) [125], influenza [126], hepatitis B [127], and human immunodeficiency (HIV) [128]. The most studied mushroom strains for producing antiviral bioactive compounds include *Coriolus versicolor*, *Lentinula edodes*, *Grifola frondosa*, *Ganoderma lucidum*, *Hericium erinaceus*, *Pleurotus ostreatus*, *Cordyceps sinensis*, *Laricifomes officinalis*, *Lenzites betulina*, *Rozites caperata*, and *Daedaleopsis confragosa* [129].

People cannot avoid harmful bacteria and viruses, but they can become ill less often and with shorter periods if the immune system is strong. The objective of enhancing immunity is attractive, but the ability to do so has proved equivocal for several reasons. The immune system is precisely that: a complex network, not a single entity. There are no scientifically proven direct links between various lifestyle changes and enhanced immune function; nevertheless, one can boost the immune system by for example sleeping well in order to release protective cytokines, taking zinc [130], vitamins A, C, and E [131], curcumin (turmeric) [132], as well as consuming mushroom dietary supplements or fresh mushroom, therefore preventing the chance of contracting viral diseases [133].

6.1. HIV/AIDS

We have previously reviewed this subject [134], and the pathogenesis of the disease is considered multifactorial. Nutrition is a fundamental part of a comprehensive package of care for people living with HIV/AIDS, and mushrooms supply bioactive molecules that may help patients [110]. To cushion the repercussion of the disease, widespread, action taken must integrate all elements involved, including nutritional care [135]. To assess and reduce the severity of the complex interaction that HIV/AIDS and malnutrition have on each other, it is essential to forecast the evolution of the disease and the probability of morbidity and death toll [136].

Mushroom β-glucans increase CD4 cells production and stimulate the immune system macrophages. Even when infected with HIV, the macrophages fight effectively and reduce HIV replication [137]. Several triterpenes from *Ganoderma lucidum* are active as antiviral agents against human immunodeficiency virus type 1 (HIV-1) [138].

In addition to polysaccharides and triterpenoids displaying a variety of medicinal properties, mushrooms contain many antimicrobial factors, which include lentinan, ganaderiol-F, ganoderic acid-β, lucidumol, PSP, coprinol, campestrin, sparassol, armillaric acid, cortinellin, and ustilagic acid [139–141]. These active compounds fight viruses in two major ways: (a) they boost the immune system: directly (specific response) and/or through various factors of humoral and cellular immunity [142]; and (b) they attack the virus directly, which prevents the proliferation of viruses and can stop viral infections from developing [143].

Direct antiviral effects include inhibition of viral enzymes, synthesis of viral nucleic acids, and adsorption or uptake of viruses [144]. Indirect antiviral effects are achieved by stimulating the immune response against the viral invasion and promoting biochemical factors, such as alkalinity, which discourage viral replication [145]. In many mushrooms, β-glucans, glycoproteins, melanins, terpenoids, and nucleosides displayed antiviral activity [146].

Through the lymphotropic nature of the virus HIV-1, it infects humans, and the function of the lymph node is disrupted, the production of dendritic cells is increased, and there is accumulation in lymph nodes, presenting exogenous microbial antigens [147].

Drug resistance to anti-HIV drugs is emerging, and many people infected with HIV have serious adverse reactions. Antiviral compounds from mushrooms (e.g., triterpenes, phenolic compounds, ergosterol peroxide, and purine derivatives) are strong biotherapeutics acting directly on the pathways of enzymatic system of the human host, regulating the interactions between viral and components of the human cell [148].

They may also act by inhibiting viral enzymes carried within the capsid and on the viral envelope, while some are only produced in the infected cell [149]. The antiviral compounds of mushrooms also may condition the virus genome intervening on the synthesis pathway of viral nucleic acids and its penetration of viruses into cells [150].

6.2. Herpes Virus

The Herpes Simplex Virus (HSV-1) co-evolved with humans for thousands of years in a constant, dynamic, and endless dance where the pathogen is present at a high prevalence, affecting globally half of the human population [151,152].

While there are more than 100 known herpes viruses, two strains occur in most β-amyloid plaques of Alzheimer's Disease (AD), as their proteins are two-thirds identical, suggesting that this common virus may be a possible risk factor for AD, showing some evidence that specific viral species directly contribute to a risk of developing AD [153].

The neurotropic virus can either remain in a dormant state, with occasional revitalization events, or eventually originate severe acute encephalitis, which is marked by aggravated neuroinflammation and extended neuroimmune activation, producing a life-threatening neurological disease [154]. HSVs also alter host cell metabolism, inducing antiviral mechanisms and reprogramming cell death in non-immune cells; they are also capable of inducing apoptosis in immune cells and the death of T cells, while allowing viral replication to occur in epithelial cells before uprising into the neural ganglia, producing a latent infection [155].

Antiviral activity of the mycelia of higher mushrooms (*Pleurotus ostreatus*, *Fomes fomentarius*, *Auriporia aurea*, *Polyporus squamosus*, and *Coriolus versicolor*) against influenza virus type A (serotype H1N1) and herpes simplex virus type 2 (HSV-2) was determined to be effective [156]. They occur in SSA but may not be edible due to their texture and bitter taste but used as medicinal and functional properties [157].

6.3. Influenza Virus

Several mushrooms in natural form or as a food supplement are effective on preventing and treating a variety of viruses such as the common cold and the flu virus. This is significant upon considering the highly infectious nature and ability of these viruses to mutate. *Boletus edulis*, *Datronia molis*, *Calvatia gigantea*, *Laricifomes officinalis*, *Suillus luteus*, *Coriolus versicolor*, *Lentinus edodes*, *Lenzites betulina*, and *Piptoporus betulinus* were shown to be effective against the flu-causing influenza viruses [116,158]

6.4. Human Papillomaviruses (HPVs)

The use of *Coriolus versicolor* biomass supplement in women for 1 year revealed a great efficacy, whether in the regression of the cervical dysplasia (LSIL) or in the disappearance of the High-Risk HPV. This dietary supplementation showed positive therapeutic impact either in the reversion of LSIL (with High-Risk HPV+) or in those HSIL patients who have undergone surgery, but the High-Risk HPV viral count continued to increase [159].

This was subsequently replicated with active hexose correlated compound (AHCC), which is a fermented extract of cultured *Lentinula edodes* mycelia that is administered for at least 6 months with a 60% successful elimination of human papillomavirus (HPV) infections in women with positive PAP smears [160]. A recent study involving 42 patients showed that a combination of administration of *Coriolus versicolor* biomass provided positive outcomes in cases of primary or recurrent genital warts [161].

Mushroom biomass forms may be given as a complement in aggregation with surgery, chemo-, or radiotherapy, with a significant influence on NK cell activity when induced by the presence of a viral infection.

6.5. The Novel Coronavirus (SARS-CoV-2)

Currently, no specific treatment has been identified for COVID-19. The interesting thing about this SARS-CoV-2 virus is the symptoms, which can range from no conceivable symptoms all the way to having severe cases of all major symptoms, lower respiratory tract infection with fever, dry cough, and dyspnoea, spreading the virus. There are a vast number of studies that have been done with mushrooms as a potential antiviral treatment but very few yet specifically with this new virus [162]

Recently, *Cordyceps sinensis* and *Cordyceps militaris* were claimed be effective agents for the prevention and treatment of COVID-19 by immunomodulating, reducing the proinflammatory cytokines, preventing lung fibrosis, improving tolerance to hypoxemia, and inhibiting the viral enzymes [163]. *Lentinus edodes*, *Grifola frondosa*, and *Inonotus obliquus* are considered to have therapeutic potential as a natural antiviral treatment against SARS-COV-2, opening the research into this field.

In Norway, *Agaricus blazei*, *Ganoderma lucidum*, *Hericium erinaceus*, and *Grifola frondosa* were considered to have preventive or curative effect against the severe lung inflammation and acute pneumonia that often complicates COVID-19 infection [164].

A recent study in Iraq showed that *Ganoderma lucidum* uptake on some hematological and immunological response in patients with Covid-19 had a significant role in helping in the treatment of COVID-19 infections [165].

Mushrooms are the highest dietary source for the unique sulfur-containing antioxidant ergotheionine. This amino acid is a Generally Recognized as Safe (GRAS) product by the FDA and gets into the food chain mainly through mushroom consumption. There is a recent study revealing ergotheionine's potentially beneficial role in SARS-CoV-2 cases [113].

The above claims must not be generalized to the recent SARS-CoV-2 infection [166], and the immediate priority is to harness innate immunity to accelerate early antiviral immune responses.

7. Antitumour Activity of Mushrooms

Usually, the causes of cancer are multifactorial, and they include genetic, environmental, and other risk factors. A recent meta-analysis of 213 studies, including 77 clinical studies, showed that *Ganoderma lucidum* or *Coriolus versicolor* mushrooms enhanced the efficacy and ameliorated their adverse effects, which lead to an improved quality of life in cancer patients [108,167].

Mushroom lectins are a group of proteins/glycoproteins that can possess immunomodulating as well as direct cytotoxic activity toward tumour cell lines. In mushroom extracts and biomass, there are also some anticancer haemolysing proteins [168], enzyme laccase [169], ribosome-inactivating proteins [170], and ubiquitin-conjugated proteins, which also display direct cytotoxic activity [171,172].

Polysaccharides of mushrooms have antitumor activity, which is associated with the immunostimulatory effect that they can exert, since they activate foreign body reactions from the immune system [173]. This antitumor activity is not caused by a direct cytotoxic effect but via activation of the innate immune system of the host. The mechanism of action is related to the presence of pattern recognition receptors that can recognize the polysaccharides as pathogen-associated molecular patterns (PAMPs), due to its high molecular weight [174].

Consequently, proinflammatory cytokines are produced in a cascade, including tumour necrosis factor alpha (TNF-α), which are members of the IL-1 family that regulate immune homeostasis and the mechanisms against infections in recognition of foreign cells and tumour cells [175].

Some structures of mushroom β-glucans are better adapted to specific receptors, which suggests a relationship between the structure and antitumor activity of polysaccharides, and it was found that mostly β-1,3-glucans have the highest antitumor activity [176,177]. Triterpenes, the secondary compounds found in mushrooms, cause tumour cells to self-destruct (apoptosis) [178,179].

Polysaccharide extracts from *Hericium erinaceus* are active against liver cancer cells in vitro and in vivo [180,181]. The highest consumption of dietary mushrooms, including *Agaricus bisporus* and *Lentinula edodes*, is associated with a decreased risk of breast cancer in premenopausal women and postmenopausal women [182].

Maitake mushroom (*Grifola frondosa*) is one of the most popular edible medicinal mushrooms. The natural killer (NK) cells, which have the ability to eliminate target cells without prior immunization, show an important role in controlling viral infections and high cytotoxic activity in oncologic patients administered *G. frondosa*, and they significantly restrain tumour growth. This is achieved by an increased release of TNF-α and IFN-γ from the spleen and a significant boost in IFN-γ and TNF-α expressed in NK cells [183].

8. Prebiotic Activity of Mushrooms

Prebiotics act as food for probiotics, and some health benefits of prebiotics, such as reducing glucose levels in the blood and improvement of the bowel function, have been medically proven and recognized by health authorities [184].

Endogenous β-glucans show better prebiotic properties than exogenous β-glucans. We have discussed the role of some bacteria responsible for the degradation of mixed linked β-glucans in the small intestine and in the hind gut [185].

Currently, inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), lactulose, and polydextrose are recognized as the well-established prebiotics, but there is evidence that β-glucans can also be a source of long chain prebiotics [186]. *Pleurotus ostreatus* and *Pleurotus eryngii* have a potential stimulator effect on the growth of probiotic bacteria [187]. *Pleurotus* and *Cyclocybe* mushrooms, studied in terms of their prebiotic potential, exhibited a beneficial influence on the composition of gut microbiota of apparently healthy and elderly subjects [188].

The prebiotic effect of mushroom biomass (e.g., *Coriolus versicolor*) on human gut populations of total aerobes and anaerobes showed that dietary mushroom inclusion beneficially affected gut homeostasis performance and exerted changes in intestinal microbial communities [189].

9. Mushrooms and Neurological Disorders

One of the most challenging public health problems in Africa is data collection, and only very few epidemiological studies have been carried out in Sub-Saharan Africa, but generally, there is a high reported prevalence of neurological morbidity and disorders (e.g., epilepsy, dementia, stroke), which have been escalating [190,191].

When cells generate energy, they use oxygen and yield free radicals as a consequence of ATP generation by the mitochondria, which is a cell organelle that has a critical role in the development of neurodegenerative disorders [192]. The human body has various mechanisms to prevent oxidative stress by either yielding inner natural antioxidants (e.g., catalase, enzymes glutathione peroxidase, superoxide dismutase) or having them provided through foodstuffs and/or dietary supplements [193].

Neurodegeneration caused by disruptions of crucial homeostatic interactions between circulation and the brain may be mediated by microbial products that modulate the gut–brain axis, causing neuro-inflammation and neuronal dysfunction [194]. Neuro-inflammation can be caused by virus DNA/RNA infection, which challenges the host immune system, and continued exposure to the inflammatory mediators (e.g., cytokines, chemokines, and ROS) can result in neuronal dysfunction and degeneration [195].

People who incorporate mushrooms into their diets, even in small amounts (more than twice a week), seem to have a lower risk of mild cognitive impairment, usually

preceding Alzheimer's disease [196]. Mushrooms contain many other substances whose exact role in brain health is not yet clear, but they include hericenones, erinacines terpenoids, scabronines, isoindolinones, sterols, and dictyophorines, which are a series of compounds that could contribute to the growth of nerve and brain cells [197].

Hericium erinaceus has been studied as a precursor of acetylcholine, which has neuroprotective and anti-neurodegenerative properties. *H. erinaceus* mycelium shows great promise for the treatment of Alzheimer's and Parkinson's diseases [198].

We have previously discussed how abnormal redox homeostasis and oxidative stress causes diverse neuropsychiatric disorders and the immunomodulation role of mushroom biomass of *Coriolus versicolor* [199]. Presently, the interest is to focus on mediator markers of oxidative stress and neuroinflammation in progressive neurodegenerative disorders and distinct configurations of chronic mental illness [200].

Oxidative stress and altered antioxidant systems have been considered an important factor underlying the pathogenesis of Alzheimer's disease. Brain inflammation has been linked to many diseases, including amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Parkinson's disease (PD) and, particularly, Alzheimer's disease (AD) [201].

We have previously discussed [186] the emerging role of lipoxin A4 and inflammasome in neurodegeneration and the potential therapeutic role of mushroom *Coriolus versicolor*. Integrated survival responses exist in the brain, which are under the control of redox-dependent genes, called vitagenes, including heat shock proteins (HSPs), sirtuins, thioredoxin, and lipoxin A4. The activation of LXA4 signalling and modulation of stress-responsive vitagene proteins could serve as a potential therapeutic target for AD-related inflammation and neurodegenerative damage [202,203].

Mushrooms through their powerful antioxidant characteristics have the potential to protect neurons in mitochondrial dysfunctions-associated aging and neurological disorders [204].

Anxiety symptoms and disorders, more common than depression, are among the most common primary care challenges in medical practice. L-theanine is an amino acid (an analogue of amino acids L-glutamate and L-glutamine) found most in tea leaves and in mushrooms. It should be noted that health claims for L-theanine as a supplement are not recognized in the European Union but are approved by the FDA. However, mushrooms, as well as dietary biomass supplements, not extracts, as food containing L-theanine do not need any approval, becoming a functional food secondary component of medical treatment [205].

Coriolus versicolor, a common healthful mushroom, has been receiving increasing attention by its antitumoral, anti-inflammatory, antioxidant, antibacterial, and immunomodulatory properties, including in the hippocampus. Our data unveiled a so far unexplored neurogenic potential of *Coriolus versicolor* supplementation as a possible preventive strategy for different neurological conditions [110,203].

Role of Mushrooms in Autism

According to the World Health Organization, one child in 270 worldwide suffers from an autism spectrum disorder. Autism is more common in Africa than initially believed, and it is a growing global public health concern. Most Africans are largely unaware of autism, which is a highly heritable neurodevelopmental disorder that is often confused with witchcraft, curses or spells, and demons, and children with autism in SSA tend to be diagnosed only around age 8, some 4 years later than worldwide.

Many African children with autism, more often boys than girls, are usually hidden away at home, and the prevalence is unknown, while only few clinicians have the skills or experience to identify the condition. Indeed, maternal and child mental health services have not been a priority, since child mortality and malnutrition are more urgent concerns [206,207].

Autism Spectrum Disorder (ASD) is a disorder still very poorly understood that is caused by genetic or environmental factors; it is first recognized in early childhood in the form of a multi organ system disability caused by impaired neurogenesis and apoptosis,

impaired synaptogenesis and synaptic pruning or an imbalanced excitatory–inhibition system [208].

A few studies with a handful of cases have been dealt with in South Africa, Nigeria, Ethiopia, and Kenya, fostering the conviction that ASD is more severe in African children than elsewhere with up to 4% of children having the condition [209]. Recent epidemiological studies revealed a possible important link between mycotoxin exposure and neurodevelopmental disorders with regard to ASD [210].

We have previously discussed dietary mushrooms and supplements, which have specific effects on gastrointestinal inflammation in ASD patients. The most commonly used mushrooms as potent health-boosters, which may bring some hope to autistic children and families, include Chaga (*Inonotus obliquus*), Reishi (*Ganoderma lucidum*), Turkey Tail (*Coriolus versicolor*), Shiitake (*Lentinula edodes*), Lion's Mane (*Hericium erinaceus*), Cordyceps (*Cordyceps militaris*), and oyster mushroom (*Pleurotus giganteous*). They have shown beneficial to symptoms relating to anxiety and depression, which are related to both autism and attention deficit–hyperactivity disorder (ADHD) [211].

The therapeutic potential of *Hericium erinaceus* bioactive and bioavailable components that pass the blood–brain barrier has been demonstrated [212]. They condition several functions, including triggering the production of nerve growth factor, the obstruction of the cytotoxicity of an extracellular heterogeneous mixture of small peptides plaque deposits, and the shielding against neuron lysis [213].

10. Concluding Remarks

The right to adequate food of acceptable quality has not been achieved in SSA. Hunger and malnutrition in the general SSA population remained a challenge even prior to the pandemic. COVID-19 is affecting food systems globally and has negative combined impacts on agricultural markets, economic recession, food insecurity, acute malnutrition, high levels of childhood illnesses, and water-borne diseases further threatening the health and life of people, namely children. Among the recovery measures to adopt, there is the need to maximize macro- and micronutrient intakes.

It is necessary to valorise indigenous foods and detect novel local food sources to aid and promote healthy lifestyle, income, wellness, and wellbeing. Authorities and international aid should aim helping the country's smallholder farmers make the transition move from subsistence to community commercial farming on a long-term ambitious plan.

Africa constitutes at least 25% of the total mushroom biodiversity in the world, but it has been barely researched. The abundant agricultural waste found in SSA offers opportunity for mushroom production. Mushrooms have distinct nutritional and bioactive profiles and have been absent in global dietary guidelines despite its high biological value. There is limited information about the nutritive, therapeutic, and biomedicinal uses of mushrooms in Africa. Mushrooms complement the human diet and microbiota requirements with various ingredients not found or deficient in food items of plants and animal origin, being considered an ultimate health food for the prevention of various human diseases.

Recognizing mushrooms as good sources of bioactive components, in strengthening human immune system, enhancing natural body resistance, and lowering proneness to disease with little scope of toxicity or overdose, along with their minimal side effects, make them ideal candidates for developing novel foods, dietary supplements, and therapies.

Author Contributions: Conceptualization, T.F.; validation, J.F. and C.G.; writing-original draft preparation, T.F.; writing-review and editing, J.F., V.B., C.G., and T.V.; supervision, T.F. and T.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: All authors declare no conflict of interests.

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Article

Cytotoxic Influence of Khat (*Catha edulis (Vahl) Forssk. ex Endl)* on Oral Fibroblasts, Squamous Carcinoma Cells, and Expression of α Smooth Muscle Actin

Azeem Ul Yaqin Syed [1], Muhammad A. Ahmed [2], Eman I. AlSagob [3], Mansour Al-Askar [4], Abdulrahman M. AlMubarak [4], Rizwan Jouhar [2], Abdul R. Ahmed [5], Sameer A. Mokeem [4], Nada Aldahiyan [6], Fahim Vohra [7] and Tariq Abduljabbar [7,*]

[1] Academic Unit of Restorative Dentistry, The School of Clinical Dentistry, University of Sheffield, Claremont Crescent, Sheffield S10 2TA, UK; azeem.ajaz@gmail.com

[2] Department of Restorative Dentistry and Endodontics, College of Dentistry, King Faisal University, Al-Ahsa 31982, Saudi Arabia; mshakeel@kfu.edu.sa (M.A.A.); rjouhar@kfu.edu.sa (R.J.)

[3] Preventive Dental Sciences Department, Community Dentistry Division, College of Dentistry, Princess Nourah Bint Abdulrahman Universty, Riyadh 11671, Saudi Arabia; eialsagob@pnu.edu.sa

[4] Department of Periodontics and Community Dentistry, College of Dentistry, King Saud University, Riyadh 11545, Saudi Arabia; malaskar@ksu.edu.sa (M.A.-A.); amalmubarak@ksu.edu.sa (A.M.A.); smokeem@ksu.edu.sa (S.A.M.)

[5] Department of Prosthodontics, College of Dentistry, King Khalid University, Abha 61421, Saudi Arabia; a_razzaq1@hotmail.com

[6] Department of Restorative Dental Science, College of Dentistry, King Saud University, Riyadh 11545, Saudi Arabia; naldahian@ksu.edu.sa

[7] Department of Prosthetic Dental Science, College of Dentistry, King Saud University, Riyadh 11545, Saudi Arabia; fvohra@ksu.edu.sa

* Correspondence: tajabbar@ksu.edu.sa; Tel.: +966-143744555

Citation: Syed, A.U.Y.; Ahmed, M.A.; AlSagob, E.I.; Al-Askar, M.; AlMubarak, A.M.; Jouhar, R.; Ahmed, A.R.; Mokeem, S.A.; Aldahiyan, N.; Vohra, F.; et al. Cytotoxic Influence of Khat (*Catha edulis (Vahl) Forssk. ex Endl)* on Oral Fibroblasts, Squamous Carcinoma Cells, and Expression of α Smooth Muscle Actin. *Appl. Sci.* **2021**, *11*, 3524. https://doi.org/10.3390/app11083524

Academic Editor: Theodoros Varzakas

Received: 19 March 2021
Accepted: 9 April 2021
Published: 14 April 2021

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Abstract: The aim was to determine the cytotoxicity of Khat *(Catha edulis (Vahl) Forssk. ex Endl)* on normal oral fibroblasts (NOFs) and SCC4 (squamous carcinoma cells) along with expression of α-smooth muscle actin (α-SMA) in fibroblasts. Khat filtrate was prepared to obtain a concentrated viscous solution. NOFs and SCC4 cells were cultured in biological cabinets and were grown in Dulbeccos' modified Eagles medium. Frozen cells were thawed at 37 °C and cell seeding was performed. NOFs and SCC4 cells were seeded on 96 well plates and allowed to attach. The medium was removed and a fresh medium containing different concentrations of Khat was added. The group without Khat served as a negative control and 4% paraformaldehyde as the positive control. Cell viability was assessed using the MTT assay and effect of Khat on fibroblast and SCC4 phenotypes was evaluated by immunostaining. Analysis of variance was used to assess data ($p < 0.05$). NOF 316 showed cell death in response to 4% paraformaldehyde, 12.5, 6.25, and 3.12 mg/mL of Khat. The highest concentration of Khat (25 mg/mL) failed to cause cytotoxicity of NOF 316. NOF 319 and NOF 26 displayed cell death at all concentrations of Khat, however, cytotoxicity was not dose dependent. NOF 18 and SCC4 cells showed dose-dependent cell death. NOF 316 showed α-SMA expression after 1 mg/mL of Khat exposure. Not all fibroblasts were α-SMA-positive, suggesting specific activation of a subset of fibroblasts. Khat is cytotoxic to NOF and SCC4 cells. Furthermore, it can also cause activation and phenotypic changes in oral fibroblasts, indicating a potential role in progression of oral squamous cell carcinoma.

Keywords: normal oral fibroblast; squamous carcinoma cell; Khat; myofibroblasts; cytotoxicity; cell death

1. Introduction

Oral cancer is considered to be a significant health problem, as around ninety percent of all oral cancer cases are oral squamous cell carcinomas (OSCC) [1]. It has a worldwide

distribution and is the sixth most commonly occurring cancer globally [2]. The major risk factors associated with oral cancer are tobacco smoking and alcohol consumption. Other established risk factors i.e., betel quid chewing, use of smokeless tobacco, human papilloma viruses (types 16 and 18), nutrient deficiency, exposure to solar radiation, and genetic predisposition also causes OSCC [3]. However, there exists a great difference in inter- and intra-country distribution of these causative factors, which possibly explains the different geographical pattern of the disease [1]. One such product was reported to cause malignant and pre malignant oral lesions and is routinely used in the Arabian Peninsula as Khat [4].

The increasing use of Khat *(Catha edulis (Vahl) Forssk. ex Endl)* is associated with grave health hazards [5]. Chronic consumption of Khat affects almost every organ of the human body. It also leads to oral histopathological changes i.e., hyperkeratosis, epithelial hyperplasia, and mild dysplasia [6]. Lukandu et al. revealed that Khat induces abnormal epithelial differentiation and decreases basal cell proliferation [7]. Similarly, a study conducted by Al-Ahdal et al. reported that the cytotoxicity and mutagenicity of Khat leaves extract on human cells [8]. Khat performs its genotoxic activity by inhibiting de novo synthesis of proteins, RNA, and DNA, to reduce free radical metabolizing enzymes, induces oxidative stress, and provokes caspase-dependent apoptosis in various leukemic cells [9,10]. However, studies on its toxicological potential remain scarce.

Previously, it was believed that epithelial genetic changes were the sole cause of oral pre-cancer and cancer, but recent reports showed that progression of carcinoma is derived from the epithelial component as well as mesenchymal tissue [11]. The connective tissue stroma in the vicinity of the tumor plays an important part in oral cancer progression [12]. Normal oral fibroblasts (NOFs) are cells of mesenchymal origin and are omnipresent in almost all tissues within the stroma or connective tissue [13]. In precancerous and cancerous lesions, NOF are modified or 'activated' into myofibroblasts (MFs) and express α-smooth muscle actin (α-SMA) due to mechanical cell stress and tumor stimulating factors [14]. These MFs tend to differentiate in the presence of the transforming growth factor (TGF)-β, another factor that increases in fibrotic lesions. Studies showed that the stroma surrounding the tumor assists in metastasis and malignant progression [15]. However, the role of mesenchymal tissue and NOFs in disease progression due to Khat is not yet determined.

Considering the available indexed literature, it was found that numerous studies showed adverse effects of Khat on oral mucosa. Whereas, its role in OSCC, particularly in relation to connective tissue/stromal changes is largely uncharacterized. Therefore, it is hypothesized that Khat is cytotoxic to the NOFs and SCC4 cells (squamous carcinoma cells). It is also hypothesized that it will exhibit positive expression for α-SMA in fibroblasts. Hence, the aim of the current study was to determine the cytotoxicity of Khat on NOFs and SCC4 cells, along with the expression of α-SMA in fibroblasts.

2. Materials and Methods

2.1. Khat Preparation

Khat (mature) used in the present study was commercially obtained from Sana, Yemen. The Khat extraction method was similar, as previously explained by Aziz et al. [16]. Small pieces of frozen Khat leaves (100 gm) were dissolved in 100 mL of 95% ethanol. The prepared solution was centrifuged at 5000 rpm for 5 min and filtered using Whatman filter paper (no.1). After filtration, 100 mL of ethanol were added to the remaining leaves and the procedure was repeated. Rotary evaporator (EYELA, N-1001S-W, Tokyo, Japan) at 70 rpm was used to concentrate ethanolic Khat at 30 °C, until 30% of ethanol was left. The concentrated viscous solution obtained was then diluted using 100 mL of distilled water and stirred at ambient temperature for 1 h. The filtrate was freezed for 24 h by keeping it at −80 °C and then dried by lyophilization (Labconco, Kansas City, MO, USA). Every 100 g of dried Khat leaves gives 8 g of Khat in powder form. Liquid chromatography was performed to confirm the presence of alkaloids, i.e., 80% cathine and 20% norephedrine. Cathinone was not discovered in the analysis. All materials and equipment used in the

study are presented in Appendix A. Dimethyl sulfoxide (DMSO) was used a vehicle for suspension of Khat extract.

2.2. Culture of NOFs and SCC4 Cells

2.2.1. Cell Lines and Culturing

NOFs in the present study were obtained after ethical approval from patients undergoing third molar extractions at Charles Clifford Dental Hospital. NOFs and SCC4 cells (American Type Culture Collection/ATCC CRL-1624) were cultured in Biological Class II sterile laminar flow cabinets. In order to maintain cell viability, the confluence medium was routinely changed and cells were maintained at 37 °C in a 5% CO_2 incubator. Details of cells used in this study are provided in Table 1.

Table 1. Cells used in the study.

Cell Line	Type	Origin
NOF 18	Primary cells	Normal Oral Fibroblasts
NOF 26	Primary cells	Normal Oral Fibroblasts
NOF 316	Primary cells	Normal Oral Fibroblasts
NOF 319	Primary cells	Normal Oral Fibroblasts
SCC4	Cell line	Squamous Cell Carcinoma

2.2.2. Cell Growth Medium

All cells were grown in Dulbeccos' Modified Eagles Medium (DMEM) (Invitrogen) with supplements (Table 2).

Table 2. Medium used for growing primary cells and SCC4 cell line.

Composition	Volume	Remarks
Dulbecco's Modified Eagles Medium (DMEM) supplemented by 4500 mg/L glucose, GlutaMAX™ I and Sodium Pyruvate	450 mL	Invitrogen, UK lot- 1250148 lot- 1122288 lot- 1369047
10% Fetal Bovine Serum (FBS)	50 mL	Biosera, East Sussex, UK
Pen/Strep (Antibiotics)	5 mL 100 IU/mL Penicillin, 100 μg/mL Streptomycin	Sigma Aldrich, Dorset, UK

2.2.3. Thawing and Seeding Cell Lines

Frozen cell vials were removed from liquid nitrogen and thawed in a water bath at 37 °C. Thawed cells were moved to a sterile universal tube containing 9 mL of pre-warmed medium and centrifuged at 1000 rpm for 5 min. The supernatant was discarded, the cell pellet was re-suspended in 10 mL fresh culture medium, and moved to a 75 cm^2 tissue culture flask.

2.2.4. Adherent Cell Sub-Culturing and Passaging

Inverted microscope (Axioinvert Carl Zeiss) was used to visualize the cell growth. Subculturing was performed when 80% of confluence was reached. To passage the cells, the medium was removed; cells were washed twice in a 10 mL solution of Ca^{2+} and Mg^{2+} free PBS. Trypsin/EDTA solution was added and flasks were placed in the incubator for 10 min. Trypsin was neutralized by fresh medium and the cells were centrifuged, resuspended, and counted using a hemocytometer.

2.3. MTT Assay

2.3.1. Seeding Cells in 96 Well Plates

1.5×10^4 NOFs and 1×10^4 SCC4 cells (in 100 µL per well) were seeded on 96 well plates and allowed to get attached for 24 h.

2.3.2. Khat Exposure

Khat at a concentration of 25 mg/mL was used throughout this study, which was diluted accordingly. The extract was filtered using 0.2 µm Merck Millipore express filters in a liquid form. After 24 h, 96 well plates were checked under the inverted microscope to ensure cell attachment. The medium was removed and a fresh medium containing 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.19 mg/mL of Khat was added. The group without Khat served as a negative control and 4% paraformaldehyde as the positive control. Paraformaldehyde was dissolved in DMSO and the control wells were also treated with it. The 96 well plate was incubated for a further 24 h at 37 °C in a 5% CO_2 incubator. In addition, a triplicate of each concentration was run and the average values were taken.

2.3.3. Determination of Cytotoxicity/Metabolic Activity

Cell viability was assessed using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay, a yellow tetrazole. MTT solution (Sigma Aldrich) (0.5 mg/mL in PBS–100 µL/well) was added after removing the medium and washing the cell, using PBS solution. The unreacted MTT solution was removed after one hour and newly formed purple intracellular formazan salt was solubilized using acidified iso-propanol (50 µL/well). Absorbance was measured at 540 nm with a reference at 630 nm, using a spectrophotometer (Tecan) and Magellan software. Background subtraction was done and optical densities were normalized to a control sample of the untreated cells. Data were entered and analyzed through Microsoft Excel. Analysis of variance (ANOVA) was used to determine the significance of data obtained from MTT assays.

2.4. Effect of Khat on Fibroblast Phenotype

2.4.1. Immunostaining Preparation

2×10^4 cells per well were seeded on sterile coverslips in 24 well plates. The plate was incubated for 24 h in 5% CO_2 at 37 °C. the old medium was removed from all wells and the cells were washed with 1 mL of medium. Cells were then exposed to Khat concentrations of 1 mg/mL and 0.5 mg/mL; and the 40 µg/mL of Transforming Growth Factor- β (TGF-β) acted as a positive control. A total of 500 µL of medium without Khat or TGF-β acted as negative control.

2.4.2. Immunofluorescence for SMA

The medium from the 24 well plate was removed and the cells were washed twice in PBS solution, followed by fixation in 100% 1 mL methanol for 10 min. The coverslips were washed once in 0.5 mL of 4 mM sodium deoxycylate in PBS and permeabilized in the same solution for 10 min. Sodium deoxycylate was removed and the coverslips were blocked using 500 µL of 2.5% Bovine Serum Albumin (BSA) in PBS for 30 min, followed by an incubation with 0.5 mL of FITC-conjugated anti-alpha smooth muscle actin antibody (Sigma, Clone1A.4, dilution 1:1000) for one hour at 37 °C, in dark. An IgG isotype antibody was used as a negative control. After 1 h, the coverslips were washed in PBS and placed on glass slides containing 50 µL DAPI (vectorized). Glass slides were kept at 3–4 °C for a day and were viewed using a fluorescence microscope (Zeisss Axioplan 2, imaging with software Proplus 7.0.1. Presence of a green staining was considered to be positive.

3. Results

3.1. MTT Assay

3.1.1. NOF 316

MTT Assay was performed to determine the viability of NOF 316 primary cells, after being exposed to different concentrations of Khat. Cell death was seen in response to 4% paraformaldehyde (positive control), 12.5, 6.25, and 3.12 mg/mL concentrations of Khat, as compared to the negative control ($p < 0.05$). However, the lower doses of Khat (1.56, 0.781, 0.39, and 0.195 mg/mL) did not display significant cytotoxicity. Interestingly, at the highest dose, i.e., 25 mg/mL, Khat did not display a significant increase in cell death (Figure 1).

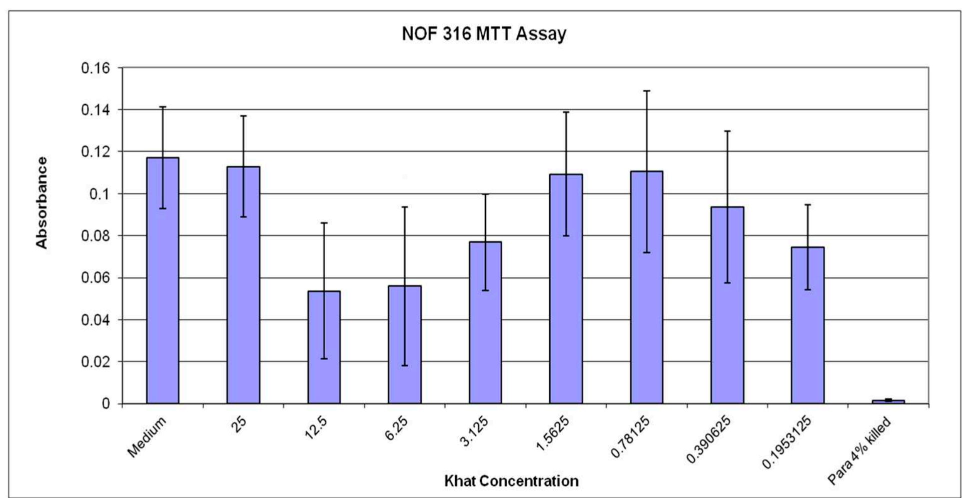

Figure 1. Absorbance from MTT Assay for NOF 316 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 316 was used as a negative control while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.2. NOF 26

Results for NOF 26 were somewhat different from NOF316, as nearly all concentrations of Khat caused a significant increase in cell death, as compared to the negative control ($p < 0.01$). A similar response was observed in the positive control and cytotoxicity appeared to be dose dependent (Figure 2).

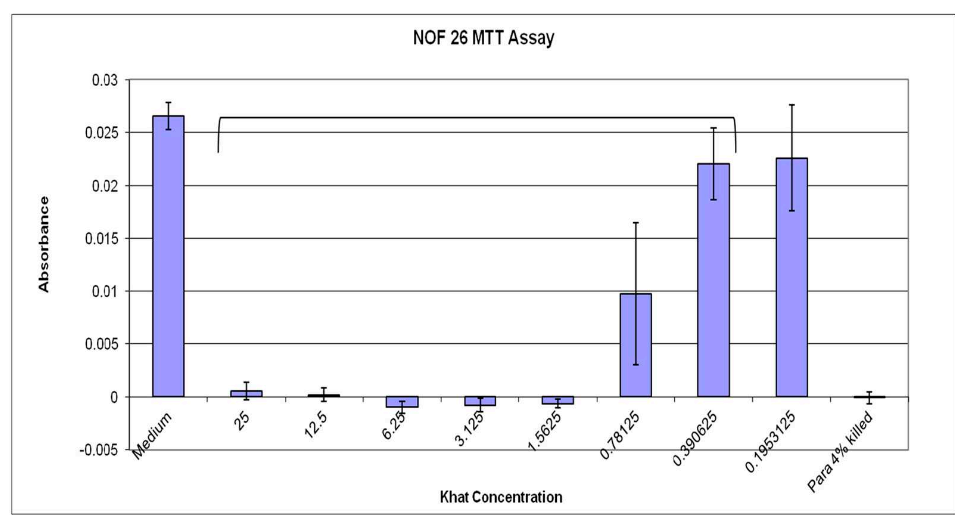

Figure 2. Absorbance from MTT Assay for NOF 26 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 26 was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.3. NOF 319

NOF 319 cells showed a similar pattern to NOF 26 cells, as all concentrations of Khat caused significant cell death ($p < 0.01$). However, cytotoxicity did not appear to be dose-dependent. However, the positive control caused significantly more cell death, as compared to Khat ($p < 0.01$) (Figure 3).

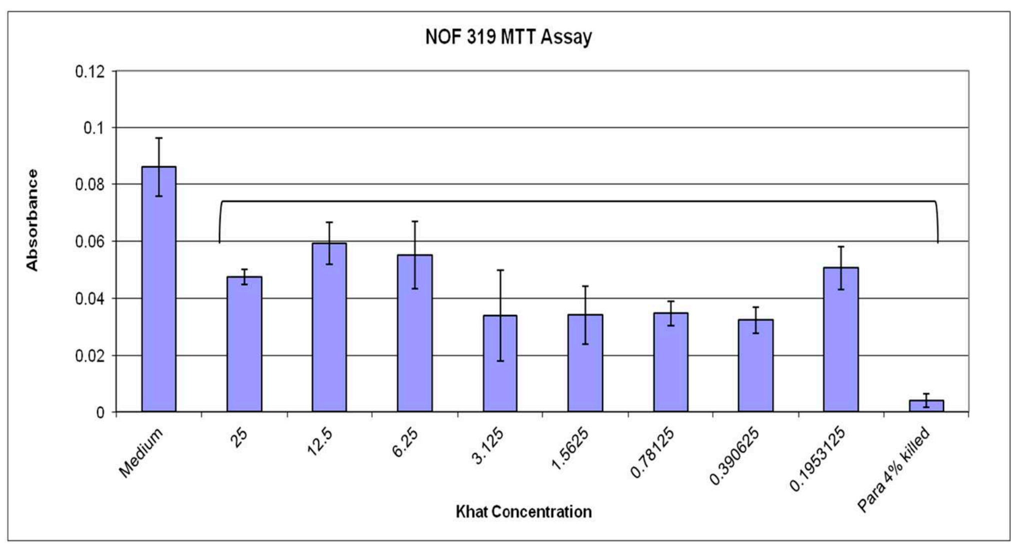

Figure 3. Absorbance from MTT Assay for NOF 319 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 319 was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.4. NOF 18

Exposure of NOF 18 cells to different Khat concentrations resulted in a significant increase in cell death, as compared to the negative control ($p < 0.01$). In addition, the extent of cell cytotoxicity was dependent on Khat concentration, showing a dose-dependent response (Figure 4).

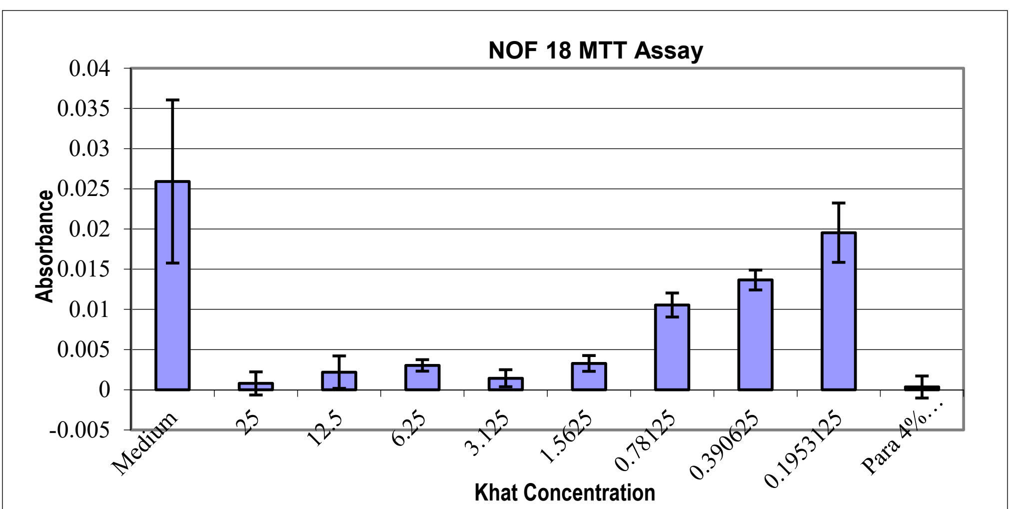

Figure 4. Absorbance from MTT Assay for NOF 18 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only NOF 18 was used as a negative control while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.1.5. Cytotoxicity of Different NOF's

A comparison of the different batches of NOFs used showed a somewhat similar trend with the highest Khat toxicity seen between 1.56 to 12.5 mg/mL concentrations. Surprisingly, in some cells, the highest concentration (25 mg/mL) failed to cause cell death. However, a significant variation between the different cells was observed (Figure 5).

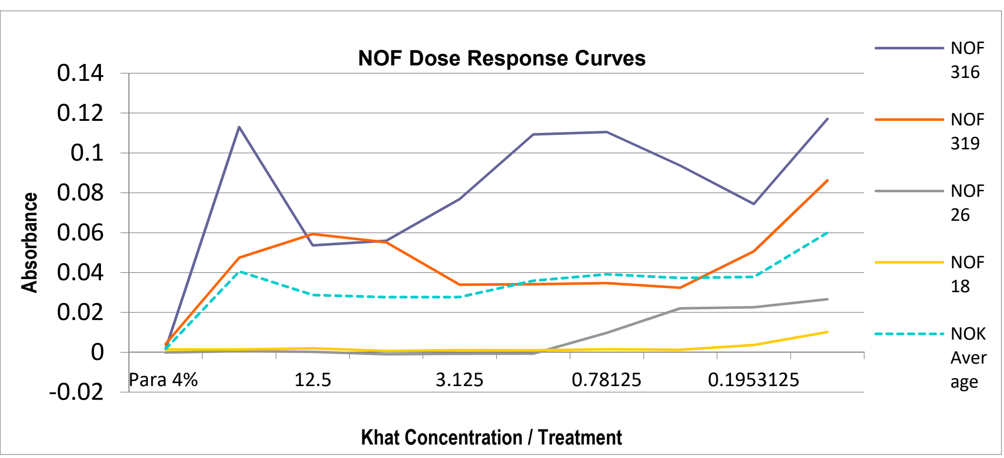

Figure 5. Comparison of absorbance values from MTT Assay for NOF 18, NOF 26, NOF 319, and NOF 316 cells treated with different concentrations of the Khat solution (25 mg/mL to 0.195 mg/mL). Medium only with cells specific for each experiment was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death. Aqua dotted curve = average of the 4 assays.

3.2. SCC4 Cells

The SCC4 cells also displayed concentration-dependent cytotoxicity. The highest cytotoxicity was seen after cells were incubated with 25–3.125 mg/mL of Khat, which was significantly different from the negative control ($p < 0.005$). However, lower doses of Khat (1.56–0.19 mg/mL) failed to elicit a significant response, with an increase in number of cells seen with two concentrations (1.5625 and 0.1953125) (Figure 6).

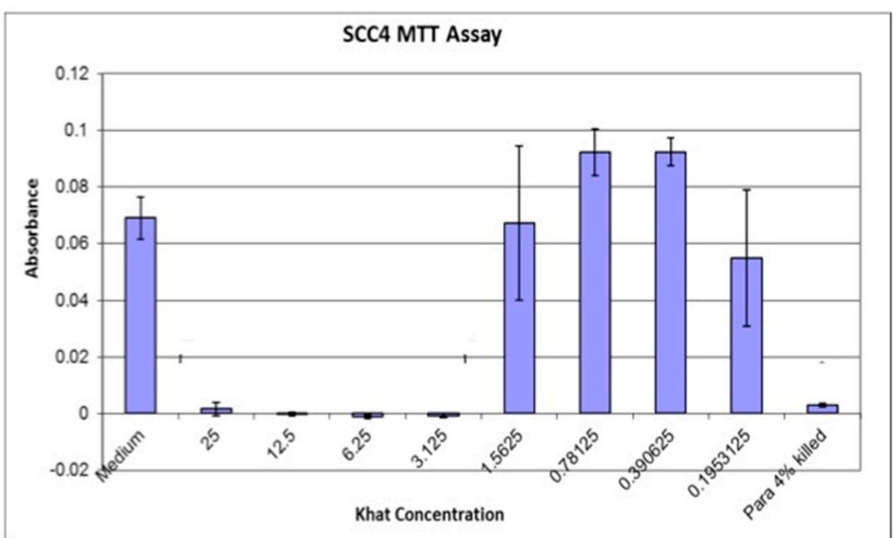

Figure 6. Absorbance from MTT Assay for SCC4 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only SCC4 cells was used as a negative control while 4% paraformaldehyde was used as a positive control, showing complete cell death.

SCC4 (Late Passage)

Somewhat different results were obtained when late passage of SCC4 cells were performed. A significant reduction in absorbance was seen with all doses of Khat (except 1.56 mg/mL) indicating toxicity ($p < 0.05$). No obvious difference was seen between the different doses used, suggesting variability in cell behavior in late passages (Figure 7).

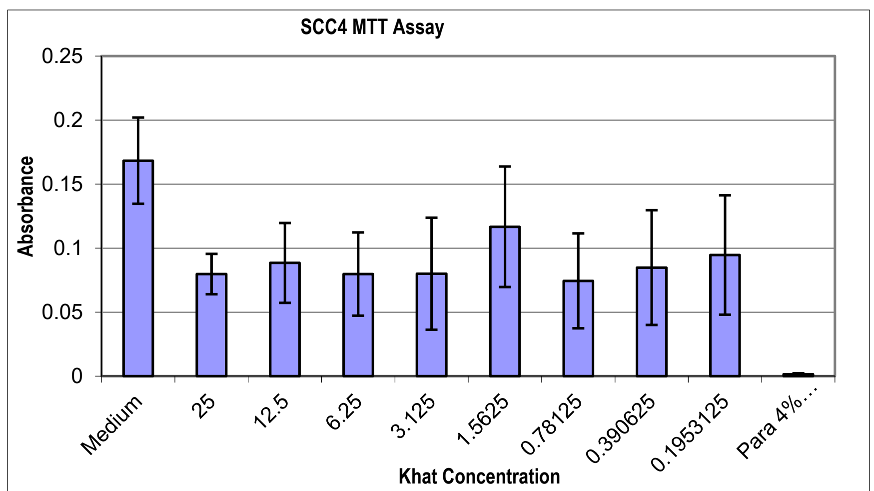

Figure 7. Absorbance from MTT Assay for SCC4 cells (late passage) treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium with only SCC4 cells was used as a negative control, while 4% paraformaldehyde was used as a positive control showing complete cell death.

3.3. Comparison of NOF's and SCC4 Assays

Comparison between the average cell number for all NOFs and repeat SCC4 assays showed no significant difference between the two cell types, with a similar dose response curve (Figure 8).

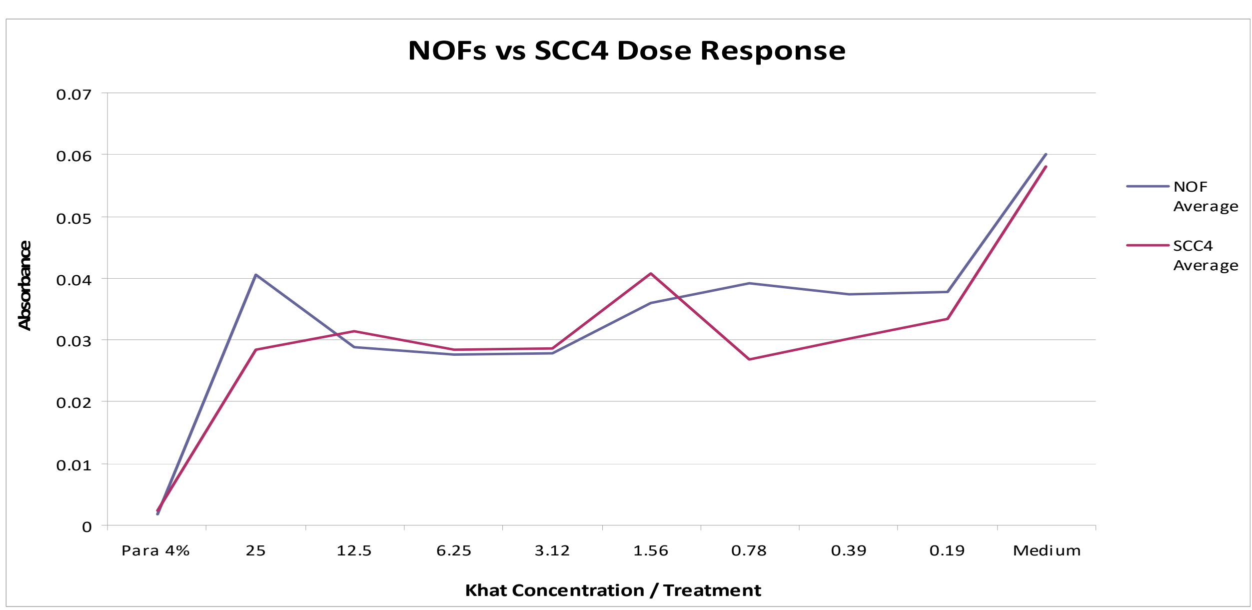

Figure 8. Comparison of dose response curves (MTT Assay) for NOFs and SCC4 cells treated with different concentrations of Khat solution (25 mg/mL to 0.195 mg/mL). Medium was used as a negative control while 4% paraformaldehyde acted as a positive control.

3.4. Detection of α-SMA

No α-SMA staining was seen with the negative control IgG isotype antibodies for NOF 316 (Figure 9). This suggested that 1 mg/mL of Khat concentration was sufficient to alter the fibroblast phenotype. Figure 10 shows a clear positive staining of α-SMA fibers running as vertical strands throughout the cell body of a fibroblast. Not all fibroblasts were α-SMA-positive, suggesting specific activation of a subset of fibroblasts. Similar results were seen when cells were stimulated with TGF-β, as this is known to increase the expression of α-SMA. SMA staining was seen in NOF 316, after incubation with 40 μg/mL of TGF-β (Figure 11). TGF-β is a protein that regulates proliferation and cellular differentiation, and causes apoptosis in tumor cells.

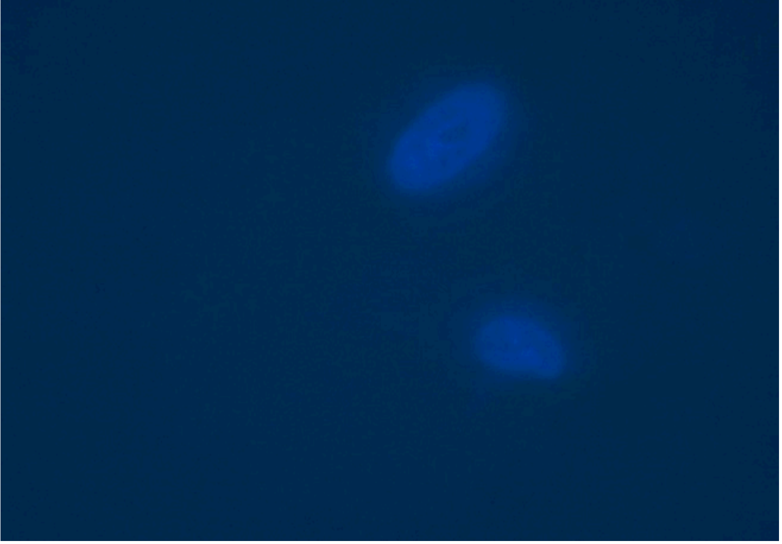

Figure 9. NOF 316 showing nuclear DAPI staining with the IgG antibody. (magnification ×100, Fluorescence at 1.3 s, DAPI at 0.07 s).

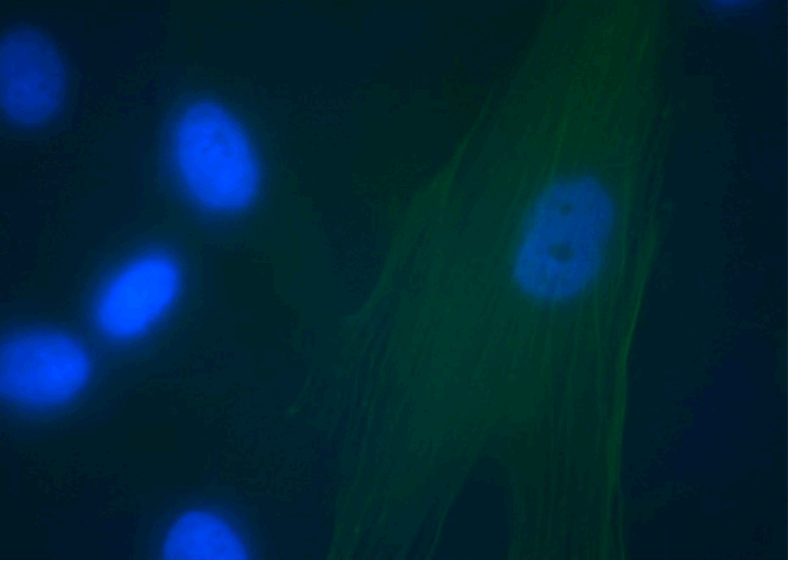

Figure 10. NOF 316 showing SMA expression after stimulation with 1 mg/mL Khat (magnification ×100, Fluorescence at 1.3 s, DAPI at 0.07 s).

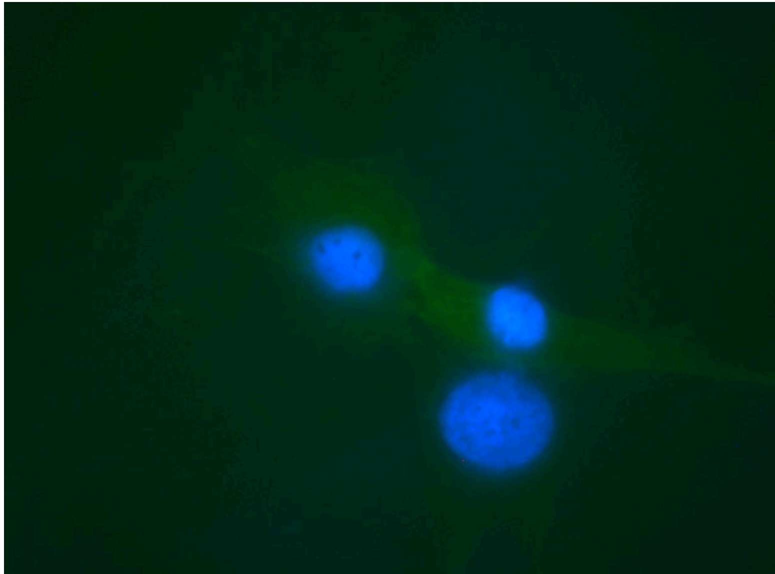

Figure 11. SMA expression in NOF 316 after incubation with 40 μg/mL of TGFβ (magnification ×100, Fluorescence at 1.6 s, DAPI at 0.100 s).

4. Discussion

The present study was based on the hypothesis that Khat is cytotoxic to NOFs and SSC4 cells. It was also hypothesized that Khat would stimulate positive expression of α-SMA in fibroblasts. Interestingly, the study revealed the cytotoxicity of Khat for both NOFs and SSC4 cells, and increased cell death occurred as compared to the negative control. Moreover, fibroblasts also stained positive for α-SMA after Khat exposure. Therefore, both postulated hypotheses were accepted. For cytotoxicity assessment, MTT assay was employed, as the technique was convenient, rapid, and reproducible [17]. Similarly, immunofluorescence was used to determine α-SMA positive staining in fibroblastic cells, as it showed high sensitivity and specificity over other techniques [18].

Khat was studied in relation to its damaging effects on oral mucosal keratinocytes, but its potential influence on the oral fibroblasts and cancer cell line was not studied. Multiple studies stated that Khat chewing is associated with oral hyperplasia, hyperkeratinization, and oral cancers [19]. The results of the present study showed that Khat caused cell death of both NOFs and SCC4 cells. The exact mechanism for Khat-induced cell death is not known. However, the available literature focused on the presence of alkaloids (cathine and other phytochemicals) as a cause of its cytotoxicity and suggests that Khat could induce cell death even in short tissue exposure [6]. In addition, Khat also triggers intracellular generation of free radical, i.e., reactive oxygen species (ROS) and Glutathione (GSH) depletion, which initiates programmed cell death (apoptosis) by causing injury to the DNA [20]. Furthermore, in the present study, it was also observed that higher concentrations (25 mg/mL) of Khat displayed variable outcomes of cytotoxicity, not showing significant cell death of NOF 316 cells, as compared to the other tested cells.

Interestingly, Khat showed dose-dependent cell death of NOF 18, NOF 26, and SCC4 cells, indicating higher toxicity at higher doses. In contrast, NOF 316 and NOF 319 did not exhibit dose-dependent cytotoxicity due to Khat. A possible explanation for this might be derived from the fact that Khat (25 mg/mL) used in the present study was in the frozen from and might have lost some of its activity [21]. Moreover, the variations in backgrounds and oral habits of the patients from which NOFs were obtained, could have resulted in such outcomes [22]. Similar findings were also reported in studies by Abderrahman and Modallal [23] and Lakundu et al. [24]. Furthermore, it was reported that Khat caused genetic material damage and its genotoxic effect on normal keratinocytes and fibroblasts increased with higher dose [25]. Their findings supported the outcomes of the present study. Therefore, clinically speaking, oral lesions including mucosal keratosis,

pigmentation, and leukoplakia were not only induced by Khat, but could also progress and differentiate due to increased frequency and the extent of Khat chewing habit. Hence, patients should be educated that the Khat chewing increases the risk and incidence of oral lesions and a cessation of the habit should be encouraged. The present study reports a positive expression for α-SMA staining in fibroblast, indicating a phenotypic change. SMA is a well appreciated marker for MFs differentiation in tumor stroma, particularly in relation to OSCC [26]. MFs display intermediate physical characteristics between fibroblasts and smooth muscle cells [27]. Various laboratory-based studies demonstrate the role of cancer cell line in acquisition of fibroblast transformation in MFs, by secreting various extracellular matrix proteins, matrix-degrading enzymes, and growth factors [28]. These MFs modulate the growth, adhesion, migration, invasion, and differentiation of cancer cells. MFs tend to differentiate in the presence of TGF-β, another factor that increases in fibrotic lesions [29]. Recently, studies showed that suppression of α-SMA stops the connective tissue growth factor (CTGF) activity linked with reduced nuclear factor kappa B (NF-κB) translocation in different areas of progenecity, indicating the importance of MFs in tissue fibrosis and cell death [30]. In addition, a study conducted by Etemad-Moghadam et al., reported that MFs appear more frequently in OSCCs and are detected more towards the invasive front [31]. These findings indicate that Khat influences the progression and development of pre-malignant and malignant lesions, as it express α-SMA in fibroblasts. Weak and anecdotal evidence exists in relation to Khat chewing and oral cancer, in light of findings from retrospective and descriptive studies [32,33]. Our findings further the clinical notion, that Khat usage (expression of α-SMA) facilitates differentiation and progression of pre-malignant lesions. Therefore, Khat users (with tobacco use) should be warned of the risk of possible development of pre-malignant lesions and their progress to malignancy. Hence, further randomized controlled trials investigating the effect Khat usage with and without tobacco smoking, on the incidence of malignant and pre-malignant lesions are recommended.

Khat showed cytotoxicity at almost all concentrations, as compared to the negative control but there are certain limitations, which should be considered in the interpretation of the outcomes of the present study. This investigation was in-vitro, and cell cytotoxicity was performed under ideal conditions. In the present study, decreased cell viability was observed, which indicated induction of differentiation or cell cycle arrest not necessarily indicating cell death. Therefore, for strictly assessing cell death, other assays including trypan blue exclusion assay is recommended in future studies. For the cytotoxicity of cells, MTT assay, and for α-SMA, immunofluorescence was performed. However, VELscope® (Visually Enhanced Lesion scope) is a relatively new method to screen oral lesions and is recognized as an efficient tool by World Health Organization (WHO) in oral cancer prevention [34]. Therefore, further clinical trials investigating the influence of Khat on inducing oral lesions using VELscope® as a tool are recommended. In addition, confounding factors, i.e., smoking, tobacco chewing, oral hygiene, and chronic diseases are critical in the development of OSSC, and result in accelerated cell death in the presence of Khat. However, these factors were not assessed in the present study. Moreover, this study used control, Khat, and TGF-β (as a positive control), therefore, an interaction of Khat and TGF-β by incubating cells pretreated with TGF-β with Khat should be performed in future studies. Additionally, the effect of dose-, duration-, and friction-associated trauma in Khat chewers is of great importance in assessing its clinical influence on mucosal differentiation. Therefore, further clinical randomized controlled trials assessing the oral cellular changes among Khat users in oral epithelial and mesenchymal cells with the associated factors (smoking, tobacco chewing, oral hygiene, chronic diseases, and trauma) are recommended to clinically translate the findings of the existing study.

5. Conclusions

Results from the present study showed that Khat is cytotoxic to oral fibroblasts. It also causes cell death of oral cancer cells (i.e., SCC4 cells). Furthermore, it can also

cause activation and phenotypic changes in oral fibroblasts, indicating a potential role in progression of OSCC.

Author Contributions: A.U.Y.S., M.A.A. and E.I.A., data collection, study design, data assessment, and manuscript writing, manuscript revision. M.A.-A., A.M.A. and R.J., data inference, experiment performance (SEM and RS), study design, manuscript drafting, data analysis, and manuscript revision. A.R.A. and S.A.M., experiment (MTBS and DC), data collection, funding, resources, data interpretation, writing, revise, editing, and final manuscript approval. N.A., F.V. and T.A., experiment (MTBS and DC), data collection, data interpretation, funding, resources, software, writing, revise, manuscript revisions, and final manuscript approval. All authors have read and agreed to the published version of the manuscript.

Funding: The authors extend their appreciation to the Deputyship for Research & Innovation, "Ministry of Education" in Saudi Arabia for funding this research work through the project number IFKSURG-1438-075.

Data Availability Statement: The study data is available from the corresponding author on request.

Acknowledgments: The authors extend their appreciation to the Deputyship for Research & Innovation, "Ministry of Education" in Saudi Arabia for funding this research work through the project number IFKSURG-1438-075.

Conflicts of Interest: The authors declare that they have no conflict of interest and all authors have read and approved the final draft.

Appendix A

Table A1. Materials and equipment used in the study.

Materials/Equipment	Company
Dulbecco's Modified Eagles Medium, Cell culture medium	Gibco by Life technologies Invitrogen
PBS solution (without Ca^{2+} and Mg^{2+})	Sigma Aldrich
Trypsin/EDTA	Invitrogen
Acidified Isopropanol	Fisher Scientific, Leicestershire, UK
MTT, Thiazolyl Blue Tetrazolium Bromide	Sigma Aldrich
Galaxy R CO_2 Incubator	Brunswick lab, (UK)
MSE Harrier 18/80 Refrigerated Centrifuge	MSE, UK
25 cm^2 and 75 cm^2 tissue culture flasks (Cellstar®)	Greiner Bio-One Ltd. (Gloucestershire, UK)
96 well tissue culture plates	Fisher Scientific UK Ltd. (Loughborough, UK)
Integra Biosciences PIPETBOYpro	Thermo Scientific, UK Ltd.
Disposable sterile pipettes (Costar®)	Corning Incorporated (USA)
Micropipettes	Thermo Scientific and Gilson lab (UK)
Labpette pipettes	Manufactured by Labnet (NJ, USA)
Pipette tips	STARLAB, Ltd. and Gilson. (UK)
Universal containers	SARSTEDT Ltd. (Leicester, UK) & Sterilin UK
Manual Desktop Counter	Ryman, UK
Cell counting chamber slides/Haemocytometer	Invitrogen
Zeiss Axiovert 200 M inverted microscope	Carl Zeiss Ltd. (Hertfordshire, UK)
Syringe and Its filter units	Millex®GP, Ireland
Eppendorf centrifuge tubes	Eppendorf, UK
24 well tissue culture plates	Fisher Scientific UK Ltd. (Loughborough, UK)
Glass slides	Thermo, UK
Axioplan 2 Imagin software	Operated by University of Sheffield

Table A1. *Cont.*

Materials/Equipment	Company
Spectrophotometer plate reader, Infinite M200	Tecan, UK
Magellan™ data analysis software	Tecan, UK
100% Methanol	Provided by tissue culture lab, Dental School, University of Sheffield
Sodium deoxycholate	Provided by tissue culture lab, Dental School, University of Sheffield
Bovine Serum Albumin (BSA)	Sigma Aldrich, UK
Monoclonal Anti-Actin, α-Smooth Muscle - FITC antibody produced in mouse clone 1A4, purified immunoglobulin, buffered aqueous solution	C2 Sigma Aldrich, UK
DAPI nuclear stain	Life Technologies, Invitrogen
Monoclonal Anti-Human IgG1−FITC antibody produced in mouse	Sigma Aldrich, UK
TGF-β	Sigma Aldrich, UK

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Communication

An Italian Innovative Small-Scale Approach to Promote the Conscious Consumption of Healthy Food

Gloria Formoso [1,2,†], Caterina Pipino [2,3,†], Maria Pompea Antonia Baldassarre [1,2], Piero Del Boccio [2,4], Mirco Zucchelli [2,3], Nicola D'Alessandro [5], Lucia Tonucci [6], Angelo Cichelli [2], Assunta Pandolfi [2,3] and Natalia Di Pietro [2,3,*]

[1] Department of Medicine and Aging Sciences, University G. d'Annunzio of Chieti-Pescara, 66100 Chieti, Italy; g.formoso@unich.it (G.F.); marbaldassarre@gmail.com (M.P.A.B.)
[2] Center for Advanced Studies and Technology-CAST (ex CeSI-MeT), University G. d'Annunzio of Chieti-Pescara, 66100 Chieti, Italy; c.pipino@unich.it (C.P.); p.delboccio@unich.it (P.D.B.); m.zucchelli@unich.it (M.Z.); cichelli@unich.it (A.C.); assunta.pandolfi@unich.it (A.P.)
[3] Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio of Chieti-Pescara, 66100 Chieti, Italy
[4] Department of Pharmacy, University G. d'Annunzio of Chieti-Pescara, 66100 Chieti, Italy
[5] Department of Engineering and Geology, University G. d'Annunzio of Chieti-Pescara, 66100 Chieti, Italy; nicola.dalessandro@unich.it
[6] Department of Philosophical, Educational and Economic Sciences, University G. d'Annunzio of Chieti-Pescara, 66100 Chieti, Italy; l.tonucci@unich.it
* Correspondence: natalia.dipietro@unich.it; Tel.: +39-0871541424
† These authors contributed equally.

Received: 9 July 2020; Accepted: 13 August 2020; Published: 15 August 2020

Featured Application: The SANI small-scale approach may offer a great methodological opportunity to improve the food environment and promote healthy and conscious food consumption through a new nutritional educational approach, which lays the foundation for future application in a wider population.

Abstract: An unhealthy diet is considered to be one of the main causes for increases in obesity and chronic diseases. Food choices are frequently influenced by food systems and environments along with the availability and affordability of healthy and sustainable food. In this context, a major contemporary challenge lies in improving these aspects in order to support healthy dietary choices. Hence, to address this issue, here, we propose a small-scale approach called SANI (Italian for "healthy") which involves experts in science and marketing. Two typical agri-foods of the Abruzzo area (center of Italy), tomato sauce and extra virgin olive oil, are characterized as high-quality products in terms of their nutrient content, absence of chemical contaminants (chromatographic, spectrophotometric, and magnetic resonance techniques), and ecological footprint (lifecycle assessment and carbon footprint). Hence, their consumption is promoted, with strict attention being paid to several aspects of the food system, such as production, processing, distribution, labeling, and promotion, as well as marketing strategies and dissemination activities. Overall, these SANI actions, especially labeling and dissemination, have proven to be a valuable learning tool for consumers moving toward more conscious consumption, which can be extended and applied to additional food products. Future applications of similar research strategies in a wider context could positively affect human and environmental health.

Keywords: healthy food; Mediterranean diet; food system; sustainability; conscious consumption

1. Introduction

In order to promote and maintain good health throughout the course of life, it is necessary to adopt a healthy diet as well as a healthy lifestyle. Unfortunately, life changes determined by industrialization, urbanization, economic development, and market globalization have had a significant negative impact on population health and nutritional status, leading to a global dietary transition [1]. While the standard of living has improved, food availability has expanded, with consequences in terms of unhealthy dietary patterns (e.g., high consumption of processed meat- and plant-based foods, sodium, sugar, and saturated fats and low consumption of fruit and vegetables, whole grains, fibers, legumes, fish, and nuts) [2]. This, together with sedentary habits, smoking, alcohol consumption, and weight gain, has led to an increase in the prevalence of obesity and numerous other chronic diseases [3].

Furthermore, as recently demonstrated, unhealthy food is often less sustainable, with a negative impact on the environment which, in turn, negatively affects human health [4,5].

In this regard, the Mediterranean diet (MD) represents a high-quality dietary pattern which, as commonly represented graphically by the MD pyramid, includes the consumption of extra virgin olive oil (main source of monosaturated fatty acids, to be used for all culinary purposes); high consumption of plant-derived foods (fresh fruits, vegetables, legumes, and tree nuts); moderate-to-high consumption of fish, whole-grain cereal, and red wine (with meals); and reduced consumption of red and processed meat, cheese, butter, whole-fat dairy, sugar-sweetened beverages, biscuits, and cake [6]. Furthermore, the MD has been recognized since the 1990s as the most healthy diet by both the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), and since 2010, it has been listed by the United Nations Educational, Scientific, and Cultural Organization (UNESCO) as an intangible cultural heritage of humanity [7,8].

The MD is also recognized as an example of sustainability, as it respects the natural environment, generally represented as a double "food and environmental" pyramid. Indeed, there is a connection between good eating habits and a positive contribution to environmental sustainability [9]. The environmental pyramid roughly mirrors the food pyramid, but upside down. The double "food and environmental pyramid" suggests that food recommended for consumption in higher quantities, especially with regard to the MD, has a lower impact on the environment. Food advised for consumption in smaller quantities has the greatest impact on the environment. Thus, two different but equally important goals—health and environmental protection—fit into a single food model (Figure 1) [10,11].

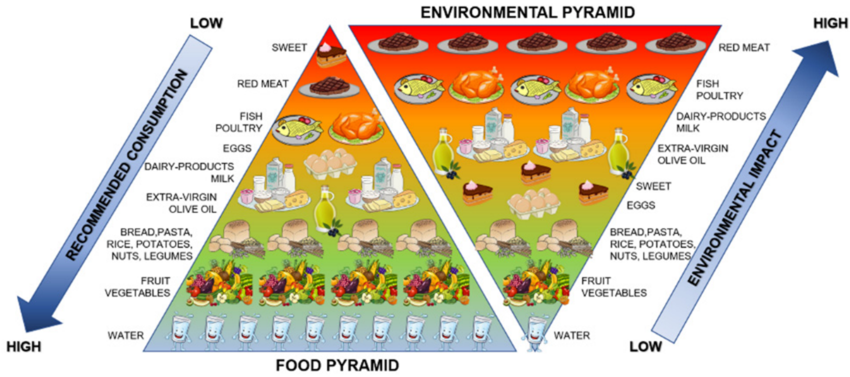

Figure 1. Double "food and environmental" pyramid.

Therefore, it is necessary to steer consumers towards an informed choice of healthy and sustainable food consumption [12]. Indeed, together with several previous studies [13–15], a recent review examining several articles published from 2017 to 2019 regarding food choices and nutrition found that, starting from childhood to adulthood, food education is the main strategy to improve choices [16].

Furthermore, nutritional knowledge is an important and decisive aspect that also influences the eating habits of the elderly [17]. Therefore, given that nutrition is an essential component of

healthy aging, a critical aspect in promoting optimal nutrition and health throughout life, including in old age, is to ensure the availability of safe and adequate foods [9]. However, alongside this, multi-level innovation—including policy, private sector, public health, general community—is needed as well as individual-level approaches. Together, these aspects would allow for the development of a new, healthier, and more sustainable food system with positive effects on human health and the environment [18]. Indeed, one major contemporary challenge is to improve the food system, which refers to the elements and activities related to food production, processing, distribution, preparation, consumption, and food environments in order to make healthy foods available and affordable to consumers [19]. Therefore, beyond paying attention to the nutritional quality of naturally healthy foods, it is necessary to develop a strategy sharpening all key aspects of food supply chains, food environments, and consumer behavior [19,20].

In this regard, cities offer great opportunities to improve food environments and the eating habits of residents [20–22], particularly in Mediterranean cities, which have unique characteristics in terms of small food shops and public markets whose accessibility is essential for stimulating eating and lifestyle habits towards more healthy profiles. Moreover, it should be taken into account that by 2050, 66% of the world's population will live in cities [23].

However, it should be considered that large cities also present many fast food and full-service restaurants, and the number of bars/pubs and liquor stores that have been found positively associated with obesity and abdominal obesity [24].

In this complex scenario, we propose an Italian innovative small-scale approach as a useful model to educate and steer consumers towards conscious and healthy food choices, providing better characterization of some Italian local agri-food products typical of the MD in terms of nutrient content, chemical food contaminants, and environmental impact, and to promote these foods through dissemination of results and via innovative marketing channels.

Previous studies have suggested that improving product quality and the appearance of the store could potentially influence residents' food purchase decisions, though these studies did not adopt a broad-spectrum approach [25].

The SANI (Italian for "healthy") approach, developed for the first time here, has the main goal of providing education on the conscious consumption of local, healthy, and sustainable products using a combination of different skills from the field of agriculture, academia, marketing, communication, and technological tools. All of this may have the following positive repercussions covering all fields: (i) producers can appreciate the quality of their products better, becoming aware of the content of specific nutrients and their environmental impact; (ii) universities can develop new experimental protocols, and professors and researchers can play an important role in the dissemination process; and (iii) there will be an increase in the sale of products considered to be healthy in local retail specialty stores. This approach might steer consumers toward conscious food choices from sustainable and local food systems and could be implemented to more products and extended to a wider population, hopefully with consequent positive feedback for human health.

Last but not least, our study, adopting the multidisciplinary approach described above, intends to fill the gaps of previous monodisciplinary research [26], which often overlooks some aspects of the complex network of determinants in the food choice system.

2. Materials and Methods

2.1. Description of SANI General Approach

The general objective of the SANI small-scale approach was to characterize some local agri-food products typical of the MD in terms of their nutrient content, chemical food contaminants, and environmental impact, and to promote them through dissemination of results and via innovative marketing channels.

To achieve this, the SANI strategy required the coordination of various activities—ranging from the agricultural sphere to the scientific and marketing spheres—that result from the knowledge and skills of the SANI partners (represented as a series of gears in Figure 2), which were as follows: (1) producers (local farmers); (2) academic research groups (universities) focused on food analysis, lifecycle assessment (LCA), and with expertise in the study of nutritional molecules [27–30]; (3) a consulting and training center (C&T Center, a local society) specialized in organizing training in communication, marketing, and professional updating courses; and (4) a local retail specialty store located in an Italian medium-sized urban area (population 200,000–500,000), which played the role of the principal investigator of the funded SANI project.

Figure 2. SANI (Italian for "healthy") partners and activities.

2.2. Evaluation of Specific Nutritional Molecules and Chemical Food Contaminants in Selected Products

As a small-scale Italian approach, the analyses focused on two highly consumed, healthy foods in the Mediterranean area and, in particular, on valuable and exclusive local product varieties of the Abruzzo region (center of Italy)—extra virgin olive oil (EVOO, produced by an oil mill located in the hills of the province of Chieti, Abruzzo) and tomato sauce (made from the tomato "pera d'Abruzzo", a typical variety of the Abruzzo region registered in the SIAN Plant Variety Register, Cod. 3399 SAAB-CRA). The research groups involved in the SANI strategy have technical scientific skills regarding chemical analysis of the nutraceutical compounds and toxic substances possibly present in food. Over the years, they have developed analytical methods for the qualitative and quantitative determination of different bioactive compounds, such as polyphenols, vitamin E, and carotenoids, using chromatographic, spectrophotometric, and magnetic resonance techniques [31–34]. For the SANI approach, attention has mainly been focused on the content of three bioactive compounds, namely, polyphenols, vitamin E, and carotenoids. These compounds have demonstrated antioxidant, anti-inflammatory, and anti-tumor activities that are responsible for the efficient prevention of numerous diseases (e.g., atherosclerosis, cardiovascular, neurodegenerative, and chronic intestinal diseases) [29,35,36]. In this regard, we determined the content of polyphenols and vitamin E in EVOO, and lycopene, β-carotene, and vitamin E in tomato sauce [32,37,38].

Furthermore, the levels of chemical food contaminants harmful for human health, including fatty acid esters of 2-chloropropane-1,3-diol (2-MCPD), 3-chloropropane-1,2-diol (3-MCPD), and glycidol, were assessed [39]. These contaminants, as we have previously shown, are mainly formed at high temperatures as a result of the refining process but are also usually derived from poor quality raw material [34].

In parallel, the AOCS Official Method Cd 29c-13 was used for the determination of 2-MCPD, 3-MCPD, and glycidol in EVOO [34,40].

2.3. Assessment of the Environmental Impact in Selected Products

In general, the environmental impact depends on many factors, including treatments during the production, packaging, marketing, and consumption processes in terms of energy expenditure and employment of resources. Usually, three indicators are used to quantify this impact, namely, the ecological footprint, measuring the Earth's capacity to regenerate the resources used to produce a single food; the carbon footprint, measuring the greenhouse gas emissions (GHGEs) during its lifecycle; and the water footprint, which measures the consumption of water for each specific food.

As the research groups involved in the SANI approach have specific experience in the analysis of the environmental impact of the process using the lifecycle assessment (LCA) and carbon footprint [41,42], the latter was determined as an index of the environmental impact of the selected products.

The carbon footprint of EVOO was evaluated taking into consideration the following processing steps: agricultural phase (all mechanical processing, and phytosanitary treatments and the management of the aerial part of the crops), collection and transport phase in oil mills, and extraction and packaging (into 5-liter steel containers) phases. The evaluation of the carbon footprint of tomato sauce included the development of the tomato seedling; cultivation, harvesting, and transport of tomatoes; washing and sauce production; and packaging, distribution, and end-of-life of packaging.

2.4. Identification of Health Claims and Development of SANI Labels

In line with the rules governing the nutrition and health claims sector [43], nutrient quality labels (SANI labels) and a sustainability label (carbon footprint label) were designed. Briefly, we chose easily distinguishable colors and icons as a result of collaboration with labeling experts, designers, and nutritionists. The carbon footprint label was designed with the same characteristics as those already available on the web and on commercial products, i.e., a foot on a green background and with the CO_2 symbol (chemical symbol for carbon dioxide) to indicate GHGEs. For the nutritional labels, they were designed on the basis of the following characteristics: (i) clear label (a transparent, comprehensive, easy to read, and information-rich label) and (ii) social label (a label at the service of people who know exactly what they are buying and are able to trace the entire supply chain). Indeed, these were equipped with a quick response code (QR code) useful for providing real-time information to consumers (Figure 3).

2.5. Determination of Customer Shopping Habits

The shopping habits of consumers who follow a balanced diet and/or require specific nutritional needs (food intolerances, sports, diabetes, aging, obesity, hypercholesterolemia, pregnancy, menopause, nephropathy, etc.), as well as the perception of the meaning of healthy food vs. unhealthy food and natural food vs. artificial food have been identified through the development of collected metadata (through testing conducted by different customer relationship management companies, like Perfex, Infusionsoft, Hubsport, and Salesforce—free trial versions) by the local retail project partner. To study customer behavior towards natural food, the local retail project shop conducted a preliminary interview on 10 randomly selected regular customers. This allowed for building the following survey to be taken by 192 adults, 78% of whom were women and 22% were men, all aged between 19 and 64 years (8% aged 19–24, 13% aged 25–34, 22% aged 35–44, 34% aged 44–54, 16% aged 55–64, and 7% older than 64 years) and with different working statuses (66% freelancer and employees, 5% entrepreneurs, 5% housewives, 10% students, 7% retired, and 7% unemployed). The survey was designed to test the interviewees' knowledge on general wellbeing and healthy eating by completing a simple questionnaire.

Moreover, the retail specialty store classified its customers by applying a psychographic method, the eight values and lifestyles (VALS), which allowed for designing an innovative marketing strategy by using several analysis tools such as Javelin Board, the Value Proposition Canvas, and selling funnel marketing. These tools focused on the following key elements for consumers: improvement of physical and economic food access through specific custom fidelity programs oriented to prize the consumption

of healthy food; innovative services useful to increase healthy food consumption, focused on the project mission; and an explicit food offer based on nutritional quality and healthy safety using the SANI label (the trademark "Bollino SANI" (SANI label), which was registered with deposit no. 302019000012369, on 21 February 2019, Figure 3) [19,20].

2.6. SANI Approach Communication, Dissemination and Marketing Strategy

The communication of the obtained data and the dissemination of the SANI small-scale strategy represent two key points of the project. They were implemented using the following tools: (1) a blog containing several informative articles (namely, referring to scientific publications) regarding the beneficial effect of the consumption of specific healthy products; (2) a dedicated SANI project web page; (3) a SANI project Facebook page; (4) web videos on the SANI project covering several topics on healthy nutrition, published on different online platforms (Facebook, YouTube, and the SANI project page); (5) several public dissemination events to promote the SANI project at local fair events; (6) distribution of informative flyers on SANI; (7) organization of educational meetings for consumers; and (8) organization of academic meetings. Physicians, nutritionists, and naturopaths as well as different types of associations (professional, consumer, and social operators' associations) were involved in the dissemination activities listed above.

3. Results and Discussion

The first activity of the SANI approach was to identify some highly consumed "km zero" agri-food products in the retail specialty store that involved local farms. Among them, local EVOO and tomato sauce were identified as being worthy of further investigation. Indeed, these two valuable and exclusive local product varieties of extra virgin olive oil (EVOO; produced by an oil mill located in the hills of the province of Chieti, Abruzzo) and tomato sauce (made from the tomato "pera d'Abruzzo", a typical variety of the Abruzzo region registered in the SIAN Plant Variety Register code 3399 SAAB-CRA) are highly consumed in the Abruzzo region, especially by people who know their organoleptic properties. However, they have never been fully characterized prior to the SANI study in terms of their specific nutrient contents, the absence of chemical contaminants, and their ecological footprints.

As shown in Table 1, these products presented a specific quantity of nutritional molecules (vitamin E, carotenoids, and polyphenols), the total absence of specific chemical contaminants (2-MCPD, 3-MCPD, and glycidol) harmful for human health, and both were within the range corresponding to a low environmental impact on the Mediterranean area, as shown by their carbon footprint evaluations.

In more detail, the results revealed a high quality of local EVOO, demonstrated by the presence of vitamin E (alpha-tocopherol 229.5 mg/kg) and an adequate polyphenol content (360 mg/kg). In the tomato sauce, the presence of specific carotenoids (beta-carotene 0.12 mg/100 g and lycopene 7.57 mg/100 g) and vitamin E (alpha-tocopherol 1.14 mg/100 g) were observed. This allowed us to confirm that the two local products naturally contain bioactive compounds beneficial for human health and, thus, can possibly be defined as "high-quality foods". This aspect is even more relevant if we consider the total absence of chemical contaminants, such as glycidyl fatty acids esters which, as demonstrated by previous studies, are harmful to human health and are generally present in processed oils and food-based products containing oil [44]. In this regard, it has been shown that palm oil has a high content of chemical contaminants as a result of the transformation process, while crude or unrefined oils and fats, such as extra virgin olive oil, either do not contain chemical contaminants or contain merely trace amounts [34,44,45].

However, it is important to specify that these evaluations were not performed for comparing the quality of the selected products to other brands, but mainly aimed to support the consumer toward conscious consumption through innovative methodology characterized by the easy readability of the specific nutritional properties of the product (e.g., through colorful SANI labels).

Beyond the nutritional value of the foods in terms of their carbohydrate, protein, fat, and fiber contents, with a direct impact on physical fitness and health, attention should also be paid to the fact that each food has an environmental impact and by considering its entire lifecycle.

The analysis performed on EVOO, together with the information gathered from the oil mill, revealed that the production of this specific local EVOO had a total emission of approximatively 6 kg CO_2eq (carbon dioxide equivalent) for 5 L (e.g., 1.2 kg per liter). Thus, EVOO emission falls in the lower part of the carbon footprint average range in the Mediterranean area (10.51 kg CO_2eq per liter of olive oil, in line with the world level) [42,46–48]. The carbon footprint measured for the tomato sauce resulted of about 1.3 kg CO_2eq per liter of product packaged in glass, thus in a good impact range considering that, depending on the packaging materials and the size used, the carbon footprint average ranged between 0.3 and 2.28 kg CO_2eq per liter [49,50]. Hence, the carbon footprint assessment of the two described products showed that both fall within the range corresponding to a low impact.

This suggests that the use of local products represents a positive factor that together with the improvement of other components (e.g., reduction in the use of fossil fuels), could contribute to a healthy environment. In this regard, our approach fits well into the contemporary challenge aimed at expanding research on the production and consumption of sustainable local food [51]. The consumption of sustainable products would have positive effects on various aspects, first environmental, but also social, economic, and human health aspects [51]. As for the last aspect, it is known that a healthy environment is the basis of a healthy life, and an unhealthy environment contributes to the onset of various non-communicable diseases (NCD), such as stroke, heart disease, cancer, and other chronic diseases [52].

Table 1. SANI products: content of valuable compounds and their carbon footprint.

PRODUCT	Vitamin E	Polyphenols	β-Carotene	Lycopene	2-MCDP 3-MCDP Glycidol	CO_2eq/L
EVOO	229.5 mg/kg	360 mg/kg	—	—	absence	1.2 kg
Tomato sauce	1.14 mg/100 g	—	0.12 mg/100 g	7.57 mg/100 g	absence	1.3 kg

Based on these results and the rules governing the health claims sector [43], nutrient quality labels (SANI labels) and a sustainability label (carbon footprint label) were designed that were easily recognizable to consumers.

As shown in Figure 3, the SANI labels consisted of specific colorful marks that were assigned to each healthy nutritional component. Interestingly, these labels were equipped with a QR code, intended to be useful to consumers by allowing them to access real-time information in order to enrich their shopping carts with many healthy products, making it possible to achieve a balanced, healthy, complete, and sustainable diet.

This, along with communication of the obtained data, the dissemination activity, an innovative online e-commerce platform, and through optimal shelf marketing, led to a considerable increase in conscious purchasing. Indeed, before the SANI survey, the results showed that people would like to live a healthy life by consuming natural food, but that their knowledge about healthy natural food was conflicting, thus affecting their choices. Indeed, an analysis of the distribution of the responses to the questionnaire (Table 2) based on age groups was conducted (Figure 4), and this revealed how age can affect certain choices and awareness. For example, most interviewees (60% of the total) considered natural food as "whole food, without any human intervention", or "as natural, thus you can trust" (10%). Only 30% of interviewees considered natural foods as "organic foods" rich in healthy nutritional components.

Figure 3. SANI labels and marketing approach. Following the directions of the SANI marketing approach (1–4), the shopping cart is enriched with many healthy products, each with a different label to help achieve a balanced, healthy, complete, and sustainable diet.

Table 2. SANI questionnaire.

1. Are you . . . ?	A) Female
	B) Male
2. What is your occupation?	A) A professional
	B) An employee
	C) An entrepreneur
	D) Other
3. Age	A) 25–30
	B) 31–40
	C) 41–50
	D) 51–60
	E) 61–70
	F) Other
4. What do you think about natural products?	A) I think they are good for my health
	B) I think I should consume them or use them every day
	C) I think it is okay if I consume them or use them occasionally
	D) I think industrial products convince me more
	E) Other
5. What does natural food mean to you?	A) Whole food, without any human intervention
	B) Organic food
	D) Natural, thus you can trust it
	E) A new way to sell products
	F) Other
6. What would you do to live in a state of wellness?	A) I would go to a beauty center
	B) I would go to a gym
	C) I would go on holiday in a farmhouse or in a spa
	D) I would use natural products for my diet and/or my esthetic
	E) Other

AGE	19-24	25-34	35-44	45-54	55-64	≥65
ANSWER TO QUESTION 4	A B	A B	A B C	A B C D	A B	A B D
ANSWER TO QUESTION 5	B A D	B C A	B C A D	B C A	B C A D	B A D
ANSWER TO QUESTION 6	B C D	B C D	healthy food,sport healthy environment live in nature B C D A	E B C D	B C D	B C D A

Figure 4. Distribution of responses to the SANI survey questionnaire by age group. Question 4: What do you think about natural products? Question 5: What does natural food mean to you? Question 6: What would you do to live in wellness? Refer to Table 2 for the answers.

Following the SANI approach, a significant increase in labeled SANI product sales was found during both 2017–2018 (number of sales 183; 95% confidence interval (CI): 157.45–211.52, $p < 0.05$; Poisson distribution analysis) and 2018–2019 (number of sales 245; 95% CI: 212.47–274.49, $p < 0.05$; Poisson distribution analysis) compared with the sales in 2016–2017 (number of sales 150; 95% CI: 126.96–176.02; Poisson distribution analysis) before implementation of the SANI approach. However, it is important to point out that these data are not intended to highlight an increase in sales in terms of earnings but to indicate the increased sale of products considered to be healthy as a consequence of people becoming oriented towards healthier product consumption through SANI.

Indeed, in accordance with a previously published research [53], the results of the SANI approach confirm that consumers buy certain organic and local foods because they are perceived as being healthier, fresher, more respectful of the environment, and favorable to the local economy compared with conventional products.

In this regard, one of the main future purposes will be to extend our approach to many other local products, but also for other Italian products in a wider context.

SANI was experimentally applied, as a small-scale approach, in a single city of the Abruzzo region, but given that farms located throughout the region were involved, its repercussions were wider. Furthermore, the project was part of public policies on innovation in the Abruzzo region with the aim of reducing the environmental effects of human activities in this region, and was interconnected with other projects conducted by the research groups of the SANI strategy and funded by the Italian Ministry of Scientific Research (CL.USTER A.GRIFOOD CL.AN Pros.It; MIUR, 2012) and the European Union (PEFCR pilot project on olive oil; EU, 2014). The purpose of both grants was to develop technologies and knowledge for safer food production with high-quality features and a low environmental impact of food products typical of the MD, such as olive oil, wine, pasta, and dairy products. Future application of similar research strategies in a wider context could positively affect human and environmental health.

4. Conclusions and Implications

The increasing prevalence of obesity and chronic diseases demands that action be taken in recognition of the perilous direction many consumers are taking regarding their food choices [54–56]. Many aspects of current lifestyles should be improved, together with a new and profound renewal of the food system. Recently, the High-Level Panel of Experts on Food Security and Nutrition identified three constituent elements of food systems, namely, food supply chains, food environments, and consumer behavior [19].

The SANI approach deals with all of these aspects and allows for improvements in some of these areas specific to food systems, with positive effects on human and environmental health thanks to the promotion of conscious consumption. In more detail, the specific nutrient content and environmental impact of two varieties of agri-food typical of the Abruzzo region were assessed, which allowed for creating specific labels associated with technological tools. Furthermore, a key aspect which allowed for reaching the main objective, was the dissemination of the approach and results. Finally, the SANI approach was proven to be a valuable learning and growth opportunity for all of the involved subjects (producer, academia, distributor, and customer).

This suggests that implementation of this small-scale strategy in a wider context, such as cities or countries, could be very useful. This would involve both short- and long-term goals. In the short-term, it may be extended to other cities in the Abruzzo region and to cities of other Italian regions, while as a long-term objective, this strategy could be extended to Mediterranean countries and, subsequently, to a wider territory. In conclusion, we believe that the pilot study proposed here lays the foundation for further development in a larger population, thus leading to successful improvements in all aspects of food systems and increased conscious consumption of healthy and sustainable foods.

5. Limitations

Although the SANI approach may represent a valid tool to respond to the current challenges regarding the improvement of the food system, focusing attention on human health in terms of nutrition and environmental sustainability, it has some flaws and shortcomings.

First, the possible difficulty of reorganizing an active network between various partners (producers, academic research groups, consulting and training centers, consumers, and retail specialty stores) in a scenario other than that of the SANI strategy. Second, SANI knowledge should reach a wider population that, outside the Mediterranean area, could be difficult to apply as a result of different eating habits and lifestyles [57]. These actions, as mentioned above, should comprise both short- and long-term targets. Among these, assessing the long-term potential benefits on human health and the environment could require more skills and people to be involved.

Additionally, further research will be necessary to improve the informative method on health claims (such as labeling improvement) in order to help consumers with choosing products for specific and customized dietary needs. Indeed, a weakness of SANI labels is that they indicate exclusively specific nutrients, which are contained in high quantities in the products, associated with a technological tool (QR code) that allows to get real-time information about the nutrient properties. A label that presents a complete profiling of all macronutrients and micronutrients, together with an indication of the unhealthy ones for human health, such as those already reported in the NutriScore and SAIN-LIM system [58,59], could be more useful for consumers.

Another limitation may be the ability of older people to use technological tools (such as smartphones, tablets, and computers), as these skills were required especially during the communication, dissemination, and marketing phases of the SANI project. As for our small-scale project, the elderly population was only 23%, and they had assistance from the staff of the specialty retail store in order to get information about the products of interest during purchases. However, this aspect is expected to be resolved in the near future, with directed efforts already underway [60].

In conclusion, in spite of these limitations, the SANI project offers a great methodological opportunity to improve the food environment and to promote conscious healthy dietary consumption through a novel nutritional educational approach.

Author Contributions: Conceptualization, N.D.P. and A.P.; methodology, N.D. and A.C.; formal analysis, P.D.B.; investigation, L.T. and M.Z.; resources, N.D. and P.D.B.; data curation, M.P.A.B.; writing (original draft preparation), N.D.P., G.F., and C.P.; supervision, N.D.P.; project administration, A.P.; funding acquisition, A.P. and A.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by SAper NutrIre (SANI; Por Fesr Abruzzo 2014–2020 Asse I Determinazione), grant number 39 DPG013/12222017.

Acknowledgments: The authors thank the SANI network partners: Francesco Cuddemi, Paola Renzetti, Elisa Antonioni, and Marianna Belfatto.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Review

Diet and Oral Health Coaching Methods and Models for the Independent Elderly

Maria Antoniadou [1] and Theodoros Varzakas [2,*]

[1] Dental School, National and Kapodistrian University of Athens, 15127 Athens, Greece; mantonia@dent.uoa.gr
[2] Department Food Science and Technology, University of the Peloponnese, 24100 Kalamata, Greece
* Correspondence: theovarzakas@yahoo.gr; Tel.: +30-2721045279

Received: 4 May 2020; Accepted: 2 June 2020; Published: 10 June 2020

Featured Application: Diet and oral health coaching is the emerging yardstick that will differentiate professionals, especially dentists, in order to become more effective as clinicians while it will guide the elderly patients to improve dietary habits, nutritional intake, and performance of oral hygiene for better oral health.

Abstract: Health-related behavior based on diet is an important determinant of oral health in independent elderly. Aging impairs senses, mastication, oral status, and function, causing nutritional needs and diet insufficiencies that contribute to a vicious circle of impairment. But the present needs of independent older adults suggest that health research and oral health care should shift from disease management and therapy to integral customized and personal treatment plans, including lifestyle, psychological, nutritional, and oral health coaching approaches. In this paper health coaching approaches in medical and dental settings are valued as to their effectiveness for older adults. Furthermore, coaching approaches for seniors are discussed and coaching models for better senior patient-dentist cooperation on the diet issue are suggested. Diet and oral health coaching is proven to be a modern senior patient-centered approach that needs to be incorporated at all relevant settings. It should aim to empower older adults in co-management of their oral diseases or bad diet habits affecting their oral health. This can be carried out through an incorporated educational plan for dentists either at the postgraduate or professional level since advantages seem to enhance the quality of life of the independent elderly.

Keywords: diet; nutrition; oral health coaching; older adults; senior coaching; motivational interviewing; cognitive behavioral coaching techniques; independent elderly

1. Introduction

Aging impairs senses, mastication, oral status, and function, causing nutritional needs and diet insufficiencies. The present needs of independent older adults (OA) suggest that health research and oral health care should shift from reductionist disease management and therapy to integral customized and personal treatment plans, including lifestyle, psychological, nutritional, and oral health coaching approaches [1]. The American Society for Geriatric Dentistry, the Education Research Group of the International Association for Dental Research, and the American Association for Dental Research have been committed from their part to improving oral health in OA through education and skills development [2,3]. In response to these challenges, on the other part of the Atlantic, the European College of Gerodontology (ECG) and the European Geriatric Medicine Society (EUGMS) have created a common task and Finish Group. This group reported that the development of a workforce of dentists

with knowledge about and skills for working with OA would be enhanced by interdisciplinary and interprofessional education [4]. This philosophy has been also suggested by others in the past [5–7].

The vicious circle of malnutrition and oral health, discussed in detail elsewhere [1], should be broken especially for the independent OA, who may still be active or working. For those individuals who live alone or with family members, but still cooking and preparing meals by themselves, old recipes such as the Mediterranean diet (Med-Diet) should be kept as a base [1]. Then dentists or other medical professionals should enrich this base in a customized interpersonal way according to the specific needs of one's oral and general health status [1,4] and help people incorporate it in their daily routine. That is what oral health coaching does.

Generally, health coaching has been described as "the practice of health education and health promotion within a coaching context, underpinned by psychological principles, to enhance the well-being of individuals and to facilitate the achievement of their health-related goals" [8]. Health coaching is a patient-centered approach aiming, in other words, to empower patients in co-management of their disease or bad health habits (e.g., smoking, alcohol, diet, etc.) [9]. It is a strategy that emphasizes and supports patient autonomy, learning, and action instead of compliance. It is based on shared decision making and collaborative goal setting facilitated by motivational interviewing (MI) [8,10–13]. Basically, it is described throughout the literature as a partnership between the coach and the patient/coachee [13]. Cognitive behavioral coaching techniques and strategies are often used to tackle psychological blocks to goal achievement [14] by examining the patients' health-inhibiting thinking (HITs) and then helping them to develop health-enhancing thinking (HETs) [15]. But, as simple as that sounds, change is anything but easy for most of the people. It takes drive, motivation, action, and strategy to change one's habits—especially if someone has been doing things a certain way for a long period of time. For OA, the challenge is even greater due to physical and mental impairments that cause memory lapses or forgetfulness, lengthening of response, depression, loneliness, and even more anger and frustration because of aging. But as said by B. Pascal, "people are better persuaded to change by the reasons they themselves discovered than those that come into the minds of others".

Little data can be gathered on senior coaching concerning diet for oral health and those are mainly out of studies concerning interventions on nutritional aspects for diabetes mellitus, cardiovascular diseases, multi-morbidity, or cancer in independent adults, older adults, or frail elders in hospitals and care centers [16–21]. Thus, in this nonsystematic review the process of senior coaching on diet issues for better oral health are discussed for the independent OA. Suggestions, methods, and models for relevant senior coaching interventions are also described and compared. For purposes of this article, the term *older adults (OA)* refers to individuals age 65 or older.

2. Physical and Mental Issues of OA That Resist Change of Attitude

There are certain alterations in behavior during aging that may interfere in the process of desirable changes (Table 1). These alterations are: (1) Memory lapses or forgetfulness (symptoms might include varying degrees of memory loss, language difficulty, poor judgment, and communication, problems concentrating, and impaired visual perception). (2) Low mood after experiencing loss, coming with depression and a persistent feeling of sadness that can include changes in sleep, appetite, energy level, bad hygiene, and other areas. Mood changes, apathy, confusion, agitation, fear of death, or anger may also signal early dementia. (3) Discouragement or anger as health declines. Anger or aggression—which can show up as emotional or verbal abuse lashed out at loved ones or the doctors—can be particularly difficult to handle. (4) Takes longer to learn new things. On top of a normal decline in short-term memory in OA, it is also common to see a lengthening of "response time"—meaning they learn more slowly and retain new information less effectively. Many seniors who "age well" make a conscious effort to maintain mental alertness by reading widely, learning new skills, taking classes, and/or maintaining social contacts with people from a variety of age groups but this is quite often the exception for most of them. (5) No more resilience on life's hard modalities and various life events such as loneliness, death of a spouse, physical pain, lack of social life, estrangement

from family, eating by oneself, difficulty in getting foods, lack of cooking skills, and loss of economic independence [1].

Table 1. Physical and mental issues of Older adults (OA) that resist change of attitude.

Physical and Mental Issues of OA	Symptoms	Results
Memory lapses or forgetfulness	Memory loss, language difficulty, poor judgment, absence of communication, problems concentrating impaired visual perception	Bad relationships Accidents Need of repetition Loss of orientation
Low mood or depression	Changes in sleep, appetite, energy level, denial and difficulty in oral and body hygiene	Unsocialized behavior, isolation, denial, estrangement from family
Sudden changes in mood	Apathy, confusion, agitation, fear, anger, breakdown	Difficulty or denial in supporting one's needs Social/role limiting
Discouragement or anger	Emotional or verbal abuse	Feeling of loneliness and fatality
Decline in short memory	Longer period of learning, Lengthening of response time Repetitive questioning	Loss of information, neglect of basic survival habits
Low resilience to pain and death	Lack of social life, estrangement from family, eating by oneself, loss of smiling and talking, loneliness	No visits to doctors & dentists, uncontrolled systematic diseases, high stress, bad oral health, anorexia
Lack of cooking skills and physical impairment	Difficulty in getting foods, eating only snacks	Malnutrition, bad oral hygiene
Loss of economic independence	Frustration, fear of the near future	Poverty, difficulty in getting foods, no access to health services

3. Health and Oral Health Coaching Issues

Under the above discussed impaired circumstances, OA need customized repetitive and more motivational dietary interventions for general and oral health than younger individuals, in order to achieve desired changes. Most of all, it takes support, compassion, and empathy for facilitating any coaching approach in these individuals. Those are characteristics at which senior health coaching should excel to be effective.

As said before, the health coach-coachee/patient relationship is "a goal-oriented, client-centered partnership that is health-focused and occurs through a process of client enlightenment and empowerment" [11]. So, certified health coaches or health care professionals doing health coaching are somewhat like "change agents". They should understand how habits form, know how to reverse them, and specialize in helping people overcome obstacles in pursuing their goals. Their role thus involves listening, understanding, facilitating, applauding, supporting, motivating, providing feedback, rewarding, and helping the patient to weigh options and make choices. This can be accomplished by establishing trust and intimacy with the coachee/patient, active listening, powerful questioning, direct communication, creating awareness, designing action plans and goal setting with the coachee, and managing his/her progress and accountability [22]. In this process of change for the better, it is very important to identify and overcome challenges in the first place and then clarify the patient's strengths and aspirations, listening to his/her concerns, boosting his/her confidence in their ability to change, and eventually collaborating with him/her on a plan for change.

Health coaching, in specific, guides a learning process for improved disease or diet management that, if successful, it should lead to permanent changes in patient self-management skills and behavior. But these changes in self-management skills and behavior take time to influence health outcomes [6].

Therefore, in general, the impact of health coaching on health care and cost effectiveness should be assessed in long-term follow-ups [13] for all age groups but even more for the OA due to the physical and mental alterations discussed above.

The problem in the relevant literature is that evidence on the effectiveness of health coaching is, so far, conflicting and it is based on studies for adults with short-term follow-up only (up to 24 months) [6,13,23–26]. Due to the heterogeneity of target populations and outcome measures, no systematic reviews with meta-analyses have been completed [19]. So far, individual studies show basically either small or no significant effects of health coaching interventions [6,27]. They usually include the key recommendations shown in Table 2.

Table 2. Key recommendations for health coaching in OA.

Recommendations for OA during Health Coaching	
1	Know how and when to call for help
2	Learn about the condition and set goals
3	Take medicines/nutrients correctly
4	Get recommended tests and services
5	Act to keep the condition well controlled
6	Make lifestyle changes and reduce risks
7	Build on strengths and overcome obstacles
8	Follow-up with specialists and appointments

In many cases, self-management booklets are sent to patients to support progress toward the key recommendations [28]. Further, a traffic light system, telephone, or e-application can be used in order to visualize patients' progress and support [12,25–27,29–36].

Other research data reinforce the controversial benefits from diet health coaching in OA. To date, most of the large-scale lifestyle modification randomized controlled trials (RCTs) aiming to achieve healthy weight and/or improve nutrition were conducted among noncancer populations [23,37–39]. But, further, one should think that it is more interesting to evaluate the coaching effect especially on cancer patients. Since these patients are basically faced with the risk of death, they should be expected to be more willing to change habits. Generally, all cancer survivors are advised to adhere to the World Cancer Research Funds'/American Institute for Cancer Research (AICR) recommendations [28] to maintain a healthy weight, be physically active; eat a diet rich in fruits, vegetables, and whole grains; limit consumption of red and processed meats, sugar-sweetened beverages, fast foods high in fat, starches, or sugars, and alcohol; and do not rely on dietary supplements for cancer prevention. Additionally, it is recommended to abstain from smoking and reduce excess sun exposure. The American Cancer Society (ACS) guidelines for cancer survivors similarly aim to improve overall survival, metabolic health, and quality of life [40]. To one's great surprise, only a minority of cancer survivors meet the above ACS and AICR recommendations [41–46]. In a nationally representative survey among breast, prostate, and colorectal cancer survivors, only 16% to 18% consumed five or more servings per day of fruits and vegetables, and 24% to 43% engaged in 150 min or more per week of moderate to vigorous physical activity [44,47]. Also mentioned elsewhere, female breast cancer survivors are more likely than males to meet fruits and vegetables recommendations, while male cancer survivors are more likely than females to meet the physical recommendations [48]. Further, it seems that cancer survivors are more likely to adhere to recommendations either during cancer treatment or soon after completion of it [49]. A recent systematic review of lifestyle interventions among cancer survivors, including 51 studies, reported that cancer survivors' adherence to recommendations after participation in such studies is surprisingly low, at 23% on average (range, 7–40% [49]. The authors also reported that these interventions were more effective among survivors with diagnosis in the past five years or recent

survivors compared with long-term survivors (>5 years). Finally, survivors were more likely to adhere to recommendations to not smoke or to reduce alcohol consumption, while they were less likely to meet the recommendation for dietary fiber consumption, something that future senior coaches should keep in mind, too.

Reasons for cancer survivors not following diet and physical activity recommendations include lack of knowledge, low self-efficacy, and motivational and structural barriers (i.e., lack of access to healthy food and exercise facilities) to achieving sustained change [50]. On the other hand, a study showed that 80% of breast and prostate cancer survivors stated they are motivated to make lifestyle modifications through nutrition and physical activity health promotion programs [42]. So, data on this specific issue are quite controversial. It seems that, although patients are often provided enough, if not extensive, knowledge on diet and nutrition in order to change their dietary behaviors, they have only limited success in changing them [50]. It is important to mention that, although initial changes may occur, these may not persist over the long term [51,52]. Everywhere in the literature it is highlighted that patient self-management is not always easy to accomplish. It is difficult to change a long-entrenched lifestyle, even when there is motivation to do so; however, it is much more difficult if there is no motivation. Psychosocial and financial factors are key barriers especially for OA. Many of them, usually quite independent during their lifespan, may be embarrassed about the need for help, lack resources to make changes, or may fear failure and the associated perception that they are incompetent. Of course, there has often not been a strong support system within the medical community to help OA to manage on their own nor in the community at large or even sometimes within the family. To address this gap, effective lifestyle modification programs at the clinics, dental units, and community centers and settings are needed to promote sustained behavior change for those individuals [24,27,53].

It is thus important to conclude that, so far, adherence of this aging group to professional recommendations is astonishingly low. Of course, there always seems to be a gap between what people 'know' and what they 'do'. The process that maintains the gap between knowledge and behavior is ambivalence. OA are faced with conflicting motivations and pressures; the change feels too big, the rewards too distant, motives no longer exists, the personal or financial costs are too high, or maybe it was never their idea to change in the first place [18]. Studies on adherence to health professionals' recommendations have shown that approximately 30–60% of health information provided in the clinician–patient encounter is forgotten within an hour and that 50% of health recommendations are not followed [54]. Thus, overcoming persistent noncompliance of OA can make health-behavior change one of the most rewarding and the most challenging responsibilities for dental health professionals.

4. Positive Data on Health Coaching

Health coaching has led to positive patient outcomes in several studies, including weight loss, diabetes control, decreased blood pressure, HIV, and improved health behavior and multi-morbidity [25,30,55–61]. Previous research has also demonstrated that when used in chronic disease management, wellness coaching enhances self-management skills in patients with diabetes and helps reduce readmissions in those with chronic obstructive lung disease [62,63]. In a review of 15 randomized health coaching interventions, six were able to demonstrate significant improvements in targeted behaviors such as physical activity and medication adherence [64]. It was also reported that wellness coaching was associated with improvement in three areas of psychosocial functioning: Quality of life, mood, and perceived stress [65]. Participants also improved their self-reported health behaviors and goal-setting skills [66]. It was further suggested that integrating wellness coaching within primary care practice is a feasible model for diabetes care, which can be done even without significant additional resources [67]. It is also reported as being well received by patients and physicians in primary care setting [68]. While wellness coaching conducted in health care settings has been shown to be effective in chronic disease and weight management [35,62,63], its use among OA who do not have a chronic disease but who are at high risk for it has not been widely explored. In the study of Knowler et al. [16],

the methodology focused on a well-structured curriculum that included supervised physical activity sessions supported by individual case managers who functioned as "lifestyle coaches". Also, in a systematic review of counseling interventions to change diet and physical activity behaviors among obese and overweight persons with cardiovascular disease risk factors, it was reported that there was decreased diabetes incidence and improved intermediate cardiovascular health outcomes up to two years [57].

On the part of nutrition and diet, clinical recommendations guide clinicians to support especially cancer patients in making healthy diet and nutrition choices [69,70]. Clinical assessments can provide a snapshot of the current state of dietary consumption and dietary patterns of those patients. Most dietary interventions in cancer patient populations exist within a framework of lifestyle interventions for diet and physical activity as well as weight loss [71]. Specifically, targeted dietary change interventions focus on either weight loss or encouraging weight maintenance in cancer patients with good results [71].

Prior dietary interventions have included and tested behavioral models to improve understanding of how patients change their behavior. Successful interventions have used behavior change techniques derived from theoretical behavior models [72,73]. Further, dietary change strategies have been identified to manage weight in cancer populations [74,75]. Two recent reviews [71,74] demonstrated the relevance of the social cognitive theory (SCT) behavioral model [76]. In the study of Park and Chang [61], the effectiveness of a health-coaching self-management program for OA with multi-morbidity in nursing homes was studied with success. Participants in the intervention group had significantly better outcomes in exercise behaviors, cognitive symptom management, mental stress management/relaxation, self-rated health, reduced illness intrusiveness, depression, and social/role activities' limitations. In addition, there was a significant time-by-group interaction in self-efficacy. According to the goal attainment scales, their individual goals of oral health and stress reduction were achieved.

It is also reported elsewhere that health coaching has the potential to decrease the amount of time patients spend with a physician, decrease physician follow-up, and increase satisfaction among both patients and providers as a result of the delivery of more personalized care [77,78]. As an example, one study reported increased patient trust in their physician when health coaching was provided [79].

Furthermore, evidence demonstrates that targeted motivational interviewing in the treatment of chronic diseases and conditions prevalent in OA achieves positive outcomes and reduces health-related costs [80]. It was also reported that when patients receive collaborative self-management support, they have fewer hospitalizations, improved quality of life, and improved clinical outcomes in several ambulatory-sensitive conditions [81–83]. Especially, health coaching provided by nurses has shown promise as a strategy for facilitating behavior change that can lead to improvement in OA with chronic illnesses [84]. It is reported that based on a humanistic and holistic perspective, health coaching is compatible with nursing ideals, and a coaching strategy holds promise for helping OA to achieve their health goals [9]. Coaching by nurses may motivate OA with chronic illnesses to move forward, to act towards making lifestyle changes, and to increase their understanding [85]. So it seems that health coaching could be an expected competency not only for nurses but also for dentists, doctors, and other medical professionals who could help OA promote their self-management skills, prevent complications, lessen their health and oral health care costs, and appreciate a better quality of life [9,86,87].

It is further reported elsewhere that OA are more likely to benefit from a series of health education sessions followed by tailored feedback from the counselor [88]. All that is needed is absence of criticism, patience, empathy, and total acceptance by the dentist/professional coach. Empathy is said to be the necessary element for effective communication between patients and providers to achieve optimal clinical outcomes. Empathy has been defined as a "predominantly cognitive attribute that involves an understanding of patients' experiences, concerns and perspectives combined with a capacity to communicate this understanding and an intention to help" [89–91]. Higher empathy scores have been positively associated with clinical competence and better patient outcomes in physicians [92]. The nature of empathy has been studied extensively in medical students but less so

in dental students [93]. Sherman and Cramer (2005) [94] found that the psychometric properties of empathy in a sample of dental students were comparable to those found in medical students [94]. Four factors emerged, such as perspective taking, compassionate care, standing in the patients' shoes, and efforts to ignore emotions in patient care. Waldrop et al. [7] studied dental students' knowledge about aging and found that, although information is readily consumed by dental students, positive attitudes are not as easily taught [95,96]. It was also reported that attitudes are significantly influenced by the amount of exposure to older people [97], but that is not the case elsewhere [7]. However, attitudes and knowledge may only partially contribute to the development of a caring professional [7]. It is interesting to know that women are scoring higher in empathy than males among dental students concerning communication with OA [7,94].

After all the above, it seems that dental and medical professionals should spare time to explore the factors mentioned before and attach them to the character of the OA. Then oral health coaching based on empathy could be very effective in encouraging, inspiring, and empowering them to reach their maximum health potential [98].

To do so, professionals need training in coaching strategies [9,99,100]. For this reason, coaching modalities were sparingly investigated for their effectiveness in the program of studies both in dental and medical schools with promising effects [101–103]. In a study where medical students were enrolled in the role of health coach for patients with diabetes it was shown that patients accepted the procedure as an opportunity to learn a great deal about the management of their diabetes. Several participants mentioned that the student was so persistent that they eventually altered their exercise and dietary behaviors. In addition, several patients mentioned they often felt uncomfortable asking their regular physician questions due to time constraints. Because of their relationship with student health coaches, patients expressed feeling more comfortable talking to their coach who, in turn, would obtain answers to their health questions. In addition, several patients mentioned that working with students on their health goals improved their motivation to change health behavior [104]. However, although the use of medical students as health coaches to increase patient activation is a novel approach, there seems to be an indication that health coaching by medical students can improve health care communication and disease awareness among patients regarding their disease and overall health [104]. No such data exist yet on dental education, making it a promising research field.

Generally, it can be assumed that improvement in communication between patients and their health care providers can allow for higher utilization of health care, better adherence to treatment recommendations, and improved management of chronic disease, such as diabetes [53,104–107]. From the above, the suggestion was derived that dental and medical professionals could be trained to serve as health coaches [108] with great success if time is found for them to be educated on and perform it, together with their other responsibilities.

5. Methodology for Behavior Change during Coaching

Although no single theory or conceptual model dominates health behavior research or practice of coaching [109], it is well recognized that interventions to modify health behaviors are enhanced through reliance on health behavior theory [72,87,110–112], including foundational behavior change theories such as social cognitive theory [113], the health belief model [113,114], the theory of reasoned action and the theory of planned behavior [115], the integrated behavioral model [116], the precaution adoption process model [117], health locus of control theory [118], and the transtheoretical model of behavior change [119]. Due to overlap among these and other foundational theories, and because only a limited number of variables are relevant to consider when promoting health behavior change [120], Fishbein proposed the integrative model [55] to unite a volume of theory from years of interdisciplinary work into a coherent model to support health behavior change practices [120].

Further to be discussed here, social cognitive theory is a unified conceptual framework, which taps into patients' beliefs in their capability to engage in a new behavior and their expectations of how engaging in that new behavior will influence their health (i.e., the outcome of interest) [121]. Beliefs about capabilities, beliefs about consequences, and social influence are important determinants of adopting and maintaining dietary behavior change. These beliefs and attitudes are then targeted by behavior modification techniques (i.e., the intervention), which then leads to changes in behavior and subsequent changes in health outcomes [76].

The Transtheoretical Model stages of change construct complements by describing the five stages individuals move through as they make behavioral changes [122–125]. It has been effectively used to target and adapt behavioral interventions and to measure the magnitude of effective interventions. This theory has been used extensively across cancer survivor populations, within different cultural settings and applied to variety of behaviors (e.g., diet, physical activity, and weight management) [71,74].

Approaches to behavior change broadly consist of individual- and group-level interventions, and a combination of approaches has been shown to be more effective than one approach or the other [74,75]. Changing dietary behaviors in cancer patient populations adopts variations in behavior change models, with success being driven by a unique combination of behavior change techniques. Five general techniques frequently emerge as effective within published interventions: Goal setting, action planning, social support, instruction on how to perform behavior, and motivation. Self-monitoring of behavior and feedback on behavior are common in interventions, but these techniques were less effective [75].

Behavior change models attempt to explain why patients may change their behavior, with an emphasis on how these internal and external factors mediate the relationship of change to improve health outcomes (e.g., diet). However, most successful interventions for cancer patients include not only dietary change but also physical activity and behavior modification support in the form of materials to assist in change [126]. Lifestyle behavior modification interventions have previously focused on cancer survivors, but more recently a change has taken place to shift focus to supporting patients with dietary change during treatment.

Therefore, targeting theory-based factors is improving dietary and physical activity lifestyle interventions in cancer patients, although additional development is necessary to inform better intervention programs for longer-term maintenance of weight change. Evidence exists on the benefits of such interventions to achieve and maintain healthy weights and to adhere to nutrition and physical activity recommendations for improving cancer prognosis and survival. Examples included below demonstrate first the need to make sure there are multiple strategies to support behavior change, as patients have differing needs. Second, in cancer survivors, targeting behavioral motivation factors (i.e., self-confidence, goal setting, self-monitoring, feedback, taste preferences) can improve healthier food choices. Third, there is a need to consider more pragmatic approaches using adaptive communication strategies in person and via electronic messaging (i.e., text messaging, interactive websites). In the study of DeJesus et al. [34] the coaching methodology that was followed was 12 weeks of one-to-one coaching conducted mainly on a face-to-face basis. Alternative methods of delivering behavioral interventions by web or mobile devices are showing promise [25], as well as a combination of personal and group coaching [30]. Other wellness coaching studies mention that the duration of the intervention program [16,17,122] consisted of at least 12 weeks of sessions, while even shorter duration, such as six weeks or shorter, posed also feasibility with significant changes in outcome measures [53].

Furthermore, the implementation of the new technology seems promising in achieving this. For example, Kima et al. [53] developed a new mHealth version of "the Self-Help Intervention Program (SHIP)," by incorporating the principles of persuasive technology [29]. In addition, to address the relatively slow "technology readiness" of the target population, they incorporated human interaction into the intervention using community health workers (CHWs) as facilitators. This hybrid intervention, called model hSHIP, which combines digital and human touch, was inspired and influenced by the collective work of B. J. Fogg, who coined the term "persuasive computing "(later broadened to "persuasive technology"), and his colleagues at the Stanford Persuasive Technology Laboratory.

Persuasive technology is a new, evolving branch of implementation science that acknowledges the ubiquitous yet invisible influences of technology on behavioral change. Fogg postulates seven primary task support principles that, when incorporated into systems, applications, and technologies, support and enable behavior change without coercion [99]. For the hSHIP, a chronic disease management system (CDMS) was developed that combined all processes of project management (recruitment and enrollment, monitoring, questionnaires, messaging, reporting, etc.) in real time and delivers the intervention's components (education and training, monitoring and counseling, messaging, goal setting, etc.) into a web application. Thus, research nurses and CHWs communicated with program participants in real time using smartphone modules for Short Message Service (SMS) and notifications in the CDMS. The findings suggested that it is possible to sustain motivation to engage in self-care behaviors over the long term, so that those behaviors will be translated into optimal clinical outcomes. The key to sustaining motivation is constant and immediate feedback through a combination of digital and personal touch, because positive, real-time feedback helps to eliminate uncertainties, fear, or reluctance in self-care behaviors. Furthermore, utilizing the most innovative technology in an accessible, personalized, self-help intervention that will proactively reduce potential heath disparity gaps is consistent with the movement towards precision medicine/health [53].

So far it seems that continued efforts to further refine wellness coaching programs through new technological interventions will help optimize their role in OA health prevention measures [72]. Also, it is unlikely that there is any or only one health theory that works ideally to promote health in all contexts, by all providers, for all types of OA [127]; further, all theories are not constant but in flux and evolving over time [128].

6. Oral Health Coaching

The benefits of oral health coaching, however, have been reported mostly anecdotally. Complete understanding of effective behavior changes in the dental setting and coaching research, especially in OA group, is in its infancy [72,87,112,122,127,129]. Moving forward, it is important to learn what behavior change approaches work best in the dental setting, as well as for whom, how, and when such approaches work [128,130]. This will require study designs that can measure, isolate, and validate health theory mechanisms of action [127]. To date, it is understood that, in the dental and other health care settings, providing information alone appears to have little long-term impact on promoting behavior change [122,131]. Why is this? It is because this kind of approach is based on many assumptions—e.g., that people want to know this information (they perceive it as being relevant and important to their lives); that they understand this information; that they are ready, able, and motivated to apply this information; and, further, that they can address any challenges that should arise in implementing this information both in the short and long term.

The contemporary field of oral health behavior and oral health education has shifted considerably and now reflects a blending of theory, strategies, models, and approaches between the social, dental, and medical sciences. Additionally, theory and research on integrative health coaching and intentional change coaching suggest that it is critical for the provider to communicate hope, trust, and genuine optimism to the patient (both verbally and nonverbally) in order to ground the provider-patient exchange in the patient's intrinsic hope, motivation, and vision of health and well-being [100,132]. Even in an emergency care condition, the provider can "plant the seeds" to raise the patient's oral health self-awareness in the near- or long-term future. Indeed, establishing a connection based on shared hope, trust, and respect may enhance the likelihood that the patient will return for follow-up (and, ideally, ongoing routine) dental care [98,133,134]. This increased interest in the psychosocial aspects of behavior change was evident in a recent systematic review of interventions to improve oral hygiene based on psychological models [135].

6.1. Oral Health Coaching Techniques and Models for OA

The general layout needed for an effective oral health senior coaching intervention, addressing diet needs for better oral health, should be based on certain characteristics employed by certified coaching associations, like International Coach Federation (ICF) [22] or Association for Coaching [136], translated, and incorporated into the oral health sector from dental professionals.

Then the basic procedure for engaging patients in self-management for better diet habits towards better oral health should be: (1) *Preparation for the visit*. It is important for both the patient and the dentist to prepare for the visit. Patients who can share their concerns with a care coordinator or provider are less anxious and show more improvement, even if they just provide a written list of those concerns. So, it is crucial for the dental professionals to help patients understand their central role in managing their conditions and that the entire health care team is there to help. Time must be found for the practice of self-management by gathering clinical and patient experience data in the chart and encouraging patients to bring questions and concerns on their next visit. (2) *Scheduling an agenda together*. At the start of the visit with the patient, a list should be written down of the things that each of the parties hope to achieve during the visit and prioritize the most important items needed to be addressed first. Working together to build the agenda demonstrates that the patient's concerns are valued, and time will be given in order to hear them. The patient feels appreciated, which is a strong motivational feeling. (3) *Asking open-ended questions.* Encouraging the patient to share their experience with the dentist by asking questions that require more than a yes/no response. This can also be done in the form of a statement such as "Tell me more about that". (It will be discussed further later.) (4) *Practicing reflective listening in order to build a trusting relationship with the patient*. It is important to practice reflective listening, without interruption, and respond by rephrasing what it was heard without adding meaning or judgment. (5) *Recognizing and eliciting "change talk"*. Change talk is any statement that expresses a desire to change. The professional "catches the moment" in order to enhance possible alterations in behavior. (6) *Affirmation and celebration of what works.* At the end of the session and during the recall appointment of the patient, the dentist should make time to acknowledge and talk about what has worked and what success will look like. Time should be spent in discussing how it will look and feel to accomplish the patient's goals. Celebrations of even small achievements are crucial in the coaching process, making people feel proud of themselves and enhance their motivation. (7) *Making a specific and realistic plan.* Identification of the concrete steps that will be taken to address diet and oral hygiene habits should be made in partnership with the patient. Discussion of the different options and selection of the best one that is consistent with the patient's lifestyle and that the patient is confident he/she can implement should take place. Also, there should be a timeline and talking about the way that the monitoring of the progress will take place. This procedure builds patient's confidence in his/her ability to reach these goals. A written care plan or visit summary, which includes goals and action plans and ensures patients and families on what to do when they leave the visit, should be made. (8) *Following up.* There should always be time for arranging support services that will help the patient to be successful in achieving his/her goals. For some people this may mean a phone call in the next 24–48 h, while for others a follow-up phone-call in 1–2 weeks [122,136–139].

6.1.1. Motivational Interviewing in the Service of Senior Oral Health Coaching

The use of motivational interviewing (MI) is suggested in health settings [1,122]. MI is a person-centered, goal-directed method of communication for eliciting and strengthening intrinsic motivation for positive change [140]. It is predicated on a 'spirit' of rapport, based on partnership, empathy, and acceptance. As such, the MI counselor must be willing to hear, accept, and respond to a patient's personal perspective rather than recite a predetermined set of prescribed instructions and guidelines. It is important to mention that the information-giving approach seems to have no effect on behavior change but behavior change with self-monitoring and goal setting is a better approach [135]. A key component of a MI conversation for OA is to acknowledge that they have every right to make no change. Acceptance of the situation as it is does not mean though that a guiding communication style,

which invites people to consider their own situation and find their own solutions to situations that they identify as problematic, would not work towards change [141]. The patient's view is elicited by the clinician in order to help them understand the situation from the client's perspective including their goals and values. This is a collaborative approach in which the expertise of the practitioner plays a part, but it is the patient's journey as he/she decides where to go and if and how to get there [18]. MI has shown good results in different dental settings [135,142]. However, these results are transitory, have negligible impact on the incidence of dental caries [133,142], and are not yet searched for effectiveness in OA.

OARS Model in MI

A MI model well discussed in the relevant literature, supposed to work in OA, is OARS (Open-ended Questions-Affirming-Reflective Listening-Summarizing). OARS is the acronym for the four core communication skills – open-ended questions, affirmations, reflections, and summary—that are integral to the collaborative, client-centered, motivational interviewing approach. While many of these are not new concepts, their collective and strategic use is the essence of the spirit of MI. As described above, many aspects of the dental visits are routinely closed-ended. Medical history questions seek yes/no answers, whereas the types of oral hygiene used generate short categorical responses. Often in response to the presence of disease, traditional instructions consist of a prescribed explanation of the disease process that is entirely a one-way communication. By comparison, the OARS approach not only provides key strategies to shift the conversation so that the patient is doing more of the talking, it also provides an opportunity to discover what is uniquely meaningful to each individual, gauging their oral health understanding and their desire and ability to change. This is important for OA who seek acceptance and trust in order to overcome their fears. To achieve this insight, it is necessary to use the OARS approach to get OA talking. The R in OARS stands for reflective listening. Reflections of patients' responses to open-ended questions serve two main purposes. First, it develops the partnership by showing the patient we really hear what they have to say. The intent is to listen for responses that represent change talk (in the direction of the desired change) or sustain talk (avoiding change) that will be discussed further in other models. Second, if the provider is unsure, he/she understood the patient correctly, it provides an opportunity to clarify meaning. Varying levels of reflections, from simple repetition of what the client said to amplified reflections that exaggerate the response, help direct the patient in the direction of the health-behavior change wished to achieve. Skilled reflections allow the provider to interpret the meaning of the patient's responses [141]. It is important, though, that the reflection is made as an interpretive statement, not a question. A good method to use when beginning to use reflective statements is the phrase, "*Sounds like* ... " (e.g., "Sounds like the deep pockets worry you"). Once the provider become accustomed to using reflections, he/she can simply drop the 'Sounds like' expression.

The S in OARS is summary. Summaries reiterate the fact that the dentist was truly listening, while setting the stage for behavior change. The art is to summarize any aspects of the conversation, allowing OA to hear any contradictions in their own responses with a focus on what they want to do next.

The four processes of OARS model—engaging, focusing, evoking, and planning—strengthen a patient's own motivation for, and commitment to, change. Beyond OARS, more sophisticated motivational interviewing strategies are aimed specifically at evoking and planning intrinsic motivation for behavior change. Once again, the plan ultimately originates from the patient with direction from the clinician [142]. Decisional balance is another useful strategy for evoking and planning. It is a means of allowing the patient to examine the pros and cons of a behavior change. This strategy is particularly helpful for OA who are ambivalent, uncertain, fearful, or reluctant about making a change [110,142].

6.1.2. Other Models and Tools for Immediate Senior Oral Health Coaching

For time-management reasons, in a private dental practice or senior center, dentists and other professionals acting like coaches should be introduced only to certain models for quick patient interference, like the following.

Dental PAM (Patient Activation Measure)

Patient activation (PA) refers to a person's ability to manage their health and health care. An activated patient has knowledge, skill, and confidence to manage his/her health and health care in wellness and illness. The chronic care model was built with the understanding that the patients would learn how to manage their care on a day-by-day basis. However, the level of PA varies considerably, as was mentioned already. Clinicians, so far, strongly encourage patients to follow medical advice but are less likely to endorse that patients should be able to make independent judgements or take independent actions. Discussing a practice's culture around patient self-management is a critical first step. Stratifying patients according to activation level using the evidence-based tool, Patient Activation Measure® (PAM®), provides an effective method to: (1) Guide resource allocation at the practice level, (2) tailor support to a patient's abilities, and (3) improve patient safety and satisfaction [143]. The Patient Activation Measure (PAM) is a global assessment of an individual's self-management competency. PAM quickly evaluates three key personal health domains—knowledge, skills, and confidence—and segments patients into one of four activation levels along an empirically derived continuum. Coaching for activation focuses on seven core areas of self-management—condition and symptom understanding, medication adherence, diet and nutrition, physical activity, stress and coping, information seeking, and smoking cessation. Each area of self-management is tailored to health status, addressing diabetes, asthma, Chronic Obstructive Pulmonary Disease (COPD), Congestive Heart failure (CHF), Coronary Artery Disease (CAD), hypertension, and high cholesterol, as well as disease prevention through a lifestyle module. Within each self-management core category, information, goals, and related action steps are tailored to an individual's health status and level of activation. Goals and steps are supported with self-care resources suitable for coaching use. Potential contribution of an oral health PAM instrument–not yet implemented-is suggested for the dentistry field [143].

Tell-Show-Do

The most common model in dentistry, well proposed in children and young adolescents, is the model of tell-show-do. The steps described for this model are: (1) Tell or explain the procedure, (2) show or demonstrate the procedure, and finally, (3) the learner can do or practice the technique until he/she has mastered the skills involved. The last step is the most important one if the learner is to develop proficiency. For OA, this model is expected to bring direct conscientiousness of the present situation and is simple to practice for both the dentists and the patients [144]. It will be well performed in cases of memory weakness and in OA with sudden mood changes or depression.

Balloons' Diagram

In the balloons' diagram [145], the patient can place in the balloons the problems and worries he/she must face in order to release them one by one. Some helpful questions might be: *"What do you think is going on?" or "What is your understanding of this (condition, issue)?" or "What worries you the most?" or "What else are you concerned about?" or "What do you know about (treatment, self-management)?" or "Which balloon do you want to release first?"* The balloons' form could be provided to the patient at the reception area; so the front office staff should be trained in introducing the form and asking questions like: *"Which of the healthy change activities seems most important to you right now? or "Which bad diet habit could you put into the balloon to release first?"* (if none does, ask what other area they might choose to address) *or "We are working on improving our care for people with (mention the oral health problem). Dr. X would like to discuss your health goals with you. This form has some ideas you might consider placing in*

these balloons." Since this model engages the vision, it might be effective for OA with other physical impairments such as listening or memory problems (Figure 1).

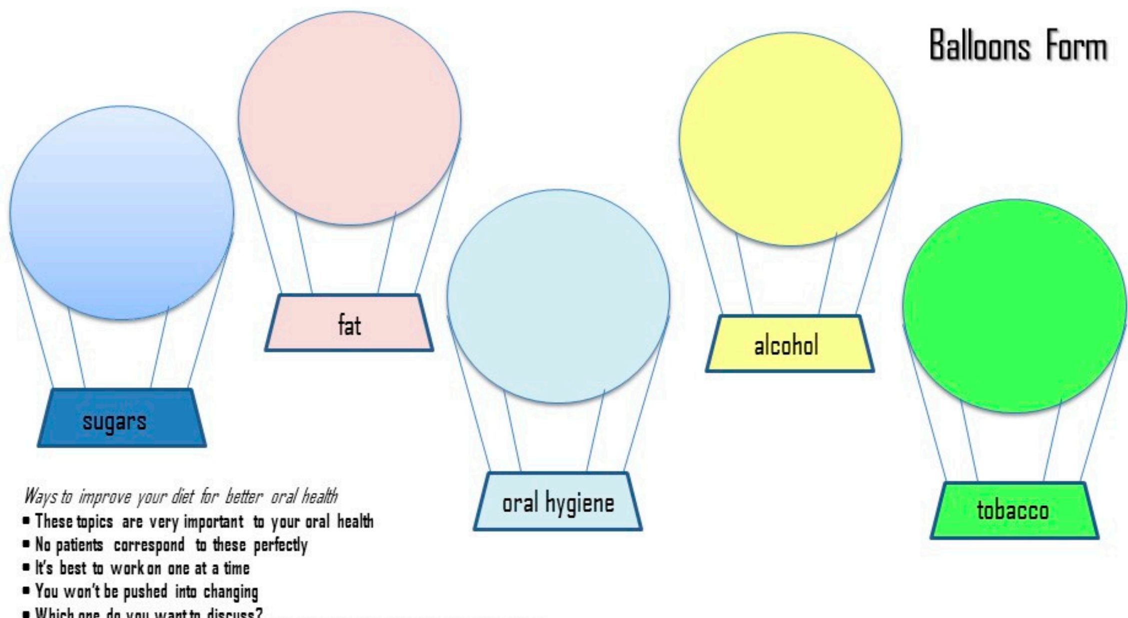

Figure 1. Balloons' form. Adapted for diet oral health coaching from [145,146].

Ask-Tell-Ask-Close the Loop

In this model, the coach should ensure OA understanding and recall. This must be done in a way that the patient feels respected, accepted, and asked rather than being told to change. It should be effective for strong-minded OA who do not accept their impairments due to aging.

Ask permission: The provider asks permission to give information about a topic of importance to the patient (*"Would you like to hear more about.."* or *"Is it ok if I share some information about the importance of physical activity?" or "I'd like to show you how to check your teeth. Would this be a good time?"* or *"There are several things I want to tell you about the new periodontal therapy. Ready?"* or *"Can we talk a bit about your (insert risky/problem/unhealthy behavior)?"* or *"I noticed that you have (insert conditions). Do you mind if we talk about how different lifestyles affect (insert condition)?"* (Diet, exercise, smoking, and alcohol use can be substituted for the word "lifestyles" so as not to provoke guilt).

Tell: Explanations and written material are most effective when given in response to the patient's expressed agenda and tailored to their ability to understand. Simple visual aids can be very effective. Powerful questions might be the following: *"Do you mind if we spend a few minutes talking about . . . ?"or "What do you know about . . . ?"or "Would you like to share with me your emotion on this matter?" or "What do you know about how your (insert a health behavior) affects your (insert health problem)?"or "Are you interested in learning more about . . . ?"or "What do you know about the benefits of including Med-Diet in your diet plan?" or "So you said you are concerned about gaining weight if you stop smoking; how much do you think the average person gains in the first year after quitting?"*

Ask for understanding: The dentist then should provide information, considering the following tips: (1) Address gaps in understanding; (2) use language the patient can understand and avoid jargon; (3) share information in small bits, tailored to patients' questions or concerns; (4) use graphics, charts, models leaflets, etc.; (5) monitor whether the patient is tracking nonverbally; (6) encourage family/significant other involvement; finally, (7) ask for understanding *("What questions do you have?" or "Please tell me what do you now understand about diet and how you think we need to proceed to get this under control?" or "When you go home, what will you say to (family member, other caregiver) about what we talked*

about today and what you plan to do?)" In this point, one should be aware that people often have either little or incorrect information about their behaviors. Research has shown that telling people what to do does not work well [142,146]. Most individuals prefer to be given choices in making decisions to change behaviors. By presenting information in a neutral and nonjudgmental manner empowers a person to make informed decisions about quitting or changing a risky/problem/unhealthy behavior [146].

Close the loop: The physician asks the patient to restate the information as the patient understands it. The provider can then tailor the information for the patient's needs and level of understanding. It is important to mention here that, as reported elsewhere, diabetes patients recalled and comprehended only 12% of new concepts introduced during the visit. Those patients whose recall and comprehension were assessed were more likely to have hemoglobin A1c levels below the mean [147]. Finally, the Closing the Loop technique was not found to add time to the visit duration [147] and, as such, could be of service at a busy dental office or senior center.

Rating of Change Check

It is said that patients are more likely to succeed with a health behavior change when the change can be related to a matter that is important to them and other people they care about (grandchildren, friends in certain activities, etc.) and when they are confident that they can achieve the change. This is a point that is very important for OA who feel their competencies to decrease. Simple ratings of level of importance and confidence using a scale of 0 to 10 can give a quick indication of readiness and next steps. Ratings of less than 7 on either measure signify less likelihood of success and the need to explore concerns and barriers with the patient, or even to select a different topic for health behavior change [10,122]. In this modality, it is important to know that there should be avoidance of the use of scare tactics, lectures, or direct warnings, as some people might pretend to agree in order to not be further attacked. Effective questions on this part of the session would be: *"Why would you want to make this change?" or "How might you go about it, in order to succeed?" or "Do you think it will help you to have strong teeth when you talk or smile?" or "How about being able to talk without embarrassment of having no teeth in front of your friends/grandchildren?" or "Do you understand that by eating so much sugar you'll end up having no teeth when you will need them most? "What are the three best reasons for you to do it?" or "How important is it for you to make this change?" or "So what do you think you'll do towards … ".*

Goal Setting and Action Planning

Goal setting and action planning can be performed by filling up action planning forms, which are problem-solving forms addressing patients' barriers to achieving success with behavior change and put them in action. Once a health goal is chosen that is important and meaningful to OA, the next step is collaborative work with the dentist/coach to create an action plan, framing small steps that have high likelihood of success. In one study, action planning was found to take as little as 1 or 2 min or as long as 20 min. The average was 6.9 min [137]. Some OA may require a longer visit or additional contacts to help achieve their self-management goals. However, most of them would be best served by a short process that is revisited, improved, and modified over short periods of time due to memory loss. Questions that might be asked in this modality are: *"When will you know you have succeeded in your goal?" or "If you had a magic wand, what would you ask for first?" or "How do you imagine yourself in six months' time concerning (mention the problem)?"*

Problem-Solving Check List

The problem-solving check list provides a quick assessment tool for grading the steps needed in order to change a habit. OA should write down, with the help of the dental professionals, the identified problems and the proposed solutions in order to overcome barriers [122]. (1) *"On a scale of 0 to 10, with 0 being not at all confident and 10 being as confident as you can be, how confident are you that you can (describe the activities on the action plan here)?".* Depending on the patient's answer, ask follow-up questions. *("What makes you say 6?" or "What led you to rate it as high as a 6?" or "What has helped you to*

be confident in the past?" or "What might help you get to a 7 or 8?"or "What could I do to help you feel more confident?") (2) Anticipate barriers and consider strategies to overcome them. (*"What might get in the way of completing your action plan?" or "Anything else?" or "What might help you to overcome... (barrier)?" or "What has helped in the past?" or "What else?" or "What or who might help you?" or "Here is what others have done..."or "How will having no teeth interfere with being with your friends?" or "What keeps you away from the possibility to talk and smile with safety with your friends/grandchildren?").*

Follow-Up Strategies

In this modality, OA will be assisted in completing a checklist action plan form and given then a copy to take home. ("This form has helped many people begin to make healthy changes by spelling out small, doable steps and anticipating problems. I see you have decided with Dr. X to work on being more active. Would you be willing to work with me to complete the form and establish goals for becoming more active?" or "I'd like to call to see how you are doing with your action plan. Would that be OK with you? When would be a convenient time?" or "What would you like to do in the next few weeks on behalf of your diet?" or you could assess how convinced the patient is for the change "On a scale of 0 to 10, with 0 being not at all confident and 10 being as confident as you can be, how convinced are you that it is important to (insert patient's goal)?" and depending on the patient's response one might say, "What makes you say 3?" or "Why 3 and not zero? or "What might lead you to rate this as a 4 or 5?" or "What would have to happen for you to rate it higher?" or "How about raising it one point higher?" (prefer small raises that do not scare the patient).)

Collaboratively set goals. Creation of an action plan and high OA confidence for making behavior changes are not, of course, enough to guarantee healthy change. Follow-up with patients, during subsequent visits and between visits, to assess progress and adjust plans as needed is an essential part of self-management support [148–150]. The establishment of healthy habits, like getting enough sleep, choosing Med-Diet food, staying in touch with family and friends to keep the spirits up, eating in company, joining a walking group or other social groups, and surrounding oneself with loved ones and happy people, should be checked on follow-up [144].

Laser Coaching in OA

In this rapid "laser effective" coaching approach [151], the following steps have to be carried out in almost a 15–20-min session: (1) Giving permission to do coaching (*"Would you like coaching on this subject*?), (2) helping make the results clear (*"What do you really want? How will you know when it has been achieved? How do you have success in mind?"*), (3) identification of the importance *("Why is this so important to you*?"), (4) identification of the consequences, in case no action is taken (*"What is the cost, does it cost, or will it cost you if you continue on the same path?"*), (5) identification of the obstacles (psychological, emotional, natural*) ("What limits you so that you do not face the situation? What excuses or rationalizations have you used to prevent you from moving forward*?"), (6) decision and taking action as the very next step (*"What is the next step* (try to give a simple action) *that will motivate you as quickly as possible?* (Today!) (within the next 15 min)), (7) giving responsibility (*"Apart from me, who or what else can you use as a lever to ensure that you continue your commitment*?"), and (8) recognition and reward. This final step is the most important of the model since it supposes to have a direct impact in helpless and frustrated, due to mind impairments, OA.

Ask Me Three Questions Model

This model is also an elegant quick oral health model approach since it is based mainly in only three questions that the patient should ask: (1) What is my main problem? (2) What do I need to do? (3) Why is it important for me to do this? So basically, it can be effective in a 15–20-min coaching session and can be applied right before or after the dental therapy [122,152]. It is comprised of the following questions, in the mentioned order: "*What is most important for you to accomplish during your visit today*?" (agenda); "*What do you think is the problem here*?" (knowledge, beliefs); "*What ideas do you have about*

what is contributing to your problem?" (knowledge, beliefs); "*What ideas do you have about treatment or things you can do to manage your condition?*" (knowledge, preferences); "*How important do you think it is to do* ... ?" (X treatment or self-management task) or " ... *to manage or treat your condition?*" (ideas, values, preferences); "*What would you like to know about your condition?*" (knowledge, Preferences); "*What concerns you the most about your condition*?" (feelings); "How do you feel about trying to ... ?" (feelings); "*What specifically would you like to work on to manage your condition*?" (goals); "*What is that you want for yourself in six months' time?*" (goals); "*What would help you to manage your condition*?" (needs, preferences); "*Who do you think will help achieve this?*" (needs, preferences); "*How confident are you that you could do* (X treatment or self-management task)?" (feelings); "*From 1 to 10, how much you believe you can achieve* (X treatment or self-management task)?" (feelings); "*What might get in the way or keep you from being successful*?" (barriers); "*How do you think you can surpass this?*" (knowledge, beliefs).

All proposed models and their effects and characteristics for the OA are seen synoptically in Table 3.

Table 3. Diet and oral health coaching models for OA.

Model	Method	Type of OA	Results
OARS	Open-ended questions, affirmations, reflections, summary	OA who like talking and communicating with others	Discovery of goals, clarification of wishes, acknowledgement of contradictions, strengthening patient' own motivation
Dental PAM	13-questions Questionnaire	Ambivalent, fearful, uncertain, reluctant to change, untrusting OA	Evaluation of knowledge, skills and confidence Stratification of patients according to activation level
Tell-Show-Do	3 simple and quick steps: share information, show how to do it, let patient do it	OA with physical impairments or with short memory loss	Quick evaluation of perceived information, achieving results through often repetition and exercise
Balloons Diagram	Balloons form	OA with hearing or other physical difficulties, optical way of learning	Sudden realization, visualization, metaphorical release of problematic situations and habits
Ask-Tell-Ask-Close the Loop	Ask for permission, give information through written materials, brochures, etc., ask for understanding and rephrase goals	OA with sensitivities, depression, negative feelings, isolated, strong-minded, unwilling to accept age impairments	Specification of goals, feeling trusted and accepted
Rating of Change Check	Change Check List	OA who likes numbers and numerology	Determination for achieving small steps, summarization of change
Goal setting and Action Planning	Action Planning Form	OA who still can write, with good vision but memory loss, those who like order and organization	Stratification and empowerment of goals, strengthening of motivation
Problem Solving	Problem-Solving Check List	Impatient, stressed, economic dependent OA	Lower guilty behavior and stress
Follow-ups	Follow-up Check List	Lonely OA with memory loss or fear of incompetence	Self-acceptance and lower stress
Laser Coaching	Short, compact communication based on reward	For OA who need recompense and like prizes, bonus, presents and gifts	Giving responsibility, feelings of self-realization and value
Ask-Me-3-Questions	The patient makes the questions and the answers	For reluctant OA	Accountability

7. Discussion

So far, it has been shown that individually tailored oral health education program is better than traditional education [135,153]. Thus, the after-sales' service is very important in the situation of OA. All over the relevant literature discussed already, the dental and medical professionals should organize follow-up support to help OA sustain healthy behaviors between visits. They should be persistent in short breakthroughs and often follow-ups due to the memory issues of OA. They should also extend care into the community by linking elders to community programs. They should further build a team of people trained to make coaching interventions and assign responsibility for self-management tasks to all team members, extending the work out from the dentist. Finally, they should use daily team huddles to review the schedule of patient charts, anticipate care needs, and enhance the flow of care in an aging population.

Sustaining healthy behaviors for a lifetime requires courage and tenacity, most often involving small, incremental changes that build over time into bigger successes. Even the best plans of action require adjustment from time to time in order to work effectively. For these reasons, making regular contact with OA after a visit or change in diet protocol or dental treatment is central to sustaining positive change. Studies in depression document the need to follow up with patients to assist them in succeeding with their action plan. Helpful as it seems might be the connection of OA with sources of support in the community such as recreation or senior centers, support groups, and voluntary community organizations. Finally, quite appealing might be to locate or develop a peer program in the dental clinic or community involving them actively with other people.

Possession of preventive knowledge and skills alone will not ensure the OA's attainment of the goal of preventive counseling, that is, maintenance of optimal diet and oral health status. The dental professional and patient must establish a therapeutic alliance, whereby each is committed to performing the activities necessary to achieve this goal. OA must be convinced that ultimately only they can help themselves by adhering to the recommended preventive measures. It might thus be helpful if the provider frames his or her oral health messages in terms of the senior patient's overall health, as this may lend to more trust, credibility, and urgency for the patient to take such messages seriously and, finally, act [98,100,130].

Professionals should work to dispel the misconception that oral disease is an inevitable consequence of aging, and that, consequently, the attempt to prevent oral disease is a futile effort. Park and Chang [24], mentioned that change comes not only by the capacity of the participants to engage in behavior change but also on the performance of the individual health coaches. According to the spirit of MI, the therapeutic relationship is more like a partnership or companionship than expert/recipient roles [140]. It is, therefore, essential that health coaches are supported in their role. It is recommended also that adequate training budgets and adequate reimbursement of health care providers for their time and commitment will help with the sustained recruitment of program participants, the effective running of these types of programs, and, ultimately, the outcomes [24].

Thus, the oral health services, dental schools, and medical faculties should be organized and developed to secure adequate early detection and prevention as well as treatment of oral health problems for all OA, whether living at home or in hospitals and health care facilities. The achievement of such a service goes beyond what a dentist can do alone. It requires the involvement of other health professionals and health care workers. This presents a realistic goal that could assure good quality of life and a reduction in the dental expenses for the elderly patients.

It is suggested that dentists should implement health coaching programs as a package in their services, containing coaching on diet and oral health prevention, goal setting, attainment, and adherence promotion. In addition, respecting each participant's autonomy and resisting the urge to push against any resistance put up by them, dentists might have a better chance to reach positive outcomes [142,154]. In the study of Park and Chang [61], participants reported a high level of goal achievement. The results are consistent with previous studies for OA with multi-morbidity where it was reported that health

coaching intervention enhanced residents' participation in intervention programs, resulting in a significant increase in their self-efficacy and self-management behaviors [155].

The health provider thus becomes a colleague, offering guidance and support instead of solely telling patients what to do to manage their oral health. In the context of a collaborative relationship with shared decision making, dental professionals can provide the elements of self-management support, including self-monitoring and problem solving, goal setting, action planning, and rewarding. To reinforce this outcome, it is interesting to know that when patients receive collaborative self-management support, they have fewer hospitalizations, improved quality of life, and improved clinical outcomes in several ambulatory-sensitive conditions [81,82]. Further, it has been shown that a short form that elicits patient concerns or needs, either mailed in advance of the visit or completed in the waiting room, can be sufficient [156,157]. Patients often leave the office visit without understanding or remembering important care instructions and medication information [158], which may lead to worse outcomes such as higher hospitalization rates [159]. Twenty percent of patients read at a fifth-grade level or below, for which written health care information is not often tailored. Physicians cannot expect that patients will spontaneously reveal their lack of understanding. Also, physicians may not provide basic information that patients need. In one study, physicians explained the adverse effects of medications or instructions about one-third of the time [160]. Despite these data, by using simple methods of coaching, like the ones mentioned here, a senior nutrition and oral health coach can help improve communication and patient understanding towards healthier nutritional habits that correspond to the OA-specific needs.

So, it seems that for the diet-changing behavior and oral health prevention scope, the team members and care givers should ensure the practical and psychological part of a good meal in order for the elderly to keep enjoying food despite any physical impairment or "being on your own", highlighted in many studies [161–163]. It is promising in this way, the fact that current older patients are better educated, more politically aware, and have more remaining teeth than in previous generations [164]. However, the older population is not homogenous. OA who have lower incomes have poorer oral health and more limited access to services [165], even more to senior coaching sessions, a fact that should sensitize the political leadership nationwide.

In the economic recession period that will follow the COVID-19 crisis, care givers, nurses, dentists, and other medical professionals should find their original motive in doing what elderly care needs despite the practical and economic difficulties and should be urged through coaching to estimate their values in taking care of the elderly. People who love others and have a good level of emotional intelligence should be better candidates for elderly units and dental offices for seniors [7,96,166].

In conclusion, dental and other medical professionals should reevaluate their role as health coaches in order to improve dietary habits and nutritional intake of the OA. By reminding themselves that dentistry is a helping profession, they will see more value in "oral health coaching" as a desired and supportive means to an end. In fact, they are helping people to make decisions that can add to the quality of their lives. By altering their thinking and approach slightly they can easily shift the focus from "us" and our procedures to "the patient" and the quality-of-life impact their services can have on their lives. This shift in thinking will enable them to communicate with their senior patients in a more mentorship-based, collaborative, and inspiring way.

Modern dentistry is bright and filled with opportunities when someone chooses to expand his/her clinical excellence while concurrently taking the time to grow as an "oral health coach". "Oral health coaching" is the emerging yardstick that will differentiate professionals, especially dentists, to become more effective as clinicians while feeling more trusted and valued in the eyes of their patients.

8. Conclusions

In this nonsystematic review the process of senior coaching on diet issues for better oral health were discussed for the independent elderly *or older adults (OA),* referring to individuals of age 65 or older. It can finally be concluded that:

(1) There are certain mental and physical issues resisting change in habits and behavior of OA.
(2) OA are more likely to benefit from a series of quick health education sessions followed by tailored feedback that is based on the absence of criticism, patience, empathy, and total acceptance by the dentist/professional coach.
(3) Overcoming persistent noncompliance of OA through specific educational training can make health-behavior change one of the most rewarding and the most challenging responsibilities for dental health professionals.
(4) Coaching models based on filling out forms or lists of goals, tasks, recruiting small steps, and rewarding are suggested as being more effective in OA due to their mental and physical issues.
(5) Health professionals should reevaluate their role as health coaches in order to improve dietary habits and nutritional intake of the OA.
(6) "Oral health coaching" will enable professionals to communicate with their senior patients in a more mentorship-based, collaborative, and inspiring way.

Author Contributions: Authors declare their equal contribution in the work reported. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

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

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