Recent Advances and Future Trends in Fermented and Functional Foods

Edited by
Jayanta Kumar Patra, Han-Seung Shin and Spiros Paramithiotis

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About the Editors

Jayanta Kumar Patra

Dr. Jayanta Kumar Patra, M.Sc. Ph.D., PDF, is currently working as Associate Professor at Dongguk University, the Republic of Korea. His current research is focused on nanoparticle synthesis by green technology methods and their potential application in biomedical and agricultural fields. He has about 14 years of teaching and research experience in the field of pharmacology and nano-biotechnology. Dr. Patra has published more than 150 papers in various national and international peer-reviewed journals along with around 32 book chapters in different edited books. Dr. Patra has also authored 16 books on the topic related to nanomaterials, medicinal plants, ethnopharmacology, biotechnology, etc., in various publications. Besides, he is an editorial board member of several International Journals. In addition, Dr. Patra is listed under World's Top 2% Scientists based on citation impact during the single calendar year 2019-2020 & 2020-2021. He is a fellow member of the American Nano Society.

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Spiros Paramithiotis

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Preface to "Recent Advances and Future Trends in Fermented and Functional Foods"

Health and wellness are among the core segments of quickly-changing consumer goods, with ever-increasing health consciousness among consumers around the globe. Functional foods and beverages, formulated from natural ingredients with targeted physiological functions, are at the heart of research and development in the food industry. The application of modern biotechnology methods in the food and agricultural industry is expected to alleviate hunger today and help avoid mass starvation in the future. Modern food biotechnology has in recent years been transforming existing methods of food production and preparation far beyond the traditional scope. Currently, at the global level, food biotechnological research has focused on traditional process optimization (starter culture development, enzymology, fermentation), food safety and quality, nutritional quality improvement, functional foods, and food preservation (improving shelf life). The fermentation of substrates considered for human consumption has been applied for centuries as a process that enhances shelf life, sensory properties, and nutritional value. Special emphasis has also been given to newly growing concepts, such as functional foods and probiotics. The application of biotechnology in the food sciences has led to an increase in food production and has enhanced the quality and safety of food.

The guest editors are grateful to the researchers that published their work in this Special Issue and the MDPI editorial team.

Jayanta Kumar Patra, Han-Seung Shin, and Spiros Paramithiotis
Editors

Article

Effect of Fermentation Duration on the Quality Changes of Godulbaegi Kimchi

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Abstract: Fermentative and antioxidative characteristics of Godulbaegi kimchi (LGK), a traditional, fermented Korean food, were conducted. For the study, LGK kimchi was made of Godulbaegi kimchi with pepper powder, salted shrimp, refined salt, green onions, and so on, and fermented at 5C for 6 months. The pH was decreased, and total acidity was increased during fermentation. Furthermore, lactic acid bacteria and yeast were increased, while the total viable count was decreased. The LGK showed the highest DPPH-scavenging activity, phenol content, and nitrite-scavenging activity with methanol extract among methanol, ethanol, and water. In addition, we screened strains among LGK kimchi with high antimicrobial activity and isolated them. We tested antimicrobial activity for 20 lactic acid bacteria, and we separated and identified nine strains of lactic acid bacteria with high antimicrobial activity. Given these results, LGK is expected to be an effective food in considerable antioxidative activity with an antimicrobial effect. These results are expected to serve as basic data for the study of Godulbaegi kimchi.

Keywords: Godulbaegi kimchi; antioxidant activity; antimicrobial activity; kimchi quality

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1. Introduction

Recently, natural products have become increasingly popular in the prevention of various diseases. Particularly, the anti-cancer properties of natural compounds are of interest, with research focusing on the discovery of anti-cancer and immunity-boosting substances. Moreover, studies have investigated the antioxidant activity of natural substances and their extraction process [1–6].

In the southern province of Korea, *Ixeris sonchifolia* Hance is a wild vegetable with a strong bitter taste, known as Godulbaegi (Korean lettuce) and belonging to the dandelion genus (*Taraxacum*) of the Asteraceae (also termed Compositae) family. Various types of Godulbaegi are grown in the mountains and fields of Korea [7]. There are a total of nine types, including *I. sonchifolia*, *Ixeris denticulate* (Hottu), and *Lactuca indica* L. var. *laciniata*. *I. sonchifolia* is also widely distributed in the northeastern part of China. *I. sonchifolia* is also a folk medicine that has been used for many years in China to improve health [8].

Kimchi is a Korean fermented food made from cabbage, onions, red pepper powder, garlic, ginger, and vegetables. Kimchi is a rich source of functional ingredients, including antioxidants, such as vitamins, flavonoids, and diverse phenolic compounds, as well as abundant lactic acid bacteria (LAB) involved in a complex fermentation process. Kimchi also contains free sugars, minerals, amino acids, fatty acids, polyphenols, flavonoids, and triterpenes. The health benefits of kimchi include its ability to enhance intestinal health and prevent constipation as well as display anti-mutagenic and anticancer effects. *I. sonchifolia* is widely used in Korea to prepare kimchi pickles or kimchi. Godulbaegi kimchi has been mainly used in the southern region of Korea and has been established as a local food in the region.

Godulbaegi is eaten as raw greens in spring or soaked in kimchi in autumn and has been used medicinally, as it is known for improving blood circulation and dissipating

blood stasis to relieve blood stasis pain, among other effects [1,9–11]. Among the diverse varieties of kimchi, Godulbaegi (Korean lettuce, *I. sonchifolia*) kimchi, which is consumed as a delicacy in the southern province of Korea, contains high levels of polyphenols and dietary fiber [12].

Recently, Godulbaegi was associated with cardiovascular disease [13,14] and the mechanism of oxidative stress modulation of antioxidant capacity [15]. Currently, research on Godulbaegi is mainly aimed at its antioxidants [10,16] and antitumor compounds [17] and its component analysis by HPLC/MS. The efficacy of Godulbaegi is highlighted by the various compounds present in Godulbaegi, red pepper powder, garlic, and ginger, which are the main ingredients, as well as substances produced by LAB (Lactic acid bacteria) fermentation process [18]. The growth of LAB in Godulbaegi acts as a beneficial probiotic, causing the production of various substances [19]. Various antioxidant benefits have been reported for metabolites produced by LAB during fermentation, and thus, there is an increasing interest in Godulbaegi as a functional food [20]. Therefore, in this study, we comparatively analyzed the physicochemical properties, antioxidant activity, and antibacterial activity of Godulbaegi kimchi according to storage period. In addition, we investigated the potential of Godulbaegi kimchi as a healthy functional food material.

2. Materials and Methods

2.1. Material and Preparation of Godulbaegi Kimchi

The main material in the kimchi used in this experiment was Godulbaegi. Auxiliary materials consisted of dried red pepper powder, anchovy fish sauce, salted shrimp, refined salt, garlic, scallions, onions, ginger, carrots, green onions, and white sugar. All materials were produced in Korea, purchased from a large shopping mall (Emart, Seoul, Korea) in the Gwangjin District of Seoul and delivered to the laboratory within 30 min. Godulbaegi was soaked in 5% brine for 48 h to bring out the bitter taste, then washed 3 times under running water and drained for 30 min. The materials for kimchi were as follows: Godulbaegi (83.0%), dried red pepper powder (4.0%), anchovy fish sauce (3.5%), salted shrimp (2.3%), refined salt (2.3%), garlic (1.6%), scallions (1.4%), onions (0.5%), ginger (0.5%), carrots (0.4%), green onions (0.4%), and white sugar (0.1%). The dried ingredients were freeze-dried using a freeze dryer (Freeze Dryer-5, Ilsin Engineering, Co., Dongducheon, Gyeonggi, Korea), sealed and stored in a freezer maintained at −20 °C, and then ground and used whenever necessary. Subsequently, 2 kg of each were placed in a plastic container (25 × 15 × 20 cm) and stored at 5 °C, and samples were taken on the 6th month of storage. The samples taken were short-term fermented Godulbaegi kimchi (SGK, fermented for 7 days) and long-term fermented Godulbaegi kimchi (LGK, fermented for 6 months). Additionally, due to the acidic environment and the presence of many LAB during the fermentation process, kimchi is very durable [21]. Godulbaegi kimchi is generally eaten within six months of purchase. Therefore, we evaluated Godulbaegi kimchi after a week and after six months of storage.

2.2. Sampling, pH, and Total Acidity

Five grams of samples were added to 45 mL of sterile distilled water, blended with a stomacher (Stomacher ® 400 circulator; Seward Inc., West Sussex, UK), and filtered using filter paper (Whatman, Kent, UK). The pH of the SGK and LGK solutions was measured using a pH meter (pH Basic+; Sartorius AG, Göttingen, Germany). A homogeneous solution was obtained and filtered using filter paper (Whatman), and 0.1 mol/L NaOH was used to neutralize the kimchi solution. Ten milliliters of NaOH were used for total lactic acid content conversion and to determine the acidity (%, *w*/*v*) of each solution by titration of SGK) and LGK.

2.3. Number of LAB, Total Bacteria, and Yeast

LAB, total bacteria, and yeast were incubated by homogenizing the initial fermented Godulbaegi kimchi and LGK individually. LAB was cultured using de Man, Rogosa, and Sharpe (MRS) agar (Difco, Detroit, MI, USA) and bromocresol purple agar (BCP) agar.

Cultures were incubated under anaerobic conditions at 37 °C for 48 h [22]. Plate count agar (Eiken Chemical, Tokyo, Japan) was used for the total bacterial count, using the homogenized solution, and incubated at 30 °C for 72 h [23]. Yeast was counted using potato dextrose agar (Difco) at 25 °C for 5 days and identified by its shape and size [24].

2.4. Sample Preparation and Extraction Yield

Godulbaegi kimchi was separated and cut into small pieces and freeze dried using a freeze dryer (EYELA N-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at −50 °C, 35 mm Hg. Samples were stored in an air-tight container at −20 °C prior to further use. The extracts were prepared according to the method described by Mohd-Esa et al. (2010), with modifications [25]. *I. sonchifolia* was extracted using either distilled water (J. T. Baker, NJ, USA), methanol (J. T. Baker), or ethanol (J. T. Baker) for 24 h at room temperature, using an orbital shaker (JeioTech, Daejeon, Korea).

The mixture was filtered through a filter paper (Whatman no. 4, WM1004090). The filtrate was considered to be Godulbaegi kimchi extract and used for the antioxidant activity assays.

The extract was vacuum concentrated in each solvent, and the dried extract was weighed. The extraction yield was calculated using the following equation:

$$\% \text{ Yield} = (\text{extract (g)}/\text{raw material (g, dry weight)} \times 100$$

2.5. Determination of Total Polyphenols

The total polyphenol contents in diverse sample extracts were determined using the Folin–Ciocalteu colorimetric method [26]. A 20 mL sample of each extract filtrate was mixed with 1.58 mL of water, and 100 μL of Folin–Ciocalteu's phenol reagent was added to the mixture. Then, in a 3 min reaction, 300 μL of 20% (*w*/*v*) sodium carbonate solution was added, and the mixture was incubated for 30 min at 40 °C. The absorbance of each sample was determined with a spectrophotometer at 765 nm (Beckman du 530, Brea, CA, USA). The total polyphenol content was calculated as gallic acid equivalent (μg of GAEs/mg extract) and calibrated. The total polyphenol content was calculated using the following equation:

$$\text{Phenolic content} = 0.031 \times \text{A sample} + 0.159$$

2.6. Measurement of Free-Radical-Scavenging Activity

Free radical scavenging was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical by Shimada et al. [27]. Four milliliters of each sample extract were added to 1.0 mL of 1 mM DPPH solution. Then, 20, 40, 60, 80, and 100 mg/mL samples were prepared by adding 1 mL of DPPH solution to 5 mL of 80% methanol. After a 30 min incubation at 25 °C, the absorbance at 517 nm (UV-1601, Shizuoka, Japan) was recorded. The inhibitory of DPPH was expressed by the following equation:

$$\text{DPPH free radical-scavenging activity (\%)} \ (1 - \text{A sample}/\text{A blank}) \times 100$$

2.7. Nitrite Scavenging Activity

Nitrite scavenging was generated from sodium nitroprusside and was measured according to the method by Gray and Dugan [28]. The nitrite-scavenging activity was conducted under various range of pH 1.2, 3.0 and 6.0, respectively by measuring absorbance at 520 nm using 1 mL of sample extract and Griess reagent. This solution was incubated for 1 h at 37 °C and subsequently mixed with 5 mL of 2% acetic acid and 0.4 mL Greiss reagent (1% sulfonic acid in 30% acetic acid and 1% naphthylamine in 30% acetic acid) and kept at room temperature for 15 min. The absorbance of the chromophore formed during the diazotization of sulphanilamide and nitrite, and subsequent binding with

napthylethylenediamine was measured in 520 nm. The activity of nitrite scavenging (%) was determined by the following equation:

$$\text{Nitrite scavenging activity (\%)} = [1 - (A - B)/C] \times 100$$

A. The absorbance of 1 mM $NaNO_2$ added sample after allowing to stand for 1 h;
B. The absorbance of control;
C. The absorbance of 1 mM $NaNO_2$.

2.8. Antimicrobial Activity

After analyzing antioxidant activities, we selected LGK with excellent antioxidant activity and conducted an antimicrobial test. A total of 20 colonies from LGK, of different shapes and sizes, were selected and incubated at 37 °C for 48 h in MRS broth. *Escherichia coli* KCCM 21052, *Salmonella typhimurium* p99, *Staphylococcus aureus* KVCC BA1100335, and *Listeria monocytogenes* KVCC BA0001449) were used as indicators to determine the antibacterial activity of 20 separate strains isolated from LGK. The agar disc method was used to determine the antibacterial activity of compounds produced by each isolate against the aforementioned food-borne pathogens. Paper disks were impregnated with 50 μL of bacterial suspension containing approximately 10^7 CFU/mL of LGK from isolated bacteria. The paper disks containing the bacterial suspension were placed on the plates and incubated at 37 °C for 48 h. The antibacterial activity was expressed according to determining the diameter of the clear zone of growth inhibition.

2.9. Identification of LAB from Godulbaegi Kimchi

Colonies with the highest antibacterial activity were selected for taxonomic identification. DNA was extracted using the Power-Prep DNA Extraction Kit (Kogene Biotech, Seoul, Korea). The extract of DNA was used for polymerase chain reaction (PCR) with the two primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′, forward) and 1492R (5′-GGCTACCTTGTTTACGACTT-3′, reverse). Subsequent identification was performed using the 16S rRNA gene sequence analysis provided by SolGent (SolGent Co., Ltd., Daejeon, Korea). The phylogenetic tree was determined with the neighbor-joining method with the Molecular Evolutionary Genetics Analysis (MEGA) 7 software (Available online: https://www.megasoftware.net/ accessed on 20 February 2022).

2.10. Statistical Analysis

All experiments were conducted in triplicates and expressed as mean ± standard error. Statistical analyses were determined in the SPSS program (SPSS version 12.0, SPSS Chicago, IL, USA) using unpaired *t*-tests repeated measures analysis of variance (ANOVA) when appropriate. If the data was statistically significant by ANOVA ($p < 0.05$), differences in the means were determined using Duncan's multiple range tests.

3. Results and Discussion

3.1. pH and Acidity Value of Godulbaegi Kimchi

Usually, in the early stage of kimchi fermentation, the pH, acidity, and microbial composition change very actively [29]. These parameters can be used as indicators to judge the quality of kimchi while also affecting the flavor of kimchi. The pH and acidity value changes in SGK according to the fermentation period are shown in Table 1. Early on, the average pH of SGK was 4.43, and the acidity value ranged 0.46%. Then, at 4 °C, pH tended to decrease, and the acidity value increased as fermentation proceeded. Therefore, by 6 months, the average pH and acidity value of LGK had reached 4.20 and 0.92%, respectively. Various conditions such as the composition of subsidiary materials, salinity, and storage temperature affect the pH of matured kimchi [30]. In addition, our results showed that the increase in acidity of LGK was due to the production of organic acids, which also affect kimchi taste [20,31,32]. The pH results were similar to those of typical

fermentation processes reported previously [33]. Changes in pH or total acidity content are commonly used as indicators of ripeness during Godulbaegi kimchi fermentation. A pH of approximately 4.2–4.4 and acidity values ranging between 0.5–0.75% are optimal conditions for Godulbaegi kimchi consumption [34,35].

Table 1. Changes in pH, acidity value, and numbers of LAB, total bacteria, and yeast.

	SGK [a]	LGK [b]
pH	4.43 ± 0.14 [c]	4.20 ± 0.13
Total acidity (%)	0.46 ± 0.02	0.92 ± 0.03
Lactic acid bacteria (CFU/mL)	6.06 ± 0.28	6.59 ± 0.10
Total viable count (CFU/mL)	5.85 ± 0.34	4.66 ± 0.13
Total yeast count (CFU/mL)	1.24 ± 0.14	3.06 ± 0.03

[a] short-term fermented Godulbaegi kimchi (SGK, fermented for 7 days); [b] long-term fermented Godulbaegi kimchi (LGK, fermented for 6 months); [c] the values are expressed as mean ± standard deviation (n = 3).

3.2. Microbial Load Analysis

The changes in the LAB, total bacteria, and yeast during the storage of SGK and LGK are shown in Table 1. Changes in LAB counts during SGK fermentation showed a time-dependent increase from 6.06 $\log_{10}$ CFU/g to 6.59 $\log_{10}$ CFU/g. Other studies have shown that the number of LAB in kimchi increases rapidly during the early stages of fermentation until the peak is reached after approximately 8 days, then slowly decreases and gradually stabilizes [36]. Nonetheless, as fermentation progressed, the antibacterial substances secreted by certain LAB gradually increased [37]. This inhibited the growth of other bacteria, decreasing the total bacterial count from 5.85 $\log_{10}$ CFU/g to 4.66 $\log_{10}$ CFU/g. Conversely, as fermentation progressed, yeast counts gradually increased from 1.24 $\log_{10}$ CFU/g to 3.06 $\log_{10}$ CFU/g, indicating that the fermentation process of kimchi is suitable for yeast growth. In general, the fermentation pattern of kimchi is such that as fermentation progresses in the early stages, the microbial quantity increases. Fermentation then gradually decreases as microbial counts reach the maximum level. This process gives kimchi a unique taste and aroma, accompanied by biochemical changes.

3.3. Extraction Yield

Table 2 shows the extract yield of LGK using either methanol, ethanol, or water. The extraction yield varied in the following order depending on the solvent used for extraction and duration of fermentation, namely methanol extraction > ethanol extraction > water, extraction and ranged from 48.54% to 61.51%.

Table 2. The yield of extraction and total phenol content from Godulbaegi kimchi by fermentation duration by diverse solvents.

Extract Solvent	Yield Extraction (%)	Total Phenolic Content (µg of GAEs/mg Extract)
	LGK [1]	
Ethanol	52.58 ± 2.06 [b]	77.40 ± 1.42 [a]
Methanol	61.51 ± 1.21 [a]	79.01 ± 2.36 [a]
Water	48.54 ± 0.97 [c]	38.02 ± 0.80 [b]

[1] long-term fermented Godulbaegi kimchi (LGK, fermented for 6 months). Values are mean ± standard deviation (n = 3). Means in the same column and with different lower-case letters ([a–c]) indicate significant difference ($p < 0.05$).

Methanol extracts have been reported to have higher antioxidant capacities. Our results were consistent with previous research, showing the following order: methanol > ethanol > water extracts [38].

3.4. Total Phenolic Content

The total phenolic content was conducted using the Folin–Ciocalteu method. The content of phenolic compounds was determined using a regression equation of the calibration curve (y = 0.031x + 0.1593, R2 = 0.9922) and expressed in GAE. Table 2 shows that the total phenolic content of LGK extracts in methanol, ethanol, or water was 38.02, 77.40, and 79.01 µg of GAE/mg extract, respectively. A significantly higher total phenol content was determined in samples that underwent higher fermentation periods. That is, phenol content increased with increasing duration of fermentation because phenolic acids, such as coumaric and ferulic acids, form ethyl or vinyl phenol derivatives through reactions with microorganisms. Generally, the total phenol content increases as fermentation proceeds, and this phenomenon was consistent with the results of this experiment [39].

Moreover, the methanol and ethanol Godulbaegi extracts had higher polyphenol content than the water extract. Therefore, methanol and ethanol were the most efficient solvents for extracting the antioxidants from the samples. Therefore, LGK is high in total polyphenols and is expected to play a role as an antioxidant.

3.5. DPPH Radical Scavenging

The results in Figure 1 show the scavenging activity on DPPH radicals of the different LGK extracts increased in a dose-dependent manner with DPPH doses varying between 20 and 100 mg/mL. The scavenging activity on DPPH radicals of LGK ranged from 17.56% to 88.37%. These variations may have been caused by differences either in potency or in the concentration of reducing substances, mainly phenolic compounds, which reflects the effect of red pepper seed on Godulbaegi kimchi antioxidant activity during fermentation. DPPH-radical-scavenging activity studies reported that fermented kimchi in different solvent extracts contains active materials, such as phenolic compounds, vitamin C, and phenolic acid, which can show scavenging activity on DPPH radicals. The scavenging activity on DPPH radicals of LGK was significantly higher ($p < 0.05$) in methanol ethanol extracts than in water extracts. Particularly, the DPPH-radical-scavenging activity of the LGK methanol extract ranged from 58.12% to 88.35% using DPPH doses between 20 and 100 mg/mL. These data highlight the antioxidant properties of Godulbaegi kimchi. Thus, Godulbaegi kimchi possesses antibacterial and antioxidant activities that could be useful for the development of various pharmaceutical and food products through future research. Aarti et al. [40] reported that the DPPH-radical-scavenging activity of *Lactobacillus brevis* LAP2 increased in a concentration-dependent manner (18.8–68.35% at 108–109 CFU/mL). Additionally, the scavenging activity on DPPH radicals of the extracts increased according to the duration of fermentation.

3.6. Nitrite Scavenging in Godulbaegi Kimchi

Table 3 shows the change in the quantity of nitrite scavenging depending on the time of fermentation and solvent used for extraction at various pH levels (pH 1.2, 3.0, and 6.0). The degree of degradation was higher at acidic pH conditions, being highest at pH 1.2 in all extraction solvents ($p < 0.05$). Furthermore, the result showed that the methanol and ethanol extracts had the highest nitrite-scavenging activity among the extracts, followed by the water extract. The nitrite-scavenging effect was highest at pH 1.2 (81.90%) for methanol extracts. This was likely because of the various phenolic substances in Godulbaegi kimchi. This result was similar to that of a previous study wherein *Lactobacillus sakei* was able to deplete nitrite and degrade N-nitrosodimethylamin (NDMA) in MRS broth, and LAB was able to deplete $NaNO_2$. In addition, the inoculation of *L. sakei*, *L. curvatus*, and *L. brevis* into kimchi resulted in a marked reduction in the nitrite levels, which might be due to the nitrite-scavenger effects of LAB. Green onion, garlic, and dried red pepper contain a variety of active compounds, such as allyl sulfides, carotenes, phenolic compounds, and ascorbic acid, which prevent nitrosation [41,42].

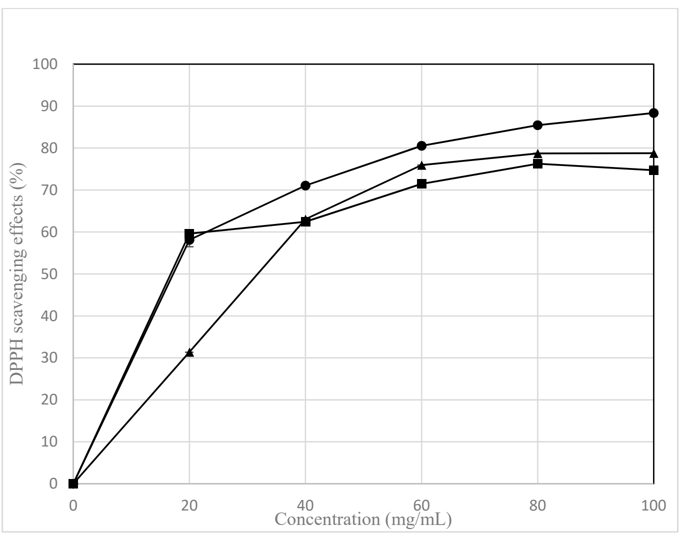

Figure 1. Scavenging activity on DPPH radicals of the methanol, ethanol, and water extracts in LGK (six months fermented Godulbaegi kimchi). The symbols express the following samples: closed circle, methanol extract; closed triangle, ethanol extract; closed square, water extract.

Table 3. The activity of nitrite scavenging (%) of Godulbaegi kimchi by fermentation duration using diverse solvents.

Concentration (%)	pH	Ethanol	Methanol	Water
LGK; Long-term fermented Godulbaegi kimchi	1.2	80.97 ± 0.87 ^{aA}	81.90 ± 2.17 ^{aA}	74.86 ± 2.05 ^{bA}
	3.0	72.30 ± 1.96 ^{aB}	72.80 ± 1.50 ^{aB}	54.44 ± 2.03 ^{bB}
	6.0	26.70 ± 0.96 ^{aC}	30.49 ± 2.04 ^{aC}	21.32 ± 1.25 ^{bC}

Values are mean ± standard deviation (n = 3). Means in the same column with different capital case letters ($^{A–C}$) and same row with different lower-case letters ($^{a–b}$) were significantly different (p < 0.05).

3.7. Antimicrobial Activity of Lactic Acid Bacteria Isolates against Target Bacteria

Preliminary screening of the antimicrobial activities of LAB were verified for 20 strains isolated from LGK. Among them, we selected nine strains with antibacterial effect and numbered them 1–9. The results in Table 4 show the LAB strains' antimicrobial activities were effective against Gram-negative bacteria but not against Gram-positive ones. For instance, sample 1 had strong inhibition zones of >1.5 mm for *E. coli* (KCCM 21052) and of 1.2–1.5 mm for *S. typhimurium* P99. Samples 4, 6, and 9 had clear inhibition zones of over 1.0 mm for *E. coli* (KCCM 21052) and *S. typhimurium* P99. However, the strains had no inhibition against Gram-positive bacteria such as *S. aureus* KVCC BA1100335 and *L. monocytogenes* KVCC BA0001449. This may be because the cell walls of Gram-positive bacteria hamper the entry of antimicrobial compounds. A previous study showed that *L. sakei* have a weak antimicrobial effect on *L. innocua* and *S. aureus* [43]. In this study, we found that antimicrobial compounds produced during LGK fermentation by LAB have high antibacterial activity against two Gram-negative food-borne pathogens. Therefore, LAB isolated from LGK can be used to make natural preservatives, which are important for food safety and for the food distribution industry [44,45].

3.8. Identification by 16S rRNA Sequencing

In total, nine LAB strains were selected for 16S rRNA sequencing based on their antimicrobial activity. 16S rRNA sequencing revealed that strain 4 showed 100% homology with *L. sakei* NBRC 15893. Strain 9 showed 99.09% homology with *Lactobacillus graminis* G90. Sample strains 1, 2, 3, 5, 6, 7, and 8 exhibited more than 99.86% homology with *L. sakei* subsp. DSM 20017. Figure 2 shows the phylogenetic tree constructed with the neighbor-joining method and the 16S rRNA gene sequences of the strains. In conclusion,

various types of LAB were present in LGK, but *L. sakei* and *L. graminis* were the main types exhibiting the antimicrobial activity found in the LGK isolates in our study. These strains showed similar morphological features, and both secrete antioxidant molecules. In particular, *L. sakei* had a key role in meat preservation and fermentation mainly producing antioxidants with antibacterial effects, similar to our results [46–48]. The isolated strains can be widely used in the manufacture of natural preservatives and as natural additives in the production of certain fermented foods. Moreover, we found that the fermentation period affected the extraction yield, DPPH scavenging, content of total phenol, and antioxidant activity of Godulbaegi kimchi. These data describe the basic aspects of Godulbaegi kimchi. Nonetheless, to establish the value of Godulbaegi kimchi, more diverse functional ingredients and various physiological functional comparative studies are needed.

Table 4. Comparison of antimicrobial activities of long-term fermented Godulbaegi kimchi.

Strain No.[a]	Negative Control [b]	*Escherichia coli*	*Salmonella typhimurium*	*Staphylococcus aureus*	*Listeria monocytogenes*
1	- [c]	+++	++	-	-
2	-	++	-	-	-
3	-	++	-	-	-
4	-	++	++	-	-
5	-	++	-	-	-
6	-	++	++	-	-
7	-	++	-	-	-
8	-	++	-	-	-
9	-	++	++	-	-

[a] *Escherichia coli* KCCM 21052; *Salmonella typhimurium* P99; *Staphylococcus aureus* KVCC BA1100335; *Listeria monocytogenes* KVCC BA0001449. [b] Each of the extraction solvent. [c] Degree of clarity of clear zone by growth inhibition: +++: Strong inhibition (≥15 mm), ++: clear inhibition (≥12 mm, <15 mm), +: slight inhibition (<10 mm), -: No inhibition.

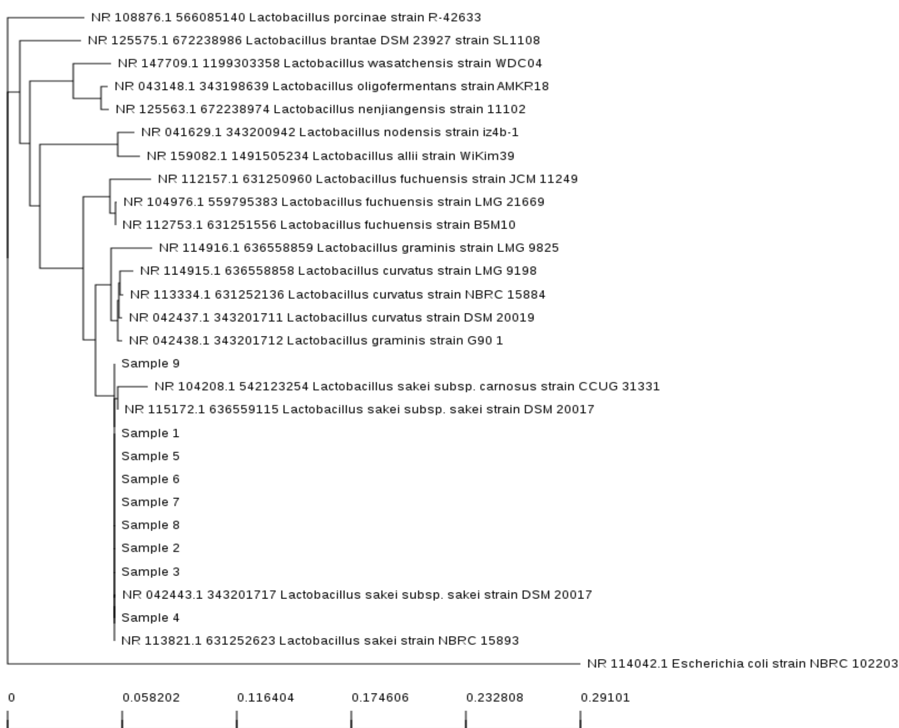

Figure 2. Phylogenetic tree construction using the neighbor-joining method and gene sequences, based upon 16S rRNA sequencing. The figure shows the positions of strains and other closely related lactic acid bacteria (LAB) isolated from long-term fermented Godulbaegi kimchi (LGK).

4. Conclusions

This study was conducted to investigate microbial and antioxidative characteristics of Godulbaegi kimchi (LGK). The pH was decreased, and total acidity was increased during fermentation. The effects obtained from the extraction of methanol, ethanol, and water from fermented Godulbaegi kimchi on antioxidant activity were investigated. In DPPH scavenging activity, phenol content, and nitrite-scavenging activity, methanol extract in Godulbaegi kimchi has the highest antioxidant activity among ethanol and water. The results of this study suggested that fermented Godulbaegi kimchi has a high antioxidant content. Additionally, Lactic acid bacteria such as *Lactobacillus sakei* NBRC 15893, *Lactobacillus graminis*, and *Lactobacillus sake,* with antibacterial activity, were identified in fermented Godulbaegi kimchi, traditional Korean kimchi. However, to establish the value of Godulbaegi kimchi, it is judged that more diverse functional ingredients and various physiological functional comparative studies of Godulbaegi kimchi are needed in the future.

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Article

Diversity of a Lactic Acid Bacterial Community during Fermentation of Gajami-Sikhae, a Traditional Korean Fermented Fish, as Determined by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Abstract: Gajami-sikhae is a traditional Korean fermented fish food made by naturally fermenting flatfish (*Glyptocephalus stelleri*) with other ingredients. This study was the first to investigate the diversity and dynamics of lactic acid bacteria in gajami-sikhae fermented at different temperatures using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). A total of 4824 isolates were isolated from the fermented gajami-sikhae. These findings indicated that *Latilactobacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Weissella*, and *Leuconostoc* were the dominant genera during fermentation, while the dominant species were *Latilactobacillus sakei*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Weissella koreensis*, and *Leuconostoc mesenteroides*. At all temperatures, *L. sakei* was dominant at the early stage of gajami-sikhae fermentation, and it maintained dominance until the later stage of fermentation at low temperatures (5 °C and 10 °C). However, *L. plantarum* and *L. brevis* replaced it at higher temperatures (15 °C and 20 °C). The relative abundance of *L. plantarum* and *L. brevis* reached 100% at the later fermentation stage at 20 °C. These results suggest that the optimal fermentation temperatures for gajami-sikhae are low rather than high temperatures. This study could allow for the selection of an adjunct culture to control gajami-sikhae fermentation.

Keywords: gajami-sikhae; MALDI-TOF MS; microbial community; culture-dependent method; fermentation; identification; fermentation temperature

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1. Introduction

Sikhae is a traditional fermented food in Korea, commonly served as a side dish. Gajami-sikhae, which uses flatfish belonging to the species *Glyptocephalus stelleri* as the main ingredient, is made by mixing salted flatfish with ingredients such as cooked grains, salt, red pepper powder, white radish, garlic, ginger, and green onion [1]. In recent years, gajami-sikhae has been well accepted by Korean consumers due to its unique flavor and potential health benefits, such as anticancer and antioxidant effects, attributed to the fermentation of various microorganisms [2,3].

The spontaneous fermentation of foods is mainly affected by the microorganisms present in the food at various stages of the fermentation process [4]. Lactic acid bacteria (LAB) are the most prominent microorganisms responsible for fermenting vegetables, meat, dairy products, and fish [5]. Some studies showed that the main taxa of fermented fish are related to *Latilactobacillus* and *Weissella*, including *Latilactobacillus sakei* and *Weissella koreensis* [6,7]. The quality of fermented foods correlates to the various microorganisms that occur naturally during the fermentation process; they produce bacteriocins, organic acids, and flavor compounds responsible for the flavor formation of fermented foods [8]. Since the growth of LAB in fermented foods is affected by fermentation conditions, such

as the fermentation temperature and period, it is necessary to investigate the change in the microbial community under various fermentation conditions to improve the quality of gajami-sikhae made for consumption.

Investigations of the entire microbial community present in many foods became possible with the advent of next-generation sequencing [4]. This technology has been successfully applied to study microbial communities in a fermented food matrix. Reportedly, it provides deeper, more precise information on the microbial community than polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) [4,9]. Additionally, there are approaches to investigate viable microbial communities using metagenetics (e.g., after total RNA extraction and reverse PCR or through the use ethidium monoazide (EMA) treatment PCR to only amplify DNA from viable cells) [10]. However, the species identification obtained using the metagenomic approach might be limited.

Culture-based approaches are still widely used to analyze microbial communities. The development of new tools, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), has allowed a reduced time to detection as compared to conventional culture-based methods [11]. Currently, MALDI-TOF MS is an alternative to the sequencing method for identifying microorganisms [12]. This high-throughput technique compares mass spectral patterns, including ribosomal protein obtained from microbial cells, with a reference spectral database [13]. This technique has emerged as a new method for the relatively rapid, simple, and effective identification of microorganisms based on its reliance on microbial fingerprints [12]. Moreover, MALDI-TOF MS is superior to the 16S rRNA gene for taxonomic resolution at the species or subspecies level for some closely related species such as *Lacticaseibacillus casei*/*L. paracasei* and *Lactobacillus acidophilus* group species [14–17]. However, MALDI-TOF MS relies on a spectral database, so only species present in the database can be identified.

Many studies addressing its use in experimental approaches related to pathogenic bacteria have been published [12,13]. Some studies have applied MALDI-TOF MS technology to observe changes in the culturable microbial community in fermented foods, but there has been no study examining the microbial community of gajami-sikhae [18]. Moreover, although the microbial community of gajami-sikhae has been investigated by pyrosequencing [19], the effect of temperatures on this fermentation process has not yet been investigated.

In this study, we aimed to analyze the microbial community during the fermentation of gajami-sikhae using MALDI-TOF MS. The results provide a deeper understanding of the correlation between fermentation conditions and the microbial community. In addition, they will lay a foundation for standard gajami-sikhae manufacturing systems and quality improvement.

2. Materials and Methods

2.1. Sample Preparation

A gajami-sikhae sample was purchased from traditional manufacturers in Korea in December 2020. A sample prepared using the traditional method, in which the salted flatfish (*Glyptocephalus stelleri*) was mixed with radish, red pepper powder, boiled millet (*Setaria italica*), chopped garlic, and NaCl, was purchased [19]. This mixture was fermented at 5 °C, 10 °C, 15 °C, or 20 °C for 60 days in a poly cyclohexane-1,4-dimethylene terephthalate plastic bowl (20 × 11 cm). Samples were collected at from 8 to 11 points at each fermented temperature. Thirty-nine samples in total were obtained. Table S1 provides detailed information about the obtained samples.

2.2. pH and Acidity Measurements

The gajami-sikhae sample was ground for 2 min using a blender and filtered through gauze to remove large particles and measure the pH and acidity. The pH value of the filtered gajami-sikhae samples (50 mL) was measured in triplicate using a pH meter (Thermo Fisher Scientific, Waltham, MA, USA). Also, 10 mL of the filtered sample was titrated with 0.1 N

NaOH to a final pH of 8.2 to measure the acidity of the gajami-sikhae [20,21]. The acidity was calculated by substituting the measured volume of 0.1 N NaOH into the percentage (%, *v*/*v*) of lactic acid produced.

2.3. Cultivable Microbial Community

2.3.1. Isolation of LAB

For the isolation of LAB, 25 g of gajami-sikhae sample and 225 mL of sterilized phosphate-buffered saline (PBS) were placed in a sterile stomacher bag (Seward Limited, London, UK). The mixture was homogenized for 2 min at 230 rpm using a peristaltic homogenizer (Circulator stomacher 400; Seward Limited). Subsequently, serial dilutions of the homogenate were prepared, followed by isolation of LAB on MRS (Difco) agar incubated at 20 °C and 30 °C for 72 h. After incubation, the colony-forming units (CFU) were counted. All colonies from countable plates with lactic acid bacterial growth of between 30 and 300 CFU/plate on MRS agar were selected. The harvested colonies were subsequently subcultured on MRS agar and incubated under similar conditions to those described above.

2.3.2. Analysis of the Microbial Community by MALDI-TOF MS

A single colony was smeared onto a polished steel MALDI target plate (Bruker Daltonics, Bremen, Germany). The spot was covered with 1 µL of 70% formic acid and dried at room temperature. Subsequently, 1 µL HCCA matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) (Bruker Daltonics) in acetonitrile, water, and trifluoracetic acid (50:47.5:2.5 (*v*/*v*/*v*)) was added to the spot and dried again. The polished steel MALDI target plate was introduced into the Microflex LT bench-top MALDI-TOF mass spectrometer (Bruker Daltonics). The mass spectra of isolates were identified by comparing the mass spectra to those in the Bruker MSP database version 4.0, containing 5627 reference spectra, using the Bruker software. The Bruker MSP database consists of 98 species and 236 spectra of LAB (*Lactobacillus*-related species). The identification score was interpreted according to the manufacturer's criteria. Thus, a score between 2.0 and 3.0 indicated highly probable species identification, between 1.7 and 1.999 indicated probable genus identification, and lower than 1.7 indicated unreliable identification. For lactic acid bacterial identification, the following reference strains obtained from the Korean Agricultural Culture Collection (KACC, Jeonju, Korea) and the Korean Collection for Type Cultures (KCTC, Daejeon, Korea) were used: *Lactiplantibacillus plantarum* KACC 11451, *L. sakei* KCTC 3603, *Latilactobacillus curvatus* KACC 12415, *Levilactobacillus brevis* KCTC 3498, *Leuconostoc mesenteroides* KCTC 3100, *Leuconostoc inhae* KACC 12281, *Leuconostoc gelidum* KACC 12256, *Weissella cibaria* KCTC 3746, *Weissella confusa* KCTC 3499, and *W. koreensis* KACC 11853.

2.4. Statistical Analysis

The pH and acidity values are expressed as the means ± standard deviations. The statistical analysis for pH and acidity values was performed using R v.4.1.0. Significant differences ($p < 0.05$) between the sample means were determined by Duncan's multiple range test. In addition, the relationships between the major species in fermented gajami-sikhae and the pH, acidity, fermentation temperature, and fermentation period were determined by calculating the Pearson correlation implemented in R. Benjamini–Hochberg correction was used to correct the p values [22].

3. Results and Discussion

3.1. Physicochemical Properties

The pH and acidity affect fermented foods' anaerobic fermentation efficiency and should be monitored during food fermentation [23]. During fermentation, the gajami-sikhae tended to decrease in pH and increase in acidity as the fermentation progressed. Figure 1A shows the change in pH during the fermentation of gajami-sikhae. The pH value was 6.25 ± 0.01 immediately after gajami-sikhae production and further dropped during

fermentation (Table S2). During the first 15 days, the pH values of samples fermented at 5 °C and 10 °C rapidly decreased to 4.54 ± 0.01 and 4.44 ± 0.01, respectively. However, the pH value remained stable from then until the end of fermentation. In samples fermented at 15 °C and 20 °C, the pH values rapidly decreased to 4.81 ± 0.01 and 4.50 ± 0.01, respectively, in the first two days and gradually decreased thereafter. The pH value of gajami-sikhae at 20 °C was the lowest after fermentation (Figure 1A).

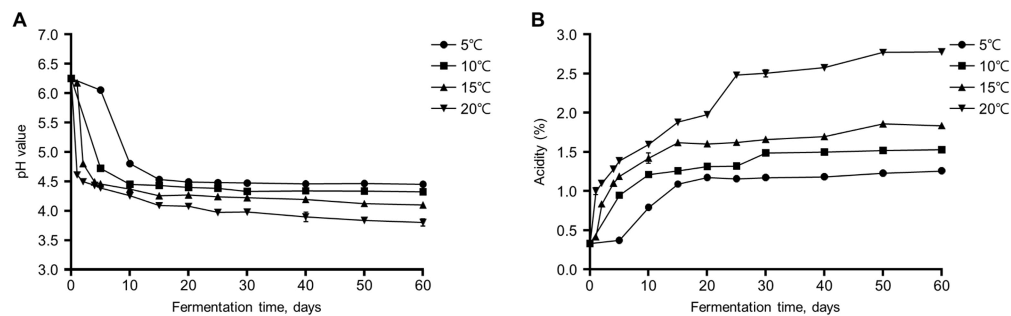

Figure 1. Changes in the pH and acidity of gajami-sikhae samples during the fermentation at different fermentation temperatures: (**A**) pH profiles; (**B**) acidity profiles. Error bars represent the mean ± standard deviation.

The acidity was 0.33 ± 0.03% at the beginning of fermentation and then increased during the fermentation process at all temperatures (Figure 1B). The acidity of gajami-sikhae fermented at 5 °C and 10 °C rapidly increased and reached about 1.18 ± 0.02% and 1.32 ± 0.01%, respectively, after 20 days. From this point, it remained stable. After 50 days, the acidity value of gajami-sikhae fermented at 15 °C gradually increased to 1.86 ± 0.03%. Also, the acidity of gajami-sikhae fermented at 20 °C continued to increase, showing 2.77 ± 0.02% acidity at 50 days of fermentation. During fermentation, gajami-sikhae fermented at relatively low temperatures (5 °C and 10 °C) showed low acidity, whereas samples fermented at high temperatures (15 °C and 20 °C) showed high acidity. Compared with the control, a lower pH value occurred in the sample fermented at 20 °C, indicating that the fermentation temperature affected the acidity of the gajami-sikhae. Fermented food quality is usually unacceptable when the acidity is about 1.6–2.0% [9]. In this study, the acidities at 15 °C and 20 °C reached unacceptable levels after 10 and 15 days, respectively. However, samples fermented at 5 °C and 10 °C did not reach unacceptable acidity (1.6–2.0%) until the end of the fermentation period.

The LAB population of the gajami-sikhae was estimated by plate counting on MRS agar. Our findings indicated that the number of viable cells rapidly increased and then slightly decreased throughout the fermentation. The initial lactic acid bacterial count in gajami-sikhae was 4.0 log CFU/g. Also, at 5 °C and 10 °C, LAB counts reached the maximum in 10 days, with an average of 8.2 log CFU/g and 8.0 log CFU/g, respectively. Then, the counts slightly decreased until the end of fermentation (Figure 2A,B). Finally, samples fermented at 15 °C and 20 °C reached the maximum cell counts with an average of 8.4 CFU/g and 7.9 CFU/g after three and two days of fermentation, respectively.

3.2. Identification of Isolates Using MALDI-TOF MS

The traditional culture method is often intensive and time-consuming. However, alternative molecular ecological methods are widely used to rapidly and efficiently observe the microbial composition in food fields [4]. Earlier, numerous studies used metagenome sequencing techniques based on 16S rRNA gene fragments to analyze the microbiome in traditional fermented foods [18,21,24]. However, the 16S rRNA gene provides low taxonomic resolution for some species [18]. Some LAB, such as *Lactiplantibacillus* species, *Latilactobacillus* species, and *W. cibaria*/*W. confusa*, which are mainly involved in vegetable or fish fermentation, were not distinguished by this method at the species level [18]. In

contrast, MALDI-TOF MS-based ribosomal protein accurately identified these species [18]. Therefore, in this study, MALDI-TOF MS was evaluated as a high-throughput method for identifying microorganisms isolated from gajami-sikhae.

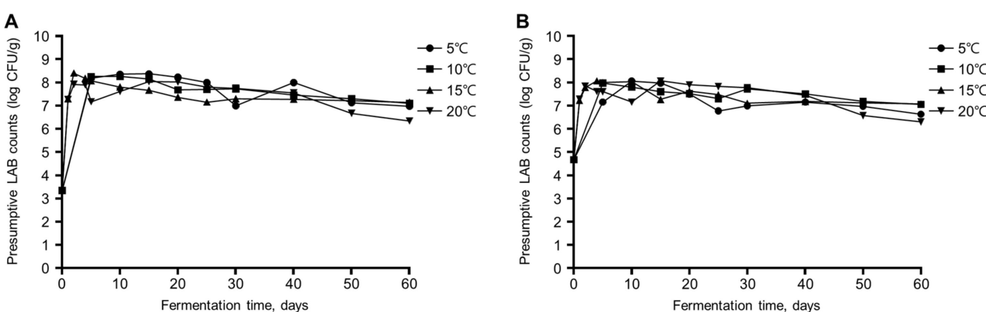

Figure 2. Changes in presumptive LAB counts (log CFU/g) of gajami-sikhae samples during fermentation at different fermentation temperatures: (**A**) presumptive LAB count incubated at 20 °C; (**B**) presumptive LAB count incubated at 30 °C.

A total of 4824 isolates were obtained during the fermentation of gajami-sikhae. The accuracy of MALDI-TOF MS mainly depends on the reference database [25]. Before identifying the isolates, 10 reference strains mainly involved in gajami-sikhae fermentation were analyzed using the bioTyper database. All reference strains were identified with score values of 2.0 or higher (data not shown). The mass profiles of the isolates were compared to the reference spectra in the database, and then the isolates were identified at the species level based on the given score values. In addition, 4824 isolates of gajami-sikhae were identified as belonging to various genera such as *Bacillus*, *Enterobacter*, *Enterococcus*, *Lactiplantibacillus*, *Lactobacillus*, *Latilactobacillus*, *Levilactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*. This finding resulted in 3805 isolates (78.88%) with a score of ≥2.000, corresponding to highly probable species identification (Table 1). This suggests that the MALDI-TOF MS method can identify most isolates related to gajami-sikhae fermentation. Furthermore, 1019 isolates (21.12%) delivered scores between 1.700 and 2.000, corresponding to probable genus identification (Table 2). While low identification scores (<2.000) may have different causes, such as species not present in the reference databases, a low number of representative isolates of a given species, or problems in sample preparation, they could reveal other LAB species. Therefore, strains not identified at the species level by MALDI-TOF MS should be further analyzed using housekeeping genes such as *pheS* and *rpoB* genes.

Table 1. Number of isolates with correct identification to the species level by MALDI-TOF MS.

Species (No. of Isolates)	No. of Isolates with Results
Bacillus pumilus (1)	0 (0%)
Bacillus subtilis (1)	0 (0%)
Enterobacter cowanii (1)	0 (0%)
Enterococcus faecium (28)	22 (78.57%)
Enterococcus hermanniensis (2)	0 (0%)
Lactiplantibacillus plantarum (661)	504 (76.25%)
Fructilactobacillus fructivorans (1)	1 (100%)
Latilactobacillus curvatus (217)	155 (71.43%)
Latilactobacillus sakei (1965)	1873 (95.32%)
Levilactobacillus brevis (643)	563 (87.56%)
Lactococcus lactis (7)	7 (100%)
Leuconostoc citreum (118)	47 (39.83%)
Leuconostoc gelidum (140)	69 (49.29%)

Table 1. *Cont*.

Species (No. of Isolates)	No. of Isolates with Results
Leuconostoc inhae (30)	3 (10%)
Leuconostoc lactis (3)	0 (0%)
Leuconostoc mesenteroides (329)	238 (72.34%)
Leuconostoc pseudomesenteroides (2)	0 (0%)
Pediococcus acidilactici (1)	0 (0%)
Pediococcus pentosaceus (7)	7 (100%)
Weissella cibaria (52)	52 (100%)
Weissella hellinica (6)	1 (16.67%)
Weissella kandleri (3)	2 (66.67%)
Weissella koreensis (599)	256 (42.74%)
Weissella viridescens (7)	5 (71.43%)
Total (4824)	3805 (78.88%)

Table 2. Number of isolates with correct identification to the genus level by MALDI-TOF MS.

Genus (No. of Isolates)	No. of Isolates with Results
Bacillus pumilus (1)	1 (100%)
Bacillus subtilis (1)	1 (100%)
Enterobacter cowanii (1)	1 (100%)
Enterococcus faecium (28)	6 (21.43%)
Enterococcus hermanniensis (2)	2 (100%)
Lactiplantibacillus plantarum (661)	157 (23.75%)
Fructilactobacillus fructivorans (1)	0 (0%)
Latilactobacillus curvatus (217)	62 (28.57%)
Latilactobacillus sakei (1965)	92 (4.68%)
Levilactobacillus brevis (643)	80 (12.44%)
Lactococcus lactis (7)	0 (0%)
Leuconostoc citreum (118)	71 (60.17%)
Leuconostoc gelidum (140)	71 (50.71%)
Leuconostoc inhae (30)	27 (90%)
Leuconostoc lactis (3)	3 (100%)
Leuconostoc mesenteroides (329)	91 (27.66%)
Leuconostoc pseudomesenteroides (2)	2 (100%)
Pediococcus acidilactici (1)	1 (100%)
Pediococcus pentosaceus (7)	0 (0%)
Weissella cibaria (52)	0 (0%)
Weissella hellinica (6)	5 (83.33%)
Weissella kandleri (3)	1 (33.33%)
Weissella koreensis (599)	343 (57.26%)
Weissella viridescens (7)	2 (28.57%)
Total (4824)	1019 (21.12%)

3.3. Bacterial Community Dynamics during Fermentation

In previous studies, microbial communities were analyzed using culture-dependent (MALDI-TOF MS) and culture-independent (metagenome sequencing) approaches [18,26,27]. Both identification systems produced almost identical results. However, MALDI-TOF MS could not identify microorganisms absent from the reference databases. Although metagenome sequencing could not accurately identify some closely related species at the species level, this approach allowed for the detection of higher biodiversity than the MALDI-TOF MS. Both approaches provided complementary information by producing a comprehensive view of the microbial ecology in environmental or food samples.

Although gajami-sikhae is a very intriguing traditional Korean fermented fish, there is little information about its microbial composition compared to other fermented foods such as kimchi and jeotgal. Moreover, Kim et al. (2014) is the only study that has identified the composition of microorganisms in different gajami-sikhae samples using pyrosequencing

analysis [19]. However, the study did not report any microbial community change in gajami-sikhae during fermentation. Since gajami-sikhae is fermented in an unsterilized natural environment, leading to the growth of various microorganisms, it is necessary to investigate changes in the microbial community to standardize the quality of gajami-sikhae. Therefore, we used MALDI-TOF MS to identify the microbial community in gajami-sikhae based on the effect of varying fermentation conditions.

The most dominant genera were found to be *Latilactobacillus* (45.23%), *Leuconostoc* (12.89%), and *Weissella* (13.83%), which were present at all fermentation temperatures (Table S3). The other major genera present include *Lactiplantibacillus* (13.70%) and *Levilactobacillus* (13.33%). This result was consistent with the findings of the previous study that confirmed the microbial community composition in gajami-sikhae using pyrosequencing [19]. The species, including *L. sakei*, *L. plantarum*, *L. brevis*, *Leu. mesenteroides*, and *W. koreensis,* were identified with high abundance during fermentation at different temperatures. The predominant species in the microbial community of gajami-sikhae belonged to lactic acid bacterial species responsible for variations in the sensory qualities of other Korean fermented foods, such as kimchi and jeotgal [28,29]. The discovery that the microbial community in gajami-sikhae is similar to that in kimchi for fermenting vegetables is probably because of the similar production methods of sikhae and kimchi [19].

Microbial communities demonstrated a similar pattern in gajami-sikhae fermented at 5 °C and 10 °C, and a similar pattern for fermentation at 15 °C and 20 °C. Figure 3 and Figure S1 represent the microbial communities identified at the species and genus level, respectively. As shown in Figure 3, *L. sakei*, *Leu. mesenteroides*, *Leu. gelidum*, *Leu. citreum*, and *W. koreensis* were abundant at the beginning of fermentation (Sample C), suggesting that these species were a major component of the microbial community of the raw materials. *Weissella* species, such as *W. koreensis* and *W. cibaria,* were isolated in samples fermented at all temperatures. These species were found only at the early stage of fermentation because their growth was affected by acid [30]. *Leu. mesenteroides*, major LAB in fermented vegetables, are mainly used as a starter in commercial food fermentation because they produce mannitol. Mannitol is a naturally occurring 6-carbon diabetic polyol that provides a refreshing taste [31]. In the low-temperatures fermented samples, *Leu. mesenteroides* existed until the later stage of fermentation, but in the high-temperatures fermented samples, it decreased rapidly as the fermentation process continued. Also, these findings showed that *L. sakei* predominated the early stage of gajami-sikhae fermentation in all temperatures. This species is the dominant species in the microbial community of fermented fish and may play some role in the fermentation process [24]. During the fermentation process, *L. sakei* increased and predominated the later stage of gajami-sikhae fermentation at 5 °C and 10 °C. However, *L. brevis* and *Lactiplantibacillus plantarum* replaced it at 15°C and 20°C. *L. plantarum* and *L. brevis* increased in samples fermented at 15 °C and 20 °C and stabilized in the later fermentation stage, becoming the only dominant species. At low temperatures (5 °C and 10 °C), this species was not identified. A rapid increase in acidity and establishment of anaerobic conditions toward the later stage of fermentation is favorable for the growth of *Lactiplantibacillus* species since they adapt well to anaerobic and highly acidic conditions [20]. Thus, the relative abundance of *L. plantarum* and *L. brevis* reached 100% at the end of fermentation in the gajami-sikhae fermented at 20 °C.

3.4. Relationship between Environmental Factors and the Microbial Community

The correlations between the relative abundance of species involved in the fermentation of gajami-sikhae and factors such as the pH, acidity, fermentation temperature, and fermentation period were analyzed to determine the effect of these factors on the microbial composition (Figure 4). *L. sakei* was identified as the major species of the microflora in gajami-sikhae. Previously, *L. sakei* has been reported as the major microorganism found in Korean fermented foods such as kimchi [18,20]. This species showed a negative correlation with fermentation temperature (Pearson coeffect $r = -0.505$, $p = 1.384 \times 10^{-3}$), with a tendency to decrease when the temperature increased. This finding was consistent with

previous reports that *L. sakei* adapts well at low temperatures [18]. In contrast, *L. plantarum* (Pearson coeffect r = 0.614, p = 5.914 $\times$ 10^{-5}) and *L. brevis* (Pearson coeffect r = 0.523, p = 8.663 $\times$ 10^{-4}) demonstrated a positive correlation with fermentation temperature. Also, these LAB species showed a strong positive correlation with acidity (*L. plantarum*, Pearson coeffect r = 0.819, p = 1.986 $\times$ 10^{-9}; *L. brevis*, Pearson coeffect r = 0.600, p = 8.589 $\times$ 10^{-5}). Therefore, *L. plantarum* and *L. brevis* are well adapted to acidic environments and high temperatures [32].

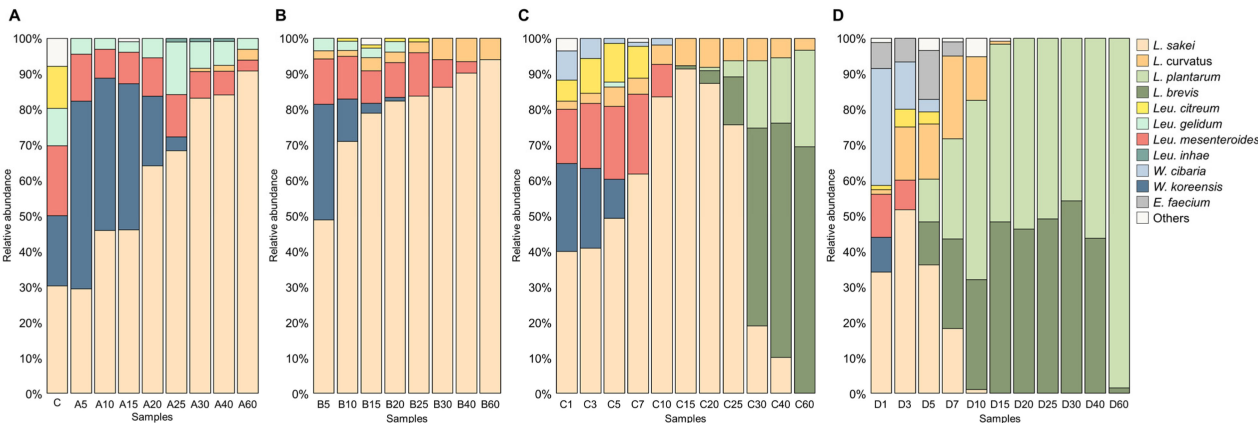

Figure 3. Changes in the LAB communities during the fermentation at (**A**) 5 °C, (**B**) 10 °C, (**C**) 15 °C, and (**D**) 20 °C. The graphs were generated by considering only the 3805 cultures identified at the species level (score ≥ 2.000). "Others" indicates species with a prevalence of 0.15%, including *P. pentosaceus*, *Lc. lactis*, *W. viridescens*, *W. hellenica*, *Leu. lactis*, and *W. kandleri*.

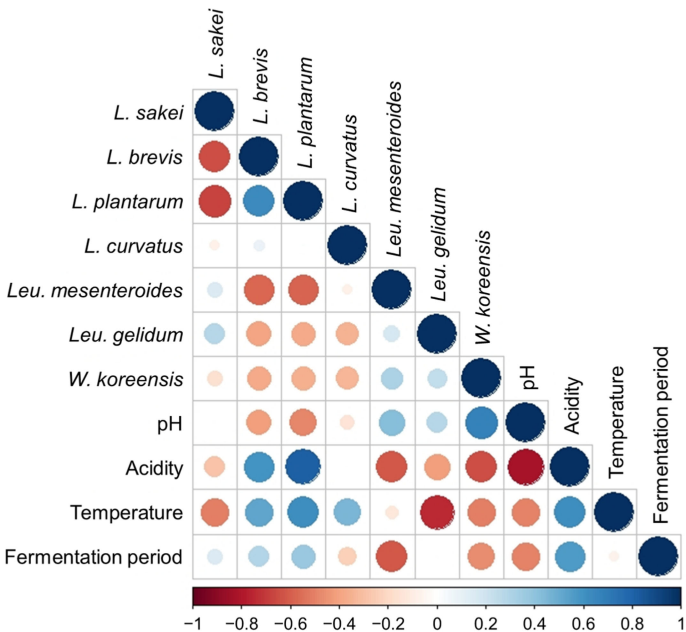

Figure 4. Pearson correlations calculated for the environmental factors (pH, acidity, fermentation temperature, and fermentation period) and relative abundance of major lactic acid bacterial species. The blue and red colors correspond to positive and negative correlations, respectively. The circle size and color intensity are proportional to the correlation coefficient.

Leu. mesenteroides and W. koreensis are often isolated from meat or vegetable products fermented at low temperatures and under weak acidic conditions [33,34]. *Leu. mesenteroides* and *W. koreensis* had a negative correlation with acidity (*Leu. mesenteroides*, Pearson coeffect

r = −0.614, *p* = 5.914 × 10^{-5}; *W. koreensis*, Pearson coeffect *r* = −0.647, *p* = 3.197 × 10^{-5}) and fermentation period (*Leu. mesenteroides*, Pearson coeffect *r* = −0.618, *p* = 5.914 × 10^{-5}; *W. koreensis*, Pearson coeffect *r* = −0.470, *p* = 2.791 × 10^{-3}). They demonstrated a tendency to decrease as the acidity and fermentation period increased. *Leu. gelidum* (Pearson coeffect *r* = −0.748, *p* = 2.463 × 10^{-7}) and *W. koreensis* (Pearson coeffect *r* = −0.501, *p* = 1.409 × 10^{-3}) showed a negative correlation with fermentation temperature. Therefore, the abundance of *Leu. gelidum* and *W. koreensis* decreased as the fermentation temperature increased, whereas that of *L. plantarum* and *L. brevis* increased. These results suggest that gajami-sikhae should be fermented at low temperatures to increase the proliferation of *Leuconostoc* and *Weissella* species. In addition, *Leuconostoc* and *Weissella* species are beneficial bacteria that provide the flavor of fermented foods [31].

The correlation analysis between the major species and environmental factors showed that *Leu. mesenteroides* and *W. koreensis* did not adapt well in an acidic environment, whereas *L. plantarum* and *L. brevis* adapted well. In addition, *L. sakei*, *Leu. gelidum*, and *W. koreensis* grew well at low temperatures (5 °C and 10 °C), whereas *L. plantarum* and *L. brevis* grew well at high temperatures (15 °C and 20 °C). This finding corresponds to the previous studies, which showed that heterofermentative LAB such as *Leu. mesenteroides* predominate under weaker acidic and lower anaerobic conditions during fermentation. Furthermore, heterofermentative LAB, such as *L. plantarum* (facultative heterofermentative species) and *L. brevis* (obligate heterofermentative species), become dominant as food fermentation conditions change to more anaerobic and acidic conditions [21,31,35].

Since only one batch was used in this study, variation between batches cannot be expected. According to a previous study, pyrosequencing data showed variation in the microbial compositions between gajami-sikhae samples from eight different manufacturers; the microbial compositions of two out of the eight gajami-sikhae samples were distinct from those of the rest [19]. In another study, 88 samples of kimchi, which has a similar microbial composition to gajami-sikhae, were examined to identify their microbial communities, and it was reported that there was little variation in microbial communities due to the shared ingredients and standardized manufacturing process [36]. Further research will be needed to observe the batch-to-batch variation in the microbial community in gajami-sikhae.

4. Conclusions

In this study, for the first time, we analyzed changes in the lactic acid bacterial community in gajami-sikhae using MALDI-TOF MS. Our studies accurately identified the LAB in gajami-sikhae at the species or genus level using MALDI-TOF MS and observed the dominant species. *L. sakei*, *L. plantarum*, *L. brevis*, *Leu. mesenteroides*, and *W. koreensis* were the key fermentative microbes in gajami-sikhae fermentation. The dominant species differed depending on the fermentation temperature and period, suggesting that the fermentation temperature and period are important indices determining the quality of gajami-sikhae. These results provide information on the fermentation conditions (fermentation temperature and period) of gajami-sikhae. Also, the information provided in this study will be useful in developing effective strategies for selecting bacterial strains. Future research should focus on sensory analysis and volatility profile analysis to improve the quality of gajami-sikhae.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods11070909/s1, Table S1: Information on sample type (fermented temperature and fermented period), Table S2: Difference in pH and acidity values according to the fermentation period, Table S3: Number of strains isolated from gajami-sikhae at the genus level, Figure S1: Changes in the LAB communities during the fermentation at (A) 5 °C, (B) 10 °C, (C) 15 °C, and (D) 20 °C. A graph generated by considering only for the 1019 cultures identified at the genus level (1.700–1.999). The others indicate species with a prevalence of 0.15%, including *P. pentosaceus*, *Lc. lactis*, *W. viridescens*, *W. hellenica*, *Leu. lactis*, and *W. kandleri*.

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Article

Protective Effects of Fermented Soybeans (*Cheonggukjang*) on Dextran Sodium Sulfate (DSS)-Induced Colitis in a Mouse Model

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Abstract: Inflammatory bowel disease (IBD) is a chronic inflammatory disease, and the incidence of IBD is increasing every year owing to changes in dietary structure. Although the exact pathogenesis of IBD is still unclear, recent evidence suggests that gut dysbiosis is closely associated with IBD pathogenesis. *Cheonggukjang* is a traditional Korean fermented soybean paste produced using traditional and industrial methods, and contains probiotics, which affect the gut microbiota composition. However, the protective effect of *Cheonggukjang* against IBD is unknown. In this study, we investigated the bacterial community structure of traditional and commercial *Cheonggukjang* samples, as well as the protective effect of *Cheonggukjang* on a dextran sulfate sodium (DSS)-induced colitis mouse model. Traditional and commercial *Cheonggukjang* were found to contain various type of useful probiotics in their bacterial community structure. *Cheonggukjang* reduced the progression of DSS-induced symptoms, such as body weight loss, colonic shortening, disease activity index, and histological changes. Further, *Cheonggukjang* improved the intestinal epithelial barrier integrity on DSS-induced colitis mice. In addition, *Cheonggukjang* suppressed the expression of proinflammatory cytokines and inflammatory mediators through the inactivation of NF-κB and MAPK signaling pathways. These results indicate that *Cheonggukjang* exerts protective effects against DSS-induced colitis, suggesting its possible application as a functional food for improving inflammatory diseases.

Keywords: inflammatory bowel disease; *Cheonggukjang*; dextran sulfate sodium (DSS)-induced colitis; protective effect; functional food

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1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestine, and its incidence is increasing every year owing to changes in the structure of diets [1–3]. Patients with IBD are known to have a lower quality of life than healthy individuals due to abdominal cramping, diarrhea, bloody diarrhea, fever, fatigue, symptoms of weight loss, and a higher risk of colitis-associated colorectal cancer [4]. Although many studies have shown that multiple factors, including genetic, microbial, environmental, and immune-mediated factors, are associated with IBD, its exact pathogenesis is complex and still unclear [2,5]. However, recent evidence suggests that gut dysbiosis is associated with IBD pathogenesis [6]. Under normal conditions, the mucosal immune system is precisely regulated; however, disruption of normal mucosal immunity to commensal microbiota results in chronic intestinal inflammation, and, consequently, IBD [7,8]. Proinflammatory cytokines, such as *IL-1β*, *IL-6*, and *TNF-α*, which are activated by the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, play a crucial

role in the colonic mucosal immune response in intestinal inflammation in patients with IBD [9–11].

Cheonggukjang is a traditional Korean fermented paste made by short-term fermentation of soybeans [12]. Various enzymes and physiologically active substances, such as dietary fiber, phosphatide, isoflavone, flavonoids, phenolic acids, saponins, trypsin inhibitors, and poly glutamic acid, are produced during *Cheonggukjang* fermentation [13]. These components show various biological activities, such as antioxidant, anti-atherosclerosis, anti-obesity, anti-diabetes, blood pressure-lowering, and osteoporosis prevention properties [13–15]. Additionally, probiotic strains, *Bacillus* and *Lactobacillus*, which were the dominant microbes at the genus level, have been reported in *Cheonggukjang* [16]. These probiotics are known to exert beneficial effects, including immune modulation, modulation of gut microbiota, displacement of pathogens, and production of bioactive compounds in the gastrointestinal tract of the host [17,18]. However, the protective effect of *Cheonggukjang* against IBD is unknown.

Cheonggukjang is typically produced using traditional or commercial methods, and its physicochemical and functional properties differ depending on the manufacturing method, soybean variety, microorganisms, and fermentation time [19]. Nowadays, consumers are highly interested in traditionally made *Cheonggukjang* products due to their consistent outstanding sensory quality. However, while traditionally made *Cheonggukjang* fermented with various regional microorganisms has better taste and aroma than commercial products fermented using certain strains, the functional difference is unknown [20]. Therefore, this study aimed to evaluate the protective effect of *Cheonggukjang* in a dextran sulfate sodium (DSS)-induced colitis mouse model, and the functional differences between traditional and commercial *Cheonggukjang*.

2. Materials and Methods

2.1. Antibodies

In this study, the following antibodies were used: anti-iNOS, anti-COX-2, anti-p-p38, anti-p38, anti-p-ERK, anti-p-JNK, anti-p-p65, anti-p65, anti-occludin, and anti-β-actin from Cell Signaling Technology (Danvers, MA, USA); anti-ERK and anti-JNK from Santa Cruz Biotechnology (Dallas, TX, USA); anti-ZO-1 from Abcam (Cambridge, UK).

2.2. Preparation of Cheonggukjang Samples

For this study, four different types of *Cheonggukjang* were obtained from the Microbial Institute for Fermentation Industry (Sunchang-gun, Jeollabuk-do, Korea). Moisture content and sample information of the *Cheonggukjang* samples were as follows: (1) S1 (60.94%, Sunchang-gun, Jeollabuk-do, Korea), (2) S2 (53.15%, Kangjin-gun, Jeollabuk-do, Korea), (3) S3 (48.45%, Paju-si, Gyeonggi-do, Korea), and (4) S4 (51.57%, Sunchang-gun, Jeollabuk-do, Korea). The *Cheonggukjang* samples S1–S3 were traditionally made, whereas the *Cheonggukjang* sample S4 was a commercial brand sample. The samples were dissolved in distilled water at 500 mg/kg, and then stored at −20 °C before oral administration to mice.

2.3. Bacterial Community Analysis of Cheonggukjang by Next-Generation Sequencing (NGS)

Bacterial community analysis of *Cheonggukjang* was performed using an NGS, as described previously [21]. Briefly, the total DNA from the collected *Cheonggukjang* samples was extracted by DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), and amplified with V3-V4 regions of 16S rRNA gene targeting primers. Libraries of the PCR amplicon were prepared by Nextera XT DNA Library Prep Kit (Illunina, San Diego, CA, USA), and sequencing was performed using 300 bp paired-end reads on the Illumina Miseq platform at the Microbial Institute for Fermentation Industry (Sunchang, South Korea). Obtained raw fastq data were analyzed using Mothur package v. 1.36. Chimeric, low-quality, and non-bacterial reads were removed, and the remaining sequences were grouped into single operational taxonomic units (OTUs) against the SILVA bacterial database v. 12350, and all

reads within 97% similarity were clustered by a single OTUs sequence. Sequences were taxonomically classified at different levels (phylum, class, order, family, genus, and species). The bacterial clustering of each sample collected from different regions was performed by principal component analysis using the R package. The α-diversity indices, such as Chao and Shannon, were calculated by the Mothur program.

2.4. Experimental Animals

Specific pathogen-free (SPF)-grade BALB/c mice (male, 5-week-old, n = 35) were purchased from Damool Science (Daejeon, Korea), and acclimated for a week. The mice were housed in a room maintained on a 12 h light/dark cycle at 22 ± 2 °C and a relative humidity of 55 ± 5%. All animals were cared for according to the guidelines of the Animal Care Committee of Jeonju AgroBio-Materials Institute (Jeonju, Korea). All experimental procedures were approved by the Animal Care Committee of Jeonju AgroBio-Materials Institute (JAMI IACUC 2021001, Jeonju, Korea).

2.5. DSS-Induced Colitis and Cheonggukjang Treatment

The animals were divided into seven groups (five mice/group) according to the treatment: NOR group (normal control), DSS group [5% DSS (MP Biomedicals, Irvine, CA, USA)], PC group [positive control; 5% DSS + 50 mg/kg/day of 5-aminosalicylic acid (5-ASA; Sigma-Aldrich, St. Louis, MO, USA)], S1 group (5% DSS + 500 mg/kg/day of S1 sample), S2 group (5% DSS + 500 mg/kg/day of S2 sample), S3 group (5% DSS + 500 mg/kg/day of S3 sample), and S4 group (5% DSS + 500 mg/kg/day of S4 sample). The NOR and DSS groups were administered distilled water. *Cheonggukjang* samples (S1–S4) were orally administered at a dose of 500 mg/kg once a day for 15 days. To induce colitis, mice were administered 5% DSS in drinking water for seven days, and then sacrificed after one day.

2.6. Disease Activity Index (DAI)

DAI was evaluated, as described previously [22]. Briefly, the severity of colonic inflammation was assessed by summing the scores for weight loss, stool viscosity, and stool bleeding status, as shown in Table 1.

Table 1. Disease activity index (DAI) score.

Score	Body Weight Decrease (%)	Stool Consistency	Fecal Bleeding
0	0	Normal	No bleeding
1	1–5		
2	5–10	Soft stools	Slight bleeding
3	11–15		
4	>15	Diarrhea	Gross bleeding

2.7. ELISA

Serum levels of *TNF-α*, *IL-6*, and *IL-1β* were determined using ELISA kits (R&D Systems R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's protocol.

2.8. Quantitative Real-Time PCR (qRT-PCR)

Mouse colon tissues were homogenized using ice-cold TRIzol reagent (MRC, Cincinnati, OH, USA). cDNA was synthesized by reverse transcription of 1 μg of RNA samples using a cDNA Synthesis Kit (Bio-Fact, Daejeon, Korea). The relative mRNA levels were calculated using the comparative Ct method. B-actin was used as the reference gene. The primer sequences are listed in Table 2.

Table 2. Primer Sequences.

Gene	Forward (5′-3′)	Reverse (5′-3′)
TNF-α	AACTAGTGGTGCCAGCCGAT	CTTCACAGAGCAATGACTCC
IL-6	TGTCTATACCACTTCACAAGTCGGAG	GCACAACTCTTTTCTCATTTCCAC
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
iNOS	CGAAACGCTTCACTTCCAA	TGAGCCTATATTGCTGTGGCT
COX-2	TTTGGTCTGGTGCCTGGTC	CTGCTGGTTTGGAATAGTTGCTC
MUC-2	GCAGTCCTCAGTGGCACCTC	CACCGTGGGGCTACTGGAGAG
MUC-3	CGTGGTCAACTGCGAGAATGG	CGGCTCTATCTCTACGCTCTC
β-actin	CGGTTCCGATGCCCTGAGGCTCTT	CGTCACACTTCATGATGGAATTGA

2.9. Western Blot Assay

Colon tissues were homogenized in a lysis buffer (Thermo Scientific, Rockford, MD, USA) containing a protease inhibitor cocktail (GenDEPOT, Katy, TX, USA). The total protein samples (25 μg per lane) were separated by SDS-PAGE, and electroblotted onto a PVDF membranes (Merck Millipore, Billerica, MA, USA). Membranes were analyzed using the specified antibodies using ECL kit (GE Healthcare, Buckinghamshire, UK), and the images were captured using an Amersham Imager 600 (GE Healthcare).

2.10. Histological Analysis

Colon tissues were fixed with 10% formalin and embedded in paraffin. Tissue sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and Alcian blue. Images were analyzed using a microscope (Olympus, Tokyo, Japan). Colon tissue damage was scored, as described previously [23].

2.11. IHC Staining

Paraffin sections (4 μm thick) were deparaffinized with xylene three times for 7 min, and rehydrated using ethanol and water. Peroxidase activity was blocked using 0.3% H_2O_2 for 15 min. Antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0) in a microwave for 15 min. The tissue sections were pre-blocked with 4% bovine serum albumin for 30 min, and then incubated overnight at 4 °C with antibodies, followed by an anti-Rabbit Envision plus polymer kit (Dako, Glostrup, Denmark). The sections were stained with hematoxylin. Images were analyzed using a microscope (Olympus).

2.12. Statistical Analysis

Statistical analyses were performed using Tukey's post-hoc tests with GraphPad Prism (version 5.0; GraphPad Software, Inc., San Diego, CA, USA). Data are presented as the mean ± standard deviation (SD). For all experiments, a *p*-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Bacterial Community Structure in Cheonggukjang Samples

The gut microbiota plays an essential role in the progression of intestinal inflammation in IBD, and it is known that IBD is associated with an imbalance of intestinal bacteria [24]. Probiotic strains can be used in the treatment and prevention of IBD in animal models of colitis, although the exact mechanism is unknown [18]. Various biological and pharmacological properties of *Cheonggukjang* have been demonstrated in animal models [13–15]. Furthermore, probiotics in cheonggukjang have been reported to relieve gut dysbiosis, which is closely associated with IBD [6]. However, several studies reported that traditionally made *Cheonggukjang* showed better bioactivity, such as glucose dysregulation, memory impairment, and immunity, compared to commercial *Cheonggukjang* [20]. These differences have been reported to be associated with bacteria-driven changes in the fermentation

process [15]. Therefore, we performed 16S rRNA sequencing using NGS to analyze the bacterial community of traditionally made *Cheonggukjang* samples from different regions (S1, Sunchang-gun, Jeollabuk-do, South Korea; S2, Gangjin-gun, Jeollabuk-do, South Korea; S3, Paju-si, Gyeonggi-do, South Korea) and a commercial *Cheonggukjang* brand sample (S4). The relative abundances of different bacteria in the *Cheonggukjang* samples (S1–S4) at the phylum and class levels are shown in Figure 1a,b.

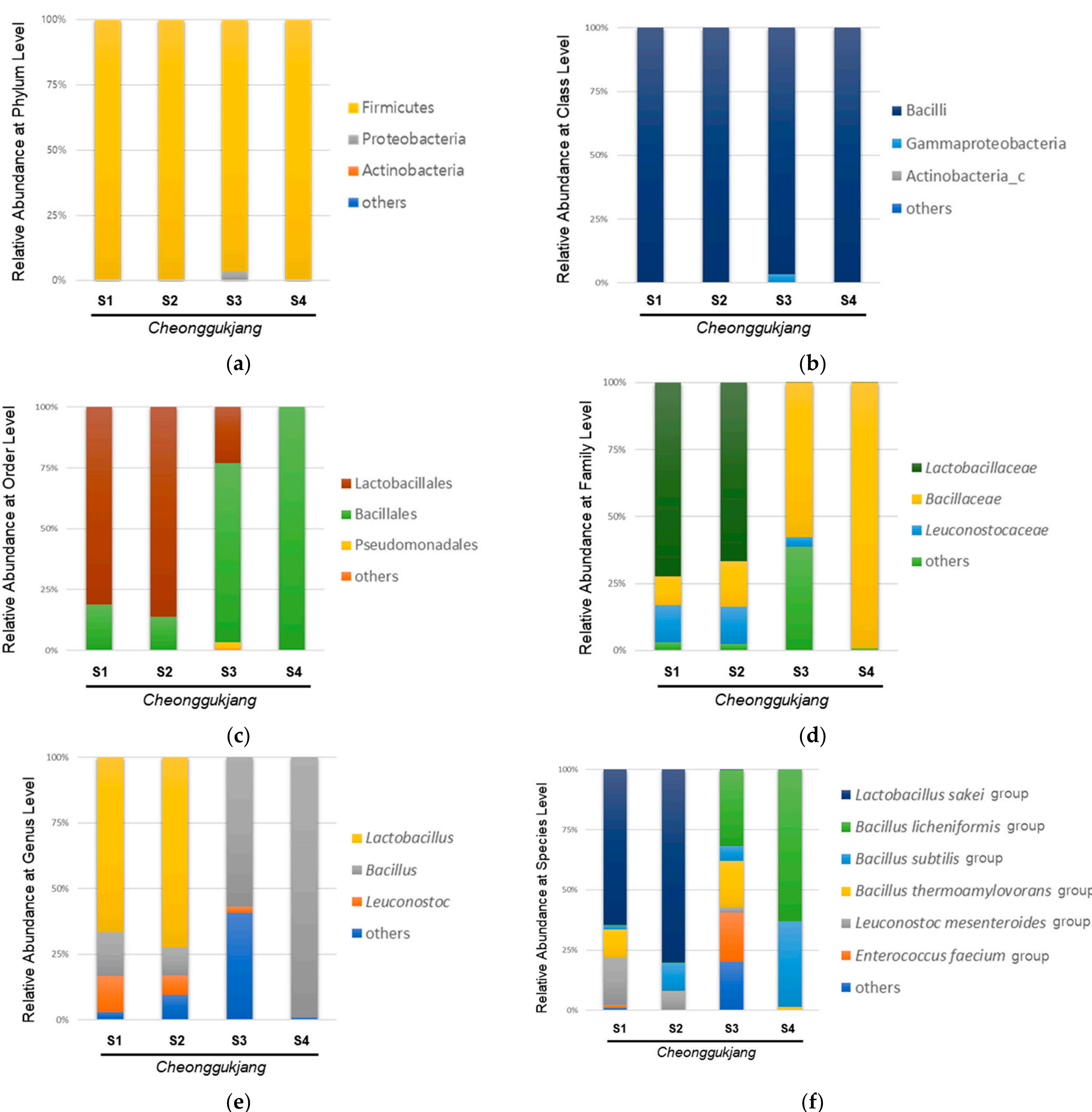


Figure 1. Microbial composition in the cheonggukjang samples (S1–S4): Relative abundance (%) at (**a**) the phylum level; (**b**) class level; (**c**) order level; (**d**) family level; (**e**) genus level; (**f**) species level.

All *Cheonggukjang* samples (S1–S4) were dominated by *Firmicutes* and *Bacilli* at the phylum and class levels, respectively. As shown in Figure 1c, the commercial (S4) and traditionally made *Cheonggukjang* (S1–S3) samples showed different bacterial community

structure at the order level. *Lactobacillales* showed the highest abundance in the traditionally made *Cheonggukjang* samples (S1, 81.1%; S2, 86.2%), except for S3 (*Lactobacillales*, 23.1%; *Bacillales*, 73.5%), whereas, *Bacillales* showed the highest abundance in the commercial cheonggukjang sample (S4) (99.9%). As shown in Figure 1d,e, the traditionally made *Cheonggukjang* samples S1 and S2 were dominated by *Lactobacillaceae* (S1, 66.6%; S2, 72.3%) and *Lactobacillus* (S1, 66.5%; S2, 72.3%) at the family and genus levels, respectively. The traditionally made S3 and commercial *Cheonggukjang* (S4) samples were dominated by *Bacillaceae* (S3, 57.7%; S4, 99.2%) and *Bacillus* (S3, 56.9%; S4, 99.1%) at the family and genus levels, respectively. At the species level, the traditionally made *Cheonggukjang* samples S1 and S2 were dominated by the *Lactobacillus sakei* group (S1, 44.8%; S2, 72.1%), while the traditionally made S3 and commercial *Cheonggukjang* (S4) samples were dominated by the *Bacillus licheniformis* group (S3, 30.0%; S4, 62.0%). Among the traditionally made *Cheonggukjang*, the *Enterococous faecium* group was observed to be higher in S3 (Gyeonggi-do, Korea) than S1 and S2 (Jeollabuk-do, Korea). Since *Cheonggukjang* is fermented by various microorganisms present in the manufacturing environment [16], the difference of bacterial community in traditionally made *Cheonggukjang* samples could be due to region-specific microorganisms. These results suggest that the bacterial community of *Cheonggukjang* may differ depending on the manufacturing method used and region.

3.2. Cheonggukjang Attenuates the Progression of DSS-Induced Colitis

To evaluate the protective effect of *cheonggukjang* against DSS-induced colitis, we designed an animal experiment, as shown in Figure 2a. To evaluate the disease progression in DSS-induced colitis, we first measured body weight lost. As shown in Figure 2b,c, mice in the DSS-treated group had a considerable body weight loss compared to the mice in the normal group (NOR), and the reduced body weight after treatment with DSS was considerable restored by administration of 5-ASA (PC) and *Cheonggukjang* samples S1 and S2. Symptoms of DSS-induced colitis were evaluated using the DAI, which is based on body weight loss, rectal bleeding, and stool consistency (Table 1). As shown in Figure 2d, the DAI of the DSS group was considerably higher than that of the NOR group. However, treatment with PC and *Cheonggukjang* samples S1 and S2 considerable ameliorated the DAI compared to the DSS only group. Next, to confirm the protective effect of *Cheonggukjang* samples against the progression of DSS-induced colitis, we evaluated the reduction in colon length, an indicator of the severity of intestinal inflammation in DSS-induced colitis. As shown in Figure 2e,f, colon length was considerable shorter in the DSS group than in the NOR group. However, this phenomenon was considerable alleviated in the PC and *Cheonggukjang* (S1, S2) treated groups. According to an analysis of bacterial community, the S1 and S2 samples were dominated by *Lactobacillus* and the S3 and S4 samples were dominated by *Bacillus* at Genus level. *Lactobacillus* and *Bacillus* strains are widely used as probiotics, and are known to benefit the gut environment [17,18]. Therefore, we found that *Lactobacillus* may have a more protective effect than *Bacillus* in DSS-induced colitis. These findings suggest that *Cheonggukjang* containing probiotics exerts a protective effect on the progression of DSS-induced colitis. We also investigated whether cheonggukjang could affect the gut environment, but did not find any significant differences of bacterial community between the groups (data not shown).

3.3. Cheonggukjang Improves Histological Changes on DSS-Induced Colitis

Under normal conditions, the colorectal tissue consists of the epithelium, crypt structure, mucosa layer, and mucosa substratum. However, colonic inflammation induced by DSS induces histological changes, such as irregular surface epithelium, depleted goblet cells, distorted and shallow crypt structures, and increased inflammatory cell infiltration [25]. To evaluate histological changes, we performed H&E staining and determined the histological score, as previously described [23]. As shown in Figure 3a, the DSS group showed depleted goblet cells and increased inflammatory cell infiltration in colon tissue. These histological changes were considerably ameliorated in the PC- and *Cheonggukjang* (S1–S4)-treated

groups. As shown in Figure 3b, the increased histological score in the DSS group was considerable reduced in the PC- and *Cheonggukjang*-treated groups. These results suggest that *Cheonggukjang* improves histological changes in mice with DSS-induced colitis.

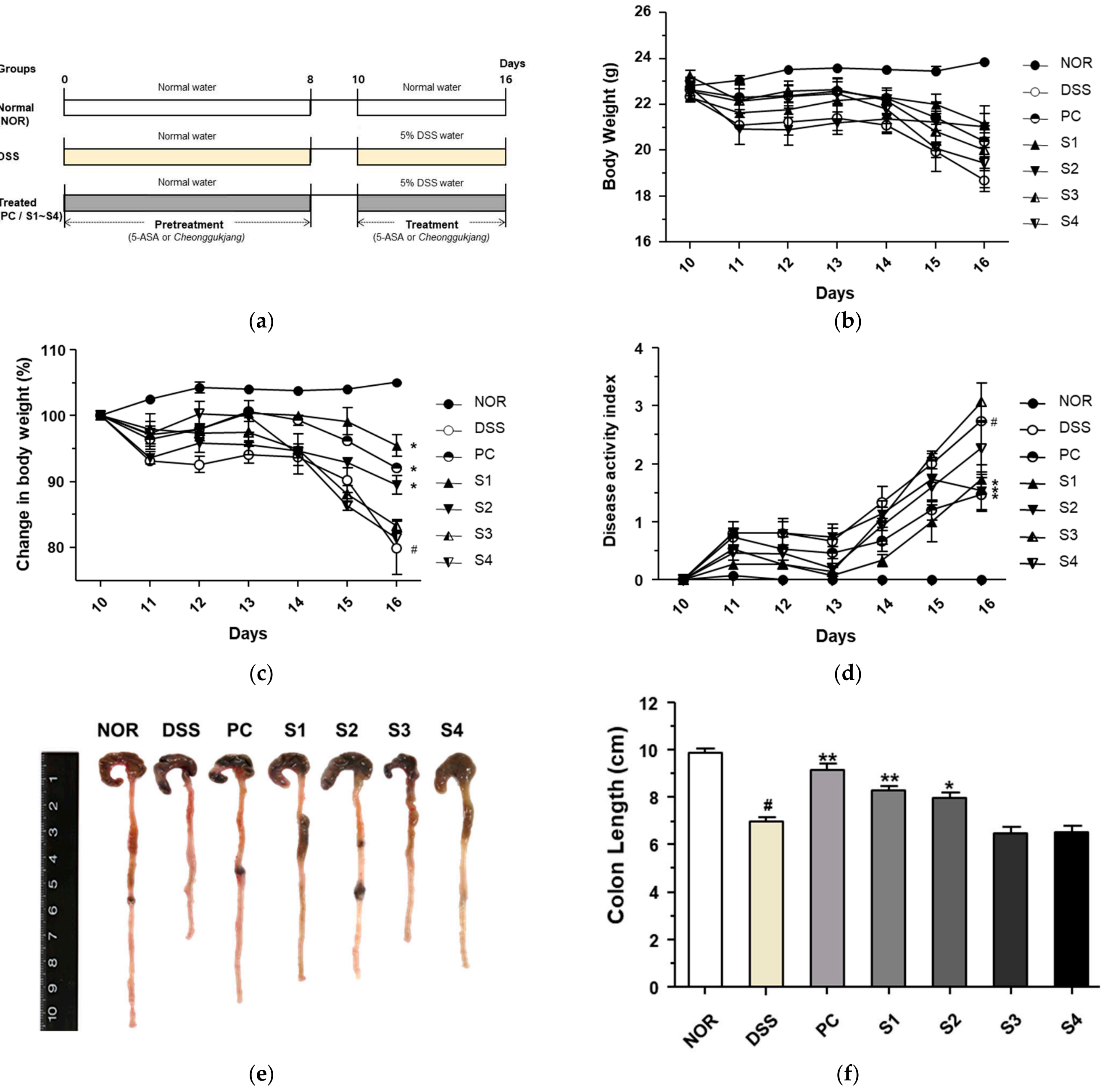


Figure 2. Protective effect of *Cheonggukjang* on the progression of dextran sulfate sodium (DSS)-induced colitis. (**a**) Schematic representation for animal experiments; (**b**) Body weight (g); (**c**) Change in body weight (%); (**d**) Disease activity index; (**e**) Representative images of colon tissue in each group; (**f**) Colon length (cm). Values are presented as the mean ± standard deviation (SD) (n = 5); #, $p < 0.005$ versus normal group; **, $p < 0.005$; *, $p < 0.05$ versus DSS group.

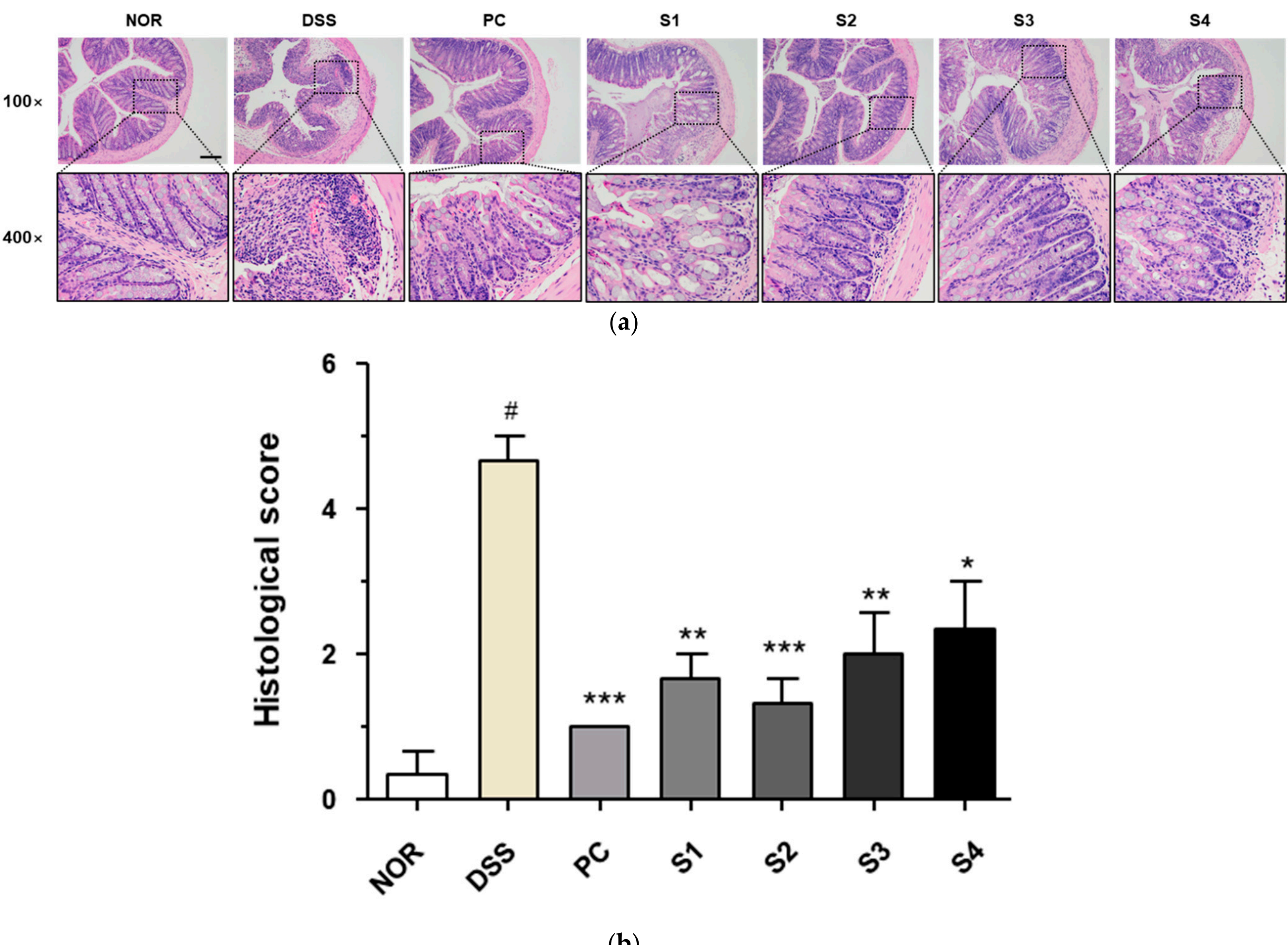


Figure 3. Histological changes in colorectal tissues of DSS-induced colitis mice: (**a**) Representative H&E images of colon tissue. Scale bar; 100 µm; (**b**) Histological score. Values are presented as the mean ± SD (n = 5); #, $p < 0.005$ versus normal group; ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$ versus DSS group.

3.4. *Cheonggukjang* Reduces the Expression of Proinflammatory Cytokines on DSS-Induced Colitis

DSS-induced colitis is associated with the induction of proinflammatory cytokines, such as *TNF-α*, *IL-6*, and *IL-1β* [26], and its expression is modulated by probiotics [27]. Thus, we investigated the expression of these markers to evaluate the effect of *Cheonggukjang* samples (S1–S4) containing probiotics on inflammation in DSS-induced mice. As shown in Figure 4a–c, the DSS group showed increased mRNA levels of *TNF-α*, *IL-6*, and *IL-1β* in colonic tissue. These increased mRNA levels were reduced significantly in the PC- and *Cheonggukjang*-(S1–S4) treated groups. Next, to confirm these effects, we evaluated the secretion of *TNF-α*, *IL-6*, and *IL-1β* in the blood. The secretion of *TNF-α*, *IL-6*, and *IL-1β* in the blood was increased in the DSS alone group, compared to those in the control group, as shown in Figure 4d–f. This increased secretion was significantly reduced in the PC- and *Cheonggukjang* (S1–S4)-treated groups, similar to the findings at the mRNA level. These results suggest that *Cheonggukjang* inhibits the mRNA and protein secretion of proinflammatory cytokines in mice with DSS-induced colitis.

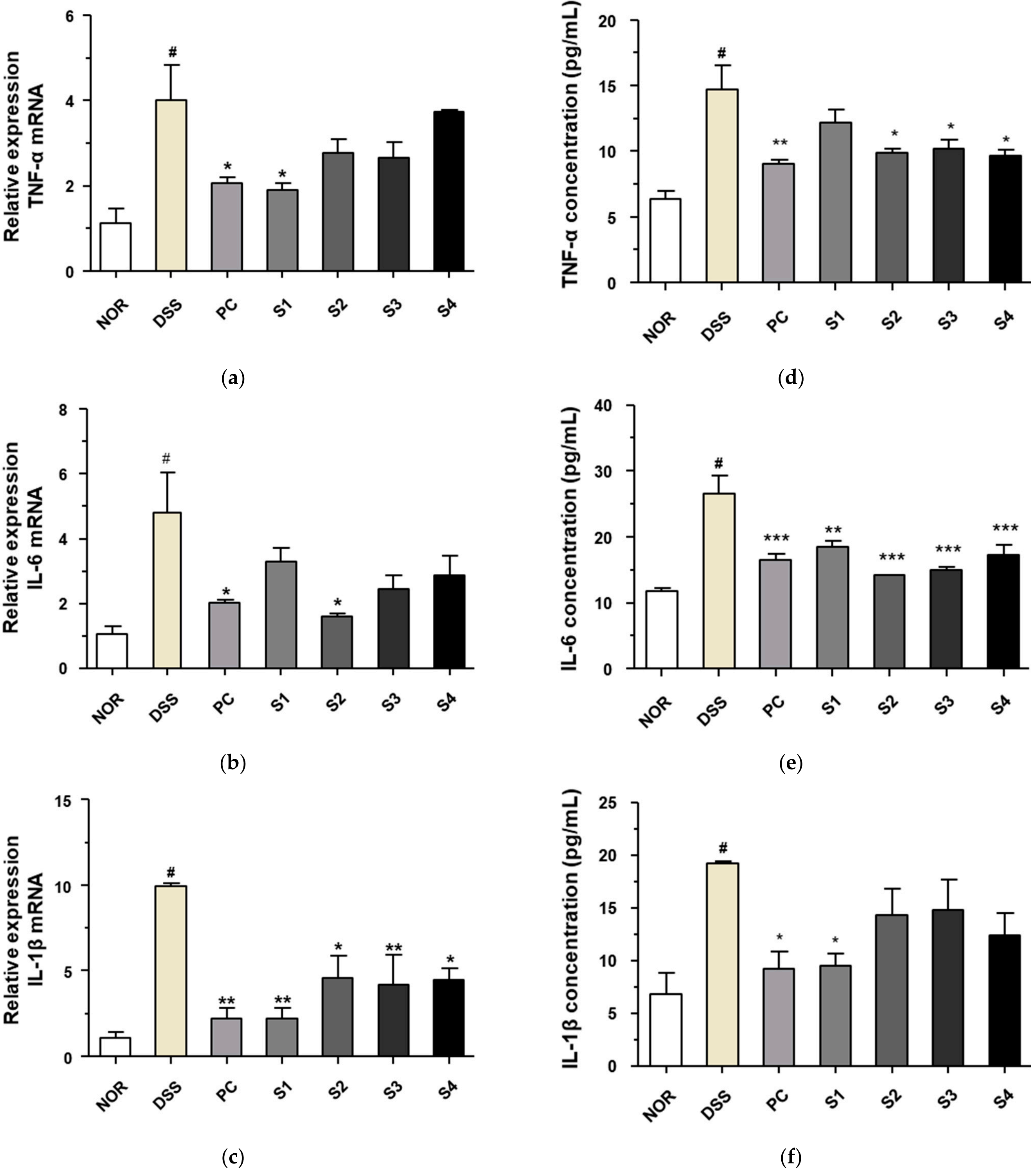


Figure 4. Inhibitory effect of *Cheonggukjang* on the mRNA and protein secretion of proinflammatory cytokines in DSS-induced colitis mice: mRNA levels of (**a**) *TNF-α*, (**b**) *IL-6*, and (**c**) *IL-1β* in colonic tissues; Protein levels of (**d**) *TNF-α*, (**e**) *IL-6*, and (**f**) *IL-1β* in the serum. Values are presented as the mean ± SD (n = 5); #, $p < 0.005$ versus normal group; ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$ versus DSS group.

3.5. *Cheonggukjang* Suppresses Activation of NF-κB and MAPKs Signaling Pathways on DSS-Induced Colitis

NF-κB and MAPK are important signaling pathways that play a role in inducing the inflammatory response in DSS-induced colitis models [28]. Therefore, we measured the expression of these signaling molecules to evaluate the inhibitory activity of *Cheonggukjang* against DSS-induced colitis. As shown in Figure 5a, the level of phosphorylated NF-κB p65 was significantly increased in the DSS alone group compared to that in the control group. DSS-induced phosphorylation of NF-κB p65 was significantly decreased upon treatment with PC and *Cheonggukjang* (S1–S4). Levels of inflammatory enzymes, such as COX-2 and iNOS, are controlled by the proinflammatory transcription factor NF-κB p65 [29]. Thus, we evaluated the protein and mRNA levels of COX-2 and iNOS. The protein levels of COX-2 and iNOS were increased in the DSS alone group compared to those in the control group, similar to the expression of NF-κB p65. DSS-induced protein levels of COX-2 and iNOS decreased significantly upon treatment with PC and *Cheonggukjang* (S1–S4). We also confirmed the mRNA levels of COX-2 and iNOS after treatment with PC and *Cheonggukjang* (S1–S4) (Figure 5b,c). Next, we evaluated the effect of *Cheonggukjang* samples (S1–S4) on the MAPK signaling pathways. As shown in Figure 5d, the levels of phosphorylated p38, JNK, and ERK were significantly increased in the DSS alone group, compared to those in the control group. DSS-induced phosphorylation of p38, JNK, and ERK decreased significantly upon treatment with PC and *Cheonggukjang* (S1–S4). These results suggest that *Cheonggukjang* can attenuate DSS-induced colonic inflammation by modulating the NF-κB and MAPK signaling pathways.

3.6. *Cheonggukjang* Improves Intestinal Epithelial Barrier Integrity by Modulating Mucins and Tight Junction Proteins in DSS-Induced Mice

In the normal intestine, mucins are expressed and secreted by goblet cells to protect the mucus layer; however, IBD often causes a loss of the mucin layer and goblet cell mucin [30]. Therefore, to evaluate histological changes, such as mucin and goblet cell depletion, Alcian blue staining was performed. As shown in Figure 6a, the control group contained well-organized mature goblet cells. However, the DSS-treated group showed histological changes, such as mucin and goblet cell depletion, compared to the control group. These histological changes were attenuated in the PC- and *Cheonggukjang* (S1–S4)-treated groups. Muc2 is a secreted gel-forming mucin and the main structural component of the protective mucus layer of the intestine [31]. It has been reported that mucin expression, such as Muc2 and Muc3, reduces with the progression of DSS-induced colitis in various animal model [30]. Therefore, we evaluated the expression of mucins to protect the mucus layer. As shown in Figure 6b,c, the reduced mRNA levels of *Muc2* and *Muc3* by DSS were attenuated upon treatment with PC and *Cheonggukjang* (S1–S4).

IBD compromises epithelial barrier functions by reducing tight junction proteins, including transmembrane barrier proteins (occludin) and cytoplasmic scaffolding proteins (ZO-1) [32]. Therefore, to examine the effect of *Cheonggukjang* samples (S1–S4) on intestinal barrier function after DSS treatment, tight junction protein expression was determined by immunohistochemical staining. As shown in Figure 6d,e, the expression levels of occludin and ZO-1 were significantly reduced in DSS-treated mice relative to those in the control group. However, PC and *Cheonggukjang* (S1–S4) treatment elevated the protein expression of occludin and ZO-1 compared to the DSS alone group. These results suggest that *Cheonggukjang* may improve intestinal epithelial barrier integrity by modulating mucins and tight junction proteins.

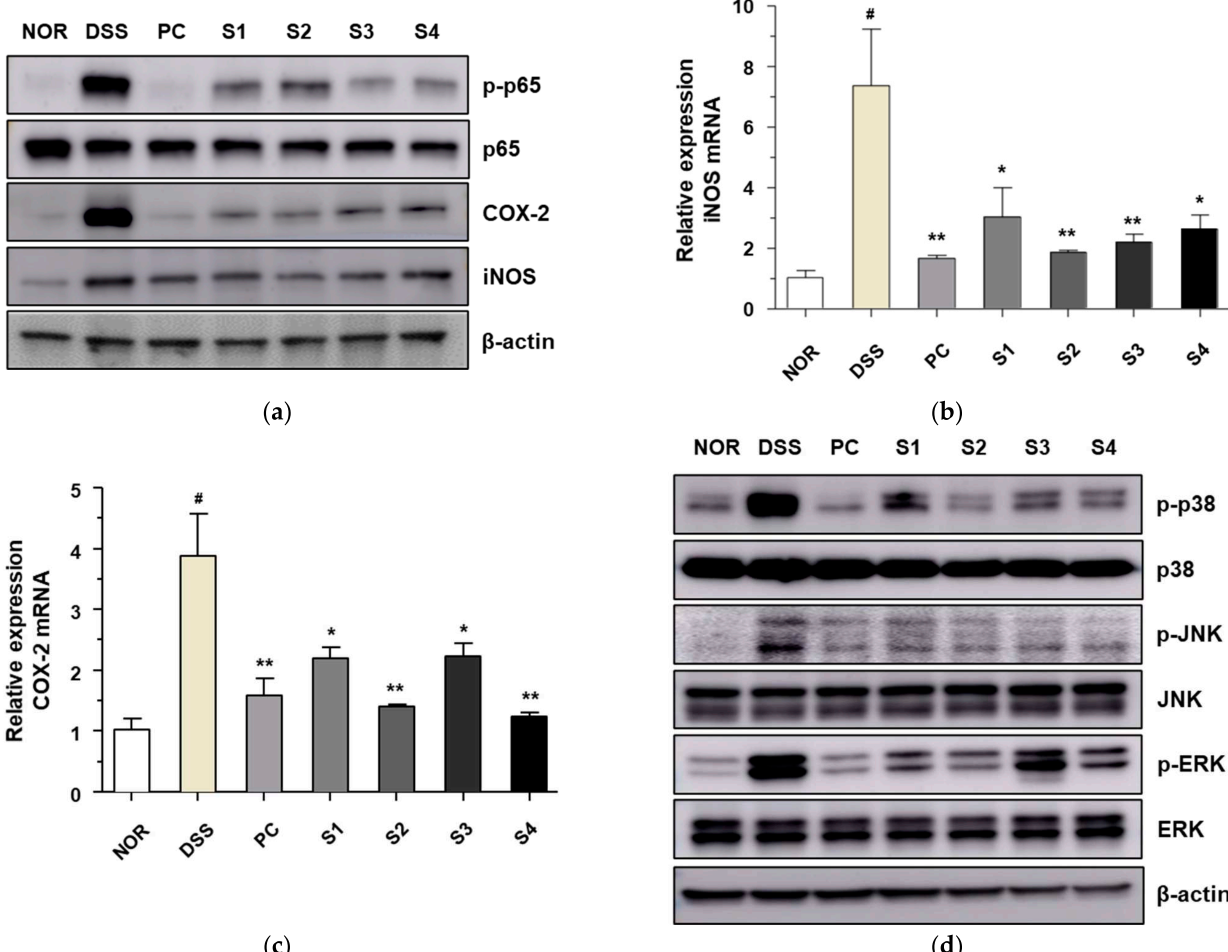
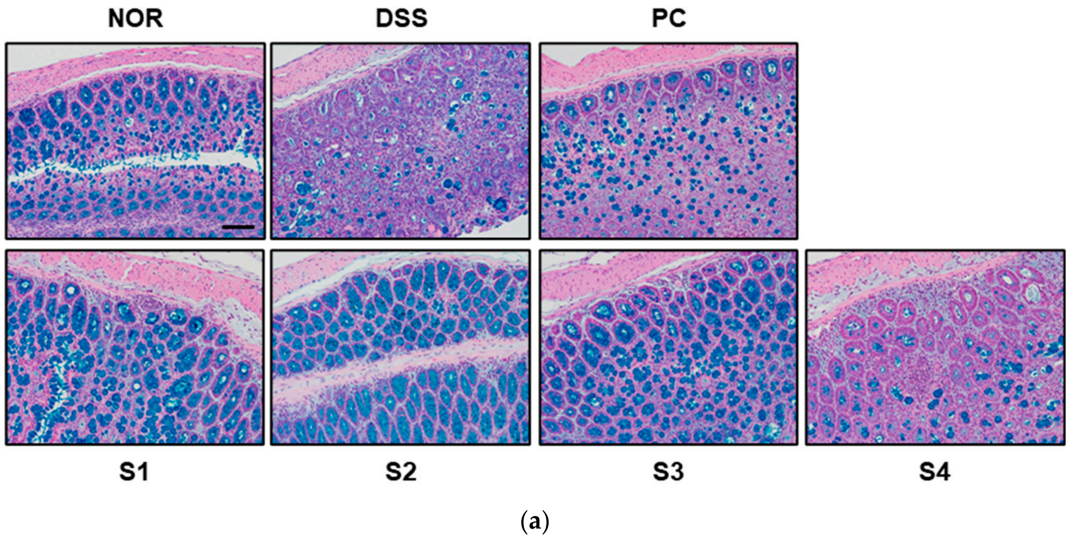


Figure 5. Inhibitory effect of *Cheonggukjang* on NF-κB and MAPKs signaling pathway in DSS-induced colitis mice: (**a**) The protein levels of phosphorylated p65, p65, COX-2, and iNOS were determined via western blot assay; mRNA levels of (**b**) iNOS and (**c**) COX-2 were analyzed by quantitative real-time PCR; (**d**) The protein levels of total and phosphorylated p38, JNK, and ERK were determined via western blot assay. Values are presented as the mean ± SD (n = 5); #, $p < 0.005$ versus normal group; **, $p < 0.005$; *, $p < 0.05$ versus DSS group.



(**a**)

Figure 6. *Cont.*

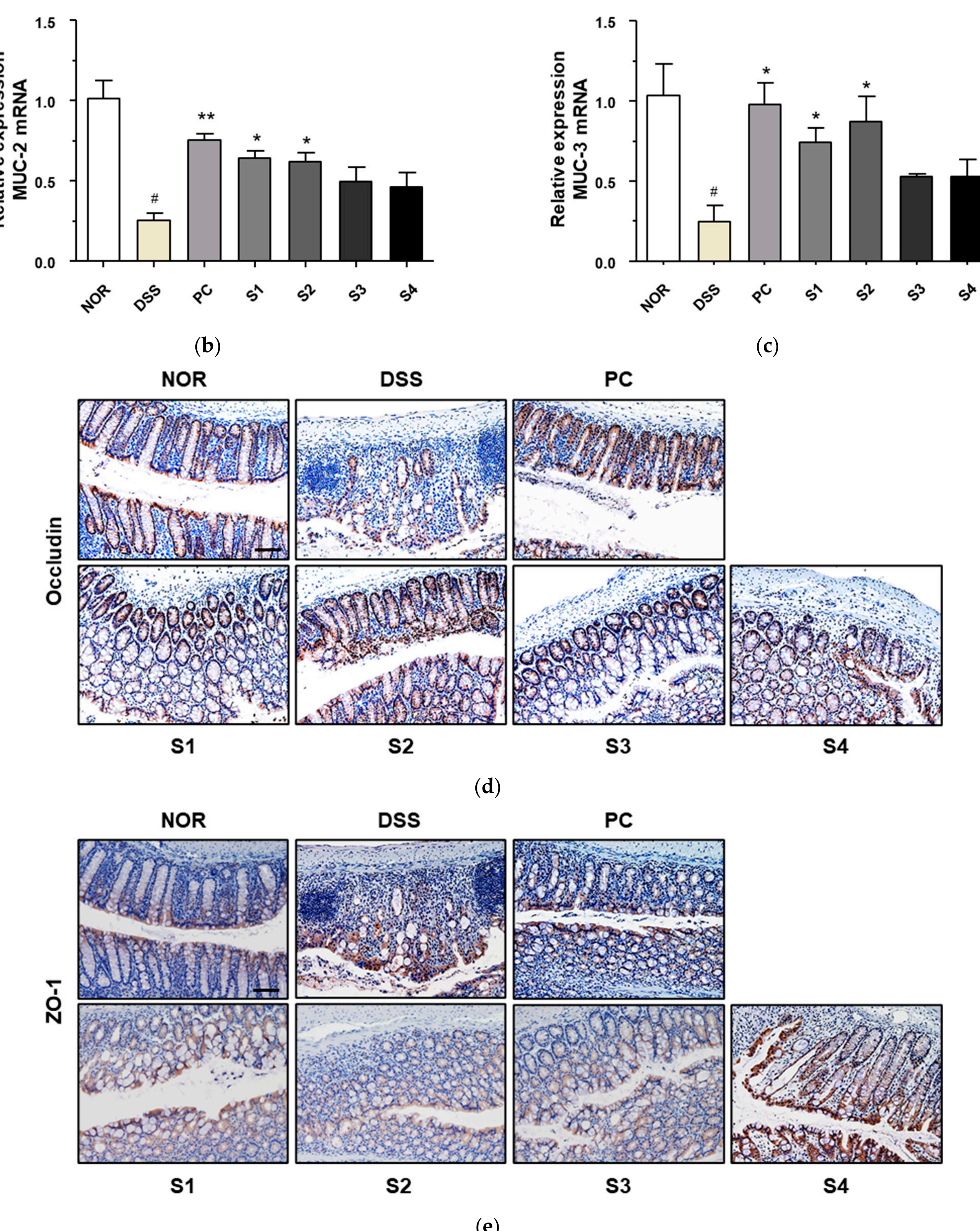
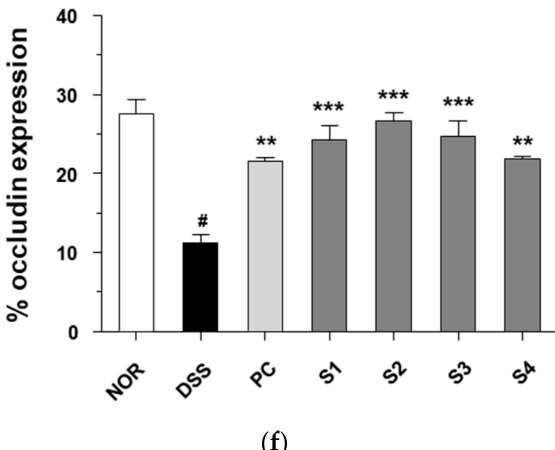
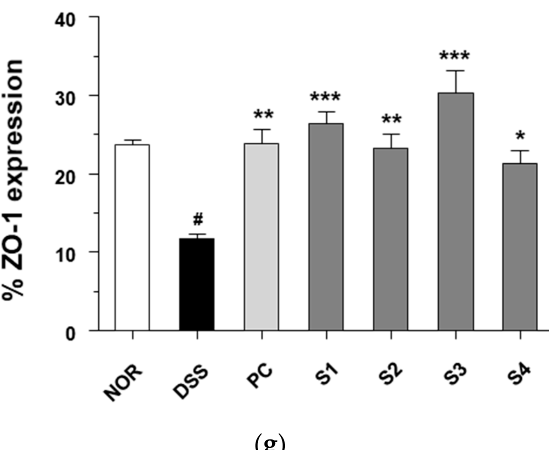


Figure 6. *Cont.*



(f)



(g)

Figure 6. Effect of *Cheonggukjang* on the expression of mucins and tight junction protein in DSS-induced colitis mice: (**a**) Representative Alcian blue staining images of colon tissue. The abundance of goblet cells and mucin production was determined. Magnification (200×), Scale bar; 50 μm; mRNA levels of (**b**) *Muc2* and (**c**) *Muc3* were analyzed by quantitative real-time PCR; (**d**,**e**) Representative immunohistochemistry images of colon tissue. Magnification (200×), Scale bar, 50 μm; (**f**,**g**) Quantification of occludin and ZO-1 expression by IHC. Values are presented as the mean ± SD (n = 5); #, $p < 0.005$ versus normal group; ***, $p < 0.001$; **, $p < 0.005$; *, $p < 0.05$ versus DSS group.

4. Conclusions

In this study, we analyzed the bacterial community structure of *Cheonggukjang* using traditionally made and commercial *Cheonggukjang* samples to evaluate whether the protective effect differs depending on the manufacturing method. We observed that both traditionally made and commercial *Cheonggukjang* contain various types of useful probiotics. We also observed that both traditional and commercial *Cheonggukjang* significantly ameliorated DSS-induced symptoms, such as body weight loss, colonic shortening, DAI, and histological changes, and improved intestinal epithelial barrier integrity on DSS-induced colitis mice. Furthermore, we showed that *Cheonggukjang* can attenuate DSS-induced colonic inflammation by suppression of NF-κB and MAPK signaling pathways. These findings suggest that *Cheonggukjang* can be useful as a functional food to improve inflammatory diseases such as colitis, regardless of the manufacturing method.

Author Contributions: Conceptualization, H.-J.Y., D.-Y.J. and S.-Y.K.; validation, H.-J.L.; formal analysis, H.-J.L.; investigation, H.-J.L., H.-R.K., S.-J.J. and M.S.R.; data curation, H.-J.L. and C.-H.J.; writing—original draft preparation, C.-H.J.; writing—review and editing, S.-Y.K. and C.-H.J.; visualization, H.-J.L. and C.-H.J.; supervision, S.-Y.K. and C.-H.J.; project administration, S.-Y.K. and C.-H.J.; funding acquisition, D.-Y.J. and S.-Y.K. All authors have read and agreed to the published version of the manuscript.

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Article

Effects of Bacillus Subtilis-Fermented White Sword Bean Extract on Adipogenesis and Lipolysis of 3T3-L1 Adipocytes

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Abstract: To investigate the adipogenesis and lipolysis effects of the Bacillus subtilis-fermented white sword bean extract (FWSBE) on 3T3-L1 adipocytes, we treated 3T3-L1 preadipocytes before and after differentiation with FWSBE and measured triglyceride, free glycerol, mRNA, and protein levels. First, FWSBE reduced the cell viability of 3T3-L1 pre-adipocytes under 1000 μg/mL conditions. Triglyceride accumulation in 3T3-L1 pre-adipocytes was suppressed, and free glycerol content in mature 3T3-L1 adipocytes was increased in the FWSBE treatment groups, indicating that FWSBE has anti-obesity effects. Further, FWSBE suppressed adipogenesis in 3T3-L1 pre-adipocytes by lowering the protein levels of C/EBPα, PPARγ, and FAS and increasing the level of pACC and pAMPK. Additionally, FWSBE promoted lipolysis in mature 3T3-L1 adipocytes by increasing the transcription levels of Ppara, Acox, and Lcad and the protein levels of pHSL and ATGL. Thus, we suggest that FWSBE can be a potential dietary supplement because of its anti-obesity properties.

Keywords: *Canavalia gladiata*; triglyceride; glycerol; AMP-activated protein kinase; peroxisome proliferator-activated receptor

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1. Introduction

Obesity is associated with various metabolic complications, such as type 2 diabetes, cardiovascular disease, high blood pressure, and dyslipidemia. The growing prevalence of obesity is a public health concern among many modern societies [1,2]. In general, obesity is caused when excessive energy is accumulated in white adipose tissue in the form of triglyceride (TG), which is composed of three fatty acids and glycerol [3]. Adipogenesis is the process of differentiation from pre-adipocytes to adipocytes caused by the stepwise action of adipogenic transcription factors. The three elements mainly involved are CCAAT-enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptors (PPARs), and sterol regulatory element-binding proteins (SREBPs) [4,5]. Among these elements, C/EBPα and PPARγ control the levels of adipocyte fatty acid binding protein (aP2), adiponectin, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS). These determine the actual adipocyte phenotype and intracellular lipid accumulation [6,7]. However, when the ratio of AMP/ATP increases, AMP-activated protein kinase (AMPK) is activated and regulates the energy balance of cells by inhibiting lipogenesis and promoting lipolysis [8]. Additionally, the lipid droplet (LD), which is the major storage organelle of TG, is degraded by enzymes such as hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) during lipolysis [9,10]. Thus, the released free glycerol and free fatty acids (FFAs) due to the breakdown of TG are important biomarkers that represent a decrease in adipocyte lipid [11]. PPARα plays a major role as a transcription factor that promotes the expression of FA oxidation genes, such as peroxisomal acyl-coenzyme A oxidase 1

(ACOX1), long-chain acyl-CoA dehydrogenase (LCAD), and medium-chain acyl-CoA dehydrogenase (MCAD) [12,13].

Since obesity is considered a key health concern in developed countries, researches are developing anti-obesity drugs (e.g., phentermine, phendimetrazine, and lorcaserin) [14]. However, anti-obesity drugs can cause several side effects, such as insomnia, headaches, and constipation [15,16]. Therefore, in recent years, interest in natural substances with safety and functionality has increased [17]. Among the natural materials, soybeans have drawn substantial attention since they have been reported to contain various bioactive compounds, including tocopherols, soyasaponins, and isoflavones [18,19]. These functional substances not only improve metabolic and cardiovascular functions but also help to prevent obesity [20–23].

The sword bean (Canavalia gladiata) belonging to the legume family is cultivated mainly in tropical and subtropical regions of Asia [24]. In Korea and Japan, the sword bean was used in folk remedies for the treatment of purulent inflammation. Sword beans have also been effective as antioxidants [25] and have anti-cancer [26], antibacterial [27], anti-diabetic [28], and anti-gastritis [29] properties. In addition, the sword bean has a significantly lower fat content (1.2 ± 0.13%) than the soybean (16.5 ± 0.29%) and black soybean (16.1 ± 0.15%), and the total flavonoid content is significantly higher (493.2 ± 21.2 mg/100 g) in sword beans than in soybeans (71.8 ± 6.3 mg/100 g) and black soybeans (97.5 ± 14.9 mg/100 g) [30]. However, research on the anti-obesity effects of sword beans is still limited.

In this study, to investigate the effect of Bacillus subtilis-fermented white sword bean extract (FWSBE) on adipogenesis and lipolysis of 3T3-L1 adipocytes, FWSBE was used to treat adipocytes before and after differentiation. TG, cell differentiation, and adipogenesis biomarkers were measured in the premature adipocyte, and free glycerol and lipolysis biomarkers were measured in the mature adipocyte.

2. Materials and Methods

2.1. Sample Preparation and Proximate Analysis

B. subtilis-fermented white sword bean extract (FWSBE) was provided by the Korea Food Research Institute (KFRI, Wanju-gun, Jeollabuk-do, Korea). The general ingredients of FWSBE were ascertained by following the general test method of the Korean Food Standards Codex [31]. The composition of freeze-dried powder samples of the fermented white sword bean was 24.09% soluble dietary fiber, 13.26% carbohydrate, 60.32% crude protein, 1.79% crude fat, and 6.88% crude ash.

2.2. Cell Culture and Differentiation

The cell line used in this study was 3T3-L1, a pre-adipocyte cell line, distributed from Korean Cell Line Bank (KCLB, Jongno, Seoul, Korea). Cells were cultured in a 5% CO2 incubator at 37 °C using Dulbecco's Modified Eagle's Media (DMEM; WELGENE, Gyeongsan, Gyeongsanbuk-do, Korea), with 10% bovine calf serum (BCS; Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (PS; WELGENE, Gyeongsan, Gyeongsanbuk-do, Korea). Cells were dispensed into a 6-well plate at a concentration of 3 × 104 cells/mL. Changing the media every 2 days, the cells were cultured to 100% and then cultured for 2 days to achieve a post-confluent state (day 0). After that, MDI solution containing 1 µM dexamethasone (DEX), 0.5 mM 3-isobutyl−1-methylxanthine (IBMX), 10 µg/mL insulin (Sigma-Aldrich Co., St. Louis, MO, USA), and 10% fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) and DMEM containing 1% PS was used to treat cells for 2 days to induce cell differentiation (days 1 and 2). Next, to promote differentiation, DMEM containing 10 µg/mL insulin, 10% FBS, and 1% PS was changed every 48 h for a total of 6 days (days 3 to 8). Cells were then treated with DMEM containing 10% FBS and 1% PS for an additional 6 days (days 9 to 14). To confirm the inhibitory effect of FWSBE on adipocyte differentiation, cells were treated with FWSBE and MDI for 48 h (days 1 and 2) at each of the four concentrations (100, 200, 400, and 1000 µg/mL).

Additionally, to check the lipolysis and FA oxidation effects of FWSBE, the mature 3T3-L1 adipocytes were treated at each of the four concentrations every 48 h for a total of 96 h after differentiation was completed (days 15 to 18). All experiments were performed in triplicate.

2.3. Cell Viability Assay

The cytotoxicity of the FWSBE in 3T3-L1 cells was evaluated. 3T3-L1 pre-adipocytes were dispensed into a 12-well plate at a concentration of 3 × 104 cells/mL and then incubated for 24 h in DMEM containing 10% BCS. After 24 h, the culture solution was removed and each of the four concentrations of FWSBE (100, 200, 400, and 1000 µg/mL) was used to treat cells, which were then incubated for 24 h. Cells were then washed with PBS and DMEM-MTT reagent (1:9) and were incubated for 4 h in a 5% CO_2 incubator at 37 °C to generate formazan. Centrifugation (LABOGENE 1248, multi-purpose centrifuge set, LABOGENE, Seoul, South Korea) at 4 °C at 3000 rpm for 3 min followed, and 1 mL of DMSO was added to each well to dissolve the produced formazan. Absorbance was measured at a wavelength of 540 nm using an ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All experiments were performed in triplicate.

2.4. Quantification of Triglyceride Content

AdipoRed™ assay reagent (LONZA, Walkersville, MD, USA) was used for measuring the TG content and was performed according to the manufacturer's instructions. Cells were treated with FWSBE and MDI for the first 48 h (days 1 and 2) at each of four concentrations (100, 200, 400, and 1000 µg/mL) and were allowed to differentiate for 14 days. After removing the culture medium and washing with PBS, each well was treated with 2 mL of PBS and 60 µL of AdipoRed at room temperature for 10 min. Thereafter, fluorescence was measured at excitation 485 nm and emission 590 nm wavelengths using a fluorescence spectrophotometer (Synergy™ HTX Multi-Mode Microplate Reader, BioTek, Sinooski, VT, USA). All experiments were performed in triplicate.

2.5. Quantification of Free Glycerol Content

To measure the effect of FWSBE on lipolysis, free glycerol was measured according to the manufacturer's instructions using a cell-based glycerol assay kit (Cayman Chemical, Ann Arbor, MI, USA). After completing differentiation at a concentration of 3 × 104 cells/mL, the mature 3T3-L1 adipocytes were treated at each of the four concentrations every 48 h for a total of 96 h after differentiation was completed (days 15 to 18). The supernatant of the medium was collected after 96 h. To a new 96-well plate, 25 µL of glycerol standards for each concentration and 25 µL of the supernatant from the control and FWSBE wells were dispensed. Then, 100 µL of free glycerol assay reagent was added to each well. After incubation at room temperature for 15 min, absorbance was measured at 540 nm. All experiments were performed in triplicate.

2.6. Quantitative RT-PCR Analysis

Total RNA was extracted with TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Quantity and purity were analyzed spectrophotometrically at 230, 260, and 280 nm (QIAxpert, Qiagen, Hilden, Germany). To synthesize cDNA using a quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), one microgram of total RNA and oligo (dT)20 primer were used (SuperScript™ II RT kit, Invitrogen, Carlsbad, CA, USA). For real-time RT-PCR amplification, ach reaction consisted of 1 µL of cDNA that had been reverse transcribed from 1 µg of total RNA and 0.2 µM of real-time RT-F/R. qRT-PCR was conducted at 95 °C for 4 min then 40 cycles of 95 °C for 10 s, 58 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min using SYBR Green as a probe (Molecular Probes Inc., Eugene, OR, USA) and a CFX96™ real-time PCR system (Bio-Rad, Hercules, CA, USA). To confirm the amplification of specific products, melting curve cycles were conducted using the settings 95 °C for 1 min, 55 °C for 1 min, and 80 cycles of 55 °C for 10 s with a 0.5 °C increase per cycle using qRT-PCR forward or

reverse primers (Table 1). β-actin gene expression, which was stable throughout the experiments, was used as an internal control to normalize expression levels between samples. All experiments were performed in technical triplicates. A relative fold-change in gene expression compared to the control was calculated by the $2^{-\Delta\Delta Ct}$ comparative method [32].

Table 1. The primer sequences used for qRT-PCR.

Target	Primer (5′→3′)	
aP2	forward	AAGGTGAAGAGCATCATAACCCT
	reverse	TCACGCCTTTCATAACACATTCC
Adiponectin	forward	GCCTGTCCCCATGAGTAC
	reverse	TCTTCGGCATGACTGGGC
Ppara	forward	ACGATGCTGTCCTCCTTGATG
	reverse	GCGTCTGACTCGGTCTTCTTG
Acox1	forward	GCACCTTCGAGGGGGAGAACA
	reverse	GCGCGAACAAGGTCGACAGAA
Lcad	forward	TCCGCCCGATGTTCTCATTC
	reverse	AGGGCCTGTGCAATTTGAGT
β-actin	forward	ACCCCAGCCATGTACGTAGC
	reverse	GTGTGGGTGACCCCGTCTC

2.7. Western Blot Analysis

After removing the culture medium from 3T3-L1 cells, the cells were washed with PBS and RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor and phosphatase inhibitor (Sigma-Aldrich Co., St. Louis, MO, USA) and were dispensed to isolate the protein. The supernatant was then obtained after centrifugation (LABOGENE 1248, multi-purpose centrifuge set, LABOGENE, Seoul, South Korea) at 4 °C at 13,000 rpm for 20 min. Protein concentration was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples for Western blot analysis were prepared by adding samples, distilled water, and 4X LaemmLi Sample Buffer (Bio-rad, Hercules, CA, USA) according to the protein quantification level. The quantified protein was subjected to electrophoresis using 8–12% SDS-polyacrylamide gel (SDS-PAGE) and then transferred using polyvinylidene fluoride (PVDF) membranes (Sigma-Aldrich Co., St. Louis, MO, USA). After blocking with 5% skim milk for 1 h at room temperature, the primary antibodies were added. The membranes were held overnight at 4 °C (Table 2). Secondary antibody reactions occurred during processing at room temperature for 1 h. ECL detection reagents (Bio-rad, Hercules, CA, USA) were applied to the membrane, and the expression levels of the protein were confirmed using ChemiDoc (Bio-Rad, Hercules, CA, USA). Each protein band was quantified using ImageJ (NIH, Bethesda, MD, USA). All experiments were performed in triplicate.

Table 2. The primary antibodies used for Western blot analysis.

Target	Secondary Host	Size (kDa)	Dilution	Company	Catalog No.
C/EBPα	Rabbit	42	1:1000	Cell signaling Technology	#2295
PPARγ	Rabbit	53, 57	1:1000	Cell signaling Technology	#2443
p-AMPK	Rabbit	62	1:1000	Cell signaling Technology	#2531
AMPK	Rabbit	62	1:1000	Cell signaling Technology	#2532
p-ACC	Rabbit	280	1:1000	Cell signaling Technology	#3661
ACC	Rabbit	280	1:1000	Cell signaling Technology	#3676
FAS	Rabbit	273	1:1000	Cell signaling Technology	#3180
HSL	Rabbit	81, 83	1:1000	Cell signaling Technology	#4107
p-HSL	Rabbit	81, 83	1:1000	Cell signaling Technology	#4139
ATGL	Rabbit	54	1:1000	Cell signaling Technology	#2138
Perilipin A	Rabbit	62	1:1000	Cell signaling Technology	#9349
β-actin	Mouse	45	1:1000	Cell signaling	#3700

2.8. Statistical Analysis

For statistical analysis, SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used, and the data are presented as mean ± standard deviation (S.D.). One-way ANOVA was used to analyze the significant differences between the control and test groups; this was followed by Duncan's test. The differences with $p < 0.05$ were considered significant.

3. Results

3.1. Effects of FWSBE on Cell Viability in 3T3-L1 Preadipocytes

To measure the cell viability, 3T3-L1 preadipocytes were exposed to each of the four concentrations (100, 200, 400, and 1000 μg/mL) of FWSBE for 24 h and an MTT assay was conducted. Every concentration showed a survival rate higher than 99%, indicating that no cytotoxicity was induced by FWSBE (Figure 1).

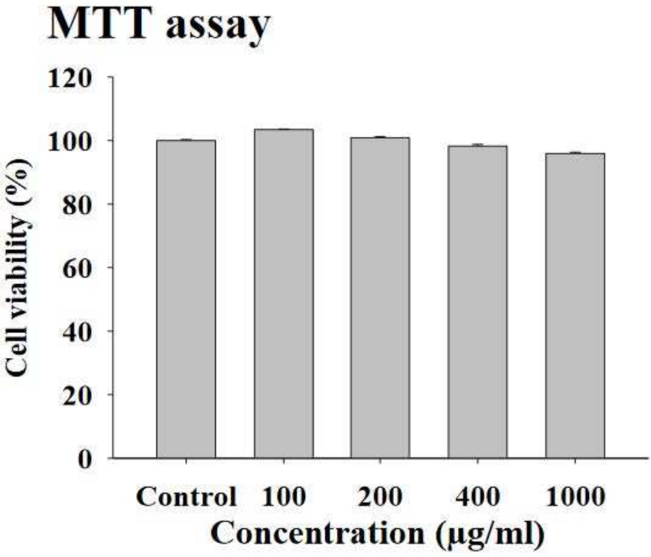
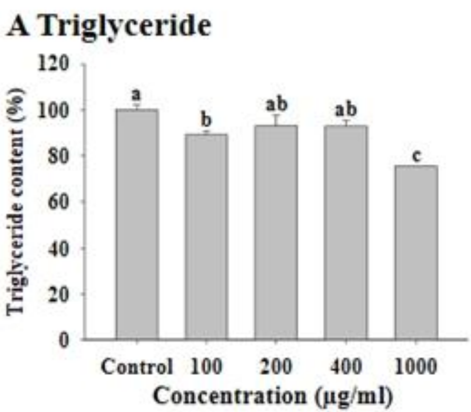


Figure 1. Effects of fermented white sword bean extract (FWSBE) on cell viability of 3T3-L1 preadipocytes. The 3T3-L1 cells (5×10^4 cells/mL) were treated with FWSBE at various concentrations (100, 200, 400, and 1000 μg/mL) for 24 h. Cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values are presented as mean ± standard deviation.

3.2. Effects of FWSBE on Triglyceride and Free Glycerol Content

To investigate the effect of FWSBE on adipogenesis, 3T3-L1 preadipocytes were exposed to each of the four concentrations of FWSBE (100, 200, 400, and 1000 μg/mL) for the first 48 h with MDI and were allowed to differentiate for 14 days. In the FWSBE-treated groups, the intracellular TG contents significantly decreased in 100 (approximately 10.5%) and 1000 (approximately 24.4%) μg/mL exposure groups ($p < 0.05$) (Figures 2A and S1). Next, to measure the effect of FWSBE on lipolysis, mature 3T3-L1 adipocytes were exposed to each concentration of FWSBE for 96 h, and the content of free glycerol in the medium was measured. The amount of free glycerol was significantly ($p < 0.05$) increased to 120.95% (100 μg/mL), 121.22% (200 μg/mL), 113.42% (400 μg/mL), and 125.57% (1000 μg/mL) compared to the control (Figure 2B).



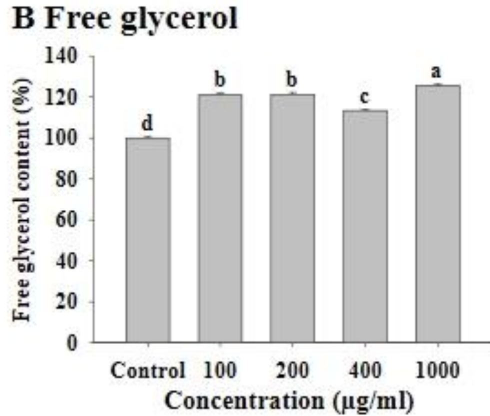


Figure 2. (**A**) Effects of fermented white sword bean extract (FWSBE) on triglyceride accumulation in 3T3-L1 pre-adipocytes. The cells were treated with FWSBE at various concentrations (100, 200, 400, and 1000 μg/mL) for 48 h during differentiation. The cumulative triglyceride content was measured with the AdipoRed assay. (**B**) Effects of FWSBE on free glycerol in mature 3T3-L1 adipocytes. 3T3-L1 cells (3 × 104 cells/mL) were exposed to different concentrations (100, 200, 400, and 1000 μg/mL) of FWSBE for 96 h. Values are presented as mean ± standard deviation. The statistically significant difference ($p < 0.05$) between groups was determined by a one-way ANOVA and Duncan's multiple range test. Different letters are indicated as significant differences.

3.3. Effects of FWSBE on the Adipogenesis in 3T3-L1 Pre-Adipocytes

To investigate the effects of FWSBE (100, 200, 400, and 1000 μg/mL) on adipogenesis, we treated 3T3-L1 preadipocytes with FWSBE for 48 h, measuring mRNA transcription and protein levels. In adipogenesis-specific gene transcription, the transcript level of aP2 and adiponectin significantly ($p < 0.05$) decreased compared to the control, except for aP2 at 400 μg/mL of FWSBE treatment (Figure 3A). Additionally, the protein levels of C/EBPα, PPARγ, and FAS were significantly ($p < 0.05$) decreased, and the protein levels of pACC and pAMPK significantly ($p < 0.05$) increased compared to the control (Figure 3B).

3.4. Effects of FWSBE on Lipolysis in the Mature 3T3-L1 Adipocytes

To confirm the effect of the four concentrations of FWSBE (100, 200, 400, and 1000 μg/mL) on lipolysis, we measured the mRNA transcription and protein levels in mature 3T3-L1 adipocytes after 96 h exposure to FWSBE. The transcript levels of Ppara, Acox, and LCAD were significantly ($p < 0.05$) decreased compared to the control at 1000 μg/mL (Figure 4A). To test whether FWSBE promoted TG decomposition in 3T3-L1 adipocytes, the protein levels of pHSL, ATGL, and perilipin A were measured. Both pHSL and ATGL showed a significant ($p < 0.05$) increase at 1000 μg/mL, but perilipin A did not show any changes in experimental groups (Figure 4B).

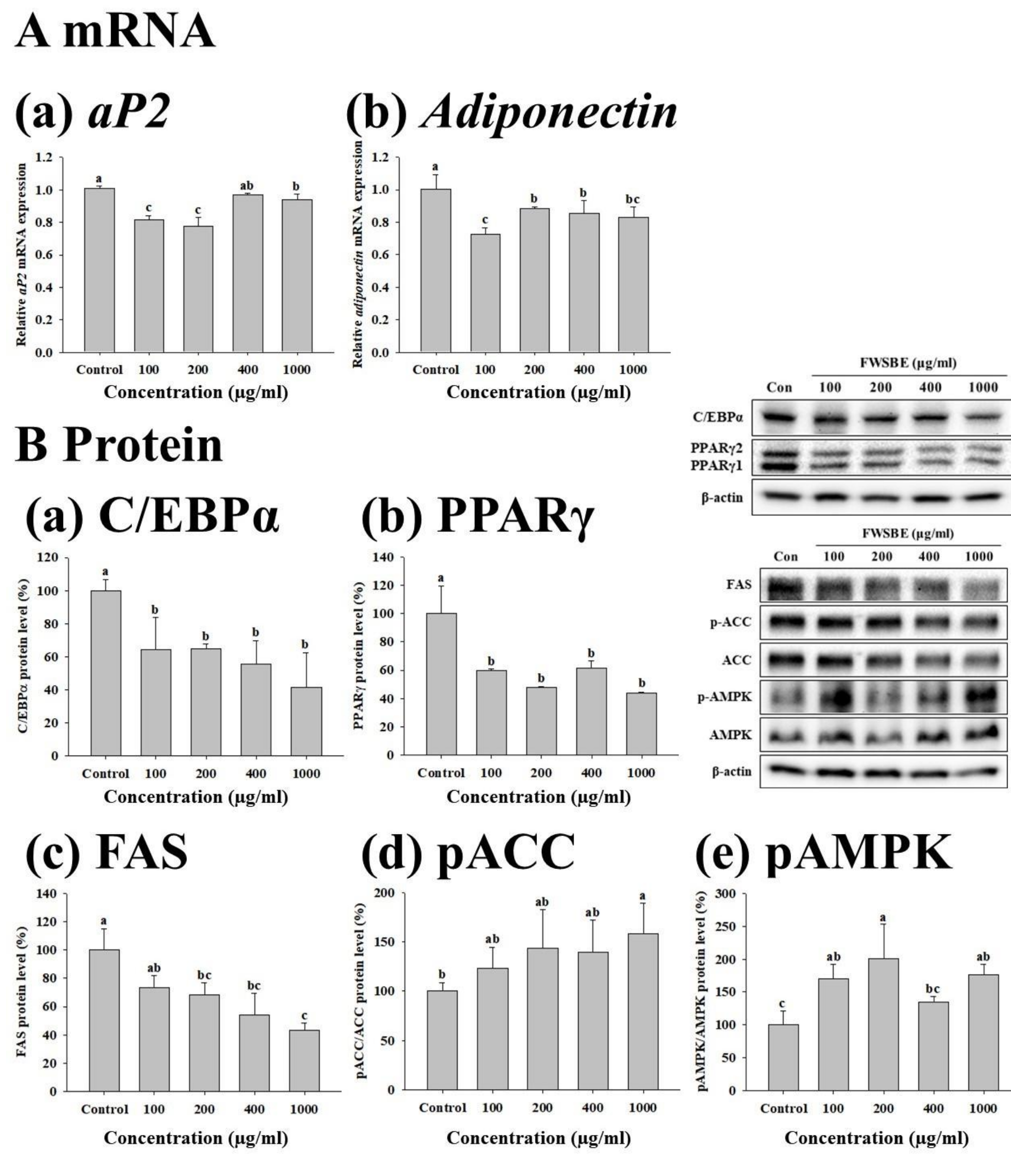


Figure 3. Effects of fermented white sword bean extract (FWSBE) on (**A**) the transcription level of (a) aP2 and (b) adiponectin and (**B**) the protein level of (a) C/EBPα, (b) PPARγ, (c) FAS, (d) pACC, and (e) pAMPK on 3T3-L1 preadipocytes. The cells were treated with FWSBE at various concentration (100, 200, 400, and 1000 µg/mL) for 48 h during differentiation. Values are presented as mean ± standard deviation. The statistically significant difference ($p < 0.05$) between groups was determined by a one-way ANOVA and Duncan's multiple range test. Different letters are used to indicate significant differences. C/EBPα, CCAAT/enhancer binding protein α; FAS, fatty acid synthase; ACC, acetyl-coenzyme A carboxylase; PPARγ, peroxisome proliferator-activated receptor γ; and AMPK, AMP-activated protein kinase.

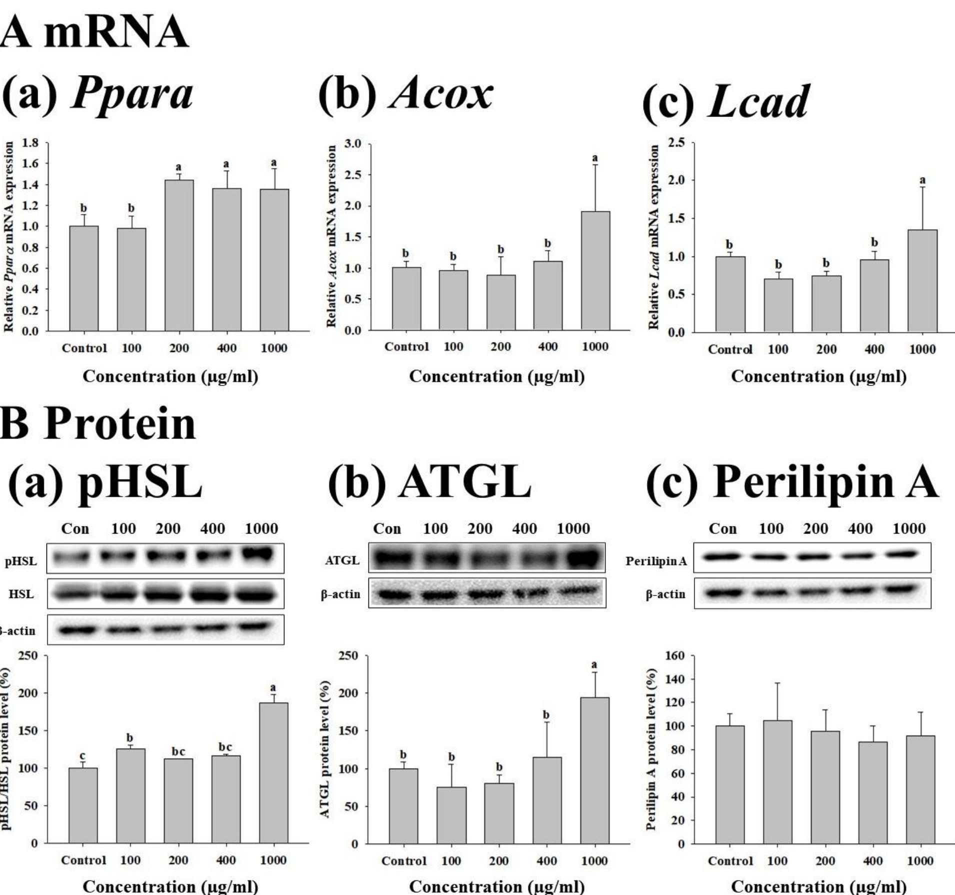


Figure 4. Effects of fermented white sword bean extract (FWSBE) on (**A**) transcription level of (a) *Ppara*, (b) *Acox*, and (c) *Lcad*, and (**B**) the protein level of (a) pHSL, (b) ATGL, and (c) perilipin A in mature 3T3-L1 adipocytes. The cells were treated with FWSBE at various concentrations (100, 200, 400, and 1000 μg/mL) for 96 h. Values are presented as mean ± standard deviation. The statistically significant differences ($p < 0.05$) between groups were determined by a one-way ANOVA and Duncan's multiple range test. Different letters are used to indicate significant differences. Acox, acyl-coenzyme A oxidase; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; Lcad, long-chain acyl-coenzyme A dehydrogenase; and Ppara, peroxisome proliferator-activated receptor α.

4. Discussion

Obesity is a state in which body fat is over-accumulated in the body, which not only affects the quality of life but is also a public health concern due to the occurrence of chronic degenerative diseases and metabolic complications. Therefore, to reduce obesity, anti-obesity studies using natural substances are attracting attention [33–35]. Previous studies have reported that various phytochemicals present in natural substances can prevent obesity through the inhibition of oxidative stress and inflammation [36,37]. Foods with antioxidant activity may help to decrease body weight and obesity-related disorders [38,39]. In general, fermentation can increase the content of functional substances [25,40] and bioaccessibility due to changes in the physiological characteristics of active substances [41]. A previous study analyzed the composition of phytochemicals in fermented and non-fermented sword beans [42]. The results showed that the total phenolic and flavonoid contents of fermented sword beans were higher than those of non-fermented sword beans, and among the fermented sword beans, fermented red sword beans showed the highest contents. As a result of antioxidant activity analysis, DPPH was similar among fermented sword bean groups; however, nitrate-scavenging activity was higher in fermented white

sword beans compared to the others. Additionally, a previous study has reported that the total flavonoid content of sword beans was about 5–6 times higher than that of soybeans and black soybeans; and the antioxidant activity of sword beans was comparable to that of α-tocopherol [43], indicating that FWSBE has higher ROS-scavenging ability through hydrogen donation [44]. Therefore, we focused on the anti-obesity effects of FWSBE, since FWSBE has the strongest antioxidant activity among sword beans and its extracts, even in our study (Figure S2), and the MTT experiment showed that FWSBE did not reduce cell viability even at the highest concentration (1000 μg/mL).

To evaluate whether FWSBE has anti-obesity effects, 3T3-L1 pre-adipocytes were treated with FWSBE for 48 h and the TG content was measured. The concentration of TG was significantly reduced by FWSBE at the highest concentration (1000 μg/mL). TG is mainly stored in LD, an inactive vesicle produced by adipogenesis surrounded by a phospholipid monolayer [45]. TG is an ester-linked form of one molecule of glycerol and three molecules of FFA. In general, TG is used as an important energy source for cells, but excess TG stored in adipose tissue causes obesity [46]. Therefore, the content of TG was mainly used to evaluate the anti-obesity effects of natural products in previous studies. For example, when 3T3-L1 adipocytes were treated with fermented soybean extract at 10, 50, and 100 μg/mL, TG significantly decreased at all concentrations compared to the control [47]. Additionally, when 3T3-L1 adipocytes were treated with fermented soybean and non-fermented soybean extracts at 50 μg/mL, the content of TG was significantly reduced in the fermented soybean extract group compared to the control [48]. However, non-fermented soybean extract did not induce any changes in TG content, indicating that the adipogenesis inhibitory effect may be increased during the fermentation process. In addition to the TG content, mature 3T3-L1 adipocytes were treated with FWSBE, and the content of free glycerol released from the cells was measured. The content of free glycerol was significantly increased in the FWSBE-treated group. The degree of free glycerol released to the cell medium can be used as a marker in anti-obesity studies since TG is decomposed into FAs and glycerol by lipolytic enzymes [49]. In a previous study on the anti-obesity effect of soymilk fermented with B. subtilis, the amount of released free glycerol increased by 43% in the experimental group [50]. Additionally, when comparing fermented and non-fermented soybean extract, free glycerol secretion was significantly increased compared to the control group [48]. Taken together, our data clearly showed that FWSBE has anti-obesity capacity in both premature and mature 3T3-L1 adipocytes.

To understand how FWSBE suppressed TG accumulation in 3T3-L1 pre-adipocytes, mRNA transcription and protein levels were measured. Results showed that the transcript levels of aP2 and adiponectin were downregulated. In the late stages of differentiation, adipocyte-specific genes are expressed, thereby allowing for differentiation into adipocytes. aP2 is a target gene of PPARγ and is specifically expressed in adipocytes [51], and its expression promotes fatty acid absorption into adipocytes [52]. Adiponectin is an adipocytokine secreted from adipocytes and plays an important role in maintaining insulin sensitivity and energy homeostasis [53]. Therefore, these genes are widely used as biomarkers in anti-obesity research. For example, in previous studies using blueberry peel extract [54] and onion peel extract [55], which are natural products rich in polyphenol content, the transcript level of adipocyte-specific genes decreased due to the decrease in the expression of adipogenic transcription factors. This suggests that those extracts have an anti-obesity effect. Conversely, overexpression of adiponectin in 3T3-L1 cells increased the expression of adipogenic transcription factors in the early and late stages of differentiation, suggesting that adipocyte-specific genes could regulate the adipogenesis process [56]. However, although adiponectin showed a decrease in adiponectin, which is one of the major biomarkers related to adipocyte differentiation, in blueberry peel extract [54] and onion peel extract [55], a decrease in adiponectin might cause side effects, such as type 2 diabetes, obesity, and cardiovascular disease in humans [57]. Therefore, further researches about FWSBE against side effects in humans are needed.

The process of adipogenesis, the differentiation from pre-adipocytes into adipocytes, is triggered by the stepwise regulation of many types of adipogenic transcription factors. Among the transcription factor products, AMPK is the key enzyme for regulating lipid metabolism. When the intracellular AMP/ATP ratio increases, AMPK is activated by phosphorylation of the threonine 172 residue of the α-subunit [58]. SREBP-1c, which is expressed by insulin in the early stages of differentiation, is an essential transcription factor for the synthesis of FAs and regulates the expression of C/EBPα and PPARγ [59]. C/EBPα and PPARγ are key regulators of adipogenesis that promote the post-differentiation process of adipocytes [60]. Lipogenesis is a process in which acetyl-CoA is converted to malonyl-CoA by the action of ACC in the cytoplasm, and FA is synthesized by the action of PPARγ-regulated FAS. Therefore, in general, when AMPK is activated in a specific situation (in this study, FWSBE treatment), activated AMPK suppresses the expression of SREBP-1c [61,62] to suppress PPARγ and the suppressed PPARγ inhibits the expression of the target genes for ACC and FAS [63–65]. Therefore, this mechanism is widely used in anti-obesity studies. For example, when 3T3-L1 cells were treated with bamboo leaf extract at 100 μg/mL [66] or siRNA of AMPK [67,68], the protein levels of C/EBPα, PPARγ, SREBP-1c, and FAS significantly decreased compared to the control. In addition, pACC and pAMPK protein expression increased, indicating that adipogenesis was suppressed. Additionally, AMPK was phosphorylated by soyasaponin Af and quercetin 3-O-glucoside from the kidney beans (Phaseolus vulgaris L.) [69], genistein and daidzein from the soybeans [70], vitexin from the mung beans (*Vigna radiata*. L.) [71], and theobromine from cocoa beans (Theobroma cacao) [72]. Our data suggest that FWSBE-activated AMPK in 3T3-L1 pre-adipocytes leads to suppression of adipogenesis through post-translational regulation of adipogenic factors.

To confirm the effects of FWSBE on lipolysis in mature 3T3-L1 adipocytes, mRNA and protein levels were measured. Results showed that Ppara, Acox, and Lcad were upregulated in FWSBE treatment experimental groups. Ppara encodes a transcription factor involved in fatty acid oxidation and peroxisome metabolism and regulates the expression of genes related to β-oxidation, such as Acox1 and Lcad [73,74]. The expression of both of these genes results in enzymes that convert FFAs to 2-trans-enoyl-CoA [75]. Therefore, this mechanism was studied in previous research studies. For example, in rats fed the flavonoid naringenin, Ppara expression was significantly increased in liver tissue, resulting in a decrease in TG [76]. Additionally, the transcript level of Acox1 increased due to the upregulation of Ppara by treatment with the flavonoid 2.4–240 μM naringenin in hepatocytes [77]. In addition to the mRNA level, the protein levels of pHSL, ATGL, and perilipin A were observed. Results showed that pHSL and ATGL were significantly increased in the FWSBE-treated group (at 1000 μg/mL) compared to the control; perilipin A did not show any changes. When HSL is phosphorylated and transferred from the cytoplasm to the surface of the LD [78], pHSL promotes the decomposition of TG in the LD. In addition, perilipin A, which presents on the surface of LD, is phosphorylated by PKA and combined with pHSL to promote the decomposition of TG [79]. ATGL, which is involved in the first step in the breakdown of TG, is also affected and promotes lipolysis [80]. Therefore, in lipolysis studies, the above biomarkers are commonly used. For example, after treatment with the flavonoid myricetin in 3T3-L1 cells, the expression level of perilipin A significantly decreased compared to the control; however, HSL increased, leading to increases in lipolysis [81]. Taken together, our data suggest that FWSBE promoted lipolysis in mature 3T3-L1 adipocytes through activation of HSL and ATGL expression.

5. Conclusions

In this study, we investigated the potential anti-obesity effects of FWSBE in both premature and mature 3T3-L1 adipocytes. In the early stage of adipocyte differentiation, FWSBE phosphorylated AMPK, which led to a decrease in the mRNA expression of aP2 and adiponectin, and the protein levels of C/EBPα, PPARγ, and FAS also decreased, resulting in suppression of TG accumulation. Additionally, in mature 3T3-L1 adipocytes, FWSBE increased the mRNA expression of Ppara, Acox, and Lcad and the protein levels of pHSL

and ATGL, promoting lipolysis in mature 3T3-L1 adipocytes. Overall, we confirmed that FWSBE affected both adipogenesis and lipolysis in 3T3-L1 cells. However, some biomarkers (e.g., adiponectin) could induce a negative impact in humans. Further studies are needed to apply to human health. We suggest that this study provides basic information on the anti-obesity effects of FWSBE.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10061423/s1, Figure S1: Lipid droplet of FWSBE on the 3T3-L1 adipocytes. Cells were observed using microscope (X200 magnification; modified from Kim, 2020). Figure S2: DPPH free radical scavenging activity of white sword bean extract (WSBE) and fermented white sword bean extract (FWSBE). Values are presented as means ± standard deviation. Different letters indicate significant differences based on a one-way ANOVA and Duncan's multiple range test ($p < 0.05$).

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Article

The Effects of Synbiotics Administration on Stress-Related Parameters in Thai Subjects—A Preliminary Study

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Abstract: Urbanization influences our lifestyle, especially in fast-paced environments where we are more prone to stress. Stress management is considered advantageous in terms of longevity. The use of probiotics for psychological treatment has a small amount of diverse proven evidence to support this. However, studies on stress management in stressed subjects using synbiotics are still limited. The present study aimed to investigate the effects of synbiotics on stress in the Thai population. A total of 32 volunteers were enrolled and screened using a Thai Stress Test (TST) to determine their stress status. Participants were divided into the stressed and the non-stressed groups. Synbiotics preparation comprised a mixture of probiotics strains in a total concentration of 1×10^{10} CFU/day (5.0×10^9 CFU of *Lactobacillus paracasei* HII01 and 5.0×10^9 CFU of *Bifidobacterium animalis* subsp. *lactis*) and 10 g prebiotics (5 g galacto-oligosaccharides (GOS), and 5 g oligofructose (FOS)). All parameters were measured at baseline and after the 12th week of the study. In the stressed group, the administration of synbiotics significantly ($p < 0.05$) reduced the negative scale scores of TST, and tryptophan. In the non-stressed group, the synbiotics administration decreased tryptophan significantly ($p < 0.05$), whereas dehydroepiandrosterone sulfate (DHEA-S), tumor necrosis factor-α (TNF-α), 5-hydroxyindoleacetic acid (5-HIAA), and short-chain fatty acids (SCFAs), acetate and propionate were increased significantly ($p < 0.05$). In both groups, cortisol, and lipopolysaccharide (LPS) were reduced, whereas anti-inflammatory mediator interleukin-10 (IL-10) and immunoglobulin A (IgA) levels were increased. In conclusion, synbiotics administration attenuated the negative feelings via the negative scale scores of TST in stressed participants by modulating the HPA-axis, IL-10, IgA, and LPS. In comparison, synbiotics administration for participants without stress did not benefit stress status but showed remodeling SCFAs components, HPA-axis, and tryptophan catabolism.

Keywords: synbiotics; stress; *Lactobacillus*; *Bifidobacterium*; galacto-oligosaccharides; oligofructose

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1. Introduction

Urbanization is a global phenomenon that transforms various aspects of everyday life. Living in a fast-paced environment drives post-traumatic stress disorder (PTSD) in parts of the vulnerable population, and its consequences can be psychotic related-events, stress-related illnesses, and/or depression [1]. The combination of the urban

environment and stress exposure can alter the biochemical-related stress system. The stress response is governed by the hypothalamus-pituitary-adrenal axis (HPA-axis). Long-term stress exposure promotes HPA-axis hyperactivation, secreting cortisol that affects behavior, and promoting low-grade inflammatory cytokines secretion, which induces improper physical function systems to cause degenerative diseases of cardiovascular and neurodegeneration [2]. In addition, the dysregulation of the HPA-axis is associated with serious mental disorders such as major depressive disorder and schizophrenia [3]. The long-term perception of uncertainty stress correlates with the greater prevalence of mental disorders [4], and highly stressful work can also cause death without cardiometabolic conditions [5,6].

The HPA-axis activity has a connection to gut conditions. The collaboration between gut and brain, called the gut-brain axis, has been elucidated by four major pathways: neurologic, endocrine, humoral/metabolic, and immune [7]. The supplementation of probiotics or synbiotics could positively regulate the above-mentioned pathways and offer health benefits to the host [8].

According to World Health Organization/ Food and Agriculture Organization (WHO/ FAO) 2002, probiotics have been defined as a living microorganism that benefits the host when administrated adequately [9]. Many clinical studies confirm the benefit of probiotics. According to the link between gut and brain on HPA-axis modulation, improvement in mental health is illustrated by a study on probiotics supplementation in preclinical and clinical studies. In the rat model study, the receiving of *Bifidobacterium infantis* could attenuate stress-like behavior and reduce corticosterone in mice [10]. In pregnant mice, the consumption of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Propionibacterium jensenii* 702 provoked stress process stimulation and enhanced stress tolerance in older adults [11]. Additionally, the consumption of *Lactobacillus farciminis* 10^{11} CFU/day for 12 weeks could attenuate low-grade inflammatory mediators IL-1β, IL-6, and LPS, finally restoring tight junction and reducing gut permeability [12].

Stress relief by probiotics intake has been the subject of academic study. The consumption of *L. casei* strain Shirota 1.0×10^{11} CFU per 100-mL bottle of fermented milk encouraged stress regulation in medical students before an examination. The test group showed lower expression of salivary cortisol than the placebo group, even though the salivary cortisol was not significantly changed throughout the intervention period [13].

Prebiotics are a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health [14]. Probiotics and prebiotics play a crucial role in physical and mental health manipulation [15,16]. The advantage of prebiotics administration on mental health has been proven in several studies [17]. Prebiotics oligosaccharides such as galacto-oligosaccharides (GOS) and fructo-oligosaccharide (FOS) have a positive effect on mental health, which has been proven in several studies [18]. The consumption of 5.5 g of GOS or FOS per day for 3 weeks reduced the waking salivary cortisol level in healthy subjects [19]. The studies suggested that the consumption of 7 g of trans-galacto-oligosaccharide improved mood and gut microbiota [20].

Furthermore, 5 g of FOS supplementation promoted a better stress index score, highlighting its role in improving anxiety [21]. Prebiotics oligosaccharides are a non-digestible carbohydrate and precursor for gut microbial metabolites short-chain fatty acids (SCFAs). The neuroprotective property is promoted by SCFAs upregulation activating G-protein coupled free fatty acid (FFAR) and/or by inhibiting histone deacetylase (HDAC) enzyme in the brain [22]. Oligosaccharides promote gut microbiota modification by enhancing commensal probiotics *Lactobacillus* and *Bifidobacterium* by suppressing infectious microbes like *Clostridium* [23].

Even though the probiotics and prebiotics administration are prone to deliver the benefits on stress regulation, the effects of both probiotics and prebiotics are still being debated [24]. Synbiotics is defined as a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on

the host [25].The subjects with moderate depression supplemented with a combination of *L. casei* (3×10^8 CFU/g) *L. acidophilus* (2×10^8 CFU/g), *L. bulgaricus* (2×10^9 CFU/g), *L. rhamnosus* (3×10^8 CFU/g), *B. breve* (2×10^8 CFU/g), *B. longum* (1×10^9 CFU/g), *S. thermophilus* (3×10^8 CFU/g), and 100 mg FOS showed better Hamilton Depression Rating Scale (HAM-D) scores after 10 weeks of administration which referred to better mental health improvement [26].

The present study aimed to investigate the effects of the synbiotics supplement containing *L. paracasei* HII01, *B. animalis* subsp. *Lactis,* and GOS with FOS on the stress modulation in the stressed participants.

2. Materials and Methods

2.1. Study Design and Participants

The study consisted of two groups (the stressed and the non-stress group) compared at baseline and after synbiotics administration. The study design and protocol followed Good Clinical Practices and fully complied with the ethical guidelines of a clinical trial conducted under the Declaration of Helsinki. The Ethical Committee of the University of Phayao approved the study (Code: 1.3/005/64).

The previous study investigated the outcome of probiotics treatment on stress, mood, and anxiety in depression [27]. The stress score at the pre-treatment was 64.3 (standard deviation = 23.9). The outcome at post-treatment was 37.9 (standard deviation = 29.0). The stress score outcome with standard deviation, α-value = 0.05, correlation = 0.50, and power = 0.80 were calculated using the statistical software package STATA 15.1 (Statacorp, College Station, TX, USA). The estimated sample size with a 5% probable drop in the sample was 12 in each group. All participants of both groups were 18–65 years old males and females working and living in Chiang Mai province, who agreed with informed consent and whose stress status was diagnosed, following the Thai Stress Test (TST) guidelines [28]. The mild stress and stressful participants were defined as the stressed group, whereas the normal mental health and excellent mental health were defined as the non-stressed group identified by the Thai Stress Test (TST). The participants were non-malignant, free from vascular diseases, psychotic disorders, and drug and alcohol addiction. Moreover, all participants were asked to abstain from probiotics and prebiotics products during the study period.

The study was conducted for 12 weeks. All participants were asked to visit the research center twice at the beginning of the study and during its 12th week to collect the samples, monitor/follow up stress status, and make essential health assessments. Salivary, venous blood, first-morning urine, and stool samples were collected after 8 h of overnight fasting. The parameters were determined at the Associated Medical Science (AMS) Clinical Service Center, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand. The study protocol is illustrated in Figure 1.

2.2. Synbiotics Administration

The synbiotics preparation used in this study comprised 2 probiotics strains and 2 prebiotics in an aluminum foil sachet. The synbiotics preparation is a mixture of probiotics strains in a total concentration of 10^{10} CFU (5.0×10^9 CFU of *Lactobacillus paracasei* HII01 and 5.0×10^9 CFU of *Bifidobacterium animalis* subsp. *lactis*) and 10 g prebiotics (5 g galactooligosaccharides (GOS), and 5 g oligofructose (FOS)). The probiotics were prepared and purchased from Lactomason Co., Ltd., Gyeongsangnam-do, South Korea. The prebiotics were purchased from New Francisco (Yunfu city) Biotechnology Cooperation Limited, Yunfu city, Guangdong, China (GOS; GOS-700-P), and Orafti®P95, (Beneo-Orafti S.A., Rue Louis Maréchal 1 4360 OREYE, Belgium) (FOS). The concentration of the probiotics strain was fixed according to the suggestions from the previous study [29].

Throughout the study, the participants were asked to consume the synbiotics preparation before breakfast every day by dissolving the content in water. The participants were on a dietary plan (as per the nutritionist's suggestions, we provided 3 meals/day to the

participants; 1200 calories per day; at the ratio of carbohydrate: protein: fat = 50:20:30) and sustained their routine medications and activities. Participants were asked to record their regular activities, and other additional medications and supplements during the study.

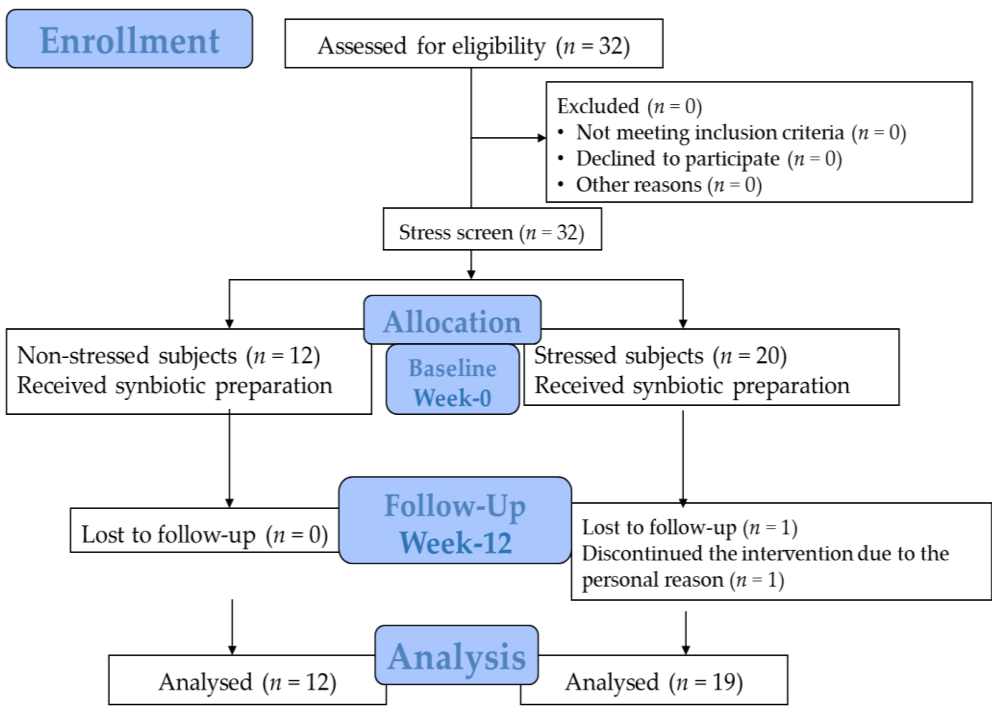

Figure 1. The allocation and follow-up chart.

2.3. Outcome Measurements

The primary outcome measurements were stress status with negative and positive feelings manipulation after the 12th week of synbiotics administration. The secondary outcome measurements were the biomarkers of HPA-axis (salivary cortisol and dehydroepiandrosterone sulfate (DHEA-S)), neuro-inflammatory cytokines (tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10)), tryptophan metabolism (kynurenine pathway; tryptophan and kynurenine, serotonergic pathway; quinolinic acid (QA) and 5-hydroxyidoleacetic acid (5-HIAA)), gut microbial metabolites (short-chain fatty acids (SCFAs) and lipopolysaccharide (LPS)), and immunoglobulin A (IgA).

2.4. Participants' Characteristic Data

Personal health data were collected, including eating habits, excretion, alcohol consumption, smoking, underlying diseases, medication, and regular supplementation. Demographic data, including sex, age, smoking habits, alcohol consumption, weight, and body mass index, were evaluated.

2.5. Stress Assessment

The study subjects were separated based on the results of the Thai Stress-test (TST). All the subjects tested for the Thai Stress Test were separated into two groups as detailed (Figure 1). The stress level was assessed via TST, developed by Phattharayuttawat et al. [28] for enrollment screening, measuring the stress status, scale scores at baseline, and the end of the study. TST has been assessed for construct validity and showed the Cronbach's alpha coefficient of 0.84 and split-half coefficient of 0.88 [28]. The test comprised 24 questions, with 12 questions for negative feelings effects and 12 for positive feelings effects. Each question needs to be answered by participants based on their personal experience. The answers have a 3-rating scale: "0" means "never", "1" means "sometimes", and "3" means "often".

Negative and positive question items were separately summarized. The matrix table has used both sides of the scale scores to identify the negative and positive scale score groups (Table 1). After grouping the negative and positive scale scores, the number in the matrix table showed the stress status. Therefore, the stress status was interpreted as follows: scoring group 1 was "Excellent mental health (if not faking)", scoring groups 2, 3, and 4 were "Normal mental health," scoring groups 5 and 6 were "Mild stress", and scoring groups 7, 8, and 9 were "Stressful".

Table 1. Matrix table for the index of TST [28].

Negative Scale Score (Sum of Item 1–12)	Positive Scale Score (Sum of Item 13–24)				
	12–36	9–11	6–8	3–5	0–2
0–1	1	2	3	4	5
2–3	2	3	4	5	6
4–5	3	4	5	6	7
6–7	4	5	6	7	8
8–36	5	6	7	8	9

2.6. HPA-Axis Assessment

Salivary samples were gathered at 7–9 a.m. for cortisol and DHEA-S measurement and stored at −20 °C before analysis. Both cortisol and DHEA-S were measured by enzyme-linked immunosorbent assay (ELISA) commercial kit (Eagle biosciences®, Amherst, NH, USA). Salivary cortisol ultrasensitive ELISA was for cortisol, and DHEA-S Saliva ELISA Assay Kit was for DHEA-S. The analysis method was followed according to the manufacturer's instructions.

2.7. Neuroinflammatory Cytokines Assessment

Serum inflammatory cytokines TNF-α and IL-10 were determined using two commercial ELISA kits: The Human TNF-α ELISA kit and the IL-10 Human ELISA kit. The measurement protocol followed the instructions of the manufacturer Thermo fisher, Sydney, NSW, Australia.

2.8. Tryptophan Metabolism Assessment

Serum level tryptophan and kynurenine were analyzed via high-performance liquid chromatography (HPLC) following the modified method of Badawy and Morgan (2010). The sample was pushed into Synergi™ 4 µL fusion-RP80 A column C18 (250 × 4.6 mm) at 37 °C with a 0.8 mL/min flow rate. Furthermore, 10 mM sodium dihydrogen phosphate (27:73 *v*/*v*) pH 2.8 was used as the mobile phase, and a UV detector at 220 nm was used [30].

The morning urine samples were collected for QA and 5-HIAA determination. The Human Quinolinic acid ELISA kit (Fivephoton Biochemicals™, San Diego, CA, USA) was used for QA and 5-HIAA-ELISA-Kit-Urine-Fast-Track (Immusmol, Bordeaux, France) for 5-HIAA determination. The measurement protocols were followed according to manufacturer's instructions.

2.9. Gut Microbial Metabolites and Immunoglobulin Assessment

The morning stool samples were used to determine SCFAs by HPLC, as detailed previously by Katoni et al. [31]. Shodex SH1011 column was used. A total of 5 mM sulfuric acid was used as the mobile phase with a flow rate of 0.6 mL/min [32].

Serum endotoxin LPS was analyzed by Human lipopolysaccharide (LPS) ELISA Kit (MyBioSource®, San Diego, CA, USA). Human IgA (Immunoglobulin A) ELISA kit (Elabscience®, Houston, TX, USA) was used for IgA determination.

2.10. Statistical Analyses

All statistical analyses were executed using STATA15.1 for Windows (Stata Corp, College Station, TX, USA). The license was for the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. All variable outcomes were displayed as mean ± standard of error (SE) and tested for normal distribution. Demographic data—age, weight, kidney function (Blood urine nitrogen (BUN) and creatinine clearance), liver function (Alanine aminotransferase (AST) and aspartate aminotransferase (ALT)), and serum cholesterol profile were assessed via *t*-test or Mann–Whitney U test, as appropriate. Sex, smoke habit, and alcohol consumption were evaluated via Fisher's exact test. The different outcomes of each group at baseline and the end of the study were calculated using a paired *t*-test or Wilcoxon signed-rank test. The results between groups were compared using a *t*-test or Mann–Whitney U test as appropriate, and Gaussian regression was used to find the relation of both groups. All parameters were also evaluated by power analysis. Statistical significance was examined as a *p*-value less than 0.05.

3. Results

3.1. Characteristics of Participants

A total of 32 volunteers were enrolled. After the stress screening, 20 participants were classified as belonging to the stressed group, with 1 drop-out for personal reasons. The other volunteers, 12 participants, were identified as the non-stressed group. The demographic data of both groups were compared statistically and demonstrated no difference in all parameters ($p < 0.05$) (Table 2).

Table 2. Characteristics of participants at the baseline.

Parameters	Non-Stressed Group (n = 12)	Stressed Group (n = 19)	p-Value *
Male, n (%)	3 (25.00)	8 (42.11)	0.282 [c]
Female, n (%)	9 (75.00)	11 (57.89)	
Age (years)	43.17 ± 3.01	45.79 ± 3.10	0.572 [a]
Weight, kg	75.20 ± 4.28	76.75 ± 3.64	0.788 [b]
Body mass index (BMI), kg/m^2	29.70 ± 1.59	29.36 ± 1.28	0.872 [b]
Blood urea nitrogen (BUN), mg/dL	12.33 ± 1.19	13.32 ± 0.80	0.481 [a]
Creatinine clearance, mg/dL	0.90 ± 0.11	0.87 ± 0.05	0.810 [a]
Aspartate aminotransferase (AST), IU/L	25.17 ± 6.01	24.37 ± 5.62	0.776 [a]
Alanine aminotransferase (ALT), IU/L	29.17 ± 9.04	27.63 ± 6.96	0.855 [b]
Smoking, n (%)			
No	12 (100.00)	19 (100.00)	-
Yes	0(0.00)	0(0.00)	
Alcohol drinking, n (%)			
No	11 (91.67)	13 (68.42)	0.143 [c]
Yes	1 (8.33)	6 (31.58)	

* p-value at 95% confidence interval. [a] p-value was calculated from the t-test. [b] p-value was calculated from Mann-Whitney U test. [c] p-value was calculated from Fisher's exact test.

3.2. Effect of Synbiotics on Stress Status and Stress Score

Stress status was identified as four statuses: excellent mental health (if not faking), normal mental health, mild stress, and stressful, according to Phattharayuttawat et al., 2000. At the baseline, the stress status of the stressed group was presented as 1 (5.26%)

participant with stressful and 18 (94.74%) participants with mild stress. The non-stressed group comprised 9 (75%) participants with normal mental health and 3 (25%) participants with excellent mental health (if not faking), as shown in Figure 2a. The changes in stress status in stressed and non-stressed participants are displayed in Figure 2b. The synbiotics administration slightly improved stress status in the stressed group but not in the non-stressed group.

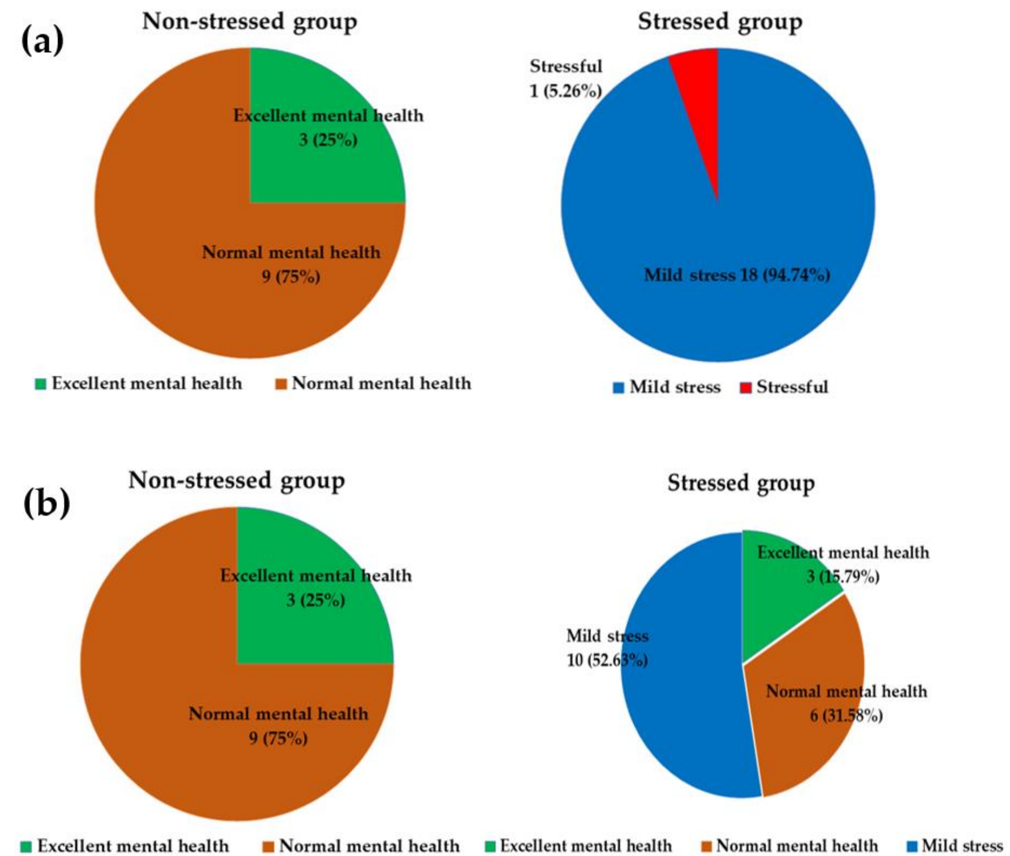

Figure 2. Stress status of the stressed and non-stressed groups (numbers of subjects (%)). The different color denotes each stress status. Green: Excellent mental health; Orange: Normal mental health; Blue: Mild stress; Red: Stressful. (**a**) Stress status at baseline, (**b**) Stress status after 12 weeks.

At baseline, the stressed group (13.53 ± 1.20) expressed a statistically significant high scale score on negative feelings compared with the non-stressed group (2.33 ± 0.54) ($p < 0.001$). In contrast, there was no statistical difference in the positive scale scores (Figure 3).

Although there was no significant change of stress status in the stressed group, the outcome was exhibited the decreasing negative scale scores of TST from 13.53 ± 1.20 at the baseline to 8.21 ± 1.33 at the 12th week; $p = 0.001$. In the meantime, there was no statistical difference in positive scale scores after the synbiotics administration in the stressed group. The positive scale scores of the stressed group were modified from 20.16 ± 1.59 to 19.79 ± 1.87, respectively ($p = 0.668$). In comparison, neither negative scale scores nor positive scale scores were modified from baseline in the non-stressed group after the 12th week of synbiotics administration. However, the negative scale scores in the non-stressed group were from 2.33 ± 0.54 to 2.67 ± 0.61, respectively ($p = 0.418$) and the positive scale scores were from 19.33 ± 2.62 to 21.00 ± 2.03, respectively ($p = 0.668$).

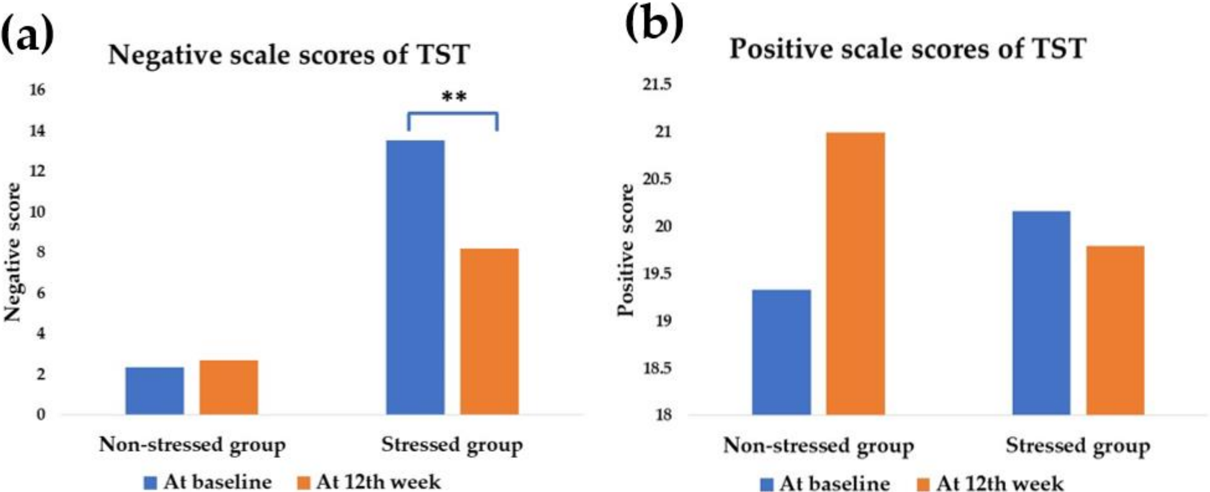

Figure 3. Modulation of mean TST scores according to either negative scale scores or positive scale scores after the synbiotics administration. The blue bar is the scores at the baseline, and the orange bar is the scores at the 12th week of synbiotics administration. ** significant difference (p less than 0.05). (**a**) The negative scale scores of TST in the stressed group were attenuated at the 12th week ($p = 0.001$, power = 0.98). (**b**) The positive scale scores of TST were not significantly changed in both groups ($p < 0.05$).

3.3. Effect of Synbiotics Administration on HPA-Axis and Inflammatory Cytokines

Before the synbiotics administration, the mean cortisol of the stressed participants and control exhibited no difference; however, a slightly higher level in the non-stressed group was observed ($p < 0.05$). At the same time, a high level of pro-inflammatory mediator TNF-α was found in the stressed group. It was modified from 89.75 ± 7.53 at baseline to 49.48 ± 5.07 ng/mL at the 12th week, respectively ($p < 0.001$).

After the administration, HPA-axis was downregulated in both groups by attenuating cortisol levels ($p < 0.05$). The synbiotics also induced DHEA-S upregulation in the non-stressed group ($p < 0.05$), as shown in Figure 4.

Salivary cortisol was improved from 219.37 ± 29.13 ng/mL at baseline to 154.77 ± 7.74 ng/mL ($p = 0.033$) in the stressed group and 275.24 ± 17.80 ng/mL at baseline to 204.16 ± 17.18 ng/mL ($p = 0.015$) in the non-stressed group. DHEA-S was also modulated from 0.99 ± 0.14 at baseline to 1.71 ± 0.23 ng/mL at the 12th week ($p = 0.012$) in the non-stressed group.

Synbiotics promoted inflammatory mediation at the end of the study. The anti-inflammatory IL-10 was significantly upregulated in both stressed and non-stressed participants. In the stressed group, IL-10 was modified from 29.82 ± 0.70 to 69.84 ± 9.44 ng/mL at the 12th week ($p = 0.002$) and the modification was found from 26.94 ± 1.85 to 82.32 ± 15.07 ng/mL, ($p = 0.015$) for the non-stressed group. Interestingly, the proinflammatory cytokine TNF-α was significantly increased in the non-stressed group from 49.48 ± 5.07 at the baseline to 205.74 ± 33.86 ng/mL at the 12th week ($p = 0.002$). In the meantime, there was no modification found in the stressed group.

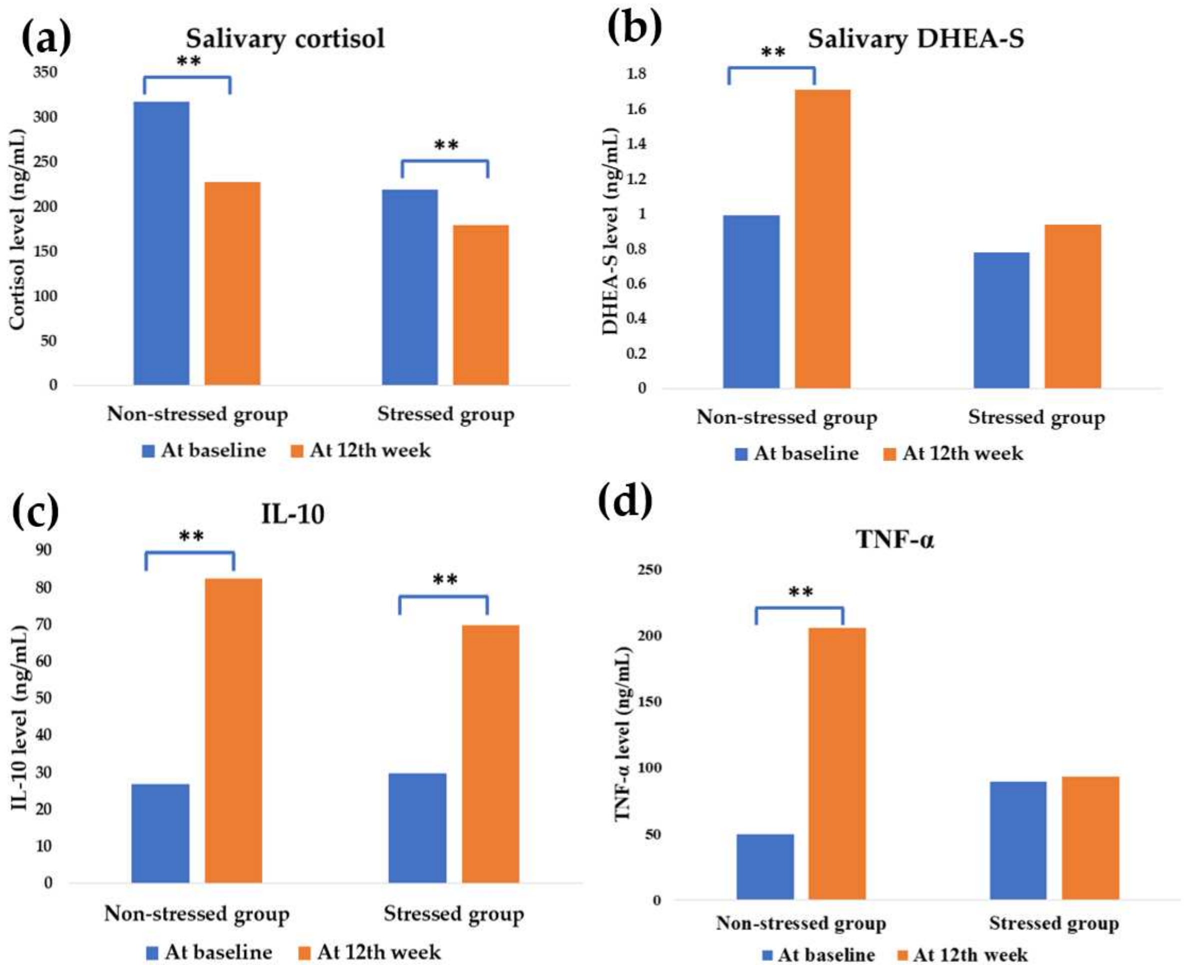

Figure 4. Effect of synbiotics administration on HPA-axis and neuro-inflammatory cytokines modulation. The blue and orange bar shows the values at baseline and the 12th week of synbiotics administration, respectively. ** significant difference (p less than 0.05). DHEA-S = Dehydroepiandrosterone sulfate; IL-10 = Interleukin-10; TNF-α = Tumor Necrosis Factor-alpha. The effect on HPA-axis: (**a**) Mean salivary cortisol value; stressed group: $p = 0.033$ (power = 0.65), non-stressed group: $p = 0.015$ (power = 0.96), (**b**) Mean DHEA-S value, non-stressed group: $p = 0.012$ (power = 0.99). The effect on inflammatory cytokines: (**c**) Mean IL-10 value; stressed group: $p = 0.002$ (power = 0.99), non-stressed group: $p = 0.015$ (power = 0.94), (**d**) Mean TNF-α value; non-stressed group: $p = 0.002$ (power = 0.99).

3.4. Effect of Synbiotics Administration on Tryptophan Metabolism

At baseline, the precursor tryptophan of the stressed group displayed lower than the non-stressed group (from 26.86 ± 1.62 to 33.64 ± 1.54 µmol/L; $p = 0.008$, respectively) and also with the catabolite kynurenine (from 1.11 ± 0.22 to 1.54 ± 0.13 µmol/L; $p = 0.033$, respectively) whereas QA (6.03 ± 0.42 and 5.83 ± 0.23 ng/mL; $p = 0.712$, respectively) and 5-HIAA (3.25 ± 0.52 and 2.34 ± 0.18 mg/L; $p = 351$, respectively) displayed a higher tendency.

The tryptophan metabolism was expressed in the difference after the synbiotics administration between groups. The precursor tryptophan was significantly downregulated in both stressed and non-stressed group (from 26.86 ± 1.62 to 21.67 ± 3.57 µmol/L, $p = 0.003$ and from 33.64 ± 1.54 to 22.94 ± 1.08 µmol/L, $p < 0.001$, respectively).

In the non-stressed group, the tryptophan was metabolized to convert all parameters at the end of the study, especially 5-HIAA. It was modulated from 2.34 ± 0.18 to 3.81 ± 0.26 mg/L, ($p < 0.001$), respectively. In comparison, there was no statistical modification found for kynurenine and QA ($p < 0.05$) (Figure 5)

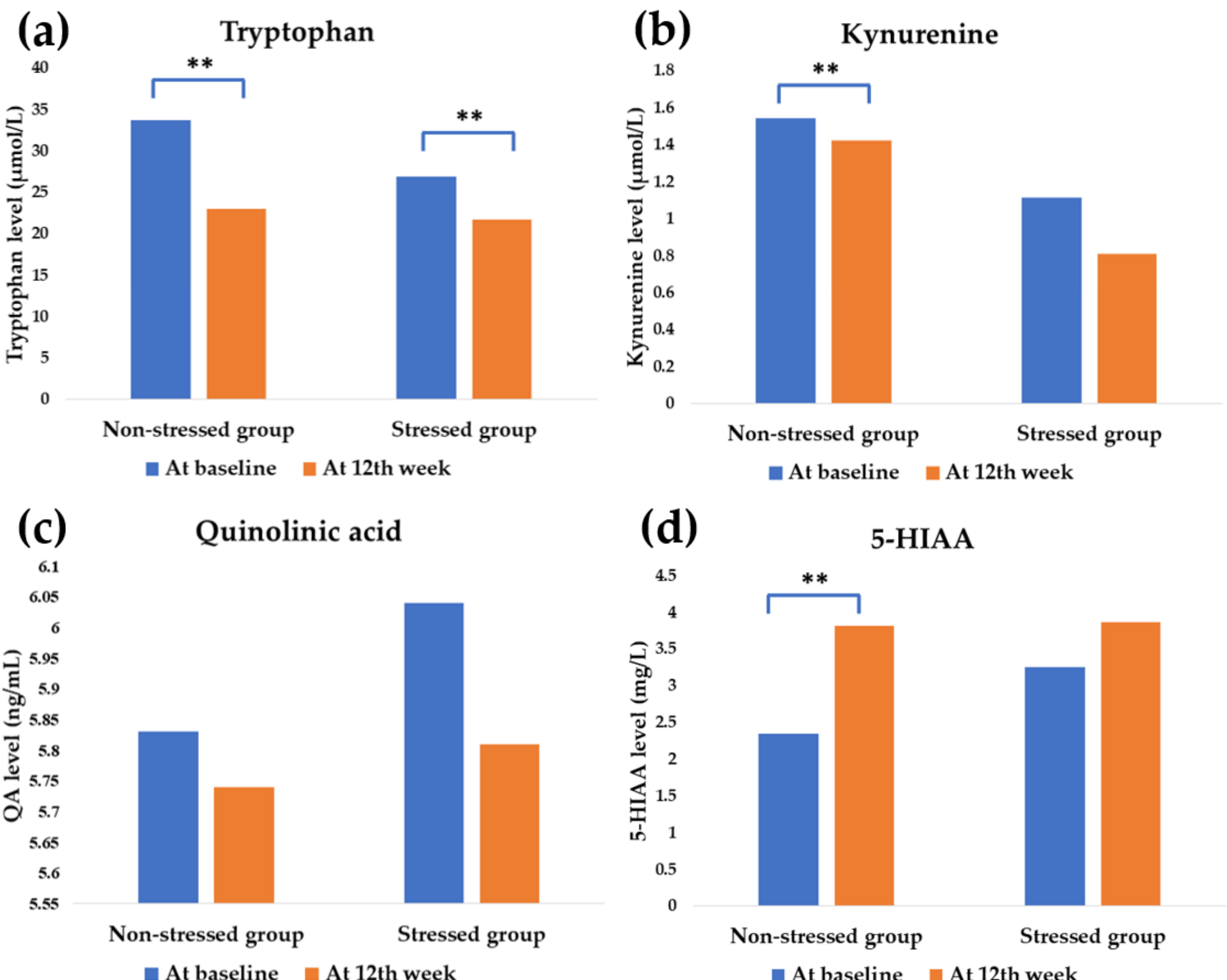

Figure 5. Effect on tryptophan metabolites. The blue and orange bar shows the values at baseline and the 12th week of synbiotics administration, respectively. ** significant difference (p less than 0.05). 5-HIAA = 5-Hydroxyindoleacetic acid. The tryptophan modulation on kynurenine pathway: (**a**) Mean tryptophan value: stressed group, $p = 0.003$ (power = 0.94), non-stressed group, $p < 0.001$ (power = 1.00), (**b**) Mean kynurenine value. The modulation of tryptophan metabolism to the serotonergic pathway: (**c**) Mean quinolinic acid value, (**d**) Mean 5-HIAA value: non-stressed group, $p < 0.001$ (power = 1.00).

3.5. Effect of Synbiotics Administration on Gut Microbial Metabolites and Immunoglobulin

Before synbiotics administration, SCFAs propionate and butyrate expressed a statistical difference. Propionate in the stressed group displayed a higher level than the non-stressed (19.99 ± 2.04 and 11.40 ± 1.57 mmol/g; $p = 0.006$, respectively) while butyrate was lower (6.38 ± 0.40 and 7.56 ± 0.24 mmol/g; $p = 0.036$, respectively). A significant difference was not observed in either LPS or IgA.

Unexpectedly, there was no statistical modification of SCFAs in the stressed group at the end of the study. In contrast, a significant modulation of acetate (4.11 ± 0.10 to 5.26 ± 0.24 mmol/g; $p < 0.001$) and propionate (11.40 ± 1.57 to 22.06 ± 0.52 mmol/g; $p < 0.001$) was found in the non-stressed group as shown in Figure 6.

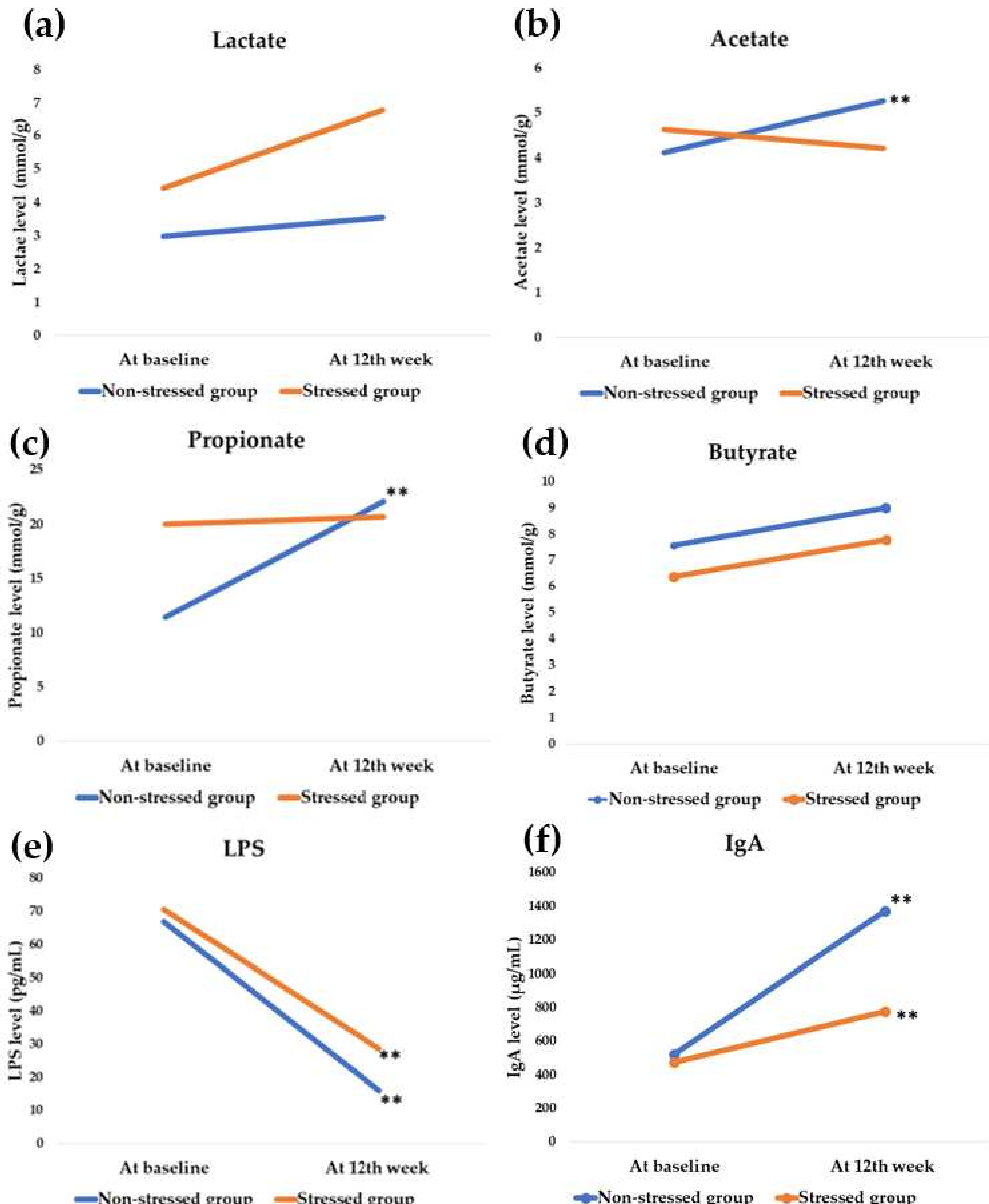

Figure 6. Effect on gut microbial metabolites. The blue and orange bar shows the values at baseline and the 12th week of synbiotics administration, respectively. ** significant difference (p less than 0.05). LPS = Lipopolysaccharide; IgA = Immunoglobulin A. Modulation of SCFAs. (**a**) Mean change of lactate, (**b**) Mean change of acetate: non-stressed group: $p < 0.001$ (power = 1.00), (**c**) Mean propionate value: non-stressed group: $p < 0.001$ (power = 1.00), (**d**) Mean change of butyrate value. The modulation of endotoxin: (**e**) Mean change of LPS value: stressed group: $p < 0.001$ (power = 0.97), non-stressed group: $p = 0.002$ (power = 0.92). Modulation of immunoglobulin: (**f**) Mean IgA value: stressed group; $p = 0.004$ (power = 0.81), non-stressed group: $p = 0.003$ (power = 1.00).

Synbiotics attenuated LPS level in both stressed participants (70.35 ± 11.43 to 28.57 ± 8.84 pg/mL, $p < 0.001$) and non-stressed participants (66.78 ± 13.73 to 16.01 ± 3.74 pg/mL, $p = 0.002$), whereas it promoted IgA elevation. In the stressed group,

IgA was elevated from 473.79 ± 56.80 to 773.17 ± 114.76 μg/mL (p = 0.004) and from 521.14 ± 65.29 to 1367.71 ± 107.15 μg/mL (p = 0.003) in the non-stressed group.

3.6. Differences in Pre- and Post-Administration Status among the Stressed and the Non-Stressed Groups

The pre-and post-administration differences showed a significant decrease in the tryptophan in the stressed and the non-stressed groups ($p < 0.05$; power = 0.77). The non-stressed group displayed higher DHEA-S levels but slightly lower than the stressed group ($p < 0.05$, power = 0.62).

The IgA, acetate, and propionate levels showed a statistical difference in the stressed and non-stressed groups. The IgA level increased significantly in both groups ($p < 0.05$; power = 0.98). Nonetheless, a higher propionate increase was observed in the non-stressed group ($p < 0.05$; power = 0.84). Unexpectedly, the negative (power = 0.94) and positive (power = 0.94) scale scores of the TST slightly increased in the non-stressed groups and decreased in the stressed group ($p < 0.05$) (Table 3).

Table 3. Comparison of the differences between pre- and post-administration.

Parameters	Differences between Pre- and Post-Administration		
	Non-Stressed Group	**Stressed Group**	***p*-Value ***
TST scores			
Negative scale scores #	0.33	−5.32	0.009 b**
Positive scale scores #	0.33	−5.32	0.009 b**
HPA-axis			
Cortisol (ng/mL)	−89.48	−40.34	0.491 b
DHEA-S (ng/mL)	0.73	−0.01	0.004 b**
Inflammatory cytokines			
IL-10 (ng/mL)	55.38	40.02	0.256 b
TNF-α (ng/mL)	156.27	3.28	<0.001 b**
Tryptophan metabolism			
Tryptophan (μmol/L)	−10.7	−5.20	0.029 b**
Kynurenine (μmol/L)	−0.12	−0.30	0.919 b
QA (ng/mL)	−0.09	−0.23	0.824 b
5-HIAA (mg/L)	1.47	0.61	0.626 b
Gut microbial metabolites & SCFAs			
Lactate (mmol/g)	0.55	2.34	0.351 b
Acetate (mmol/g)	1.14	1.31	0.016 b**
Propionate (mmol/g)	10.66	0.64	0.007 b**
Butyrate (mmol/g)	1.42	1.39	0.208 b
Endotoxin			
LPS (pg/mL)	−50.77	−41.78	0.394 b
Immunoglobulin			
IgA (μg/mL)	846.57	299.37	<0.001 b**

* p-value at 95% confidence interval, ** significant difference (p less than 0.05). # Mean of negative and positives scale scores of Thai Stress Test (TST). b p-value was calculated from Mann-Whitney U test; DHEA-S = Dehydroepiandrosterone sulfate; IL-10 = Interleukin-10; TNF-α = Tumor Necrosis Factor-alpha; QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid; LPS = Lipopolysaccharide; IgA = Immunoglobulin A.

3.7. Gaussian Regression Analysis of the Outcomes at the End of the Study

The Gaussian regression analysis of the outcomes after the 12th week of the synbiotics administration indicated that the synbiotics administration significantly modified cortisol level (power = 0.92) but downregulated IL-10 (power = 0.82). The other parameters were not altered significantly (Table 4).

Table 4. Gaussian regression analysis of the outcomes after the 12th week.

Parameters	Coefficient	95% CI	*p*-Value *
TST score			
Negative scale scores #	2.39	−3.06 to 7.84	0.372
Positive scale scores #	0.77	−8.04 to 7.09	0.897
HPA-axis			
Cortisol (ng/mL)	−72.63	−120.31 to −24.96	0.005 **
DHEA-S (ng/mL)	−0.85	−1.73 to 0.03	0.057
Inflammatory cytokines			
IL-10 (ng/mL)	−33.27	−84.60 to 18.08	0.192
TNF-α (ng/mL)	−79.85	−176.28 to 16.58	0.100
Tryptophan metabolism			
Tryptophan (μmol/L)	0.67	−4.63 to 4.96	0.942
Kynurenine (μmol/L)	−0.26	−0.77 to 0.24	0.289
QA (ng/mL)	−0.18	−1.06 to 0.70	0.670
5-HIAA (mg/L)	0.65	−1.17 to 2.47	0.466
Gut microbial metabolites & SCFAs			
Lactate (mmol/g)	3.71	−1.32 to 8.75	0.139
Acetate (mmol/g)	−1.19	−2.18 to 0.16	0.082
Propionate (mmol/g)	−1.07	−9.69 to 7.55	0.798
Butyrate (mmol/g)	−1.17	−9.87 to 7.53	0.782
Endotoxin			
LPS (pg/mL)	4.25	−26.21 to 34.72	0.774
Immunoglobulin			
IgA (μg/mL)	−554.95	−1037.47 to −72.43	0.026 **

* *p*-value at 95% confidence interval, ** significant difference (*p* less than 0.05). # Mean of negative and positive scale scores of Thai Stress Test (TST). DHEA-S = Dehydroepiandrosterone sulfate; IL-10 = Interleukin-10; TNF-α = Tumor Necrosis Factor alpha; QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid; LPS = Lipopolysaccharide; IgA = Immunoglobulin A.

4. Discussion

Stress exposure affects the gut–brain axis [33]. Stress manipulation by probiotics and prebiotics, particularly GOS and FOS, has been proven in numerous studies [34–36]. In clinical studies, the probiotics strain *L. paracasei* HII01 positively affects cholesterol, obesity, and diabetes indices modulation [37,38]. However, this study investigated the greater benefit of stress manipulation of *L. paracasei* HII01 using a synbiotics formula. The study population was small in the present study. The power analysis of each parameter was calculated, and the values are higher than 0.80 (Figures 3–6), except salivary cortisol level in the stressed group.

Our mental health status depends on our capacity for handling our feelings. Positive feelings include happiness and life satisfaction; on the other hand, negative feelings include sadness and life dissatisfaction. If someone has more negative feelings than positive thoughts, we consider them in disturbed mental states or stressed mental states [39].

Regarding stress evaluation tools, negative feelings are the most abundant. Accordingly, the appropriate tools for stress assessment should evaluate both sides of feelings. One of the common tools, such as the Perceived stress scale-10, assessed perceived distress and the ability to cope with stress [40]. In comparison, the General Health Questionnaire (GHQ) Thai version inspects the perceived stress on mental and physical symptoms [41]. Conversely, the TST is the tool to evaluate both feelings of the mind (the negative and positive feelings) corresponding with psychological well-being [28].

In this study, the stress status of the participants was classified based on TST scores. The stressed subjects had more negative scale scores and non-significant positive scale scores at baseline. We may conclude that the stressed subjects might have poor stress management ability. After the synbiotics administration, the negative scale scores were

significantly reduced, as mentioned in Figure 3. The results suggest that synbiotics administration could attenuate negative feelings in stressed participants.

Stress response corresponds to the HPA-axis, which is particularly associated with cortisol level [42]. During a stressed condition, the cortisol level increases and tries to regulate stress. However, hypercortisolemia does not mean that the subjects have excellent stress regulation. It is a sign of chronic fatigue syndrome when cortisol levels drop after acute elevation [43]. At the baseline, the cortisol level was lower in stressed subjects. No matter the cortisol level in both groups, the synbiotics administration promoted cortisol lowering effects significantly.

Synbiotics administration was found to stimulate the secretion of the anti-inflammatory cytokine IL-10 in stressed and non-stressed groups. Elevated IL-10 promotes HPA-axis modulation via adrenocorticotrophic hormone (ACTH) inhibition [44]. The low level of IL-10 is associated with prolonged stress exposure and depressive-like behavior [45]. Despite IL-10 providing an advantage for mental health modulation, the modification was found only in stressed participants. Conversely, elevated IL-10 exhibited no effect on mental modulation in the normal mental health in the non-stressed group.

Synbiotics administration promoted the TNF-α level, which mediates the indoleamine-2,3-oxygenase enzyme (IDO) [46] in tryptophan catabolism. TNF-α elevation was observed in the non-stressed but not the stressed participant group. Tryptophan is an essential amino acid correlated with mental health in animals and humans. The metabolites of tryptophan play a pivotal role in neurodegenerative diseases and mental health [47].

Surprisingly, synbiotics administration does not affect tryptophan metabolism in stressed subjects. Usually, the neurotoxic pathway originates from the catabolism of tryptophan by the IDO enzyme. Kynurenine, the metabolite of this reaction, is associated with neuropsychiatric symptoms [48]. Therefore, the several reactions after the kynurenine conversion induce QA production which is a known neurotoxin; moreover, it also induces oxidative stress, glutamine excitotoxicity, and mitochondria dysfunction [49].

Conversely, the neuroprotective route of tryptophan catabolism is via several enzymes such as monoamine oxidase (MAO) and aldehyde dehydrogenase to produce 5-HIAA, which is indicated to be a biomarker of neuropsychiatric disorders [50]. As per the results, it was found that the negative feelings attenuation of synbiotics administration might not occur via tryptophan metabolism modulation. In comparison, the low level of tryptophan and the increase of 5-HIAA level in the non-stressed group demonstrated no effect on stress status and scale scores (Figure 5).

A connection between stress regulation and gut commensal microbial metabolites has been suggested [51,52]. SCFAs such as propionate and butyrate play a role in neuroprotective and psychiatric disorder treatment [53,54]. According to the preclinical study of Maltz et al. (2019), low levels of acetate and butyrate were observed in stressed mice [55]. We observed that stressed subjects showed higher propionate levels than the non-stressed group. Unexpectedly, the effect of synbiotics on the SCFAs in the stressed participants showed no statistical difference (Figure 6). According to this result, the negative feelings modulation may not occur via SCFAs.

The gut microbial endotoxin LPS correlates with neuropsychiatric disorders [56]. LPS, by itself, can cross the blood-brain barrier and affect neurophysiology, which in turn affects emotion and behavior [57]. The rising LPS level is accompanied by negative emotion, anxiety, social disconnection, anhedonia, and fatigue [58]. LPS can influence glucocorticoid receptor expression in the hypothalamus that involves HPA-axis regulation [59–61]. The downregulation of LPS according to the synbiotics administration in the current study corresponded to the negative scale scores of TST.

The IgA is an antibody found abundantly in the intestinal epithelial and plays a role in antimicrobial activity, regulating the healthy composition and metabolic function of gut microbiota [62,63]. In addition, the alteration of IgA is also associated with stressful life events [64]. Psychological and physical stressors have a negative effect on IgA genera-

tion [65,66]. The results of this study indicate that the negative scale scores of TST were influenced by IgA elevation.

In summary, consumption of synbiotics improved negative feelings in stressed participants. According to our findings, the possible action is governed by modulating the HPA-axis, IL-10, LPS, and IgA. It is proven via the modulation of the negative scale scores at baseline after the synbiotics administration (Figure 3).

5. Conclusions

The current study was conducted with a small number of experimental subjects, and the results have to be confirmed again with extended studies. Nonetheless, the results showed that synbiotics administration reduced negative feelings in stressed participants. Furthermore, the synbiotics administration altered the HPA-axis, IL-10, LPS, and IgA levels.

Further studies are required on how the provided synbiotics administration alters the microbiota to discover the mechanism behind the positive effect of synbiotics on stress status.

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Article

Probiotics Supplementation Improves Intestinal Permeability, Obesity Index and Metabolic Biomarkers in Elderly Thai Subjects: A Randomized Controlled Trial

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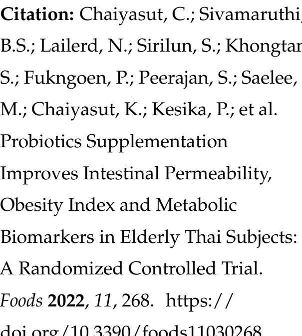

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Abstract: Intestinal integrity prevents the diffusion of allergens, toxins, and pathogens from the gastrointestinal lumen into the tissue and the circulatory system. Damage in intestinal integrity may cause mild to serious health issues, such as inflammation, gastrointestinal disorders, neurological diseases, and neurodegenerative disorders. Thus, maintaining a healthy intestinal barrier function is essential to sustain health. Probiotics are known for their ability to protect and restore intestinal permeability in vitro and in vivo. The multi-strain probiotics are more efficient than that of a single strain in terms of their protective efficacy. Therefore, the present study was planned and implemented to study the supplementation of probiotic mix (*Lactobacillus paracasei* HII01, *Bifidobacterium breve*, and *Bifidobacterium longum*) on intestinal permeability, lipid profile, obesity index and metabolic biomarkers in elderly Thai subjects. The results revealed that the supplementation of studied probiotics improved the intestinal barrier function (up to 48%), significantly increasing the high-density lipoprotein (HDL)-cholesterol. Moreover, the intervention improved obesity-related anthropometric biomarkers and short-chain fatty acid levels in human subjects. The current study strongly recommends further extended research to confirm the beneficial effect of probiotics, which may pave the way to formulate probiotic-based health supplements to adjuvant the treatment of several metabolic diseases.

Keywords: probiotics; *Lactobacillus*; *Bifidobacterium*; intestinal permeability; cholesterol

1. Introduction

The most important protective function of the intestinal epithelium is the "barrier function," which prevents the diffusion of allergens, toxins, and pathogens from the gastrointestinal lumen into the tissue and the circulatory system [1,2]. A specialized complex structure is present in the lateral epithelial membranes' apical region, which is considered to be a significant module of epithelial barrier function known as tight junctions [3].

The imbalance of gastrointestinal microbiota and its function may cause interruption of the tight junctions, which provokes intestinal permeability [4]. Thus, bacterial debris, endotoxins such as lipopolysaccharides (LPS), and other microbial metabolites breach the circulatory system and reach internal organs, which can cause mild to serious health issues such as inflammation, gastrointestinal disorders, neurological diseases, and neurodegenerative disorders [5,6].

The gastrointestinal tract, especially the gut, has a complex and bidirectional communication with the central nervous system (gut–brain axis) that communicates in health and diseases [7]. The disturbance in gut microbiota might affect neurological functions and vice versa [8]. Thus, the loss of intestinal permeability might cause various neurological diseases [9,10].

Probiotics are live microorganisms, which, when administered in adequate amounts, confer health benefits [11]. Probiotics are the most recognized method to improve gut microbiota and treat dysbiosis [12–14]. The studies reported that the probiotics had enhanced the homeostasis of intestinal permeability [15], reduced inflammation [16], and also improved several ill-health conditions in humans [17,18]. However, the mechanism behind the beneficial effect of probiotics on health benefits is not explored. Especially in the elderly, how probiotic supplementation improves leaky gut, inflammation, and gut–brain interaction is not revealed completely and is debatable. Probiotics principally modulate gut microbiota, producing several metabolites, which confers health benefits [19].

Accordingly, the present study was planned and conducted in elderly Thai subjects to understand the impact of supplementation of a mixture of probiotics on intestinal permeability, short-chain fatty acids, markers of the gut-brain axis, and lipid profile.

2. Materials and Methods

2.1. Study Design and Participants

All the participants approved the study procedure and provided their consent before the study. The ethical committee of Mae Fah Luang University agreed to the study protocol (Code: REH-62151). The study was performed according to the Declaration of Helsinki and following the Good Clinical Practices.

The effect of a probiotics mixture on intestinal permeation and other biomarkers were studied in Thai subjects in a randomized, double-blind, placebo-controlled study model.

For the screening, subjects were asked to consume mannitol and lactulose dissolved in water. Within 6 h of mannitol and lactulose consumption, subjects were required to collect their urine [20], and their intestinal permeability was analyzed using a colorimetric commercial kit (EnzyChromTM BioAssay, San Jose, CA, USA).

Any subjects with a history of cardiovascular events, suffering from kidney diseases, gastrointestinal tract (GI) disorders, or gouty arthritis were excluded from the study. In addition, those who have undergone treatment with probiotics, antibiotic drugs (or both) or any other drugs that are used to treat GI tract-related discomforts in the previous 14 days were also excluded.

Random Allocation Software was used to randomize the subjects, and the researchers and participants were blinded to the group assignment. The participants were randomized to receive either a probiotics supplement or placebo for 12 weeks and asked to come to the study center for follow-up. The screening and enrollment details are shown in Figure 1.

2.2. Treatment

The subjects in the probiotic group were provided with aluminum foil sachets containing a mixture of probiotics (2.0×10^{10} CFU of *Lactobacillus paracasei* HII01; 2.0×10^{10} CFU of *Bifidobacterium breve*; 1.0×10^{10} CFU of *Bifidobacterium longum*), which was received from Lactomason Co., Ltd., Jinju-si, South Korea, and the placebo group were provided with 10 g of corn starch in a similar package of probiotics. The instructions for the consumption of the supplement were detailed in our previous report [18].

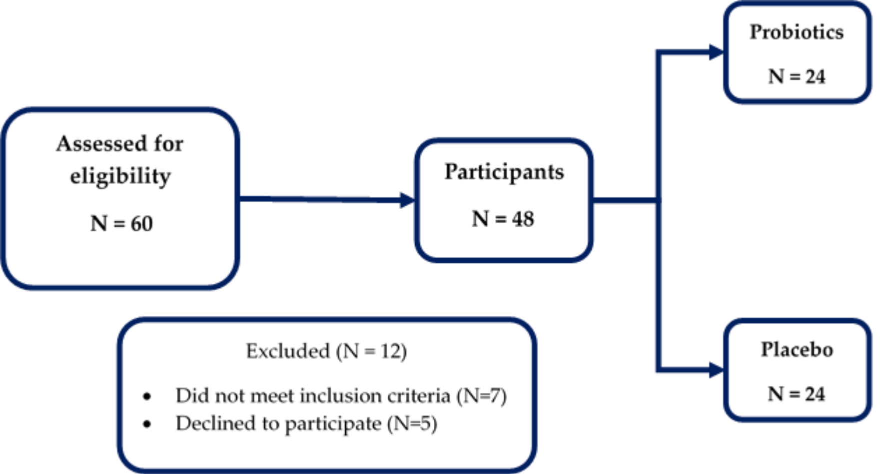

Figure 1. The enrollment and study flowchart.

2.3. Assessments

2.3.1. Clinical Data

The subjects' personal history was assessed, and their demographic characteristics were detailed. The body mass index (BMI) and weight of the subjects were measured using an electronic scale (Picooc®, Model S1 Pro, Beijing, China) [18].

2.3.2. Laboratory Data

The samples (blood, urine, and feces) were collected at the screening point, baseline, follow-up, and the end of the study (Figure 2).

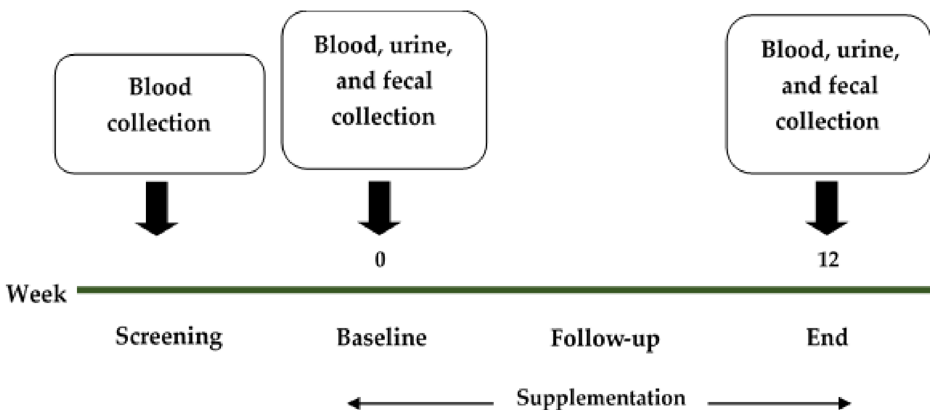

Figure 2. The timeline of this study, with sample collection points.

The biochemical parameters such as blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), and fasting blood sugar (FBS) levels were measured from blood samples using the automated machine at AMS Clinical Service Center, Chiang Mai University, Chiang Mai, Thailand.

Other biomarkers in the blood, such as Immunoglobulin A (IgA) and lipopolysaccharide (LPS), were measured using ELISA commercial kit (MyBioSource®, San Diego, CA, USA for LPS, Elabscience®, Houston, TX, USA for IgA).

Urine samples were collected from subjects to determine intestinal permeability. The samples were analyzed as detailed in our previous study [18] using a colorimetric commercial kit (EnzyChrom™, BioAssay, Hayward, CA, USA). Other biomarkers in the urine, such as quinolinic acid (QA) and 5-hydroxyindoleacetic acid (5-HIAA), were determined using ELISA commercial kit (Fivephoton Biochemicals™, San Diego, CA, USA for QA, and Immusmol, Bordeaux, France for 5-HIAA).

Fecal short-chain fatty acids content was determined using high-performance liquid chromatography (HPLC) as described previously [17,21,22].

2.3.3. Statistical Analyses

The data were evaluated using the paired *t*-test of means using STATA version 15.1 (StataCorp, College Station, TX, USA) for windows licensed to the Faculty of Pharmacy, Chiang Mai University.

A descriptive analysis of the collected parameters was expressed as an absolute number and percentage. The continuous variables were expressed as mean ± standard deviation (SD) or standard error of the mean (SEM), depending on their statistical distribution. The group's data were also evaluated using Gaussian regression and Risk difference regression. The minimum level of statistical significance was set at $p < 0.05$.

3. Results

3.1. The Study Participants

A total of 60 subjects were screened, and 48 subjects were selected for randomization. According to the study design, all enrolled subjects (men: 10, women: 38) completed the trial. The primary demographic data of the study subjects are detailed in Table 1.

Table 1. The basic characteristics of the study subjects.

Parameters	Placebo Group (n = 24)	Probiotics Group (n = 24)	p-Value
Age, years	58.79 ± 1.21	61.63 ± 0.84	0.061
Male, n (%)	7 (29.17)	3 (12.50)	0.286
Female, n (%)	17 (70.83)	21 (87.50)	
Smoking	2 (8.33)	3 (12.50)	1.000
Alcoholic	2 (8.33)	1 (4.17)	1.000
Height, cm	154.07 ± 1.57	153.40 ± 1.02	0.722
Body mass index, kg/m^2	25.13 ± 0.66	23.95 ± 0.39	0.136
BMR, kcal	1287.96 ± 32.58	1220.27 ± 21.49	0.090
Body fat, %	29.06 ± 1.57	29.19 ± 2.43	0.965
Visceral fat, %	11.00 ± 0.90	9.38 ± 0.52	0.129
Muscle, %	66.03 ± 1.62	69.57 ± 1.20	0.086
Body age, years	59.88 ± 1.83	60.57 ± 1.03	0.747
Arm circumference, cm	28.87 ± 0.59	28.25 ± 2.75	0.827
Waist circumference, cm	86.46 ± 1.74	84.77 ± 1.23	0.433
Hip circumference, cm	97.32 ± 1.23	94.63 ± 1.11	0.112

p-value at 95% confidence interval. The proportions were analyzed using an exact probability test, and the continuous demographic data were analyzed using a *t*-test.

The subjects in placebo and probiotic groups did not significantly differe at the beginning of the study. The distribution of male and female subjects did not show any significant differences ($p > 0.05$).

3.2. Changes in the Study Parameters within the Group

There were no changes in study parameters after 12 weeks of the study in the placebo group except the body fat and lactulose content compared to the baseline values, whereas significant changes were observed in: body mass index (p = 0.010); BMR (p = 0.043); waist (p = 0.011) and hip (p = 0.049) circumferences; creatinine (p = 0.013); AST (p = 0.013); ALT (p = 0.002); HDL-C (p = <0.001); LDL-C (p = 0.001); FBS (p = 0.021); IgA (p = <0.001); LPS (p = 0.001); lactulose–mannitol ratio (p = <0.001); lactulose (p = 0.006); QA (p = <0.001); propionic acid (p = 0.012); and butyric acid (p = 0.046) content in the probiotics group after 12 weeks of intervention, when compared to baseline values. Notably, the lactulose–mannitol ratio was reduced (from 0.222 ± 0.036 to 0.047 ± 0.004) after 12 weeks of treatment. The other studied parameters were not significantly changed (Table 2).

Table 2. Changes in the study parameters within-group at different times are expressed as mean ± SE.

Parameters	Placebo (n = 24)		p-Value	Probiotics (n = 24)		p-Value
	Baseline	12 Weeks		Baseline	12 Weeks	
Body mass index, kg/m^2	25.13 ± 0.66	24.53 ± 1.41	0.611	23.95 ± 0.39	23.38 ± 0.34	0.010 *
BMR, kcal	1287.96 ± 32.58	1280.05 ± 28.70	0.604	1220.27 ± 21.49	1233.36 ± 19.21	0.043 *
Body fat, %	29.06 ± 1.57	32.41 ± 1.08	<0.001 *	29.19 ± 2.43	27.72 ± 1.03	0.563
Visceral fat, %	11.00 ± 0.90	11.48 ± 0.53	0.397	9.38 ± 0.52	9.14 ± 0.48	0.234
Muscle, %	66.03 ± 1.62	62.91 ± 0.61	0.079	69.57 ± 1.20	70.36 ± 1.26	0.188
Body age, years	59.88 ± 1.83	60.75 ± 1.21	0.544	60.57 ± 1.03	60.96 ± 0.94	0.464
Arm circumference, cm	28.87 ± 0.59	28.92 ± 0.61	0.883	28.25 ± 2.75	26.87 ± 0.48	0.610
Waist circumference, cm	86.46 ± 1.74	88.00 ± 1.60	0.099	84.77 ± 1.23	81.99 ± 1.26	0.011 *
Hip circumference, cm	97.32 ± 1.23	99.20 ± 1.25	0.064	94.63 ± 1.11	87.87 ± 3.49	0.049 *
BUN, mg/dL	13.07 ± 0.58	13.30 ± 0.58	0.622	13.81 ± 0.87	13.63 ± 0.99	0.811
Creatinine, mg/dL	0.81 ± 0.03	0.82 ± 0.03	0.177	0.87 ± 0.04	0.83 ± 0.03	0.013 *
AST, IU/L	23.96 ± 1.85	27.57 ± 3.57	0.352	21.60 ± 1.29	19.70 ± 1.00	0.013 *
ALT, IU/L	20.58 ± 1.68	23.42 ± 3.36	0.840	19.35 ± 1.67	16.25 ± 1.71	0.002 *
Total cholesterol, mg/dL	215.57 ± 8.48	206.35 ± 10.27	0.234	226.35 ± 9.66	217.80 ± 8.02	0.229
HDL-cholesterol, mg/dL	51.61 ± 1.76	48.22 ± 2.12	0.074	53.25 ± 2.86	56.65 ± 2.78	<0.001 *
Triglyceride, mg/dL	141.52 ± 11.81	157.17 ± 13.80	0.330	163.55 ± 21.36	147.40 ± 20.35	0.332
LDL-cholesterol, mg/dL	136.57 ± 8.02	130.10 ± 8.62	0.367	145.46 ± 7.46	126.60 ± 6.83	0.001 *
FBS, mg/dL	99.87 ± 5.85	107.09 ± 6.84	0.130	106.53 ± 8.03	98.79 ± 7.79	0.021 *
IgA, ng/mL	739.44 ± 80.41	790.20 ± 79.52	0.200	881.79 ± 50.35	1172.34 ± 50.53	<0.001 *
LPS, pg/mL	112.62 ± 16.22	94.14 ± 10.97	0.114	99.08 ± 5.10	39.82 ± 4.76	0.001 *
hsCRP, ml/L	0.0087 ± 0.0014	0.0141 ± 0.0017	0.059	0.0117 ± 0.0046	0.0060 ± 0.0020	0.201
Lactulose–Mannitol ratio	0.156 ± 0.026	0.113 ± 0.017	0.052	0.222 ± 0.036	0.047 ± 0.004	<0.001 *
Lactulose	0.1292 ± 0.0248	0.0789 ± 0.0165	0.002 *	0.0023 ± 0.0003	0.0013 ± 0.0002	0.006 *
QA, ng/mL	28.38 ± 1.83	26.49 ± 1.21	0.513	29.84 ± 0.87	19.47 ± 0.83	<0.001 *
5-HIAA, mg/L	3.17 ± 1.12	4.94 ± 1.85	0.463	8.04 ± 2.06	8.73 ± 1.38	0.551
QA/5-HIAA ratio	0.0145 ± 0.0038	0.0092 ± 0.0025	0.463	0.0056 ± 0.0009	0.0036 ± 0.0007	0.121
Lactic acid, mmol/g sample	232.96 ± 144.52	78.58 ± 22.84	0.593	48.22 ± 8.79	96.74 ± 23.06	0.066
Acetic acid, mmol/g sample	45.11 ± 0.20	37.95 ± 1.40	0.180	37.07 ± 5.25	26.94 ± 5.66	0.128
Propionic, mmol/g sample	413.81 ± 74.29	694.21 ± 216.16	0.225	411.97 ± 28.18	682.59 ± 90.31	0.012 *
Butyric acid, mmol/g sample	5.67 ± 1.02	7.47 ± 2.27	0.913	14.62 ± 5.74	63.45 ± 15.60	0.046 *

* = Significant difference in p-value at 95% confidence interval: AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; FBS = Fasting Blood Sugar; IgA = Immunoglobulin A; hsCRP = High Sensitivity C-Reactive Protein; LPS = Lipopolysaccharide; QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid.

3.3. Changes in the Study Parameters between the Group

Significant changes were observed in some of the study parameters in the probiotics group compared to the placebo after 12 weeks of the study. In detail, the body mass index ($p \leq 0.001$), body fat ($p = 0.016$), muscle content ($p = 0.022$), waist ($p = 0.001$) and hip ($p = 0.001$) circumferences, creatinine ($p = 0.001$), AST ($p = 0.024$), HDL-C ($p = 0.001$), FBS ($p = 0.001$), IgA ($p \leq 0.001$), LPS ($p = 0.001$), hsCRP ($p = 0.029$), lactulose–mannitol ratio ($p \leq 0.001$), lactulose ($p = 0.025$), QA ($p \leq 0.008$), and butyric acid ($p = 0.014$) content showed significant improvement in the probiotics group compared to the placebo. The results indicated that the probiotics intervention improved the study parameters in experimental subjects (Table 3).

Table 3. Changes in study parameters between the group at different times, expressed as mean ± SE.

Parameters	Baseline—12 Weeks		*p*-Value
	Placebo (*n* = 24)	Probiotics (*n* = 24)	
Body mass index, kg/m^2	−0.59	−0.57	<0.001 *
BMR, kcal	−7.91	13.09	0.518
Body fat, %	3.35	−1.47	0.016 *
Visceral fat, %	0.48	−0.24	0.621
Muscle, %	−3.13	0.80	0.022 *
Body age, years	0.88	0.39	0.324
Arm circumference, cm	0.05	−1.38	0.137
Waist circumference, cm	1.55	−2.78	0.001 *
Hip circumference, cm	1.88	−6.77	0.001 *
BUN, mg/dL	0.23	−0.18	0.752
Creatinine, mg/dL	0.02	−0.04	0.001 *
AST, IU/L	3.61	−1.90	0.024 *
ALT, IU/L	2.84	−3.10	0.055
Total cholesterol, mg/dL	−9.22	−8.55	0.670
HDL-cholesterol, mg/dL	−3.39	3.40	0.001 *
Triglyceride, mg/dL	15.65	−16.15	0.154
LDL-cholesterol, mg/dL	−6.46	−18.86	0.173
FBS, mg/dL	7.22	−7.74	0.001 *
IgA, ng/mL	50.76	290.55	<0.001 *
LPS, pg/mL	−18.48	−59.26	0.001 *
hsCRP, ml/L	0.005	−0.006	0.029 *
Lactulose–Mannitol ratio	−0.04	−0.18	0.001 *
Lactulose	−0.0502	−0.0010	0.025 *
QA, ng/mL	−1.89	−10.36	0.008 *
5-HIAA, mg/L	1.77	0.69	0.837
QA/5-HIAA ratio	−0.005	−0.002	0.461
Lactic acid, mmol/g sample	−154.38	48.53	0.079
Acetic acid, mmol/g sample	−7.16	−10.12	0.558
Propionic, mmol/g sample	280.40	270.62	0.965
Butyric acid, mmol/g sample	1.79	48.83	0.014 *

* = Significantly difference in *p*-value at 95% confidence interval, AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; FBS = Fasting Blood Sugar; IgA = Immunoglobulin A; hsCRP = High Sensitivity C-Reactive Protein; LPS = Lipopolysaccharide; QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid.

The Gaussian regression analysis revealed that the body fat, visceral fat, muscle content, body age, arm, waist and hip circumferences, creatinine, ALT, HDL-C, FBS, IgA, LPS, lactulose–mannitol ratio, QA, QA/5-HIAA ratio, and butyric acid content were significantly altered in probiotics group after 12 weeks of treatment (Table 4). The risk difference analysis revealed that intestinal permeability was improved up to 48% in the probiotics supplemented group (Table 5).

Table 4. Gaussian regression analysis summary at week 12 of treatment for probiotics group.

Parameter	Coefficient	95% CI	*p*-Value
Body mass index, kg/m^2	−0.86	−4.35 to 2.62	0.612
BMR, kcal	−9.38	−35.21 to 16.45	0.458
Body fat, %	−3.65	−4.76 to −2.54	<0.001 *
Visceral fat, %	−0.84	−1.41 to −0.28	0.006 *
Muscle, %	4.23	1.83 to 6.62	0.001 *
Body age, years	−2.31	−4.07 to −0.54	0.012 *
Arm circumference, cm	−2.35	−3.99 to −0.70	0.007 *
Waist circumference, cm	−3.74	−7.07 to −0.42	0.029 *
Hip circumference, cm	−5.47	−9.96 to −0.97	0.019 *
BUN, mg/dL	−0.75	−2.89 to 1.38	0.477
Creatinine, mg/dL	−0.04	−0.076 to −0.003	0.033 *
AST, IU/L	−7.96	−16.60 to 0.67	0.069
ALT, IU/L	−8.27	−15.56 to −0.99	0.028 *
Total cholesterol, mg/dL	6.65	−15.51 to 28.80	0.546
HDL-cholesterol, mg/dL	7.62	2.80 to 12.44	0.003 *
Triglyceride, mg/dL	−18.13	−52.47 to 16.22	0.290
LDL-cholesterol, mg/dL	−1.42	−19.92 to 17.09	0.877
FBS, mg/dL	−13.63	−25.72 to −1.54	0.028 *
IgA, ng/mL	230.18	76.39 to 383.98	0.005 *
LPS, pg/mL	−58.03	−82.59 to −33.46	<0.001 *
hsCRP, ml/L	−0.008	−0.020 to 0.004	0.147
Lactulose–Mannitol ratio	−0.08	−0.12 to −0.04	0.001 *
Lactulose	−0.004	−0.030 to 0.021	0.733
QA, ng/mL	−6.97	−10.17 to −3.77	0.001 *
5-HIAA, mg/L	3.43	−3.81 to 10.68	0.307
QA/5-HIAA ratio	−0.01	−0.02 to −0.01	0.002 *
Lactic acid, mmol/g sample	60.65	−279.10 to 400.41	0.610
Acetic acid, mmol/g sample	−9.85	−89.82 to 70.12	0.649
Propionic, mmol/g sample	−19.43	−466.59 to 427.72	0.925
Butyric acid, mmol/g sample	47.79	14.54 to 81.04	0.008 *

* = Significant difference in *p*-value at 95% confidence interval. Comparison with placebo group at week 12: AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; FBS = Fasting Blood Sugar; IgA = Immunoglobulin A; hsCRP = High Sensitivity C-Reactive Protein; LPS = Lipopolysaccharide; QA = Quinolinic acid; 5-HIAA= 5-Hydroxyindoleacetic acid.

Table 5. Risk difference analysis of probiotics treatment.

Parameter	Risk Difference	95% CI	*p*-Value
Leaky gut	−0.48	−0.79 to −0.18	0.002 *

* = Significant difference in *p*-value at 95% confidence interval. Comparison with placebo group at week 12.

4. Discussion

The study subjects completed the experimental procedures successfully and showed significant clinical improvements in intestinal permeability, lipid profile, and short-chain fatty acids.

A recent study revealed that the use of *Lactobacillus* species could improve the intestinal microbiota and reduce gut permeability [23]. According to Ohland and MacNaughton [24], probiotics improved the intestinal barrier function by increasing the production of mucus, secretory IgA and antimicrobial peptides, and increased tight junction integrity of epithelial cells and competitive adherence for pathogens.

Chen et al. [25] reported that *L. paracasei* 01 protects intestinal stability by promoting intestinal epithelial cell growth and improving intestinal integrity. Furthermore, *L. paracasei* 01 treatment inhibits the inflammatory players [tumor necrosis factor-α (TNF-α), interferon-γ (TNF-α), and C-C motif chemokine ligand-20 (CCL-20)] in vitro. Similarly, *L. paracasei* JCM 1163 also improved the intestinal barrier function via its long-chain polyphosphates accumulating property [26].

Zhang et al. [27] demonstrated that the surface-layer associated proteins (SLAP) of *L. paracasei* ssp. *paracasei* M5-L and *L. casei* Q8-L protect the bacteria-mediated epithelial barrier disruption by suppressing the occludin production and inhibiting the delocalization of zonula occludens-1. Similarly, the use of *L. paracasei* ssp. *paracasei L. casei* W8® improved the intestinal barrier function and reduced the inflammation in high-fat diet-fed rats [28].

Laval et al. [29] showed that *L. rhamnosus* CNCM I-3690 enhanced intestinal integrity by increasing the level of occludin and E-cadherin.

Ahmadi et al. [19] studied the beneficial role of a human-origin probiotic (probiotics that are isolated from healthy infant gut) cocktail containing *Enterococcus* strains (*E. avium* D25-1, *E. avium* D25-2, *E. avium* D26-1, *E. raffinosus* D24-1, and *E.* INBio D24-2) and *Lactobacillus* strains (*L. paracasei* D3-5, *L. plantarum* D6-2, *L. plantarum* D13-4, *L. rhamnosus* D4-4, and *L. rhamnosus* D7-5) in aging-related leaky gut, inflammation, and metabolic dysfunctions using older C57BL/6J mice (~80 weeks mice age is equivalent to >65 years human age) as a model. The probiotic cocktail reduced physical function decline in the older mice. Furthermore, it prevented high-fat diet-induced microbiota dysbiosis by modulating the microbiota, increasing the bike salt hydrolase activity, thereby increasing the abundance of gut taurine which stimulates the tight junctions and reduces the leaky gut and inflammation [19].

Al-Sadi et al. [30] screened some probiotic species such as *Bifidobacterium bifidum, B. breve, B. longum, Escherichia coli* strain Nissle, and probiotic species or strains of *Lactobacillus acidophilus, L. brevis, L. casei, L. helveticus, L. johnsonii, L. plantarum*, and *L. rhamnosus* to identify the effective probiotic species or strain that prevents intestinal inflammation by increasing the tight junction. Among the screened probiotic bacterial species and strains, *L. acidophilus* LA1 strain showed an effective increase in Caco-2 trans-epithelial resistance and reduced paracellular permeability indicating the improvement of Caco-2 tight junction barrier function. Oral supplementation of LA1 showed TLR-2 dependent improvement of tight junction barrier and protection against intestinal inflammation, thereby preventing dextran sodium sulfate (DSS)-induced colitis in the mouse model [30].

In the present study results, the level of the lactulose-mannitol ratio and lactulose were reduced significantly in the probiotic supplemented group compared to baseline values and the placebo (Tables 2 and 3). Moreover, the intestinal permeability of the probiotic-supplemented subjects was improved up to 48% (Table 5). The increased intestinal barrier function was observed in the serum level LPS; a significant level of reduction was observed in LPS concentration after probiotic intervention (Tables 3 and 4). Accordingly, the studied probiotic mixture might have the ability to improve intestinal barrier function.

The reduction in the fecal concentrations of short-chain fatty acids (SCFAs) is associated with diseases and aging [31,32]. Cai et al. (2016) reported that the centenarians have a high concentration of SCFAs, associated with the high dietary fiber intake [33].

The probiotic strains of *Lactobacillus* and *Bifidobacterium* species were propionic, lactic, and butyric acid producers [34,35]. The consumption of *L. plantarum* P-8 significantly reduced the opportunistic pathogens and increased *Bifidobacterium* level. Moreover, the levels of propionate and acetate were increased [36]. Moens et al. reported that the growth of probiotic bacteria might increase the lactate concertation, which facilitates lactate-consuming microbial growth, subsequently increasing SCFAs production, particularly butyrate [37]. The further microbial analysis is required to confirm the association between changes in SCFAs levels and probiotic interventions. In the present study, probiotics intervention significantly increased propionic and butyric acid levels, whereas changes in lactic and acetic acids levels were non-significant (Table 2). Gaussian regression analysis revealed that butyric acid level increased notably ($p = 0.008$) after the probiotic intervention (Table 4).

The supplementation of *L. paracasei* HII01 (1.25×10^{10} CFU per day) for 12 weeks did not significantly alter IgA's level [18]. The intervention of *L. paracasei* HII01 (5×10^{10} CFU per day) for 12 weeks reduced the LPS, TNF-α, IL-6, and hsCRP levels in diabetic subjects [38]. Similarly, the supplementation of synbiotic preparation (*L. paracasei* HII01, *B. longum*, *B. breve*, inulin, and fructooligosaccharide) reduced LPS, TNF-α, IL-6, and hsCRP

levels. In contrast, IgA levels were increased in human subjects significantly. These results suggested that synbiotic intervention could improve obesity-associated biomarkers [17].

In the present study, BMI, body fat, muscle, and waist and hip circumferences were improved in the probiotic group compared to placebo (Table 3). The body and visceral fat, muscle, body age, and arm, waist and hip circumferences were significantly improved after the 12-week course of probiotic intervention, as per the Gaussian regression analysis (Table 4). The level of HDL-C was increased significantly in the probiotic-treated group, while a noted level of reduction was observed in the placebo (Table 3). No significant changes were observed in TC, TG, LDL-C, and hsCRP values after the study period in the probiotic-treated group (Table 4). These results indicated that the studied multi-species probiotic mix improved the lipid profile and obesity-related biomarkers in studied human subjects.

The gut microbiota and its secreted compounds may affect the tryptophan metabolism and gut inflammation, which affects the kynurenine and QA levels [39]. *L. paracasei* may influence the central 5-hydroxytryptamine (5-HT) system and brain-derived neurotrophic factor (BDNF) expression through butyrate. *B. breve* and *B. longum* affect the glutaminergic system and neural activities in the brain through humoral and neural routes, respectively [40].

The supplementation of *L. paracasei* HII01 increased the levels of short-chain fatty acids in obese, hypercholesterolemic, and diabetic human subjects [17,37]. 5-HIAA and QA/5-HIAA ratio was not significantly affected by the supplementation of *L. paracasei* HII01, *B. breve*, *B. longum*, inulin, and fructooligosaccharide. [18]. The present study results indicated that the supplementation of the studied probiotic mixture decreased the QA level (Tables 3 and 4). Thus, the intervention might influence the tryptophan metabolism and expression of BDNF.

The probiotic intervention improved liver aminotransferases in patients with non-alcoholic fatty liver disease [41], and alcohol-induced liver disease [42]. In this study, ALT level was reduced, and AST level was not significantly changed (Table 4).

The studies on the influence of probiotics on renal function are very limited and the reported studies showed that probiotic supplementation improved renal function through increased intestinal barrier function [43,44]. In the present study also BUN values were not changed significantly, whereas creatinine levels were reduced significantly in the probiotic treated group (Table 4).

Altogether, the results of the current study provided the basic information about the influence of studied (*L. paracasei* HII01, *B. breve*, and *B. longum*) probiotic mixture on intestinal permeability, lipid profile, body fat, liver and kidney function, neurotransmitter levels, and short-chain fatty acids in elderly Thai subjects.

5. Conclusions

The current study has limitations, such as its limited sample size, questionnaire regarding eating habits, exercise, work activity, and overcoming the disease, lack of extended follow-up, and microbiota analysis. Nevertheless, the present study represents the effects of the intervention of a probiotic mixture composed of *Lactobacillus* and *Bifidobacterium* on kidney and liver function, lipid profile, intestinal integrity, microbial metabolites, bacterial endotoxin (LPS) level, and biomarkers of gut-brain communication pathways in elderly Thai subjects.

The results revealed that the supplementation of studied probiotics improved the intestinal barrier function, the lipid profile and obesity-related biomarkers in human subjects. Further studies are strongly recommended to confirm the beneficial effect of probiotics, which may pave the way to formulate probiotic-based health supplements to adjuvant the treatment of several metabolic diseases.

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Article

Synbiotic Supplementation Improves Obesity Index and Metabolic Biomarkers in Thai Obese Adults: A Randomized Clinical Trial

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Abstract: The cluster of metabolic disorders includes obesity, dyslipidemia, hypertension, and glucose intolerance, increasing the risk of developing cardiovascular diseases and type 2 diabetes. Evolving proofs suggest an essential role of microbiota in human health and disease, including digestion, energy and glucose metabolism, immunomodulation, and brain function. The frequency of overweight is increasing, and the main causes for this are highly processed foods and less active lifestyles. Research is underway to unravel the probable relationship between obesity and intestinal microbiota. Here, we propose a method to understand and elucidate the synergistic function of prebiotics and probiotics in treating obesity. The biomarkers of obesity, such as cholesterol, gut permeability, oxidative stress, bacterial toxins, cytokines, and short-chain fatty acids, were analyzed in Thai obese individuals after being supplemented with a synbiotic preparation containing *Lactobacillus paracasei*, *Bifidobacterium longum*, *Bifidobacterium breve*, inulin, and fructooligosaccharide. The results reveal that the supplementation of synbiotics significantly altered the obesity-associated biomarkers in an appositive way. Further studies are warranted to use synbiotics as an adjuvant therapy for the management of obesity-related health issues.

Keywords: obesity; synbiotics; Lactobacillus; Bifidobacterium; inulin; fructooligosaccharide

1. Introduction

Obesity is one of the major health issues worldwide, leading to other health issues such as cardiovascular diseases, diabetes, and hypertension' which result in morbid obesity. A long-term imbalance in energy consumption, an irregular diet, altered gut microbiota, environmental factors, and genetic makeup are the primary causes of obesity [1]. According to a WHO report, about 650 million adults are obese, and 1.9 billion are overweight. Of these, possibly 38 million children (less than five years old) are obese [2].

The intestinal microbiota composition has a critical role in obesity [1]. For example, the Firmicutes to Bacteroidetes proportion was found to be higher in overweight/obese people compared to ordinary people. Energy absorption and storage may be associated with the

balance of Firmicutes in intestinal microbiota [3,4]. The dysbiosis in intestinal microbiota is associated with cell homeostasis changes and affects the integrity of tight junctions, resulting in a decline in gut permeability [5]. Dysbiosis also influences inflammation, insulin resistance, and fat deposition, leading to the development of obesity. It increases the bacterial toxic load (i.e., lipopolysaccharide) in the host [6].

Probiotic bacteria are live microorganisms that confer a health benefit on the host when administered in suitable amounts. Recent studies have highlighted the beneficial effects of probiotics supplementation in hosts with metabolic disorders, cognitive declines, and cancers via the positive regulation of gut microbiota [1,7–9]. The supplementation of synbiotics (a mixture of probiotics and prebiotics) may effectively improve intestinal microbiota composition compared to probiotics or prebiotics supplements [10].

The combination of *Lactobacillus* and *Bifidobacterium*, along with prebiotics, could provide synergic effects to the host. So far, studies on the influence of the supplementation of synbiotic preparations containing *Lactobacillus paracasei*, *Bifidobacterium longum*, *Bifidobacterium breve*, inulin, and fructooligosaccharide on cholesterol profiles, cytokines, markers of leaky gut, antioxidant levels, and short-chain fatty acids (SCFAs) contents in Thai obese adults have not yet been reported. Thus, we aimed to study the effect of a synbiotic intervention on the biomarkers of cholesterol, gut permeability, oxidative stress, bacterial toxins, cytokines, and SCFAs in Thai obese subjects.

2. Materials and Methods

2.1. Study Design and Subjects

The study on the effects of synbiotics on obesity was conducted with randomized, double-blind placebo-controlled trials of Thai obese adults. The participants of this study provided their informed consent for participation before they joined the study. The Good Clinical Practices were followed in the study. The Ethics Committee of Mae Fah Luang University approved the study protocol (Code: REH-62151).

The inclusion criteria included Thai obese adults (BMI $\geq$ 25 kg/m^2) according to the Asia-Pacific criteria, aged 18–65 years, who were willing to participate and complete the study. Subjects with kidney diseases, cardiovascular issues, gouty arthritis, and gastrointestinal tract discomforts were excluded from the study.

Randomization was conducted with computer-generated codes using Random Allocation Software version 1.0.0 (Isfahan, Iran) [11]. The researchers and participants were blinded to the group assignment. Participants were randomized to receive either a synbiotic preparation (*Lactobacillus paracasei*, *Bifidobacterium longum*, *Bifidobacterium breve*, inulin, and fructooligosaccharide) or placebo for 12 week-long supplementations. After 12 weeks of supplementation, participants were asked to return for follow-up visits. The study flowchart and enrollment are described in Figure 1.

2.2. Treatment

Aluminum foil sachets containing 5×10^{10} CFU of probiotics (2×10^{10} CFU of *Lactobacillus paracasei*, 1×10^{10} CFU of *Bifidobacterium longum*, 2×10^{10} CFU of *Bifidobacterium breve*) and prebiotics (5 g of inulin and 5 g of fructooligosaccharide) were provided to the subjects in the synbiotic group. The concentration of *Bifidobacterium breve* was decided based on the anti-obesity effects of *B. breve* reported in a randomized, double-blind, placebo-controlled trial [12]. The combination of synbiotic and the concentration of other probiotics used in this study were based on our results (unpublished data). The probiotics were received from Lactomason Co., Ltd., (Gyeongsangnam-do, South Korea), and prebiotics were purchased from BENEO-Orafti S.A., (Oreye, Belgium). Those in the placebo group were provided with 10 g of corn starch. All subjects were instructed to regularly take the supplementation by dissolving the contents of one sachet in a glass of water before breakfast.

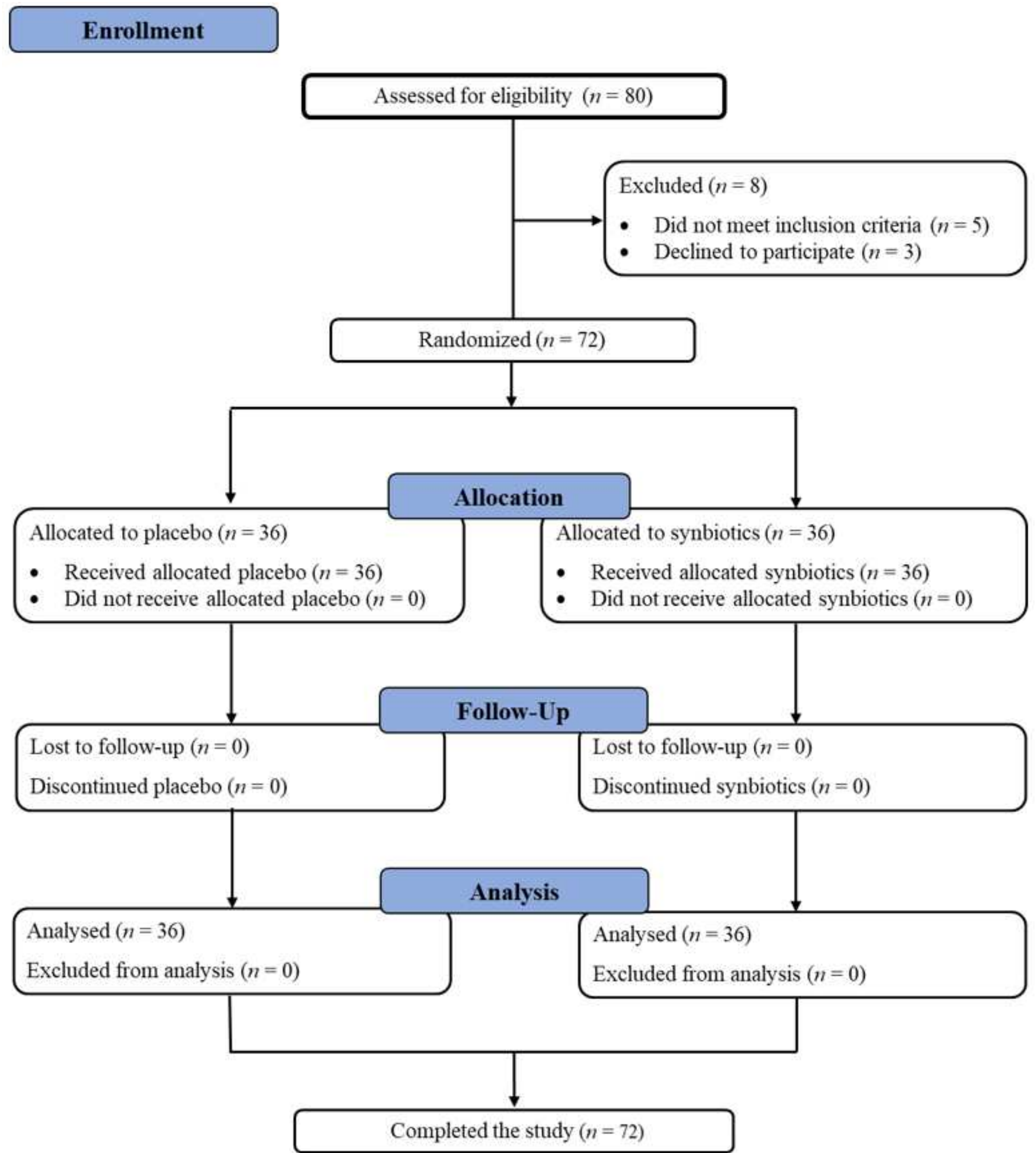

Figure 1. The study flowchart and enrollment.

2.3. Assessments

2.3.1. Clinical Data

The subjects' personal history was assessed, including education, physical activities, smoking and alcohol drinking habits, and pharmacological treatments.

Demographic characteristics, including age, diabetes, alcohol drinking, and obesity index, were recorded manually. Body weight, body mass index (BMI), body fat, visceral fat, basal metabolic rate (BMR), and muscle were measured using an electronic scale (Picooc®, Model S1 Pro, Beijing, China).

2.3.2. Laboratory Data

Blood, fecal, and urine samples were collected at baseline and the end of the study (Figure 2). The biochemical analyses including total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (TG), and fasting blood sugar (FBS) levels were determined from blood using the automated machine at AMS Clinical Service Center, Chiang Mai University, Chiang Mai, Thailand. Other biomarkers in the blood such as high sensitivity C-reactive protein (hs-CRP), immunoglobulin A (IgA), lipopolysaccharides (LPS), zonulin (ZO-1), and inflammatory chemokines/cytokines were determined

using an ELISA commercial kit (OriGene Technologies, Rockville, MD, USA for hs-CRP, Elabscience®, Houston, TX, USA for IgA, MyBioSource®, San Diego, CA, USA for LPS and IDK®, Bensheim, Germany for ZO-1). Plasma total antioxidant capacity (TAC) was determined by a 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging capacity assay [13,14]. The determination of malondialdehyde (MDA) was performed with the thiobarbituric acid reactive substances (TBARS) method [15,16]. The dismutation of superoxide radicals was determined using the assay of superoxide dismutase (SOD) [17], and reduced glutathione (GSH) in the plasma was determined using the recycling assay of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) [18].

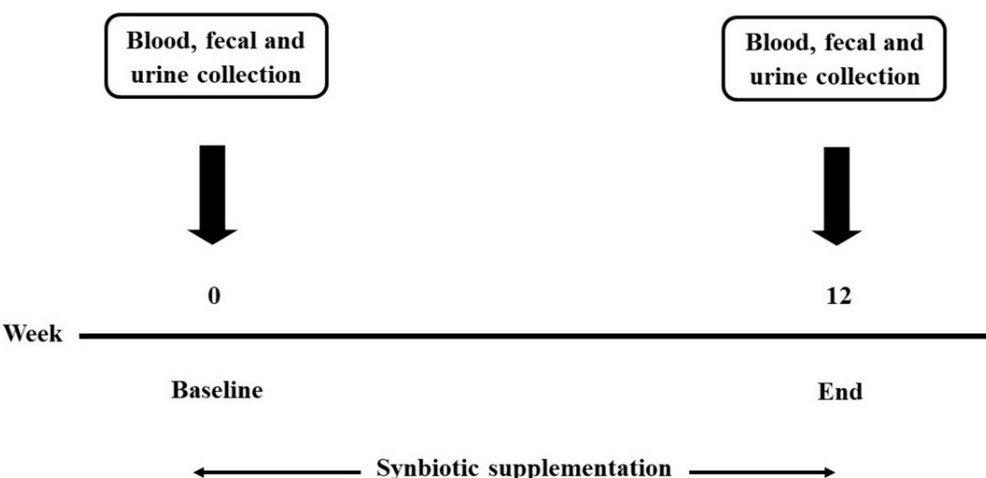

Figure 2. The timeline of this study.

Fecal samples were collected to determine the short-chain fatty acids using high-performance liquid chromatography (HPLC) according to the following conditions: Shodex SH1011 as a column, 5 mM sulfuric acid as the mobile phase, with a flow rate of 0.6 mL/min at 210 nm and 75 °C [19,20], and putrefaction using HPLC with the following conditions: C18 (4.6 mm × 15 cm) as a column, methanol: water (60:40 *v*/*v*) as mobile phase, with a flow rate of 0.5 mL/min at 200 nm [21–23].

Urine samples were used to determine intestinal permeability. The subjects were given mannitol and lactulose at a ratio of 1:2, dissolved in water. After taking mannitol and lactulose, subjects were asked to collect urine within 6 h [24]. We measured the total urine volume from each subject and analyzed the intestinal permeability using a colorimetric commercial kit (EnzyChrom™, BioAssay, Hayward, CA, USA). Neuroinflammation markers in the urine, such as quinolinic acid (QA) and 5-hydroxyindoleacetic acid (5-HIAA), were determined using an ELISA commercial kit (Fivephoton Biochemicals™, San Diego, CA, USA for QA and Immusmol, Bordeaux, France for 5-HIAA).

2.3.3. Statistical Analyses

Demographics were continuously analyzed using a t-test and discrete data using exact values. Data were analyzed using the paired t-test of means using STATA version 15.1 (StataCorp, College Station, TX, USA) for Windows licensed to the Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. A descriptive analysis of the collected parameters was expressed as an absolute number and percentage. The continuous variables were represented as mean ± standard deviation (SD) or standard error of the mean (SEM) depending on their statistical distribution. The group's data were calculated using a t-test and Gaussian regression analysis. The minimum level of statistical significance was set to $p < 0.05$ (two-tailed).

3. Results

A total of 72 subjects completed the study. There were no differences between synbiotic and placebo groups in terms of the initial measurements of age, body weight, BMI, body fat, visceral fat, muscle, arm, waist, and hip circumferences, waist/hip ratio, blood urea nitrogen content, creatinine, aspartate aminotransferase, and alanine aminotransferase, except in their BMR (Table 1).

Table 1. Basic characteristics of the study subjects.

Parameters	Synbiotic Group (N = 36)	Placebo Group (N = 36)	*p*-Value
Age (years)	54.78 ± 1.92	58.94 ± 1.32	0.078
Body weight, cm	69.09 ± 1.90	68.17 ± 1.63	0.712
Body mass index, kg/m^2	28.97 ± 0.77	30.01 ± 0.47	0.248
Body fat, %	33.09 ± 1.18	35.36 ± 0.87	0.125
Visceral fat, %	14.18 ± 0.88	15.36 ± 0.43	0.223
Muscle, %	56.48 ± 3.85	59.19 ± 1.44	0.497
BMR (kcal)	1409.42 ± 31.93	1323.04 ± 23.86	0.033 *
Arm circumference, cm	30.71 ± 0.49	30.62 ± 0.48	0.893
Waist circumference, cm	94.73 ± 1.92	95.79 ± 1.34	0.651
Hip circumference, cm	103.09 ± 1.38	104.33 ± 1.10	0.486
Waist/hip ratio	0.92 ± 0.01	0.92 ± 0.01	0.94
Diabetes, n (%)	7 (19.44%)	13 (36.11%)	0.188
Alcohol drinking, n (%)	6 (16.67%)	4 (11.11%)	0.735
Blood urea nitrogen (mg/dL)	14.89 ± 0.93	16.86 ± 1.84	0.699
Creatinine (mg/dL)	1.09 ± 0.09	1.08 ± 0.10	0.964
Aspartate aminotransferase (IU/L)	25.43 ± 4.62	24.73 ± 2.32	0.744
Alanine aminotransferase (IU/L)	27.59 ± 6.00	23.68 ± 2.97	0.925

* = Significant difference in *p*-value at 95% confidence interval. The proportion was analyzed using an exact probability test, and the continuous demographic data were analyzed using a *t*-test. BMR: basal metabolic rate.

There were no changes in all studied parameters after 12 weeks in the placebo group compared with baseline values. In the synbiotic group, significant differences were observed after 12 weeks of supplementation in body weight, BMI, body fat, waist circumference, waist/hip ratio, HDL-C, LDL-C, IL-6, IL-10, IL-1β, TNF-α, IgA, LPS, and ZO-1 values compared to the baseline values. No significant changes were observed in visceral fat, muscle, BMR, arm and hip circumferences, TC, TG, and hsCRP values in the synbiotic group (Table 2).

The antioxidant systems (TAC, MDA, GSH, total SOD, and Cu, Zn-SOD) of the subjects were documented. There were no statistically significant changes in the synbiotic and placebo groups after 12 weeks of supplementation (Table 3). The levels of butyric acid, propionic acid, acetic acid, and lactic acid were significantly changed after 12 weeks of synbiotic supplementation, whereas no changes were observed in the placebo group. The levels of lactulose, QA, the QA/5-HIAA ratio, cresol, and indole were significantly changed in the synbiotic group, which was not observed in the placebo group after 12 weeks (Table 3).

The significant changes in the studied parameters between the synbiotic and placebo groups after 12 weeks were calculated. The body weight, FBS, and cytokines, IgA, hsCRP, LPS, and QA levels were significantly altered compared to the placebo group (Tables 4 and 5). There were no notable changes in the rest of the studied parameters between the synbiotic and placebo groups.

Table 2. Changes in the studied parameters within groups at different times, expressed as mean ± SE.

Parameters	Synbiotic (N = 36)		*p*-Value	Placebo (N = 36)		*p*-Value
	Baseline	12 Weeks		Baseline	12 Weeks	
Body weight, cm	69.09 ± 1.90	67.45 ± 1.85	<0.001 *	68.17 ± 1.63	67.71 ± 1.71	0.067
Body mass index, kg/m^2	28.97 ± 0.77	28.58 ± 0.75	0.017 *	30.01 ± 0.47	30.13 ± 0.58	0.662
Body fat, %	33.09 ± 1.18	31.96 ± 1.20	0.043 *	35.36 ± 0.87	36.27 ± 1.22	0.310
Visceral fat, %	14.18 ± 0.88	13.85 ± 0.79	0.162	15.36 ± 0.43	15.56 ± 0.52	0.445
Muscle, %	56.48 ± 3.85	56.08 ± 3.81	0.284	59.19 ± 1.44	59.01 ± 1.42	0.860
BMR (kcal)	1409.42 ± 31.93	1411.27 ± 29.61	0.898	1323.04 ± 23.86	1309.68 ± 24.87	0.102
Arm circumference, cm	30.71 ± 0.49	30.59 ± 0.57	0.808	30.62 ± 0.48	30.51 ± 0.53	0.795
Waist circumference, cm	94.73 ± 1.92	92.76 ± 1.84	0.009 *	95.79 ± 1.34	95.34 ± 1.45	0.648
Hip circumference, cm	103.09 ± 1.38	102.50 ± 1.30	0.419	104.33 ± 1.10	103.84 ± 1.31	0.705
Waist/hip ratio	0.92 ± 0.01	0.90 ± 0.01	0.018 *	0.92 ± 0.01	0.92 ± 0.01	0.961
Total cholesterol (mg/dL)	200.97 ± 8.40	195.50 ± 6.48	0.171	203.30 ± 8.11	199.97 ± 7.67	0.626
Triglyceride (mg/dL)	150.24 ± 16.04	145.97 ± 14.66	0.469	148.64 ± 11.04	149.88 ± 11.20	0.893
HDL-cholesterol (mg/dL)	50.21 ± 2.42	53.10 ± 2.53	0.030 *	50.42 ± 1.47	50.91 ± 2.56	0.813
LDL-cholesterol (mg/dL)	123.93 ± 8.61	112.66 ± 6.62	0.017 *	123.35 ± 7.35	116.48 ± 7.06	0.295
FBS (mg/dL)	111.79 ± 7.44	109.00 ± 6.02	0.373	109.68 ± 6.76	118.18 ± 6.89	0.084
IL-6 (pg/mL)	11.65 ± 1.17	7.24 ± 1.63	0.017 *	11.84 ± 0.49	11.82 ± 1.16	0.116
IL-10 (pg/mL)	1.04 ± 0.19	9.91 ± 2.04	0.018 *	1.56 ± 0.13	9.20 ± 5.00	0.153
IL-1β (pg/mL)	7.79 ± 0.76	5.42 ± 0.80	0.008 *	6.97 ± 0.64	6.29 ± 0.39	0.117
TNF-α (pg/mL)	13.75 ± 2.93	7.59 ± 1.54	0.011 *	9.25 ± 0.90	9.22 ± 0.56	0.679
IgA (ng/mL)	521.02 ± 69.33	636.48 ± 79.23	0.004 *	579.40 ± 54.02	504.73 ± 60.96	0.877
hsCRP (ml/L)	0.017 ± 0.006	0.008 ± 0.002	0.086	0.012 ± 0.001	0.015 ± 0.001	0.078
LPS (pg/mL)	108.99 ± 9.62	55.00 ± 6.09	<0.001 *	93.92 ± 7.87	81.42 ± 6.18	0.054
ZO-1 (ng/mL)	1.37 ± 0.17	0.98 ± 0.18	0.032 *	1.42 ± 0.17	1.41 ± 0.16	0.551

* = Significant difference in *p*-value at 95% confidence interval. HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; FBS = Fasting Blood Sugar; IL = Interleukin; TNF-α = Tumor Necrosis Factor alpha; IgA = Immunoglobulin A; hsCRP = High Sensitivity C-Reactive Protein; LPS = Lipopolysaccharide; ZO = zonulin.

Table 3. Changes in the studied parameters within groups at different times, expressed as mean ± SE.

Parameters	Synbiotic (N = 36)		*p*-Value	Placebo (N = 36)		*p*-Value
	Baseline	12 Weeks		Baseline	12 Weeks	
Lactulose	0.16 ± 0.03	0.07 ± 0.02	<0.001 *	0.12 ± 0.03	0.08 ± 0.02	0.135
Lactulose/mannitol ratio	0.20 ± 0.06	0.09 ± 0.01	0.072	0.14 ± 0.02	0.12 ± 0.02	0.315
QA (ng/mL)	23.53 ± 2.42	13.75 ± 1.71	<0.001 *	22.44 ± 1.69	24.25 ± 1.46	0.375
5-HIAA (mg/L)	5.04 ± 1.12	9.61 ± 1.95	0.051	4.00 ± 0.66	4.95 ± 0.93	0.642
QA/5-HIAA Ratio	3.14 ± 1.60	1.04 ± 0.46	0.008 *	5.76 ± 2.23	4.71 ± 1.77	0.756
Cresol (umol/g sample)	0.24 ± 0.03	0.09 ± 0.05	0.017 *	0.31 ± 0.16	0.14 ± 0.05	0.225
Indole (umol/g sample)	0.06 ± 0.01	0.04 ± 0.00	0.035 *	0.11 ± 0.06	0.06 ± 0.02	0.18
Skatole (umol/g sample)	0.07 ± 0.03	0.04 ± 0.00	0.285	0.05 ± 0.03	0.15 ± 0.07	0.285
Butyric acid (mmol/g sample)	38.01 ± 8.59	93.80 ± 18.96	0.002 *	46.40 ± 12.29	80.03 ± 32.27	0.311
Propionic acid (mmol/g sample)	259.16 ± 38.67	624.12 ± 82.82	<0.001 *	209.44 ± 72.32	466.52 ± 178.52	0.124
Acetic acid (mmol/g sample)	202.63 ± 37.70	425.89 ± 50.86	<0.001 *	206.56 ± 61.60	400.27 ± 69.40	0.161
Lactic acid (mmol/g sample)	54.42 ± 17.98	175.81 ± 36.88	0.002 *	92.11 ± 53.12	140.03 ± 57.00	0.866
TAC (μmol/mL)	0.195 ± 0.003	0.200 ± 0.011	0.664	0.180 ± 0.012	0.193 ± 0.008	0.08
MDA (μmol/mL)	0.45 ± 0.06	0.53 ± 0.09	0.301	0.52 ± 0.04	0.45 ± 0.03	0.157
GSH (μg/mL)	44.99 ± 17.46	30.98 ± 10.63	0.075	24.87 ± 6.86	17.29 ± 8.83	0.08
Total SOD (Units/mL enzyme)	56.91 ± 5.52	57.30 ± 6.28	0.854	54.01 ± 11.78	59.29 ± 7.24	0.492
Cu,Zn-SOD (Units/mL enzyme)	16.93 ± 3.13	44.57 ± 17.96	0.345	35.28 ± 7.34	39.49 ± 19.28	0.686

* = Significant difference in *p*-value at 95% confidence interval. QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid; TAC = Total Antioxidant Capacity; MDA = Malondialdehyde; GSH = Glutathione Reduced; SOD = Superoxide Dismutase.

A Gaussian regression analysis of the data suggested that the synbiotic supplementation for 12 weeks significantly altered the body weight, body fat, muscle content, BMR, waist circumference, IL-6, IL-1β, TNF-α, LPS, ZO-1, lactulose/mannitol ratio, QA, 5-HIAA, QA/5-HIAA ratio, and butyric acid. There were no significant changes observed in cholesterol and antioxidant profiles (Table 6).

Table 4. Comparison of the changes in studied parameters between groups. Changes represent the difference between baseline and at the end of the study.

Parameters	Baseline–12 Weeks		*p*-Value
	Synbiotic (N = 36)	Placebo (N = 36)	
Body weight, cm	−1.64	−0.46	0.002 *
Body mass index, kg/m^2	−0.39	0.13	0.128
Body fat, %	−1.13	0.92	0.068
Visceral fat, %	−0.32	0.19	0.242
Muscle, %	−0.40	−0.18	0.448
BMR (kcal)	1.85	−13.36	0.483
Arm circumference, cm	−0.12	−0.11	0.809
Waist circumference, cm	−1.97	−0.45	0.113
Hip circumference, cm	−0.59	−0.49	0.51
Waist/hip ratio	−0.014	0.001	0.604
Total cholesterol (mg/dL)	−5.47	−3.33	0.695
Triglyceride (mg/dL)	−4.28	1.24	0.521
HDL-cholesterol (mg/dL)	2.9	0.48	0.066
LDL-cholesterol (mg/dL)	−11.28	−6.87	0.599
FBS (mg/dL)	−2.79	8.5	0.043 *
IL-6 (pg/mL)	−4.41	−0.02	0.010 *
IL-10 (pg/mL)	8.87	7.64	0.142
IL-1β (pg/mL)	−2.37	−0.69	0.041 *
TNF-α (pg/mL)	−6.16	−0.04	0.005 *
IgA (ng/mL)	115.46	−74.67	0.049 *
hsCRP (ml/L)	−0.009	0.003	0.002 *
LPS (pg/mL)	−53.99	−12.50	0.002 *
ZO-1 (ng/mL)	−0.39	−0.01	0.061

* = Significant difference in *p*-value at 95% confidence interval. HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; FBS = Fasting Blood Sugar; IL = Interleukin; TNF-α = Tumor Necrosis Factor alpha; IgA = Immunoglobulin A; hsCRP = High Sensitivity C-Reactive Protein; LPS = Lipopolysaccharide; ZO = zonulin.

Table 5. Comparison of the changes in studied parameters between groups. Changes represent the difference between baseline and at the end of the study.

Parameters	Baseline–12 Weeks		*p*-Value
	Synbiotic (N = 36)	Placebo (N = 36)	
Lactulose	−0.08	−0.04	0.002 *
Lactulose/mannitol ratio	−0.11	−0.02	0.508
QA (ng/mL)	−9.78	1.8	<0.001 *
5-HIAA (mg/L)	4.58	0.94	0.157
QA/5-HIAA Ratio	−2.10	−1.05	0.095
Cresol (umol/g sample)	−0.15	−0.16	0.661
Indole (umol/g sample)	−0.03	−0.05	0.379
Skatole (umol/g sample)	−0.031	0.103	0.121
Butyric acid (mmol/g sample)	55.79	33.64	0.229
Propionic acid (mmol/g sample)	364.96	257.09	0.258
Acetic acid (mmol/g sample)	223.25	193.71	0.47
Lactic acid (mmol/g sample)	121.39	47.92	0.162
TAC (μmol/mL)	0.005	0.013	0.557
MDA (μmol/mL)	0.08	−0.07	0.117
GSH (μg/mL)	−14.01	−7.59	0.584
Total SOD (Units/mL enzyme)	0.39	5.28	0.917
Cu,Zn-SOD (Units/mL enzyme)	27.64	4.21	0.251

* = Significant difference in *p*-value at 95% confidence interval. QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid; TAC = Total Antioxidant Capacity; MDA = Malondialdehyde; GSH = Glutathione Reduced; SOD = Superoxide Dismutase.

Table 6. Gaussian regression analysis summary at week 12 of supplementation for synbiotic group.

Parameter	Coefficient	95% CI	*p*-Value
Body weight, cm	−1.76	(−3.17 to −0.34)	0.018 *
Body mass index, kg/m^2	0.123	(−0.64 to 0.88)	0.744
Body fat, %	−2.55	(−4.74 to −0.37)	0.023 *
Visceral fat, %	−0.17	(−0.96 to 0.61)	0.651
Muscle, %	−5.13	(−8.82 to −1.44)	0.027 *
BMR (kcal)	57.27	(2.77 to 111.76)	0.040 *
Arm circumference, cm	−0.14	(−2.61 to 2.33)	0.909
Waist circumference, cm	−2.73	(−5.23 to −0.23)	0.033 *
Hip circumference, cm	−4.54	(−10.06 to 0.97)	0.103
Waist/hip ratio	−0.02	(−0.05 to 0.01)	0.131
Total cholesterol (mg/dL)	−8.01	(−26.91 to 10.90)	0.397
Triglyceride (mg/dL)	−0.02	(−23.02 to 22.97)	0.998
HDL-cholesterol (mg/dL)	3.22	(−1.84 to 8.27)	0.207
LDL-cholesterol (mg/dL)	−10.57	(−26.42 to 5.28)	0.186
FBS (mg/dL)	−2.24	(−16.42 to 11.94)	0.751
IL-6 (pg/mL)	−4.50	(−8.78 to −0.23)	0.040 *
IL-10 (pg/mL)	5.18	(−9.96 to 20.32)	0.477
IL-1β (pg/mL)	−1.43	(−2.78 to −0.08)	0.039 *
TNF-α (pg/mL)	−4.26	(−6.51 to −2.01)	0.001 *
IgA (ng/mL)	117.99	(−55.97 to 291.95)	0.179
hsCRP (ml/L)	−0.003	(−0.011 to 0.005)	0.497
LPS (pg/mL)	−32.59	(−53.68 to −11.49)	0.004 *
ZO-1 (ng/mL)	−0.57	(−1.08 to −0.06)	0.032 *
Lactulose	−0.02	(−0.07 to 0.02)	0.319
Lactulose/mannitol ratio	−0.12	(−0.20 to −0.04)	0.008 *
QA (ng/mL)	−8.22	(−16.04 to −0.40)	0.041 *
5-HIAA (mg/L)	8.59	(0.68 to 16.50)	0.036 *
QA/5-HIAA Ratio	−7.15	(−13.69 to −0.61)	0.035 *
Cresol (umol/g sample)	0.09	(−0.30 to 0.48)	0.583
Indole (umol/g sample)	−0.004	(−0.054 to 0.046)	0.865
Skatole (umol/g sample)	−0.47	(−2.07 to 1.12)	0.165
Butyric acid (mmol/g sample)	59.74	(20.30 to 99.17)	0.009 *
Propionic acid (mmol/g sample)	−171.28	(−541.06 to 198.51)	0.335
Acetic acid (mmol/g sample)	−111.03	(−324.73 to 102.68)	0.28
Lactic acid (mmol/g sample)	6.73	(−135.27 to 148.73)	0.919
TAC (μmol/mL)	−0.03	(−0.09 to 0.04)	0.284
MDA (μmol/mL)	0.43	(−0.36 to 1.22)	0.18
GSH (μg/mL)	7.48	(−6.37 to 21.33)	0.208
Total SOD (Units/mL enzyme)	−32.33	(−91.18 to 26.52)	0.179
Cu,Zn-SOD (Units/mL enzyme)	−74.39	(−203.46 to 54.67)	0.131

* = Significantly difference in *p*-value at 95% confidence interval. Compare with the placebo group at week 12, HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; FBS = Fasting Blood Sugar; IL = Interleukin; TNF-α = Tumor Necrosis Factor alpha; IgA = Immunoglobulin A; hsCRP = High Sensitivity C-Reactive Protein; LPS = Lipopolysaccharide; ZO = zonulin; QA = Quinolinic acid; 5-HIAA = 5-Hydroxyindoleacetic acid; TAC = Total Antioxidant Capacity; MDA = Malondialdehyde; GSH = Glutathione Reduced; SOD = Superoxide Dismutase.

4. Discussion

The synergistic blend of both prebiotics and probiotics reduces plasma fasting insulin [25]. The most-used prebiotics are arabinoxylan and fructans [26]. The synbiotic supplementation of Bifidobacteria strains along with galactooligosaccharide may improve intestinal barrier function and possess anti-obesity effects [27].

There is a need for more approaches to aid in weight loss or to control obesity. Supplementation with *Lactobacillus plantarum* in obese mice reduced the deposition of adipose and upregulated the expression of lipid oxidative genes compared to control mice [28]. In order to treat obesity, *Lactobacillus* species can be used in combination with dietary management. *L. sakei* was found to impose anti-obesity effects when used in obese murine models [29,30]. The synbiotic supplements contained *L. acidophilus*, *Bifidobacterium lactis*, *B. longum* and *B. bifidum* as well as prebiotic galactooligosaccharide mixture, which increased the abundance of gut microbiome and also improved markers of metabolic syndrome as well as immune function in obese adults [31–34]. The supplementation of *L. gasseri* SBT2055-mediated fermented milk for 12 weeks reduced the weight and the abdominal visceral and subcutaneous fat mass in obese human subjects [35].

Treating obesity has been a long-term—but not well-defined—methodology that has been linked with gut microbial management. Even though there have been numerous research works carried out on obesity, the clarification needed regarding the treatment of obesity remains lacking. The present study was performed to inspect the impact of the supplementation of a synbiotics preparation containing *L. paracasei*, *B. longum*, *B. breve*, inulin, and fructooligosaccharide on body composition and metabolic biomarkers in Thai obese subjects.

The supplementation of pro-, pre-, and synbiotics to an organism might alter the secretion of some hormones and neurotransmitters as well as inflammatory factors that inhibit the avidity towards food, therefore reducing weight gain [36]. Many systemic reviews and meta-analyses provide evidence about synbiotics intake assisting the lipid profile and improving dyslipidemia [37]. Synbiotic supplements and foods potentially modulate the gut microbiota as well as improving the metabolism of lipids, insulin resistance, and liver enzymes to a greater extent than either pro- or prebiotics alone [38].

A well-known characteristic of probiotics is their involvement in an improved serum lipid profile through immunomodulatory properties [39]. They also may reduce inflammatory cytokines and Toll-like receptor 4 (TLR-4) activation, leading to a great impact on the serum lipid profile [40]. Probiotics integrate cholesterol in their cellular membrane [41] and convert it into coprostanol [42], resulting in a reduction in cholesterol absorption and serum total cholesterol levels by means of higher bile salt excretion [43,44]. It is a well-known fact that probiotics supplementation can modulate body weight and BMI if the tested individuals are treated for a longer duration. In addition to this, previous study suggests that the outcomes in weight reduction could be effective when prebiotics and probiotics are used together [45].

Overall findings from animal and human studies revealed the more beneficial functions of synbiotics in weight reduction and the modulation of the gut microbiome [27,46] compared to prebiotics and probiotics alone [47–49].

Obese individuals showed low-grade inflammation because of the increased production of cytokines, C-reactive proteins (CRP), interleukins (IL), tumor necrosis factor (TNF), and lipopolysaccharides (LPS) [50,51], which in turn resulted in metabolic dysfunction and obesity-linked disorders [52].

The dietary supplementation of synbiotics prepared using *L. gasseri* and galactomannan and inulin fibers reduced the weight and anti-inflammatory effects of synbiotic preparations along with *L. rhamnosus* (CGMCC 1.3724), *L. plantarum*, *L. paracasei* F19, *L. acidophilus* and LactisBb12, which together with oligo fructose and inulin showed beneficial effects on waist and hip circumference and BMI in obese people [53].

The randomized controlled trails in obese and prediabetes subjects showed variable results such as reduced TC, TG [31,54], and LDL levels [54,55], and the inflammation markers hs-CRP, TNF, LPS, and MDA were also found to be reduced [55–57]. Hotamisligil [58] and Lubberts [59] demonstrated that obese individuals express more TNF-α mRNA and protein when compared to lean controls. Thus, the increase in TNF-α induced IL-6 and IL-7 gene expression [60]. So far, the gathered evidence substantiates the role of peripheral 5-hydroxyindole-3-acetic acid (5-HIAA), the derivative end product of serotonin (5-HT) that is also involved in the pathogenesis of obesity and abnormal lipid and glucose metabolism [61]. In addition, 5-HIAA is associated with chronic low-grade inflammation, which in turn leads to metabolic syndrome. There is a strong association between serum 5-HIAA and central obesity [61]. However, 5-HT has long been known to be involved in the control of appetite, energy balance, and weight control [62,63]. Kinoshita and colleagues proved that 5-HT is responsible for adipocyte differentiation and might lead to adipogenesis and obesity [64]. Kim and colleagues showed that 5-HIAA is directly correlated with low-glyceride levels. Furthermore, there is a negative correlation between HDL cholesterol and 5-HIAA. In addition, an increase in 5-HIAA concentration increases plasma triglyceride levels, but the HDL cholesterol remains unaltered. Similarly, higher 5-HT concentrations were also detected in the blood of high-fat-diet-fed mice [65]. It is

a well-known fact that zonulin is the physiological modulator of intestinal permeability and also a serum biomarker for impaired intestinal permeability [66–68]. The zonulin level was found to be elevated above the reference value in individuals with morbid obesity. S-zonulin was partially controlled after a 6-month-long conservative weight loss intervention and further reduced after bariatric surgery [69].

A meta-analysis by Ramezani Ahmadi and colleagues suggested that, compared to placebo, supplementation with pro/synbiotics pointedly reduced the serum zonulin level among selected subjects. Due to the comparison between probiotics and synbiotics, the finding of a significant level of serum zonulin reduction was only in subjects treated with probiotics [70]. The role of IL-1β in regulating adipose inflammation and fat-liver cross talk has been questioned. IL-1β regulates the lipid storage capacity in adipose tissues of the liver; however, in its absence, the adipose tissue expands, increasing in response to excess calories [71]. However, it is clear that IL-1β is a major promoter of adipose tissue inflammation in obese subjects [72].

Our results shows that 12 weeks of synbiotics supplementation significantly reduced body weight, BMI and body fat, visceral fat, BMR, and arm, waist, and hip circumferences compared to the placebo group (Table 1) in Thai obese subjects. The same parameters showed significant reductions in different time periods as well (Table 2). Reductions in IL-6, IL-1β, TNF-α, LPS, ZO-1, lactulose/mannitol ratio, QA, 5-HIAA, QA/5-HIAA ratio, and butyric acid levels were observed in the 12-week synbiotics-supplemented group (Table 6). The results support the notion that the potential use of synbiotics could be a promising choice for the treatment and/or management of obesity. This study may stimulate interest in molecular underpinnings beyond these significant results. Moreover, the study shows that synbiotic involvements in treating obesity could be a hopeful suggestive therapy in obesity and other related metabolic disorders.

5. Conclusions

The intake of synbiotics for a stipulated period of time had a moderating effect on body weight, BMI, body fat, visceral fat, BMR, and arm, waist, and hip circumference. The effects of synbiotic supplementation were proven to greatly reduce the above-mentioned parameters when administered for prolonged period of time. This evidence suggests that synbiotic supplementation produces a stronger effect compared to separate prebiotic and probiotic treatments. Additional anti-obesity effects can be obtained when obese subjects carry out synbiotic supplementation alongside any physical activity. The present study demonstrated that 12 weeks of synbiotic supplementation significantly reduced the physical parameters as well as the inflammation markers IL-6, IL-1β, TNF-α and other obesity markers including LPS, zonulin, 5-HIAA, and QA in Thai obese subjects. These obtained results offer a new platform to document other new markers and the effect of various other synbiotic supplementation combinations in the study of obesity.

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Institutional Review Board Statement: The study was conducted following the Good Clinical Practices, fully complied with the ethical guidelines of a clinical trial, and conducted according to the Declaration of Helsinki; the Ethics Committee approved the protocol of Mae Fah Luang University (Code: REH-62151).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in the manuscript is available on request from the corresponding author.

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Review

Phytochemical Profile, Biological Properties, and Food Applications of the Medicinal Plant *Syzygium cumini*

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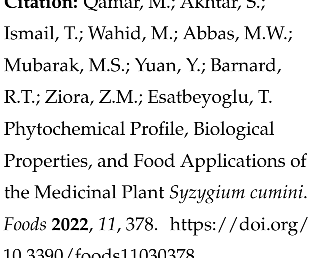
Abstract: *Syzygium cumini*, locally known as Jamun in Asia, is a fruit-bearing crop belonging to the Myrtaceae family. This study aims to summarize the most recent literature related to botany, traditional applications, phytochemical ingredients, pharmacological activities, nutrition, and potential food applications of *S. cumini*. Traditionally, *S. cumini* has been utilized to combat diabetes and dysentery, and it is given to females with a history of abortions. Anatomical parts of *S. cumini* exhibit therapeutic potentials including antioxidant, anti-inflammatory, analgesic, antipyretic, antimalarial, anticancer, and antidiabetic activities attributed to the presence of various primary and secondary metabolites such as carbohydrates, proteins, amino acids, alkaloids, flavonoids (i.e., quercetin, myricetin, kaempferol), phenolic acids (gallic acid, caffeic acid, ellagic acid) and anthocyanins (delphinidin-3,5-*O*-diglucoside, petunidin-3,5-*O*-diglucoside, malvidin-3,5-*O*-diglucoside). Different fruit parts of *S. cumini* have been employed to enhance the nutritional and overall quality of jams, jellies, wines, and fermented products. Today, *S. cumini* is also used in edible films. So, we believe that *S. cumini*'s anatomical parts, extracts, and isolated compounds can be used in the food industry with applications in food packaging and as food additives. Future research should focus on the isolation and purification of compounds from *S. cumini* to treat various disorders. More importantly, clinical trials are required to develop low-cost medications with a low therapeutic index.

Keywords: jamun; nutrition; antioxidant; inflammation; cancer; radioprotection; diabetes; hyperlipidemia; value addition; packaging



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1. Botanical Description and Traditional Uses

Syzygium cumini (L.) (synonyms: Eugenia jambolana, Syzygium jambolana, Eugenia cuminii), commonly known as jamun, jambul, jambolao, Java plum, Indian blackberry, and black plum, belongs to the family Myrtaceae. The fruit is native to South Asia, mainly Pakistan, India, Afghanistan, and Myanmar, and Pacific-Asia, including Indonesia, the Philippines, Hawaii, and Australia, and it is also cultivated in Florida and Kenya. During ripening, the fruit is greenish and at maturity pink to shining crimson (Figure 1). The harvesting period of the fruit jamun in Asia starts usually in the monsoon season (June to

July) and lasts 30 to 40 days [1]. *S. cumini* fruits (1.5 to 3.5 cm) exhibit sweet flavor and mild astringency [2]. Bitterness can be reduced by pickling, adding some salt, and standing for a minimum of 1 h [3]. *S. cumini* fruits are eaten fresh, or as chutney, and jam. *S. cumini* juice is used for preparing summer drinks such as syrup, sherbet, and squash. The squeezed fruits are normally heated for 10 min and mixed with water, sugar, citric acid, and sodium benzoate for preservation [2].

Figure 1. Leaves and fruits of *Syzygium Cumini*.

S. cumini has been traditionally used as a medicinal plant. Different parts of the plant (for example bark, leaves, seeds, and fruit) have been employed in the treatment of various diseases. *S. cumini* fruit juice has been utilized, orally, to treat gastric complaints, diabetes, and dysentery [4]. *S. cumini* seeds have been applied externally to treat ulcers and sores, and powdered seeds with sugar have been given orally to combat dysentery [5]. Powdered seeds have been reported to be effective against diabetes [6]. *S. cumini* leaves were cooked in water (concentration of 2.5 g/L) and drunk daily, where 1 L has been reported to be effective against diabetes [7]. The juice of leaves has been used as an antidote in opium poisoning, and an oral intake of leaves for 2–3 days has been reported to be effective in reducing jaundice in adults and children [8]. Traditionally, *S. cumini* leaves juice along with mango leaves and myrobalan fruit administered with honey and goat milk has been used also to combat dysentery [9], whereas bark decoction of *S. cumini* with water has been used to treat diabetes [10], dysentery, to increase appetite, to achieve sedation, and to relieve headache when taken orally [4]. Bark decoction has been given to females with recurrent miscarriages [5]. *S. cumini* bark juice with buttermilk has been reported to treat constipation, whereas an intake in the morning has been claimed to stop blood discharge in feces [11].

2. Phytochemical Profile

S. cumini fruits contain high amounts of vitamins, minerals, and fiber. They are low in calories and fat [1]. Phytochemicals of *S. cumini* including carbohydrates, proteins, fats, fiber, minerals, and vitamins are listed in Table 1.

Table 1. Content of nutrients in parts of *S. cumini* fruits, seeds, and leaves.

Nutrients	Amount	References
	Fruit	
Moisture	79.2–85.9%	[3,12–16]
Carbohydrates	7.88–22.4%	[3,12,14–17]
Proteins	0.65–6.60%	[3,12–18]
Fats	0.15–1.81%	[3,12,14–18]
Crude fiber	0.22–3.65%	[3,12,14–18]

Table 1. *Cont.*

Nutrients	Amount	References
Ash	0.30–4.50%	[3,13,15–18]
Calcium	0.02–116.0 mg/100 g	[3,13–15,19]
Sodium	3.50–141.7 mg/100 g	[3,14,16]
Potassium	172–1791 mg/100 g	[3,16,19]
Iron	0.10–4.60 mg/100 g	[3,13–16,19]
Magnesium	9.14–49.8 mg/100 g	[3,12,14,16]
Phosphorus	0.01–18.5 mg/100 g	[13–15,19]
Zinc	0.28–2.11 mg/100 g	[3,14,16,19]
Copper	0.07–6.80 mg/100 g	[12,14,16]
Chlorine	8.00 mg/100 g	[12]
Manganese	0.57–1.33 mg/100 g	[16,19]
Chromium	0.35 mg/100 g	[16]
Riboflavin	0.009–0.01 mg/100 g	[13–15]
Thiamine	0.008–0.12 mg/100 g	[12–15,19]
Niacin	0.20–0.30 mg/100 g	[12,13,15,19]
Ascorbic acid	5.70–137 mg/100 g	[3,13,14,16,17,19]
Vitamin A	80 I.U	[12]
Folic acid	3.00 mg/100 g	[12,15]
	Seed	
Moisture	47.0–52.2%	[3,18]
Carbohydrates	41.0–89.7%	[3,18,20]
Proteins	4.68–6.80%	[3,18]
Fats	0.35–1.28%	[3,18,20]
Ash	2.00–3.13%	[3,18,20]
Calcium	0.41–135 mg/100 g	[3,20]
Magnesium	111.6 mg/100 g	[3]
Potassium	606 mg/100 g	[3]
Phosphorus	0.17%	[20]
Sodium	6.10–43.9 mg/100 g	[3,20]
Iron	4.20 mg/100 g	[3]
Copper	2.13 mg/100 g	[3]
Ascorbic acid	1.84%	[3]
	Leaf	
Proteins	9.10%	[21]
Fats	4.30%	[21]
Fiber	17.0%	[21]
Ash	6.00%	[21]
Calcium	1.30%	[21]

Analysis of *S. cumini* fruits yielded moisture, protein, sugar, and ash contents as 80.8, 0.81, 12.7 and 0.70% on a fresh weight basis, respectively [22], which is in agreement with more recent results: moisture (79.2%), protein (0.65%), sugar (7.88%), ash (1.03%), and fat (0.18%) contents on a fresh weight basis [3]. Octadecane (16.9%), nonacosane (9.9%), and triacontane (9.3%) are dominant constituents of the leaf oil, whereas octacosane (7.4%), hepatcosane (4.8%), hexadecanoic acid (4.2%), and eicosane (4.02%) are also present [21–23]. Additionally, *S. cumini* seeds have fatty oils such as oleic acid (32.2%), myristic acid (31.7%), and linoleic acid (16.1%) as their main constituents. However, stearic acid (6.50%), palmitic acid (4.70%), lauric acid (2.80%), vernolic acid (3.00%), sterculic acid (1.80%), and malic acid (1.20%) were detected in small quantities [24]. The seed oil mainly comprised of 1-chlorooctadecane followed by tetracontane, decahydro-8a-ethyl-1,1,4a,6-tetramethylnapahthalene, 4-(2-2-dimethyl-6-6-methylene-cyclohexene) butanol, octadecane, octacosane, heptacosane, and eicosane in the range of 33.2%, 9.24%, 8.02%, 5.29, 5.15%, 3.97%, 1.72%, and 1.71%, respectively [23]. On the other hand, leaves contain minerals such as sodium, potassium, calcium, zinc, iron, magnesium, copper, manganese,

lead, and chromium [3]. Recently, the foliar application of zinc and boron was reported to exert profound effects on fruit weight (10.29 to 12.88 g), seed weight (1.68 to 2.55 g), and fruit length (19.55 to 25.88 mm). Other physicochemical parameters including total soluble solids and titratable acidity showed no change upon foliar treatment with zinc or boron, but it slightly influenced the reducing sugar content of *S. cumini* fruit; i.e., it increased from 6.33 to 6.64% [25].

Recent databases suggest that different plant parts including skin and pulps, essential oils, seeds, flowers, barks, and leaves have different and characteristic compositions (Table 2) [26–31].

Table 2. Phytochemicals in *S. cumini*.

Plant Part	Phytochemicals	References
Seeds	Fatty acids: oleic acid, stearic acid, octadecanal, 1-monolinoleoylglycerol trimethylsilyl ether, *n*-hexadecanoic acid Phenolic acids: gallic acid, ellagic acid Flavonoids: quercetin Phytosterols: *β*-sitoterol Tannins: corilagin, 3,6-hexahydroxy diphenoylglucose, 1-galloylglucose, 3-galloylglucose, 4,6 hexahydroxydiphenoylglucose Others: 2-bromo-cyclohexasiloxane, dodecamethyl, cycloheptasiloxane, tetradecamethyl, pyrazole[4,5-b] imidazole, 1-formyl-3-ethyl-6-beta-d-ribofuranosyl, 3-(octadecyloxy) propyl ester, benzaldehyde	[32–34]
Leaves	Alkanes: *n*-heptacosane, *n*-nonacosane, *n*-hentriacontane, noctacosanol, *n*-triacontanol, *n*-dotricontanol Terpenoids: betulinic acid, maslinic acid, α-pinene, camphene, globulol, caryophyllene, δ-cadinene, β-eudesmol, β-pinene, γ-cadinene, α-terpineol, camphor, humulene 6,7-epoxide, cubeban-11-ol, α-muurolene, epicubenol, α-copaene, viridiflorene, guanine, β-bourbonene, terpinen-4-ol, endo-borneol, levoverbenone Flavonoids: quercetin, myricetin, myricitrin, flavonol glycosides, myricetin 3-*O*-(4″-acetyl)-*α* L-rhamnopyranosides Phytosterols: *β*-sitosterol	[31,32,35]
Stem bark	Terpenoids: friedelin, friedelan-3-*α*-ol, betulinic acid Phytosterols: *β*-sitosterol, *β*-sitosterol-D-glucoside Phenolic acids: gallic acid, ellagic acid Tannins: gallotannin, ellagitannins Flavonoids: kaempferol, myricetin	[32,33]
Pulp and Skin	Anthocyanins: delphinidin-3,5-*O*-digalactoside, delphinidin-3,5-*O*-diglucoside, delphinidin-3-*O*-glucoside, petunidin-3,5-*O*-digalactoside, petunidin-3,5-*O*-diglucoside, petunidin-3-*O*-glucoside, cyanidin-3,5-*O*-digalactoside, cyanidin-3-*O*-glucoside, peonidin-3,5-*O*-digalactoside, peonidin-3,5-*O*-diglucoside, malvidin-3,5-*O*-digalactoside, malvidin-3,5-*O*-diglucoside, malvidin-3-*O*-glucoside Flavonols: myricetin-3-*O*-glucuronide, myricetin-3-*O*-galactoside, myricetin-3-*O*-glucoside, myricetin-3-*O*-rhamnoside, myricetin-3-*O*-pentoside, laricitrin-3-*O*-galactoside, laricitrin-3-*O*-glucoside, syringetin-3-*O*-galactoside, syringetin-3-*O*-glucoside,Flavanonols: DHQ-dihexoside-1, DHQ-dihexoside-2, DHQ-dihexoside-3, MDHQ-dihexoside, MDHQ-dihexoside, DHM-dihexoside-1, DHM-dihexoside-2, DHM-dihexoside-3, DHM-dihexoside-4, DHM-dihexoside-5, DHM-dihexoside-6, MDHM-dihexoside-1, MDHM-dihexoside-2, MDHM-dihexoside-3, MDHM-dihexoside-4, MDHM-dihexoside-5, MDHM-dihexoside-6, DMDHM-dihexoside-1, DMDHM-dihexoside-2, DMDHM-dihexoside-3, liquiritigenin Flavan-3-ols: catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin 3-*O*-gallate, catechin 3-*O*-gallate, epigallocatechin 3-*O*-gallate, gallocatechin 3-*O*-gallate Tannins: galloyl-glucose, 3galloyl-glucose-1, 2galloyl-glucose, 3galloyl-glucose-2, 3galloyl-glucose-3, 3galloyl-glucose-4, 4galloyl-glucose-1, 4galloyl-glucose-2, 5galloyl-glucose-1, 5galloyl-glucose-2, 5galloyl-glucose-3, 6galloyl-glucoside-1, 6galloyl-glucoside-1, castalagin, vescalagin, (2) HHDP-glucose-1, (2) HHDP-glucose-2, G-(2) HHDP-glucose-1, (2) HHDP-glucose-2, (2) G-HHDP-glucose-1, (2) G-HHDP-glucose-2, (2) G-HHDP-glucose-3, (3) G-HHDP-glucose, trisgalloyl-HHDP-glucose-1, trisgalloyl-HHDP-glucose-2 Phenolic acids: quinic acid, gallic acid, chlorogenic acid, caffeic acid, Coumarins: umbelliferon, scopoletin, Terpenoid: rosmanol	[28,32,36–38]

Table 2. *Cont.*

Plant Part	Phytochemicals	References
Flowers	Flavonoids: isoquercetin, quercetin, kaempferol, myricetin, Terpenoid: oleanolic acid, Phenolic acid: ellagic acids	[32]
Essential oils	Terpenoids: α-terpeneol, myrtenol, eucarvone, muurolol, α-myrtenal, 1, 8-cineole, geranyl acetone, α-cadinol, pinocarvone	[39]

DHQ, dihydroquercetin; MDHQ, methyl-dihydroquercetin; DHM, dihydromyricetin; MDHM, methyl-dihydrolmyricetin; DMDHM, dimethyl-dihydromyricetin. G, number (n) of Galloyl; HHDP, number (n) of hexahydroxydiphenoyl.

The purple shade of *S. cumini* fruit is due to the presence of anthocyanins, whereas its astringent flavor is imparted by a high concentration of tannins [15]. Furthermore, 3,5-diglucosides of malvidin, delphinidin, and petunidin were identified in *S. cumini* peels [26,27]. Bioactive components such as phenolic acids, gallic acid, ellagic acid, carotenoids, flavonoids, myricetin, and their derivatives were identified in fruit pulps [28,29]. About 30 compounds such as terpinyl isovalerate, dihydrocarvyl acetate, and geranyl butyrate contribute to the flavor of the purple fruits [30]. Moreover, a qualitative investigation of *S. cumini* seeds unveiled the presence of gallic acid, ellagic acid, corilagin, jambosine, quercetin, and β-sitosterol [40]. Recently, the essential oil of *S. cumini* leaves was analyzed by GC-MS, wherein τ-cadinol and τ-muurolol were found in high amounts as 21.4% and 12.4% of the total oil fraction, respectively [31].

3. Pharmacological Potential of *S. cumini*

S. cumini has been used in various ancient medicinal systems such as Siddha, Tibetan, Unani, Sri Lankan, and Ayurveda. It was employed in the aforementioned systems with a view to curing diarrhea, menstrual disorders, obesity, hemorrhage, and vaginal discharge [41]. Parts of *S. cumini* including fruit, seed, bark, leaves, pulp, and skin are known for their antioxidant [42], anti-inflammatory [43], anticancer [44], and antidiabetic activities [7]. Data are available on animals and different in vitro models to support their hepatoprotective [45], cardioprotective [46], chemopreventive potential [47], and antipyretic properties [48]. Some investigations have revealed activity against diabetes [49], obesity [50], inflammation [48], and bacterial infections [51]. Below are details about the documented pharmacological activities of *S. cumini*.

3.1. Antioxidant Activity

An imbalance between endogenous antioxidant defense and reactive oxygen species is the main reason for oxidative stress, and it has been suggested as a principal cause of the eventual inception of ailments. Antioxidants are considered to be key ingredients imparting health and protecting against various infections and degenerative diseases through radical scavenging capacity [52]. The antioxidant potential of *S. cumini* extracts has been explored by various researchers by employing a variety of in vitro assays including nitric oxide (NO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and oxygen radical absorbance capacity (ORAC) [36,53–58]. Within this context, investigations by Veigas et al. (2007) [26] revealed that anthocyanins extracted from *S. cumini* fruit peels using acidified methanol cause noteworthy protection against iron-induced lipid peroxidation. Similarly, *S. cumini* peel extract was reported to exert more potential (90.6%) against DPPH than pulp (82.5%) and seed (85.2%) extracts due to the presence of high phenolic (4812–5990 mg gallic acid equivalent (GAE)/100 g) contents on a dry weight (dw) basis [59]. Likewise, a considerable amount of tannins isolated from *S. cumini* peel using 70% aqueous acetone showed significant radical scavenging activities using the DPPH and FRAP assays [42]. Identically, freeze-dried extract of *S. cumini* pulp showed values of 670 mM Trolox equivalent (TE)/100 g (DPPH), 820 mM TE/100 g (ABTS), and 750 mM TE/100 g dw (FRAP) [60].

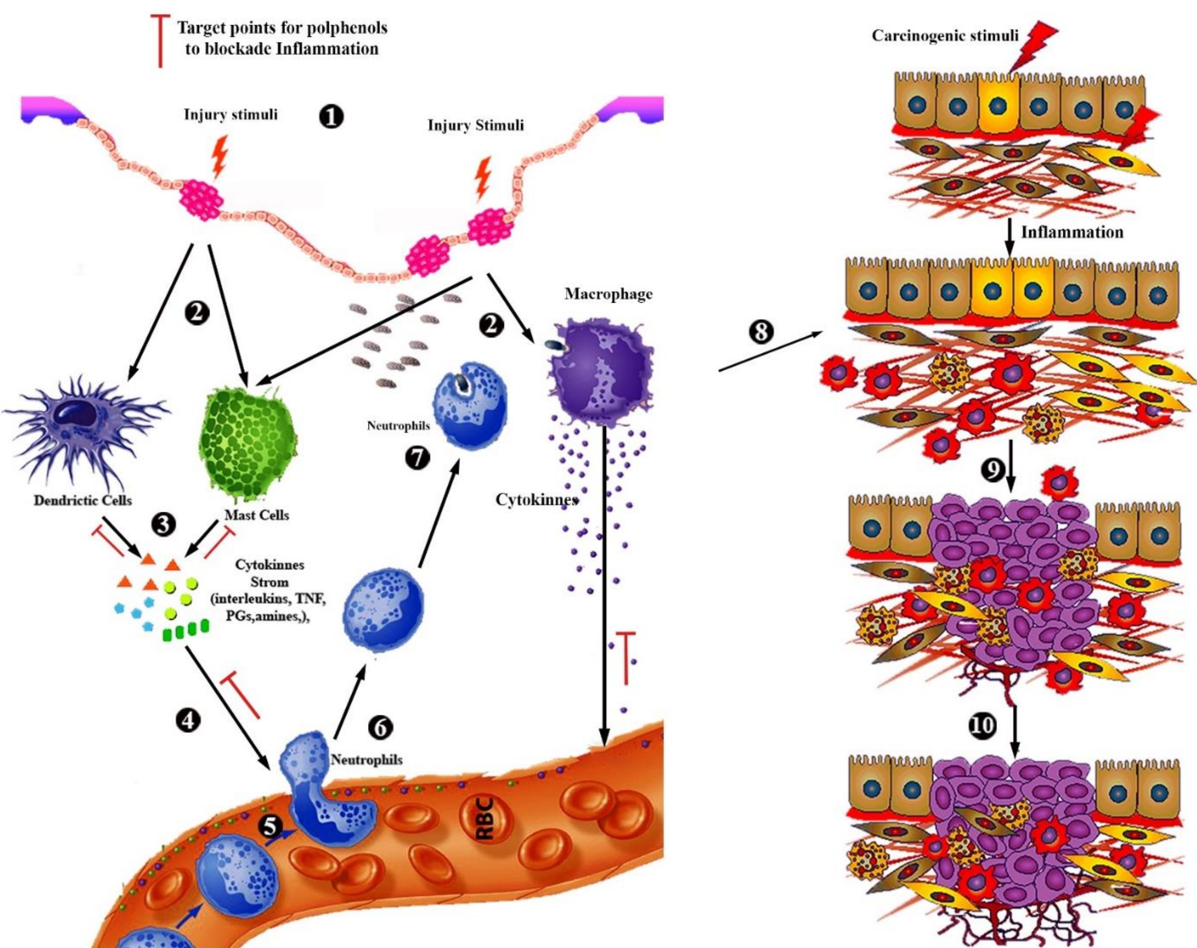
Vasi and Austin (2009) [56] reported that 50% aqueous ethanol *S. cumini* seed extract causes a six-fold greater scavenging activity against ABTS (98.9%), NO (96.8%), FRAP (94.4%), and DPPH (92.3%) as compared to the standard Trolox. Furthermore, a significant alteration was observed against the elevated level of peroxides and reduced level of antioxidant status including catalase, superoxide dismutase (SOD), glutathione (GSH), and ascorbic acid after oral administration of *S. cumini* 90% aqueous methanol seed extract in rats [61]. Water *S. cumini* seed extract showed better ORAC values (338 mM TE/100 g) than pulp extract (144.5 mM TE/100 g) as well as reducing oxidative stress [47,58]. Moreover, *S. cumini* methanol leaf extract contained significant amounts of total phenolics and flavonoids, with good antioxidant status, suggesting the potential of *S. cumini* to treat various human disorders [62].

Recent investigations have suggested that ethanol extracts of *S. cumini* parts (stem, leaf, seed) possess the protruding potential to reverse oxidation mechanism as compared to water extracts. In addition, the ethanol extracts of stem, leaf, and seed exhibited radical scavenging ability as 33%, 68%, and 98%, respectively in the DPPH assay [56]. Findings by Saeed et al. revealed that when evaluated for radical scavenging capacity, four different cultivars of *S. cumini* displayed more than 90% reduction against DPPH free radicals, which is attributed to the presence of large amounts of ascorbic acid, total phenolics, and anthocyanins [36]. In addition, the methanol leaf extract of *S. cumini* exhibited antioxidant activity at 1314 mg ascorbic acid equivalent (AAE)/100 g in the FRAP assay, whereas dichloromethane extract showed weaker activity at 122 mg AAE/100 g dw [63]. Furthermore, the ethanol leaf extract of *S. cumini* at 20 g/kg of body weight played a preventative role due to its antioxidant potential against gastric ulceration in rat stomach [64]. Similarly, the *S. cumini* bark extract can scavenge free radicals owing to the presence of a variety of bioactive compounds [65].

On the other hand, research findings showed that the *S. cumini* methanol leaf extract contains 369.75 mg GAE/g and 75.8 mg rutin equivalent (RE)/g total phenolic and flavonoid contents, respectively and exhibits notable inhibition activity with an IC_{50} of 133 μg/mL against stable free radicals i.e., DPPH comparable to the standard ascorbic acid (IC_{50} of 122.4 μg/mL) [66]. Moreover, three anthocyanins—delphinidin-3,5-*O*-diglucoside, petunidin 3,5-*O*-diglucoside, and malvidin-3,5-*O*-diglucoside—were isolated from *S. cumini* pulp using high-speed counter current chromatography; these compounds exhibited strong radical-scavenging abilities [67]. *S. cumini* leaf extract protected against paraquat-induced toxicity in *Saccharomyces cerevisiae* strains deficient in SOD owing to higher antioxidant activity [68]. Sequentially extracted ethyl acetate and *n*-butanol fractions of *S. cumini* leaves delineated notable radical-scavenging activity in the DPPH assay with IC_{50} 15.7 and 23.5 μg/mL, 1155 and 1178 μmol TE/g in the FRAP assay, and 1225 and 1314 μmol TE/g in the ORAC assay [29]. In addition, the *S. cumini* methanol fruit extract exhibited strong antioxidant properties in the DPPH (IC_{50} 81.4 μg/mL), FRAP (46.0 mmol Fe/g), and H_2O_2 (76% inhibition) assays as compared to 50% aqueous methanol and dichloromethane extracts [37]. Recently, Santos et al. (2021) [69] found that *S. cumini* water leaf extract is potent in averting the excessive generation of reactive oxygen/nitrogen species, macrophages viability loss, and foam cells formation that is caused by LDL oxidation.

3.2. Anti-Inflammatory Potential

Excess production of free radicals from activated inflammatory leukocytes, especially under conditions of chronic inflammation, may have an important role in various pathologies. Several reports have suggested that diseases associated with inflammation may be ameliorated by polyphenols (Scheme 1).



Scheme 1. Mechanism of inflammation leading to carcinogenicity (signed by the authors Saeed Akhtar and Muqeet Wahid using Adobe). 1. Carcinogenic or injury stimuli cause the injury on the epithelium layer of tissue. 2. Activation of dendritic, mast cells, and macrophages present under epithelium layer in between parenchymal cells of the tissue. 3. Dendritic, mast cells, and macrophages release the proinflammatory cytokines (TNF, interleukins, PGs, amines, amines, etc.). 4. Cytokines act on blood vessels for vasoconstriction to migrate platelets and neutrophils from blood vessels to the site of inflammation. 5. Neutrophils roll and 6. Migrate to the site of inflammation. 7. Neutrophils engulf the microbes and necrotic cells. 8. Chronic inflammation may lead to the mutagenicity and proliferation of cells 9. Inflammation and mutagenicity lead to the uncontrolled proliferation of cells with hyperplasia and dysplasia. 10. Angiogenesis with uncontrolled proliferation of cells, which leads to tumor formation in tissue.

In this regard, *S. cumini* is a well-reputed medicinal plant with anti-inflammatory potential. The *S. cumini* water fruit extract exhibited considerable anti-inflammatory activity of 68.9%, while the standard diclofenac holds an anti-inflammatory potential of 75.1% [43]. Similarly, a flavonoid-rich fraction of *S. cumini* fruits is reported to palliate inflammatory reactions in human lymphocytes, neutrophils, and monocytes against the hepatitis B vaccine [70]. In addition, ethanol, methanol, ethyl acetate, and water *S. cumini* seeds extracts are effective against carrageenan-induced paw edema in Wistar rats [71]. On the other hand, the *S. cumini* chloroform seed extract caused suppression of protein exudates, dye leakage during peritoneal inflammation, and migration of leukocytes along with inhibition of acute and sub-acute inflammation in rats [72]. Likewise, the water seed extract of *S. cumini* reduced inflammation through action on human neutrophils [73].

In a recent study, the water extract of *S. cumini* seeds alleviated inflammation by causing the suppression of ectonucleotidase, acetylcholinesterase, adenosine deaminase,

and dipeptidyl peptidase IV, and inhibited NO activities [74]. Similarly, methanol seed extract of *S. cumini* displayed greater anti-inflammatory potential compared to fruit juice in hemagglutination inhibition and membrane stabilization assays; this is attributed to the high polyphenolic content present in the methanolic fraction [75]. Moreover, *S. cumini* leaf water extracts alleviated indomethacin-induced inflammation by suppressing cyclooxygenase (COX), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-alpha (TNF-α) enzymes [76]. Recently, research findings revealed that *S. cumini* seeds alter the inflammatory responses induced by lipopolysaccharide (LPS) by suppressing jun N terminus kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 arbitrating nuclear factor-κB (NF-κb) pathways as well as down-regulation of iNOS and COX-2 [77]. In addition, food fortification with *S. cumini* seeds protected rat kidneys and liver by attenuating inflammatory cell infiltration, collagen secretion, deposition of extracellular matrix, and overload of iron, which is attributed to the presence of antioxidants [78].

Studies involving animals showed that the ethanol stem bark extract of *S. cumini* exerts significant effects against inflammation induced by carrageenan, kaolin, and formaldehyde in rats with no gastric lesions [48]. Additionally, it was reported that the ethanolic bark extract of *S. cumini* exhibited inhibition against prostaglandin E2 (PGE2), 5-hydroxytryptamine (5-HT), and histamine-induced paw edema in rats, but was ineffective against bradykinins-induced inflammation [79]. Similarly, the ethanol root extract of *S. cumini* showed anti-inflammatory potential in vitro by reducing interleukin 6 (IL-6) in RAW 264.7 macrophages [80].

In addition, the tannin-free ethyl acetate fraction of *S. cumini* leaves possessed a concentration-dependent reduction of the polymer C48/80-induced paw edema even at doses as low as 0.01 mg/kg [81]. In the same manner, *S. cumini* leaf ethyl acetate and methanol extracts showed an inhibitory effect against carrageenan-induced inflammation at doses of 200 and 400 mg/kg [82]. Other researchers showed that the *S. cumini* leaf extract exerts anti-inflammatory effects against acute and chronic inflammation in albino rats [83]. Furthermore, published research indicated that the acetone extract of *S. cumini* leaves possesses both anti-hyperglycemic and anti-inflammatory activities [84]. Similarly, the methanol leaf extract of *S. cumini* and its subsequent fractions obtained using the Kupchan partitioning method exhibited a potent action against acetic acid-induced writhing in a dose-dependent manner [85].

Another study revealed that in both acute (carrageenan, serotonin, histamine) and chronic (cotton pallet rat granuloma) models of inflammation, *S. cumini* leaf methanol extract exhibits potent activity in a concentration-dependent manner [86]. Additionally, *S. cumini* tannin-rich fraction reduced inflammation by 99.5% in the heat-induced protein denaturation assay at 100 μg/mL better than the commercially available anti-inflammatory drug aspirin that caused inhibition of 89.3% at the same concentration. A similar trend has been observed in the human red blood cell membrane (HRBC) stabilization assay where *S. cumini* tannin-rich fraction exhibited 82.9% protection of HRBC membrane at a dose of 1 mg/mL while the standard diclofenac caused protection of 70.4% as compared to the control i.e., distilled water [87].

Moreover, essential oils from *S. cumini* reduced chronic granulomatous inflammation in BALB/c mice and inhibited the migration of rat eosinophils, thus showing anti-inflammatory activity [88,89]. In addition, *S. cumini* methanol leaf extract exerted analgesic effects in rabbits as evident from decline in writhing (12.2 control vs. 3.7 treated) and anti-inflammatory response, i.e., 64.1% inhibition [66]. Furthermore, malvidin-3,5-*O*-diglucoside, an anthocyanin isolated from *S. cumini* pulp, inhibited the release of nitric oxide and pro-inflammatory mediators including IL-6, IL-1β, and TNF-α in LPS-induced RAW264.7 macrophages [67]. Similarly, methanol fruit extract of *S. cumini* exhibited significant in vitro (heat-induced hemolysis, albumin denaturation, bovine serum albumin denaturation) and in vivo (carrageenan-induced paw edema, formaldehyde-induced paw edema, PGE2-induced paw edema) anti-inflammatory activities, which were considerably

higher than 50% aqueous methanol and dichloromethane extracts [37]. In Table 3, the anti-inflammatory activities of different parts of *S. cumini* are shown.

Table 3. Anti-inflammatory activity of *S. cumini*.

Plant Part	Extraction Solvent	Species/Assays/Cell Lines	Results	References
Anti-Inflammatory Activity				
Fruit	Water	Rats	68.9% inhibition against carrageenan-induced paw edema	[44]
Fruit	Water	Lymphocytes, neutrophils and monocytes	Palliate inflammatory reactions against hepatitis B vaccine	[70]
Fruit	Methanol	Rats	70% inhibition against carrageenan-induced edema	[37]
Fruit	Methanol	Mice	72% inhibition against formaldehyde-induced edema	[37]
Fruit	Methanol	Rats	69% inhibition against PGE2-induced edema	[37]
Fruit	Methanol	Heat-induced hemolysis	67% inhibition against heat-induced hemolysis	[37]
Fruit	Methanol	Serum albumin denaturation	82% inhibition against bovine serum albumin denaturation	[37]
Fruit	Methanol	Egg albumin denaturation	75% inhibition against egg albumin denaturation	[37]
Seed	Methanol	Heat-induced hemolysis	Notable inhibition recorded against heat-induced hemolysis	[75]
Seed	Water	Neutrophils	Significant inhibition of neutrophil chemotaxis	[73]
Seed	Water	Rats	Significant suppression of ectonucleotidase	[74]
Seed	Successive	RAW 264.7	Suppression of pro-inflammatory cytokines (IL-6, IL-10, INF-γ, IL-β, TNF-α)	[77]
Leaf	Ethyl acetate	Rats	Altered C48/80 induced paw edema even at dose of 0.01 mg/kg	[81]
Leaf	Successive	Rats	Significant alteration observed against carrageenan-induced inflammation	[82]
Leaf	Methanol	Rats	Oral administration of 100 and 200 mg/kg exhibited significant anti-inflammatory activity in a dose-dependent manner	[86]
Leaf	Essential Oil	Mice	Significant apoptosis observed among inflammatory cells	[88]
Leaf	Essential Oil	Mice	67% inhibition of eosinophils migration	[89]
Leaf	Water	Mice	Significant inhibition against indomethacin-induced inflammation	[76]
Leaf	Methanol	Rats	75.2% inhibition against carrageenan-induced paw edema	[66]
Stem bark	Ethanol	Rats	40.6% against formaldehyde-induced edema	[48]
Stem bark	Ethanol	Rats	46.0% against PGE2-induced paw edema	[79]
Root	Water	RAW 264.7	Significant reduction of IL-6	[80]

3.3. Anticancer Potential

Cancer is among the most common life-threatening maladies and is a prime cause of death worldwide. According to Global Cancer Statistics, Asia including Southern Asia (India, Sri Lanka), South-Eastern Asia (Myanmar, Philippines, Indonesia, Cambodia, Vietnam), and Eastern Asia (China, Japan, Republic of Korea, Mongolia) is leading with its worldwide share of cancer deaths (57.3%). Approximately one-half of all new cancer cases and above one-half of the overall cancer deaths worldwide were reported from Asia, while breast cancer and lung cancer are the commonly diagnosed and leading cause of death in females (11.6%) and males (18.4%), respectively [90]. Various therapeutic drugs in Western medicine have been used to treat cancer-related diseases. Some of these have serious toxicity and side effects. Chemotherapy is a widely used technique for cancer treatment, but its adverse effects on normal cells cannot be neglected [91], so there is a need to discover

chemical compounds that are effective and have low off-target toxicity (that is, they possess a high therapeutic index). In this respect, the application of phytochemicals for cancer chemoprevention could be a promising approach to reduce cancer prevalence. Medicinal plants are a "herbarium" of bioactive compounds and could be used as chemopreventive agents [92].

S. cumini crude and methanol extracts were evaluated for cytotoxic activity against cultured human cancer cells, SiHa (carcinoma of uterus) and HeLa (cervical cancer). Results revealed a dose-dependent cell death against both cancer cell lines. Extracts showed more pronounced activity against HeLa than SiHa cell lines. Moreover, the 50% aqueous methanol fraction was reported to induce greater apoptosis in HeLa compared to SiHa cells [93]. A study conducted by Li et al. (2009) [27] investigated the anti-proliferative and pro-apoptotic effects of a standardized *S. cumini* fruit extract. Standardized anthocyanins fraction was reported to possess a significant effect against MCF-7 with an IC_{50} of 27 μg/mL, while an IC_{50} of 40 μg/mL was reported against MDA-MB-231 breast cancer cell lines. In contrast, the standardized *S. cumini* fruit extract was reported to cause no cell death and apoptosis against a normal breast cell line MCF-10A with an IC_{50} of >100 μg/mL. Furthermore, *S. cumini* seed ethanol extract exhibited an anticancer effect with IC_{50} values of 49, 110, 140, and 165 μg/mL against ovarian (A2780), breast (MCF-7), prostate (PC-3), and lung (H460) cancer cell lines, respectively. The ovarian cancer line was found to be most susceptible to seed extract, while non-significant results were obtained against breast, prostate, and lung cancer cell lines [51]. Furthermore, methanol and ethyl acetate fractions of *S. cumini* seeds were reported to cause concentration-dependent activity against MCF-7. In addition, the ethyl acetate extract was found to be more promising compared to a methanol extract, and a similar trend was observed for DNA fragmentation, which is considered as an apoptosis indicator [94]. In Table 4, the anticancer properties of *S. cumini* are summarized.

Table 4. Anticancer activity of *S. cumini*.

Plant Part	Extraction Solvent	Cell Line	Results	References
Peel	50% aqueous Methanol	SiHa and HeLa	Dose-dependent cell death against both cell lines	[93]
Pulp	Chloroform	PA-1	IC_{50} 27 μg/mL IC_{50} 40 μg/mL	[27]
Pulp	Methanol	OSCC	IC_{50} < 50 μg/mL	[95]
Pulp	Ethanol	Leukemia cells	Direct correlation observed between antioxidant status and anti-leukemia activity	[96]
Pulp	Acidic methanol	HCT-116	Dose-dependent cell death	[94]
Pulp	Methanol	H460	IC_{50} 35.2 μg/mL	[97]
Fruit	Ethanol	HT-29	IC_{50} 267.5 μg/mL	[98]
Seed	Ethanol	A_2780	IC_{50} 49 μg/mL	[51]
Seed	Ethyl acetate	MCF-7	Dose-dependent cell death	[94]
Seed	Methanol	HCT-116	IC_{50} 1.24 μg/mL	[99]
Leaf	Ethanol	T47D	69% inhibition	[49]
Leaf	Methanol	BM-MSCs	Concentration-dependent cytoprotective activity against H_2O_2-treated bone marrow mesenchymal stem cells of rats	[66]
Leaf	Methanol	HCT-116	IC_{50} 1.42 μg/mL	[99]

Charepalli et al. (2016) [97] reported the anticancer activity of anthocyanins isolated from *S. cumini* fruits using acidified methanol. The results revealed that *S. cumini* fruit triggered cytotoxic effects against HCT-116 colon cancer cells in a dose-dependent mode. Additionally, *S. cumini* fruit anthocyanin fractions provoked DNA fragmentation and caused apoptosis in colon cancer cell lines and colon cancer stem cells, as evaluated

through caspase 3/7 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays. Similarly, *S. cumini* methanol fruit extract elevated cytotoxicity and the suppression of cell proliferation in H460 lung cancer cells (IC_{50} of 35.2 μg/mL) in a dose-dependent manner [100]. At the same time, Aqil et al. [44] reported that *S. cumini* holds the appreciable potential to balance estrogen-mediated alterations in mammary cell-proliferation, estrogen receptor-alpha (ER-α), cyclin D1, and miRNAs, and that the modulation of these biomarkers correlated with a reduction in mammary carcinogenicity. Recently, a fraction of a *S. cumini* ethanol leaf extract, obtained from column chromatography, caused significant inhibition (69%) against the T47D breast cancer cell line [49]. Consistent with these observations, key findings of Ezhilarasan et al. (2019) [95] showed that *S. cumini* methanol fruit extract causes an inhibition of oral squamous cell carcinomas cells (OSCC). In addition, the ethanolic fraction of *S. cumini* fruit exhibited notable anti-leukemia activities, which is a direct correlation among polyphenolic contents, antioxidant status, and anticancer potential [96]. Furthermore, findings of Goyal et al. (2010) [101] revealed that pre, post, and pre–post oral administration of *S. cumini* extract induces an appreciable reduction in chemically induced tumor incidence, tumor burden, and a cumulative number of gastric carcinomas. The *S. cumini* extract also inhibits the reduction of phase II enzymes, which exhibit detoxification properties and lipid peroxidation [101]. Moreover, the *S. cumini* methanol leaf extract indicated a concentration-dependent cytoprotective activity against H_2O_2-treated bone marrow mesenchymal stem cells (BM-MSCs) [66]. Recently, *S. cumini* ethanol extract reported to inhibit the proliferation of HT-29 cells further confirmed by a DNA damage assay wherein DNA lost its integrity and went through apoptosis. The wound healing also proposed the lower change of metastasis when treated with *S. cumini* ethanol extract [98]. Recent findings showed that seed and leaf methanol extracts of *S. cumini* inhibit the growth of colon cancer cell line in a dose-dependent manner with IC_{50} values of 1.24 and 1.42 μg/mL, whereas the IC_{50} of the standard drug doxorubicin was 1.14 μg/mL [99].

3.4. Radioprotection

Phenolic compounds are known to be radioprotective with high biopotency as UV-A and UV-B blockers. Jagetia et al. (2003) [102] revealed that the application of dichloromethane and methanol leaf extracts of *S. cumini* on human peripheral blood lymphocytes before irradiation (3 Gy) results in significant protection of DNA. Additionally, protection against radiation-induced mortality and sickness in mice was also observed for the same extracts at 30 mg/kg [102]. Similarly, pretreatment with dichloromethane and methanol leaf extracts of *S. cumini* induced significant protection in mouse intestine against γ-radiation by increasing the number of regenerating crypts and rise in villus height, which is accompanied by a reduction in goblet and dead cells, which caused an increase in the life span of irradiated mice [103]. Likewise, dichloromethane and methanol leaf extracts of *S. cumini* caused inhibition against radiation-induced micronuclei generation, representing the potential against radiation-induced DNA damage [104]. Furthermore, the application of dichloromethane and methanol leaf extracts of *S. cumini* before irradiation caused an increase in GSH, catalase, and SOD levels. A dose of 50 mg/kg reduced lipid peroxidation in mouse liver [105]. Similar investigations revealed that pretreatment of mice with *S. cumini* of 50% aqueous methanol seed fraction against a lethal dose of 10 Gy alters the radiation-induced sickness and mortality. Furthermore, research findings indicated that the best protection against irradiation was at 80 mg/kg when applied intraperitoneally. The survival rate observed for the intraperitoneal route was found to be almost double (50%) compared with the oral route (22%) and 0% in the radiation alone [106]. All of these observations signify that *S. cumini* holds great capabilities to protect against radiation-induced sickness, intestinal, and DNA damage by increasing antioxidants that counter radiation-induced free radicals. *S. cumini* leaf water extract also contributed toward radioprotection by suppressing inflammatory cytokines such as TNF-α, NF-κB, iNOS, and COX enzymes [76]. Recently, a

significant increase in GSH levels and wound healing were observed after oral and topical administrations of *S. cumini* seeds ethanol extract along with topical laser therapy [107].

3.5. Hyperlipidemia and Cardioprotective Activity

An abnormal rise in blood lipid contents is known as hyperlipidemia. Any deviation in the lipid profile results in several heart issues inclusive myocardial infarction, cardiovascular diseases (CVD), stroke, and atherosclerosis [108]. Medicinal plants gained attention for the treatment of lipidemia and associated ailments due to the side effects of synthetic drugs [109]. Along this line, the *S. cumini* seeds flavonoid-rich fraction was evaluated for antilipidemic potential; results showed a reduction in low-density lipoproteins (LDL) levels and elevation of high-density lipoproteins (HDL) levels in rats [110]. Similarly, the fraction reduced the serum lipid levels [111]. Similarly, *S. cumini* seed water extract reduced triglycerides and LDL levels, whereas elevation of HDL levels was observed in alloxan-treated mice. Research findings also showed cardioprotective effects of *S. cumini* fruit ethanol extract [112]. Cardiovascular diseases are one of the important causes of death in industrialized nations, and hyperlipidemia is one of the main causes of cardiovascular ailments [109]. In this respect, work by Nahid et al. (2017) [113] revealed that methanol seed extract of *S. cumini* exhibits cardioprotective activity in isoproterenol-induced myocardial infarction in rats. Oral feeding for 30 days resulted in a concentration-dependent protection against the myocardial infarction. Application of the same extract caused alteration against cardiac and liver damage in diabetic rats. The *S. cumini* leaf methanol extract also possesses significant anticoagulant activity with a prothrombin time of 28.3 s vs. 15.8 s of control [66].

3.6. Antidiabetic Potential

In the last three decades, a considerable increase in type 2 diabetes cases, especially from developing countries where about 80% of the people have diabetes, was reported, and the rise of type 2 diabetes in South Asia is estimated to be more than 150% between 2000 and 2035 [114]. Antidiabetic properties of various parts of *S. cumini* including pulp, seed, bark, and stem have been investigated [7]. In this respect, Achrekar et al. (1991) [115] reported good antidiabetic activity of a water extract of *S. cumini* pulps; it caused a significant reduction in blood glucose level in streptozotocin-induced diabetic rats. The hypoglycemic index depended on the level of injected dose and mode of administration. Similarly, researchers showed that an excellent hypoglycemic index is achieved at about 100 to 200 mg/body weight with the intraperitoneal injection route [116]. In addition, results of rat modeling for evaluating hypoglycemic activity supported its antidiabetic potential and effectiveness against alloxan-induced diabetes [117]. Moreover, research findings indicated that the oral administration of *S. cumini* water and methanol extracts into rabbits at 200 and 300 mg/kg body weight resulted in almost the same hypoglycemic effect achieved with the standard drug tolbutamide at 100 mg/kg body weight [118].

Similarly, *S. cumini* seeds exhibit hypoglycemic activity as determined by the reduction of blood glucose levels in alloxan-induced diabetic rabbits. Results showed that ethanol extract of seeds at 100 mg/kg body weight caused a substantial reduction of fasting blood glucose levels. Histopathological analysis of various organs supported the hypoglycemic activity of *S. cumini* seed extract [117,118]. *S. cumini* seed powder [119] or extract (ethanol, methanol, ethyl acetate, and water) administered via different routes (oral, intraperitoneal) have the potential to control diabetes [117,120–124]. In a clinical trial, Sahana et al. (2010) [125] reported the effectiveness of *S. cumini* seed dosage by the oral route against diabetes (type 2) with no toxic effects on human subjects. Moreover, research findings supported the antidiabetic activity of *S. cumini* seeds due to the reduction of carbohydrate-hydrolyzing enzymes by the carotenoid luteolin, which binds at the α-amylase site and acts as an inhibitor of carbohydrate breakdown similar to acarbose [126]. This mechanism of action and results aligned with previous research studies on antidiabetic potential conducted by Karthic et al. (2008) [127] and Ponnusamy et al. (2011) [128]. These

researchers suggested that seed extracts control amylase, pancreatic amylase, glucoamylase, and other starch-hydrolyzing enzymes.

When streptozotocin-induced diabetic rats were treated with ethanol extract of *S. cumini* seeds, blood sugar levels were significantly reduced [129,130], whilst a methanol extract has been reported to control serum glucose level in alloxan-induced diabetic rats [131]. Moreover, *S. cumini* seed lyophilized powder caused a significant reduction in sugar levels in mice and rats [123,124]. Similarly, water extract of *S. cumini* seeds also controlled blood glucose levels in alloxan-induced diabetic mice [132]. Interestingly, the water-soluble seed powder of *S. cumini* with gummy fiber was found to control diabetes, whereas other preparations without fibrous matter failed to control diabetes in alloxan-induced diabetic rats [133]. Not only *S. cumini* seed extracts but also its individual bioactive compounds, such as mycaminose, play a significant role in controlling diabetes induced by streptozotocin in rats [134]. In this respect, ethanol extract of *S. cumini* seed kernel significantly reduced GSH levels in the liver and kidney of streptozotocin-induced diabetic rats [135,136].

In addition to the edible portions, including fruit and seed powder, leaves of *S. cumini* are potent in lowering glucose levels in both the blood and serum of experimental diabetic animals [137]. Sharafeldin et al. (2015) [138] extended their research by using extracts of *S. cumini* fruit and *Cinnamon zeylanicum* stem bark in combination and generated better results compared to their isolated effects. Furthermore, *S. cumini* seeds water extract at 400 mg/kg was more effective in controlling blood glucose levels in type II diabetes and in controlling peroxisome proliferator-activated receptor (PPARγ and PPARα) proteins of the liver [139]. *S. cumini* extract prepared from its stem bark was also found effective in controlling blood sugar levels against diabetic rats [140]. Accordingly, *S. cumini* seed ethanol extract was reported to reduce blood glucose levels [107]. Recently, sequentially extracted fractions of *S. cumini* leaves, i.e., dichloromethane, ethyl acetate, and *n*-butanol, were reported to cause 100% inhibition of α-amylase, wherein these fractions evinced 50% inhibition against α-glucosidase [29]. Recently, *S. cumini* seed extract (200 mg/kg) in combination with the standard drug metformin showed notable anti-hyperglycemic effects in streptozotocin-induced diabetic rats [141].

3.7. Gastroprotective, Antidiarrheal, and Antimicrobial Activity

Gastric ulcer is the most commonly diagnosed illness of the human digestive system [142]. Previous investigations revealed that the ethanol extract of *S. cumini* seeds reduces streptozotocin- and ethanol-induced peptic ulcers [129,130]. In addition, research findings showed that tannins from *S. cumini* offer excellent protection from hydrochloric acid- and ethanol-induced gastric ulceration by minimizing the gastric mucosal damage [64]. *S. cumini* seeds mixed with jaggery (non-centrifugal cane sugar) were reported to impart relief from diarrhea and dysentery. Likewise, tannins present in *S. cumini* fruits are well-known for their anti-diarrheal potential [143]. Moreover, *S. cumini* seed and flower with silver nanoparticles (AgNPs) exhibited notable antimicrobial potential when tested at concentrations between 31.2 and 2000 μg/mL against several bacterial and fungal species such as *A. naeslundii*, *C. albicans*, *F. nucleatum*, *S. aureus*, *S. epidermidis*, *S. mutans*, *S. oralis*, and *V.dispar* comparable with the activity of crude extracts tested at concentrations between 648 and 5188 μg/mL [144].

4. Value-Added Food Products and Food Packaging Material

S. cumini fruit powder was supplemented with gum arabic, which was considered to be an appropriate carrier of the food components, because it retained the maximum functional attributes i.e., total flavonoid content, total phenolic content, and total anthocyanin content, in contrast to maltodextrin and combination of maltodextrin/gum arabic [145]. Similarly, *S. cumini* pomace was added to ice cream at doses of 1, 2, 3, and 4% and evaluated the change in physicochemical features of ice cream. The addition of *S. cumini* pomace induced a considerable increase in titratable acidity, fiber contents, ash, hardness, and total soluble solids, wherein a notable decline was observed for pH, melting rate, and fat contents.

Moreover, the best sensory attributes were obtained when ice cream was treated with 3% of *S. cumini* pomace [146]. Another study conducted by Talukder et al. (2020) [147] made use of the unique feature of anthocyanins (change in color with the change in pH) and applied *S. cumini* anthocyanins as a quality indicator for meat products. Anthocyanin-rich skin extract of *S. cumini* was immobilized on filter paper strips. The strips, when attached inside a packet of chicken patties, changed color from violet to yellow as the pH varied, which was due to the reaction with volatile basic compounds produced from the meat stored at 4 °C. Observations suggested that the change in the color pattern is directly associated with the quality traits of chicken patties. For example, during a 21-day storage trial, numerous changes in the quality features of chicken patties were observed, including a decrease in pH from 6.22 to 6.04, increased level of nitrogen and ammonia, change in color, decrease in sensory attributes, and an increase in microbial count. Consequently, it has been anticipated that *S. cumini* skin extract filter paper can offer a suitable, non-toxic, visual means to check the quality of meat products during freezing and storage.

Kapoor et al. (2021) [148] prepared antioxidant-rich snacks by supplementing the rice flour with hot air-dried and freeze-dried *S. cumini* powder as 5, 10, 15, and 20% that substantially influenced the quality parameters of snacks. A considerable decrease in water absorption index and the expansion ratio was observed, along with an increase in water solubility, bulk density (g/cm^3), and hardness. The best sensory attributes were found for 10% *S. cumini* supplemented snacks (freeze-dried and hot air-dried). Moreover, freeze-dried *S. cumini* powder supplemented snacks displayed 9.52% more antioxidant potential in comparison to hot air-dried *S. cumini* supplemented snacks. In addition, the total phenolic contents were 89.7% and 80.4% in supplemented snacks comprising freeze-dried and hot air-dried *S. cumini* powder, respectively, at a 10% substitution level.

On the other hand, wheat pasta made with 30% *S. cumini* pulp was found to be the best in terms of overall acceptability based on sensory and phytochemical parameters [149]. In this case, the addition of *S. cumini* pulp enhanced the free radical-scavenging potential (5.76 to 10.2%), β-carotene level (1336 to 7624 µg/100 g), total phenolics (111 to 176 mg GAE/100 g), dietary fiber (7.08 to 16.6%), and ash content (0.59 to 2.96%). Gruel or cooking loss was more but within an acceptable range i.e., below 10% with the addition of *S. cumini* pulp. Furthermore, an inverse proportionality was observed between pulp concentration and cooking time, lightness, and yellowness while redness increased with the incremental addition of *S. cumini* pulp.

Recently, active and pH-sensitive edible films were developed by amalgamating the *S. cumini* skin extract into methylcellulose films. The addition of *S. cumini* skin extract elevated the mechanical and barrier properties of methylcellulose films. Moreover, films added with *S. cumini* skin extracts exhibited alike antioxidant potential compared to pure extracts. Analysis revealed that the resulting active intelligent films are biodegraded in seawater in two days and soil in 15 days. The developed films can offer an enhanced shelf-life, preserve product freshness, lessen atmospheric pollution, and can be applied in the meat and aquatic food industry where lipid oxidation and change in pH can spoil the food [150]. Similarly, Merz et al. (2020) [151] developed and characterized colorimetric indicator films from polyvinyl alcohol, chitosan, and most importantly anthocyanins from *S. cumini* fruit. Results revealed that the addition of anthocyanins positively influences the thickness and optical attributes of films. The developed films comprising anthocyanins presented discernable variations from red to blue color when employed to evaluate the freshness of shrimps at numerous temperatures ranging from −20 to 20 °C, which can be considered as alternative option in the food packaging industry. Kasai et al. (2018) [152] made films adopting different combinations of poly(vinyl alcohol)/*S. cumini* leaves extract and poly(vinyl alcohol)/chitosan/*S. cumini* leaves extract, adopting a solution-casting method. Films containing cassava starch, laponite, and *S. cumini* fruit anthocyanins manufactured by thermo-compression were used to monitor the meat freshness. Films showed visible changes from purple to yellow color when used to monitor the freshness of round steak stored at −20, 4, and 20 °C [153].

5. Summary and Future Perspectives

S. cumini contains valuable phytochemicals that are potential drug compounds for the treatment of a wide range of diseases. When incorporated into the diet, some of these compounds could serve as preventative treatments. Additionally, parts of the plant (skin, pulp, roots, leaves, bark) as well as their isolated compounds (quercetin, myricetin, gallic acid, caffeic acid, ellagic acid, delphinidin-3,5-*O*-diglucoside, petunidin-3,5-*O*-diglucoside, malvidin-3,5-*O*-diglucoside) can be used in the food industry with applications in food packaging and as food additives. The present review discussed in detail the health-promoting potential of the various anatomical parts of *S. cumini* and the compounds extractable from those parts. Future studies should be conducted with a view to the isolation and purification of compounds from the different parts of *S. cumini* for treating various ailments. More importantly, clinical investigations are a critical requirement for the discovery of cost-effective drugs that possess a low therapeutic index. Following the aphorism of the "Father of Medicine", Hippocrates (460–375 BC), "Let food be thy medicine and medicine be thy food", we should look back to compounds derived from nature with the application of current knowledge and technologies. We should also develop standardized in vitro 'proof-of-concept' methods for rigorous investigation of traditional use natural products, which are, unfortunately, often reported with various doses, various purities, and different exposure times, making it difficult to objectively compare and verify their potency and toxicity.

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Article

Application of Two-Dimensional Fluorescence Spectroscopy for the On-Line Monitoring of Teff-Based Substrate Fermentation Inoculated with Certain Probiotic Bacteria

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Abstract: There is increasing demand for cereal-based probiotic fermented beverages as an alternative to dairy-based products due to their limitations. However, analyzing and monitoring the fermentation process is usually time consuming, costly, and labor intensive. This research therefore aims to apply two-dimensional (2D)-fluorescence spectroscopy coupled with partial least-squares regression (PLSR) and artificial neural networks (ANN) for the on-line quantitative analysis of cell growth and concentrations of lactic acid and glucose during the fermentation of a teff-based substrate. This substrate was inoculated with mixed strains of *Lactiplantibacillus plantarum* A6 (LPA6) and *Lacticaseibacillus rhamnosus* GG (LCGG). The fermentation was performed under two different conditions: condition 1 (7 g/100 mL substrate inoculated with 6 log cfu/mL) and condition 2 (4 g/100 mL substrate inoculated with 6 log cfu/mL). For the prediction of LPA6 and LCGG cell growth, the relative root mean square error of prediction (pRMSEP) was measured between 2.5 and 4.5%. The highest pRMSEP (4.5%) was observed for the prediction of LPA6 cell growth under condition 2 using ANN, but the lowest pRMSEP (2.5%) was observed for the prediction of LCGG cell growth under condition 1 with ANN. A slightly more accurate prediction was found with ANN under condition 1. However, under condition 2, a superior prediction was observed with PLSR as compared to ANN. Moreover, for the prediction of lactic acid concentration, the observed values of pRMSEP were 7.6 and 7.7% using PLSR and ANN, respectively. The highest error rates of 13 and 14% were observed for the prediction of glucose concentration using PLSR and ANN, respectively. Most of the predicted values had a coefficient of determination (R^2) of more than 0.85. In conclusion, a 2D-fluorescence spectroscopy combined with PLSR and ANN can be used to accurately monitor LPA6 and LCGG cell counts and lactic acid concentration in the fermentation process of a teff-based substrate. The prediction of glucose concentration, however, showed a rather high error rate.

Keywords: artificial neural network; functional beverage; partial least-squares regression; probiotics; teff-based substrate; 2D-fluorescence spectroscopy

1. Introduction

Consumer demand for probiotic fermented cereal-based beverages is increasing. This is predominantly due to the limitations associated with dairy-based products, i.e., lactose and milk protein sensitivity or intolerance, fat content, and consumers' desire for foods without animal products [1]. However, analyzing and monitoring the fermentation process with conventional methods such as high-performance liquid chromatography is challenging, as it is time-consuming, costly, and labor-intensive [2]. Further challenges may arise

during sterilization, calibration, and sampling [3]. The productivity of the fermentation process and the cost of the fermented products depend mostly on the methods of monitoring and on the control over the operating conditions. On-line control of the fermentation process usually involves determination of pH and temperature. However, other key parameters of the fermentation process such as concentrations of metabolite, substrate, and cellular density need further investigation [4]. The key fermentation process parameters are not usually examined due to the expensive and time-consuming measurement methods [5].

Glucose was found to be the primarily consumed substrate while lactic acid was the primary metabolic product observed in the fermentation process of teff-based substrates inoculated with *Lactiplantibacillus plantarum A6* (LPA6) and *Lacticaseibacillus rhamnosus GG* (LCGG) [6]. Teff is a staple food crop in Ethiopia and Eritrea. It is gluten-free and an attractive source of iron (363 mg/kg flour) [7]. Thus, the fermentation industry needs an effective and efficient method to supervise the fermentation process. On-line analysis of the key fermentation process parameters assures product quality and productivity [8].

An alternative approach for this purpose is the application of a 2D-fluorescence spectroscopy. It is an ideal instrument for the on-line supervision of fermentation processes. In addition, its measurement is non-invasive and does not interfere with the fermentation medium [9]. In a 2D-fluorescence measurement, many wavelength combinations of excitation and emission are measured. A large volume of spectral data can be evaluated quantitatively using chemometric methods such as principal component regression (PCR) and partial least-squares regression (PLSR) [10]. Calibrating multivariate spectral data for quantitative spectral evaluation is becoming a standard method which allows an examination of several analytes at the same time. The PLSR and PCR are full-spectrum and are factor analyses based on multivariate calibration methods [11]. While PLSR and PCR are the most widely employed chemometric methods, PLSR usually requires fewer latent variables than PCR without influencing its predictive ability. Moreover, PLSR has superior prediction ability to PCR when there are different independent spectral components that can join with the spectral features [12].

Another method for the evaluation of the spectral data involves the use of artificial neural networks (ANN), which can be used to model a nonlinear correlation of the spectra with the variables [13]. ANN models generally contain two or more layers, each having a number of neurons. The ANN's activation functions are used to connect the neurons of the different layers to each other. One vital process in the utilization of ANN is training. It serves to minimize errors between the model output and measured values. The process of training is a continual one and consists of adjusting biases and weights at each sequence. The training process is completed when the error rate is at its lowest [14].

As of yet, there has been no work conducted on the simultaneous measurements of cell growth, glucose, and lactic acid in samples from a fermented teff-based substrate inoculated with mixed strains of LPA6 and LCGG using 2D-fluorescence spectroscopy. Therefore, this research aims to apply the potential of 2D-fluorescence spectroscopy and mathematical models of PLSR and ANN as tools for the on-line analysis of the fermentation process of a teff-based substrate inoculated with co-culture strains of LPA6 and LCGG.

2. Materials and Methods

2.1. Materials

Whole-grain teff flour was purchased from Teff-shop.de, Manuel Boesel, Homburger Str.49a, 61191 Rosbach von der Höhe, Germany. Freeze-dried strains of LPA6 (LMG 18053, BCCM, Gent, Belgium) and LCGG (LMG 18243, BCCM, Gent, Belgium) were provided by the Department of Process Analytics and Cereal Science, Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Baden-Württemberg, Germany.

2.2. Starter Culture Preparation and Storage

Freeze-dried strains of LPA6 (LMG 18053, BCCM, Gent, Belgium) and LCGG (LMG 18243, BCCM, Gent, Belgium) were activated and placed in a refrigerator (6 °C) until the

inoculation of the fermentation medium. The starter culture of LPA6 was prepared using the method described by Bationo, Songré-Ouattara, Hemery, Hama-Ba, Parkouda, Chapron, Le Merrer, Leconte, Sawadogo-Lingani, and Diawara [15]. The inoculum of LPA6 was obtained with sterilized MRS (DE MAN, ROGOSA, and SHARPE) broth by incubating in an incubator (BINDER GmbH, KB 115, Tuttlingen, Germany) for 24 h at 30 °C. Furthermore, the LCGG starter culture was prepared according to the method used by Matejčeková, Liptáková, and Valík [16]. The LCGG inoculum was collected after 24 h of incubation at 37 °C in sterilized MRS broth. Starter cultures were harvested by centrifugation (Mega star 600R, Leuven, Belgium) at 3000× *g*, 4 °C for 15 min. The LPA6 and LCGG cells were washed using a sterilized saline solution (0.9% NaCl). Finally, the supernatant was removed, and cell pellets were mixed with a sterilized saline solution to form a cell suspension of approximately 9 log cfu/mL. This was taken as an inoculum and was kept in a refrigerator (6 °C) until utilization within 48 h.

Strains of LPA6 and LCGG were stored with a medium containing 60% MRS broth and 40% glycerol in a deep freezer (−70 °C) [17].

2.3. Off-Line Measurement of Microbial Viability

The LPA6 cell count was determined by counting individual colonies on MRS agar plates (colony-forming units—cfu) according to the method used by Alemneh, Emire, and Hitzmann [6]. Each reported value represents the mean count of three plates containing 25–250 colonies. Plate agar was made by mixing 15 g agar into 1 L of MRS broth. Serial ten-fold dilutions of samples were prepared using a 0.9% NaCl solution. Fifty µL drops of diluted samples were put on MRS agar plates and incubated overnight at 30 °C. A similar procedure was followed for counting LCGG cells. However, the incubation time was nearly 48 h. For counting LPA6 and LCGG cells, the method developed by Alemneh, Emire, and Hitzmann [6] was used. Samples with co-culture strains of LPA6 and LCGG were incubated for 48 h on MRS agar plates at 30 °C. After overnight incubation, LPA6 cells were counted. Afterwards, LCGG was grown for approximately 48 h of incubation. Then, total cell counts of both LPA6 and LCGG were recorded. Finally, the difference (total cell counts of LPA6 and LCGG—cell count of LPA6) was determined as the LCGG cell count.

2.4. Fermentation Process Conditions

Overnight cultures of LPA6 and LCGG, each with an initial cell density of 6 log cfu/mL, were inoculated to the fermenting substrates, which were prepared from 4 and 7 g/100 mL of whole-grain teff flour in distilled water. Two different fermentation conditions were examined: condition 1, 7 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of LPA6 and LCGG and condition 2, 4 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of LPA6 and LCGG. Before fermentation, the substrates were heated in a water bath (GFL-1083, Burgwedel, Germany) set at 85 °C for 15 min and then sterilized in an autoclave (SHP Laboklav, 160-MSLV, Satuelle, Germany). Before the addition of microbes, the sterilized substrates were cooled down in a safety cabinet (Kendro Laboratory Products GmbH, KS 12, Hanau, Germany). Fermentations were performed without pH control for 15 h using a 2.5 L bioreactor (INFORS AG CH-4103, Bottmingen, Switzerland). The working volume was 1 L, the stirrer speed of the bioreactor was 150 rpm, and the fermentation temperature was 37 °C. Three h after the start of fermentation, samples were taken at 2 h intervals for determining LPA6 and LCGG cell counts and analyzing the concentration of glucose and lactic acid.

2.5. Off-Line Measurement of Glucose and Lactic Acid

Glucose and lactic acid content was determined using high-performance liquid chromatography (HPLC). Samples were centrifuged at 3000× *g*, 4 °C for 15 min and the supernatant filtered with a 0.45 µm polypropylene membrane (VWR, Darmstadt, Germany). After filtration, samples were analyzed by HPLC (ProStar, Variant, Walnut Creek, CA, USA), which was equipped with a refractive index detector. Twenty µL of samples were injected

into a Rezex ROA-organic acid H^+ (8%) column (Phenomenex, Aschaffenburg, Germany). The working temperature was set at 70 °C, and a 5 mM H_2SO_4 solvent with a flow rate of 0.6 mL/min was used. Lactic acid and glucose content was obtained using Software Galaxie™ Chromatography (Varian, Walnut Creek, CA, USA). Duplicate measurements were obtained for each analyte.

2.6. On-Line Measurement Using 2D-Fluorescence Spectroscopy

A BioView sensor (DELTA Lights and Optics, Venlighedsvej 4, 2970, Horsholm, Denmark) was used to collect 2D-fluorescence spectra. A fluorescence probe was attached to the sterilized bioreactor over a light guide, which connected with a 25 mm standard port. This standard port has a quartz glass window to interface with the bioreactor. Therefore, there was no contact between the fermenting medium and the actual probe. A BioView sensor measured several combinations of excitation (270–550) and emission (310–590). The observed fluorescence spectrum had 120 intensity values of wavelength combinations measured in intervals of 20 nm. Off-line measured results and the analogous fluorescence spectra data were utilized to develop calibration models of PLSR and ANN for the prediction of LPA6 and LCGG cell counts and concentrations of glucose and lactic acid. Software Unscrambler X version 10.3 (CAMO Software AS., Oslo, Norway) and MATLAB R2019a version 9.6 (The MathWorks Inc. 2019, Natick, MA, USA) were utilized to calibrate the models and to test and validate their predictive capabilities.

2.7. Examination of the Model Performance

The predicted versus measured values were plotted, and the prediction quality was estimated by calculating the root mean square error of prediction (RMSEP) and relative root mean square error of prediction (pRMSEP), which were calculated using Equations (1) and (2), respectively. The coefficient of determination (R^2) was calculated with Equation (3).

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=}^{N}(\mathrm{m_i} - \mathrm{p_i})^2}{\mathrm{N}}} \tag{1}$$

$$\mathrm{pRMSEP}\ [\%] = \frac{\mathrm{RMSEPx100}}{\mathrm{max}} \tag{2}$$

N, number of measurements; m_i, measured values; p_i, predicted values; max, maximum measured value; i, running index.

$$\mathrm{R}^2 = 1 - \frac{\mathrm{RSS}}{\mathrm{TSS}} \tag{3}$$

RSS, residual sum of squares; TSS, total sum of squares.

2.8. Statistical Analysis

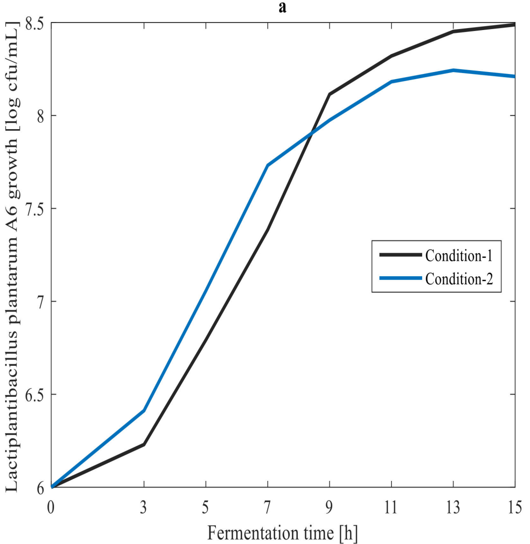
To build calibration and prediction models, Unscrambler X version 10.3 (CAMO Software AS., Oslo, Norway) and MATLAB R2019a version 9.6 (The MathWorks Inc. 2019, Natick, MA, USA) were utilized. Graphs were sketched with the same version of Matlab, which was used for model calibrations.

3. Results and Discussion

3.1. Off-Line Measurement of Cell Growth, Glucose and Lactic Acid

Off-line measured results of LPA6 and LCGG cell growth and concentrations of glucose and lactic acid are shown in Figures 1 and 2, respectively. The fermentation of the teff-based substrate (hereinafter 'substrate') inoculated with mixed strains of LPA6 and LCGG predominantly had glucose (consumed substrate) and lactic acid (produced metabolite) [6]. Under condition 1, LPA6 and LCGG growth did not decline over fermentation time. Under condition 2, however, growth of both microbes began declining after 13 h of fermentation (Figure 1). Under condition 1, LPA6 and LCGG growth increased from 6 log cfu/mL to

8.49 and 8.29 log cfu/mL, respectively. Conversely, under condition 2, growth of LPA6 and LCGG decreased between 13 to 15 h fermentation from 8.24 to 8.21 log cfu/mL and from 7.99 to 7.74 log cfu/mL, respectively. At this decreasing point of cell growth, the main substrate glucose was not consumed entirely. Thus, the reason for declining cell growth appears to be due to the development of an acidic environment.



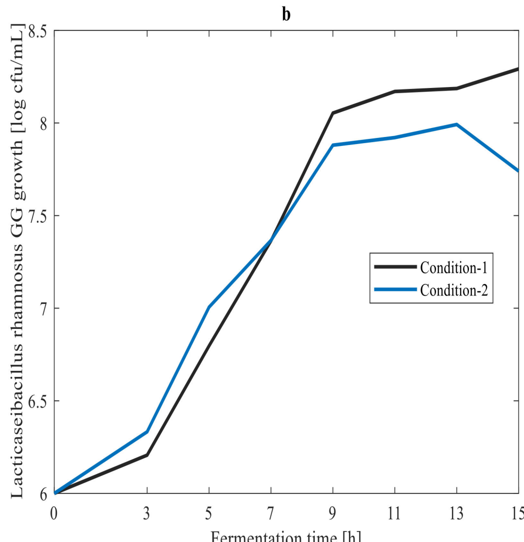


Figure 1. (**a**) *Lactiplantibacillus plantarum* A6 and (**b**) *Lacticaseibacillus rhamnosus GG* growth under two different fermentation conditions: Condition 1, 7 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; Condition 2, 4 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG.

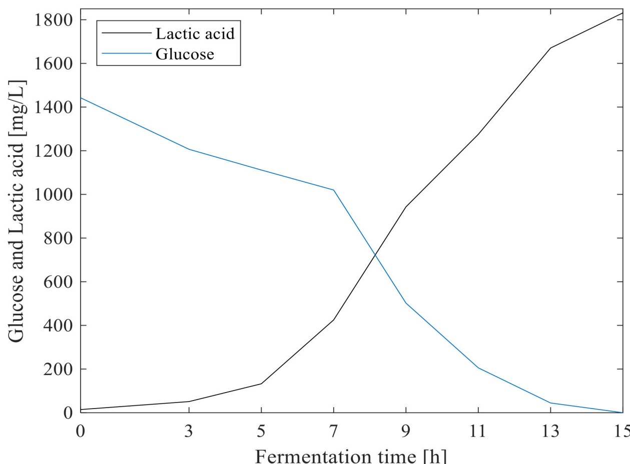


Figure 2. Glucose consumption and Lactic acid production during fermentation of 7 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG.

Under both fermentation conditions, LPA6 and LCGG cell growth was over 8 log cfu/mL. However, lower LCGG cell counts (7.74 log cfu/mL) were observed under condition 2. Both LPA6 and LCGG grew beyond the minimum level of the recommended viable probiotic of 6 log cfu/mL [18]. The minimum cell counts of probiotics must be achieved at the time of consumption to assure the probiotic effect of the product. The concentration of glucose and

lactic acid changed from 1442.5 to 0 mg/L and 14.5 to 1831.5 mg/L, respectively. Overall, consumption of glucose by LPA6 and LCGG was associated with cell growth and lactic acid production (Figures 1 and 2).

3.2. On-Line Measurement Using 2D-Fluorescence Spectroscopy

Under two different conditions after 0, 9, and 15 h fermentations, 2D-fluorescence spectra of substrate fermentation inoculated with mixed strains of LPA6 and LCGG are presented in Figures 3 and 4. As can be seen, all fluorescence spectra showed peaks in the same region. High fluorescence intensities were observed in the region of excitation (410–450 nm) and emission (510–570 nm), where riboflavin typically reaches its maximum fluorescence [19–21]. The other peak region was visible at excitation (350–390 nm) and emission (430–490 nm), which showed the presence of NADH [22–24]. Moreover, all fluorescence spectra revealed peaks in the region of excitation (270–290 nm) and emission (310–390 nm), which verified the presence of protein [19,23–25].

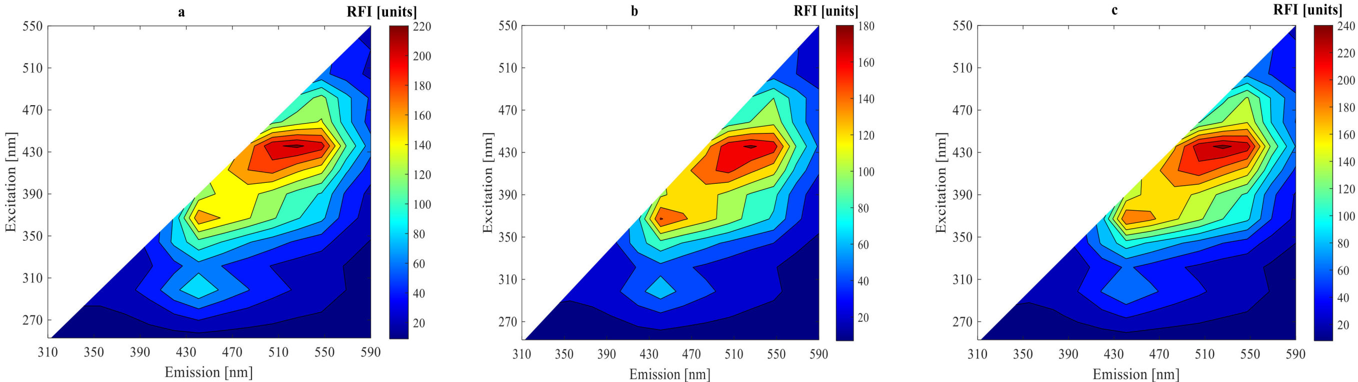


Figure 3. Original spectra after (**a**) 0 h, (**b**) 9 h, and (**c**) 15 h fermentation of 7 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG.

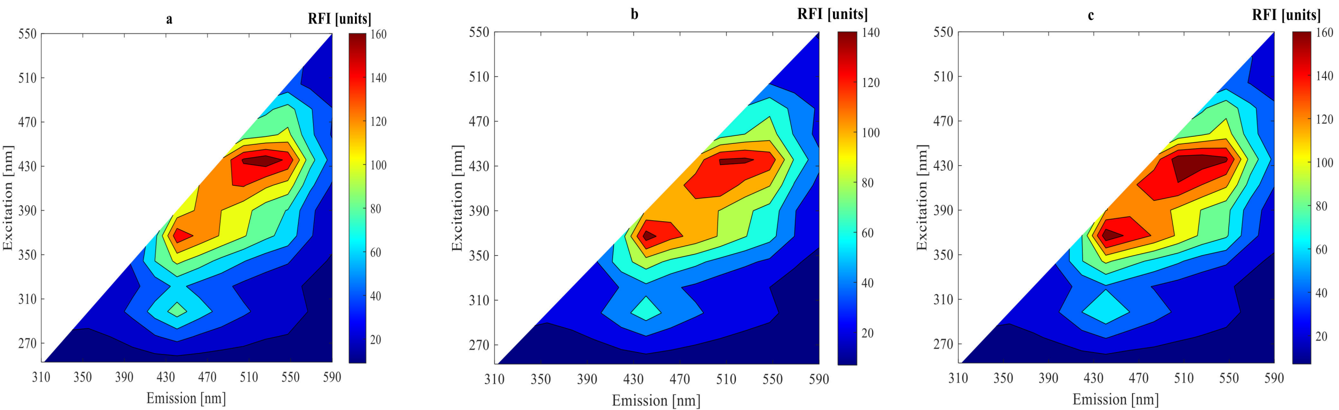


Figure 4. Original spectra after (**a**) 0 h, (**b**) 9 h, and (**c**) 15 h fermentation of 4 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG.

For a better understanding of the fluorescence spectra variations, difference spectra were calculated by subtracting the initial spectrum from the spectra of 9 and 15 h fermentations (Figures 5 and 6). The highest peak difference was observed in the protein fluorescence region at excitation (270–290 nm) and emission (310–390 nm) for all fluorescence spectra in 9 h fermentation. The other highest peak differences were observed in the fluorescence regions of riboflavin at excitation (410–450 nm) and emission (510–570 nm) and NADH for

all fluorescence spectra in 15 h fermentation. The difference fluorescence spectra under condition 1 had a higher intensity as compared to the difference fluorescence spectra under condition 2. The difference and original fluorescence spectra showed peaks in the same regions. However, different fluorescence intensities were observed. These variations in the fluorescence intensities are due to differences in substrate concentration used under the two different fermentation conditions. The fluorescence intensities decreased from their initial values during the 9 h fermentation in all fluorescence regions. However, the fluorescence intensities increased during the 15 h fermentation in the regions of riboflavin and NADH.

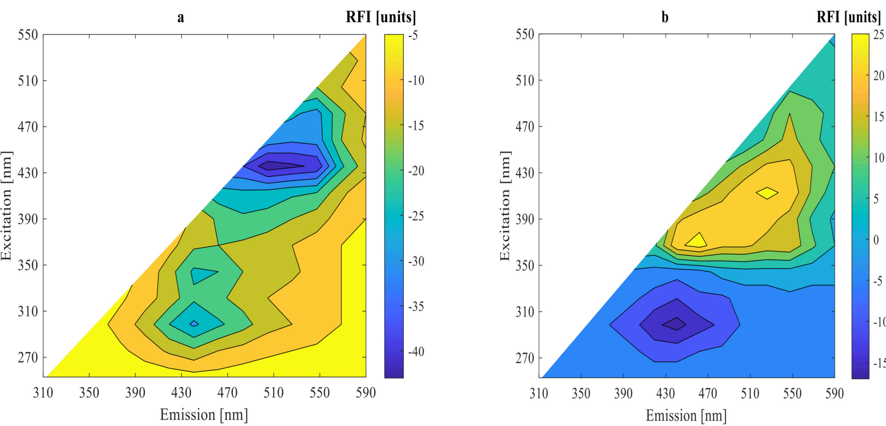


Figure 5. Subtracted spectra of the initial one from the original spectra after (**a**) 9 h and (**b**) 15 h fermentation of 7 g/100 mL substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillusplantarum* A6 and *Lacticaseibacillusrhamnosus* GG.

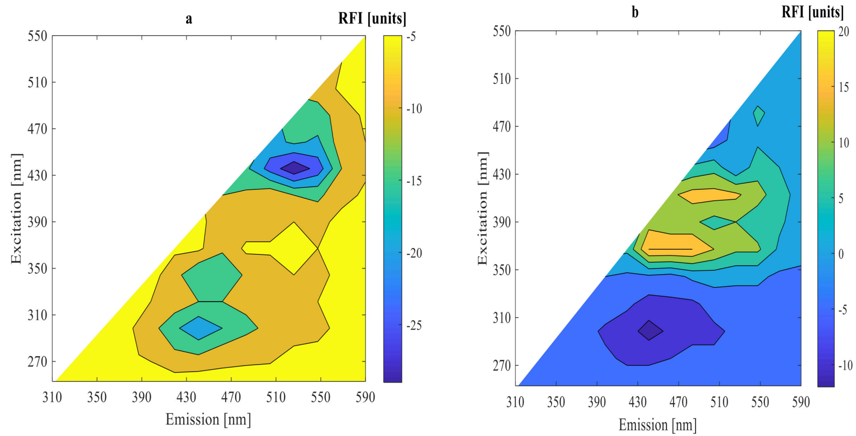


Figure 6. Subtracted spectra of the initial one from the original spectra after (**a**) 9 h and (**b**) 15 h fermentation of 4 g/100 mL substrate inoculated with mixed strains of *Lactiplantibacillusplantarum* A6 and *Lacticaseibacillusrhamnosus* GG.

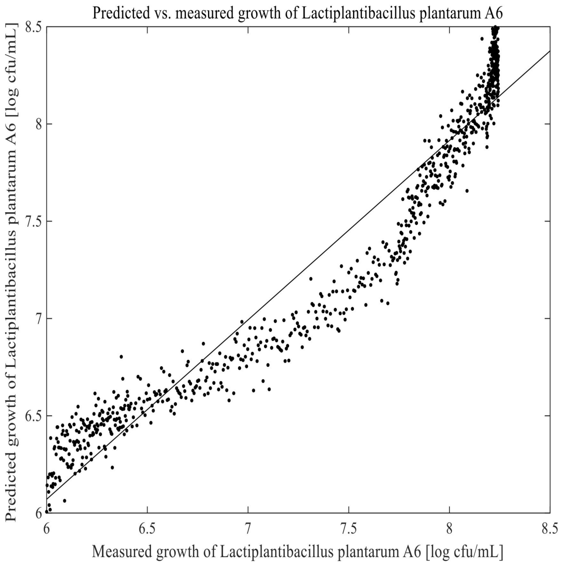
The fluorescence intensities of NADH and riboflavin decreased during the exponential growth phase of LPA6 and LCGG. Conversely, their fluorescence intensities increased in the stationary phase of LPA6 and LCGG. This shows that riboflavin consumption is inversely associated with cell growth, meaning that it is consumed during the logarithmic phase of LPA6 and LCGG, but accumulated during a stationary phase. The accumulation of NADH begins when it no longer oxidizes to form the non-fluorophore molecule (NAD^+), which results in an increase in its fluorescence intensity [3]. All fluorescence intensities decreased throughout the fermentation time in the protein region.

LCGG does not produce riboflavin [26]. Therefore, the production of riboflavin shown in the results can be attributed to LPA6 or the interaction effect of LPA6 and LCGG. Thakur

and Tomar [27] reported riboflavin production ability of *Lactiplantibacillus plantarum* in MRS media. Riboflavin (vitamin B2) is a water-soluble vitamin and is essential for human health. It must be supplemented externally from food sources since it cannot be produced in the human body [28]. Hence, it is better to use bacteria, which can produce riboflavin rather than consume it throughout fermentation [26]. Thus, the capacity of mixed strains of LPA6 and LCGG to produce riboflavin can, together with their probiotic properties, be exploited for manufacturing multifunctional foods.

3.3. Prediction of Cell Growth in the Fermentation Process

The PLSR and ANN models were built using 1670 calibration samples. Off-line measured data and the corresponding 2D-fluorescence spectra were used to develop a model for predicting LPA6 and LCGG cell growth. Two different fermentation process conditions were examined for collecting on-line data as well as the corresponding off-line results. The prediction models for LPA6 and LCGG cell counts under condition 1 and condition 2 are presented in Figures 7–10. The PLSR and ANN models were built separately for the prediction of LPA6 and LCGG cell counts.



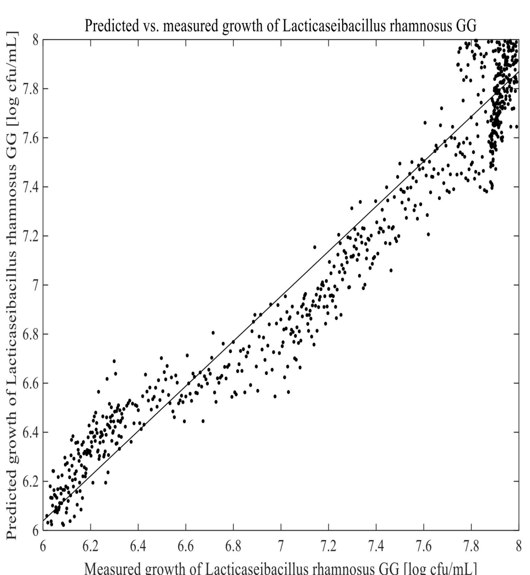
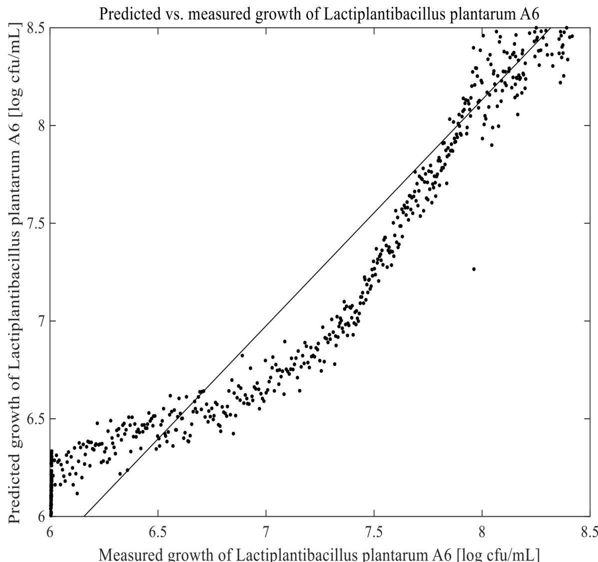


Figure 7. Predicted vs. measured cell growth in the fermentation of 4 g/100 mL of substrate inoculated with mixed strains of 6 log cfu/mL *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; predicted with partial least-squares regression using four principal components.



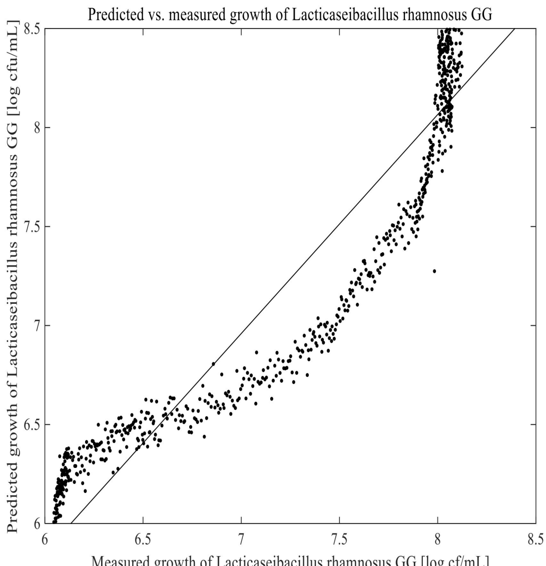
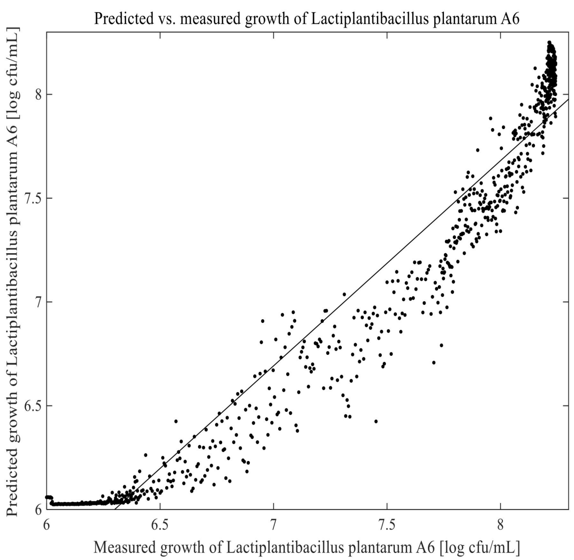


Figure 8. Predicted vs. measured cell growth in the fermentation of 7 g/100 mL of substrate inoculated with mixed strains of 6 log cfu/mL *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; predicted with partial least-squares regression using six principal components.

Analysis of Cell Growth Using Artificial Neural Networks



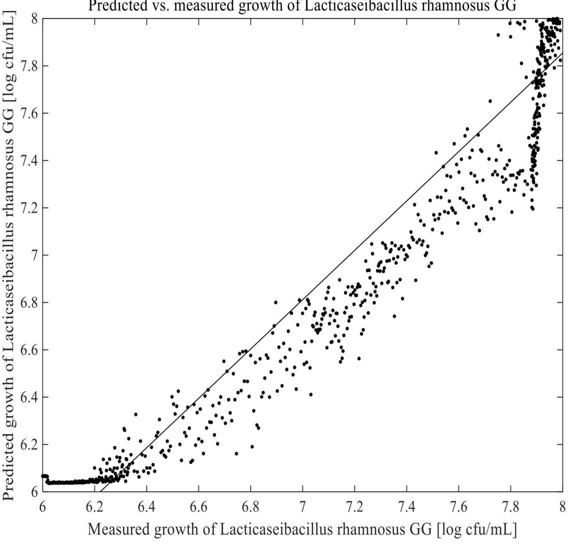
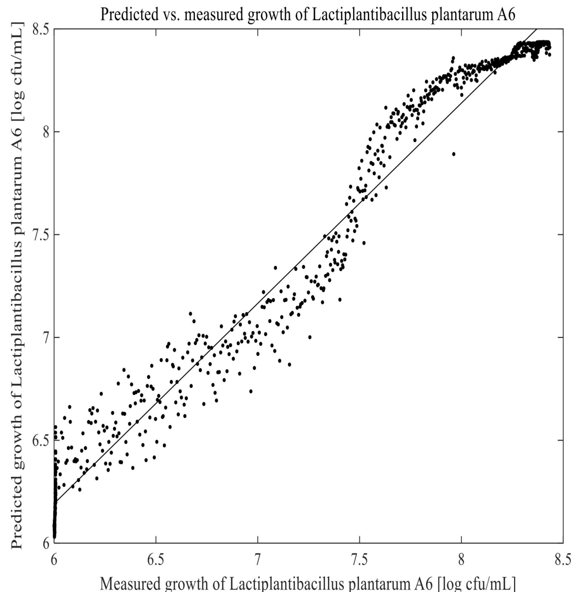


Figure 9. Predicted vs. measured cell growth in the fermentation of 4 g/100 mL of substrate inoculated with mixed strains of 6 log cfu/mL *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; predicted with artificial neural networks using five hidden neurons.



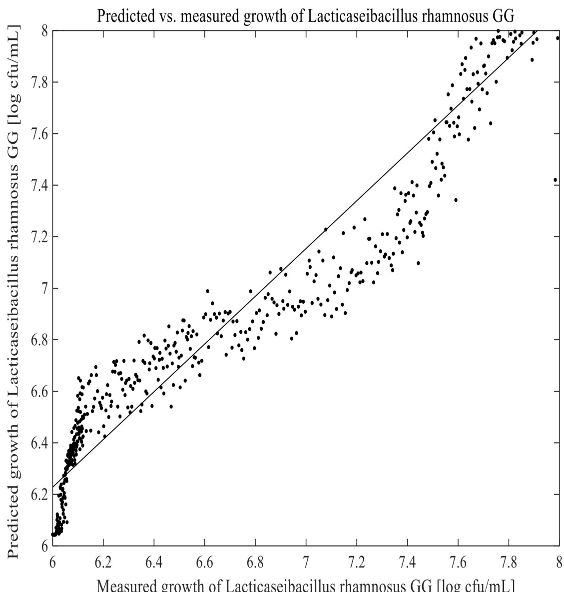


Figure 10. Predicted vs. measured cell growth in the fermentation of 7 g/100 mL of substrate inoculated with mixed strains of 6 log cfu/mL *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; predicted with artificial neural networks using five hidden neurons.

Analysis of Cell Growth Using Partial Least-Squares Regression

Models and predictions for LPA6 and LCGG cell growth under two different fermentation conditions were performed using a maximum of six principal components. The RMSEP, pRMSEP, and R^2 calculated for PLSR and ANN models are shown in Table 1. Better predictions were obtained with ANN for LCGG cell growth under condition 1. However, the predictions with PLSR were found to be better compared to ANN under condition 2. Predictions of principal component regression showed the highest rate of errors (data not shown) as compared to PLSR and ANN.

Table 1. The RMSEP, pRMSEP, and R^2 values for the prediction of LPA6 and LCGG cell growth using PLSR and ANN under two different fermentation conditions.

Predicted with PLSR	**Condition 1**			**Condition 2**		
	RMSEP (log cfu/mL)	**pRMSEP (%)**	**R^2**	**RMSEP (log cfu/mL)**	**pRMSEP (%)**	**R^2**
LPA6	0.31	3.7	0.88	0.22	2.7	0.92
LCGG	0.32	3.9	0.85	0.20	2.4	0.92
Predicted with ANN	RMSEP (log cfu/mL)	pRMSEP (%)	R^2	RMSEP (log cfu/mL)	pRMSEP (%)	R^2
LPA6	0.21	2.5	0.95	0.37	4.5	0.78
LCGG	0.20	2.5	0.94	0.29	3.6	0.83

LPA6, *Lactiplantibacillus plantarum* A6; LCGG, *Lacticaseibacillus rhamnosus* GG; RMSEP, root mean square error of prediction; pRMSEP, relative root mean square error of prediction; R^2, coefficient of determination; PLSR, partial least-squares regression; ANN, artificial neural network; Condition 1, 7 g/100 mL substrate inoculated with 6 log cfu/mL; Condition 2, 4 g/100 mL substrate inoculated with 6 log cfu/mL.

For the prediction of LPA6 and LCGG cell growth, the observed R^2 values varied between 0.78–0.95, with the lowest R^2 value being 0.78 for the prediction of LPA6 under condition 2 using ANN. In the prediction of the PLSR model, the pRMSEP was 3.7 and 3.9%, respectively, to predict LPA6 and LCGG cell growth under condition 1. Under the same condition, the ANN model had a pRMSEP of 2.5% to predict LPA6 cell growth and 2.5% to predict LCGG cell growth. Furthermore, the prediction of the PLSR model had

a pRMSEP of 2.7 and 2.4% to predict LPA6 and LCGG cell growth, respectively, under condition 2. Under similar conditions, the ANN model had a pRMSEP of 4.5% to predict LPA6 cell growth and 3.6% to predict LCGG cell growth (Table 1). The lower pRMSEP and higher R^2 values showed that the PLSR and ANN models were important for predicting LPA6 and LCGG cell counts in the fermentation of a teff-based substrate.

3.4. Prediction of Lactic Acid and Glucose in the Fermentation Process

The PLSR and ANN models were built using 1670 calibration samples. Lactic acid and glucose concentrations measured in an experiment were used with their corresponding on-line data to develop the PLSR and ANN models. To compare the PLSR and ANN models as well as to verify their performance, RMSEP, pRMSEP, and R^2 values were calculated between the predicted and measured values (Table 2). The developed PLSR and ANN models were then used to predict lactic acid and glucose in another fermentation process run using 2D-fluorescence spectra. In principle, a direct measurement of glucose concentration by using fluorescence information is not possible, as it is not a fluorescence molecule. However, its consumption is directly related to an accumulation of fluorescence molecules such as tryptophan. Thus, it is possible to indirectly measure glucose by using 2D-fluorescence spectroscopy [29].

Table 2. RMSEP, pRMSEP, and R^2 values for the prediction of glucose and lactic acid with PLSR and ANN during the fermentation process of 7 g/100 mL of substrate inoculated with 6 log cfu/mL mixed strains of LPA6 and LCGG.

Analyte	PLSR			ANN		
	RMSEP (log cfu/mL)	pRMSEP (%)	R^2	RMSEP (log cfu/mL)	pRMSEP (%)	R^2
Glucose	191.72	13.5	0.86	199.92	14.1	0.85
Lactic acid	98.41	7.6	0.96	100.09	7.7	0.96

LPA6, *Lactiplantibacillus plantarum* A6; LCGG, *Lacticaseibacillus rhamnosus* GG; RMSEP, root mean square error of prediction; pRMSEP, relative root mean square error of prediction, R^2, coefficient of determination; PLSR, partial least-squares regression; ANN, artificial neural network.

Analysis of Lactic Acid and Glucose Using Partial Least-Squares Regression

Prediction models for concentrations of glucose and lactic acid were developed using a maximum of seven principal components. The calculated results of RMSEP, pRMSEP, and R^2 for the prediction using PLSR and ANN are shown in Table 2. Glucose and lactic acid prediction models are presented in Figures 11 and 12. There were no significant differences observed between the prediction abilities of PLSR and ANN for glucose and lactic acid. Prediction of glucose and lactic acid using principal component regression showed the highest rate of errors (data not shown) as compared to PLSR and ANN. Overall, a stronger correlation was observed between 2D-fluorescence data and experimentally determined values of lactic acid than between 2D-fluorescence data and experimentally determined values of glucose.

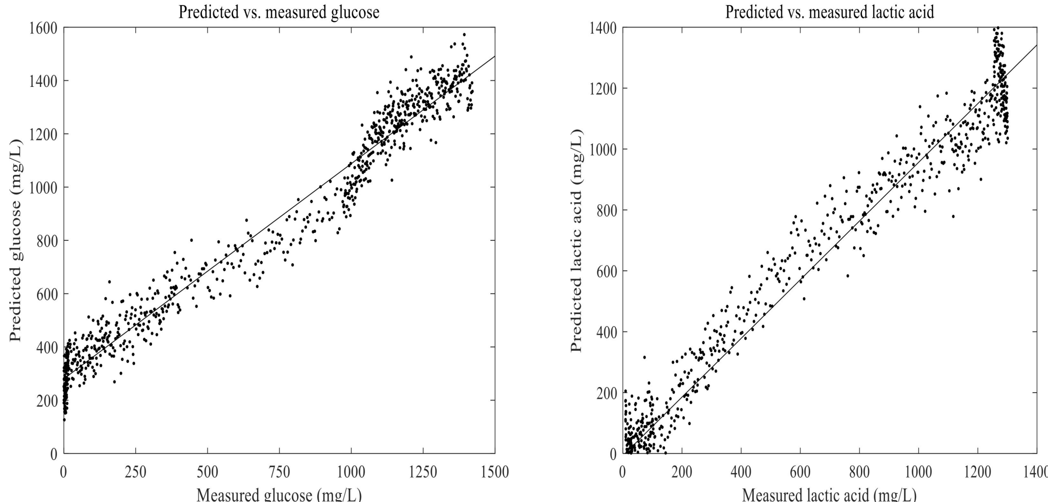


Figure 11. Predicted vs. measured glucose and lactic acid in the fermentation of 7 g/100 mL of substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; predicted with partial least-squares regression using seven principal components.

Analysis of Glucose and Lactic Acid Using Artificial Neural Networks

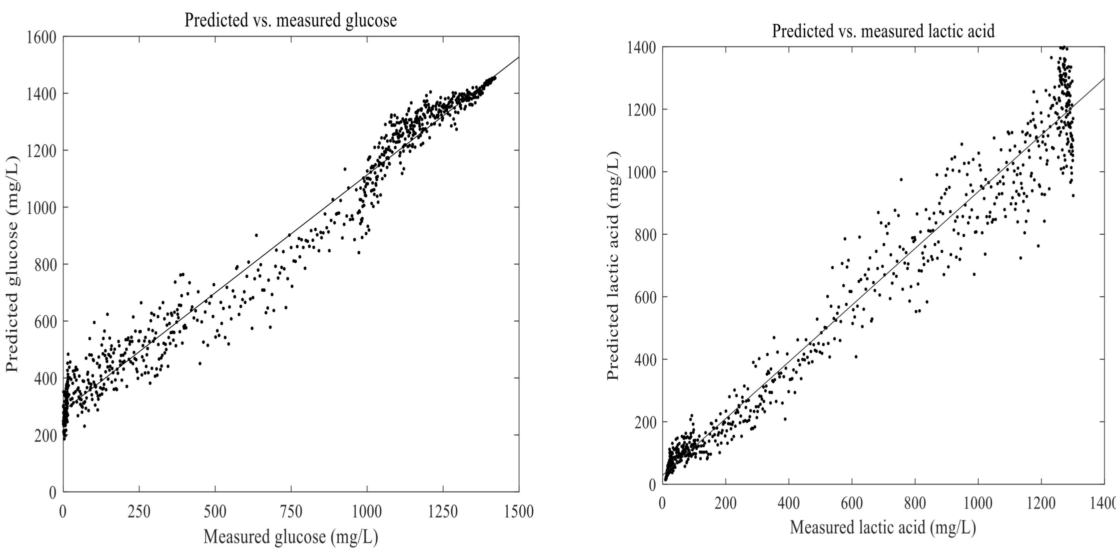


Figure 12. Predicted vs. measured glucose and lactic acid in the fermentation of 7 g/100 mL of substrate inoculated with 6 log cfu/mL mixed strains of *Lactiplantibacillus plantarum* A6 and *Lacticaseibacillus rhamnosus* GG; predicted with artificial neural networks using one hidden neuron.

Once the models were developed, it was possible to obtain results pertaining to cell counts, lactic acid, and glucose in minutes by using a two-dimensional fluorescence spectroscopy. However, it took more than three days to obtain the same results by using a plate count agar and high-performance liquid chromatography. For the analysis of lactic acid and glucose, we used expensive instruments such as a deep freezer, centrifugation, pump, filter, and fully equipped high-performance liquid chromatography. This form of analysis is time-consuming and labor-intensive. To determine LPA6 and LCGG cell counts using a plate count agar, we used several chemicals and spent a long time performing tedious work. However, without using the instruments required for the conventional analysis, similar results were obtained in minutes by using a two-dimensional fluorescence spectroscopy integrated with PLSR and ANN.

4. Conclusions

An essential vitamin (riboflavin) was accumulated in the fermentation of a teff-based substrate inoculated with mixed strains of LPA6 and LCGG. The riboflavin production ability of LPA6 and LCGG, together with their probiotic properties, could be exploited for manufacturing multifunctional food products. A 2D-fluorescence spectroscope is an ideal instrument for the rapid supervision of the fermentation process without interfering with the fermentation medium. It provides broad information about metabolic changes occurring during the fermentation process. This study has shown that 2D-fluorescence spectroscopy coupled with PLSR and ANN models can be applied to accurately monitor LPA6 and LCGG cell counts and lactic acid concentration in the fermentation of a teff-based substrate. It might even be possible to use a simple, inexpensive fluorescence sensor using light-emitting diodes and photodiodes.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

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Article

The Sensory Quality and the Physical Properties of Functional Green Tea-Infused Yoghurt with Inulin

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Abstract: The purpose of this study was to investigate the influence of the addition of inulin (3%, 6% and 9%) to green tea-infused set type yoghurt on its sensory quality and physical properties. Yogurts were made by combining green tea with milk and inulin and inoculated with freeze-dried starter cultures YO-122. Incubation was conducted at 43 °C for approximately 4.5 h until a pH value of 4.5–4.6 was achieved. For the prepared yoghurts, a panel of experts (n = 10) was selected, characterized 35 attributes and conducted a sensory quality assessment of these yoghurts using the Quantitative Descriptive Profile method. Additionally, instrumental analyses such as yield stress, adhesiveness, firmness, physical stability and color parameters were also carried out. The use of green tea infusion increased the perception of green tea flavor, bitterness, astringency, dark color of the yoghurt and the existing whey, which worsened the overall sensory quality of the yoghurt. The addition of inulin (9%) to the green tea yoghurt, increased the perception of sweet, peach flavor and aroma and improved the firmness of the yoghurt while reducing the perception of sour taste, which improved the sensory quality of the yoghurt. Both inulin and green tea affected the physical properties of the yoghurts, causing an increase in the yield stress (43%, and 20%, respectively) and deteriorated the stability of the yoghurts. Green tea affected the color of the yoghurts, causing the lightness to decrease. The L^* parameter decreased from 89.80 for the control sample to 84.42 for the green tea infused yoghurt. The use of infused green tea in yoghurt production makes it necessary to use ingredients that will neutralize its adverse effects on sensory quality and physical parameters of yoghurt, and such an additive can be prebiotic fiber–inulin at a concentration of 9%.

Keywords: yoghurt; green tea; inulin; functional product; sensory quality; physical properties

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1. Introduction

One of the most innovative food sectors in Europe is the dairy industry and it is trying to respond to consumer demands by improving its products, introducing new product formulations or technologies [1,2]. As a result, the products on offer are not only nutritious but also contain active substances which have an impact on health aspects and can be classified as functional foods. Various plant raw materials are introduced to improve the nutritional value of dairy products as well as to increase the content of substances with health-promoting properties [3]. Dairy products, including fermented drinks such as yoghurt, are a good example of this. We can find studies on enriching yoghurt with the following ingredients, among others: aloe vera gel [4], grapes [5], flaxseed [6], coconut-cake [7], pomegranate juice powder [8], dried pomegranate seeds [9], freeze-dried apple pomace powder [10], spirulina [11], saffron [12] or lotus, persimmon, rosemary, nettle, caraway, hyssop [13] and lemon balm [2,3]. The use of these ingredients can improve the health-promoting properties as well as their technological and sensory quality, although the combination of these several functions is not always possible.

Among the plant components, tea (*Camellia sinensis*) with different degrees of fermentation as green tea, white tea and black tea is increasingly appearing in studies on the production of dairy drinks with plant extracts that can be used in yoghurt to improve its antioxidant properties and maintain it during storage [13–18], as well as the influence on the overall quality; however, the studies that have been carried out so far are mainly based on hedonic tests [5,16,18–20].

The health benefits of yoghurt can be also obtained by using in its production plant-based ingredients with prebiotic properties, which could symbiotize with the bacteria presented in yoghurt. Inulin, which is found in high concentrations in chicory root (*Chicorium intybus*) [21] is one such example. When inulin is used in a product, it is also possible to use a nutrition claim "source or high fiber content" or a health claim if "native chicory inulin" is used in the product, stating "Chicory inulin contributes to normal bowel function by increasing stool frequency" [21]. While many studies have been conducted on the effect of inulin addition on yoghurt quality [22–28] to the best of our knowledge no specific studies have been conducted on the effect of both infused green tea and inulin addition on sensory quality evaluated by a panel of experts and instrumentally. The analysis of this data is of great importance in the development of new products, which translates into their purchase choice and acceptance by consumers. By using tea addition and infusion technology we can achieve a natural functional food, while the use of inulin as a prebiotic can increase both nutritional value by increasing the fiber content and the health promoting value. However, the question arises whether the product will also be attractive from a sensory point of view. Therefore, the main objective of our project process was to investigate the influence of the addition of inulin to green tea-infused yoghurt on its sensory quality assessed by a panel of experts and physical properties tested instrumentally.

2. Materials and Methods

2.1. Materials

Yoghurts were made with 3.2% fat content cow's milk, pasteurized and microfiltered (Piątnica, Poland). Milk was inoculated with freeze-dried starter cultures YO-122 (Serowar, Poland), containing Streptococcus salivarius subsp. thermophilus and Lactobacillus delbrueckii subsp. bulgaricus. In addition, in production of yoghurt with infused tea the leaf green tea (Camellia sinensis, BioFix, Tuszyn, Poland) was used. As a prebiotic ingredient Frutafit® CLR inulin (chicory root, inulin ≥85% dm, DP 2-10, sweetness 30%) (Sensus, Roosendaal, The Netherlands) was used to enrich both natural and green tea infused yoghurt.

Yoghurt Processing

The production process of natural and infused green tea yoghurt was carried out according to the methodology described by Świąder et al. [29]. Milk for yoghurt production with tea was first heated for 30 min to 85 °C. Then, green tea leaves (2 g tea/100 mL milk) were infused over the milk and steeped covered for 10 min. The resulting infusion was then filtered manually through gauze strainers and cooled. The infusion prepared in this way was inoculated with starter cultures (0.1%) and gently stirred and then bottled into 100 mL sterile, plastic containers with lids. The next step was incubation of yoghurts in an incubator (INE 500, Memmert, Schwabach, Germany) at 43 °C for about 4.5 h until a pH value of 4.5–4.6 was reached (Voltcraft PH-100ATC, Conrad Electronic Sp. z.o.o., Wrocław, Poland). The yoghurt samples were then cooled and stored at 4 °C until the structure was built. After this time, the samples were ready for sensory and instrumental evaluation. In contrast, for natural yoghurt, the difference was that no tea was added. In order to enrich the yoghurt with inulin, inulin was added to the milk at 3%, 6% and 9% levels before the heating process. An analytical balance (PS 1000/C/2, Radwag, Radom, Poland) was taken to weigh all the raw materials used to make the yoghurt. The yoghurt samples were coded as follows: control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3).

2.2. Methods

2.2.1. Sensory Evaluation

The Method

The sensory quality assessment of the produced yoghurts was carried out in accordance with the procedure described in the ISO 13299:2016 standard [30] using the Quantitative Descriptive Profile (QDP) method. The expert team selected for evaluation and defined 35 discriminators characterizing the appearance, smell, texture and taste of the evaluated samples (Table S1). Seven factors described the appearance of the samples: presence of whey, shine of surface, color intensity, adhesiveness, visual smoothness, filling the teaspoon, uniformity of consistency. Nine distinctions described the odor of the samples: sour, sweet, yoghurt, milk, fat, green tea, nectar, peach and citrus. Another seven described texture and consistency: melting, thickness in the mouth, yield stress, firmness, fat film, smoothness in the mouth, creaminess. The largest number of attributes (10) was chosen to describe the taste and flavor of the yoghurts. The taste attributes were sweet, bitter, sour, astringent, and flavor attributes were yoghurt, milky, quark, peach, green tea, nectar. In addition, body and overall sensory quality were assessed. Yoghurt aroma was first assessed by slightly tilting the lid of the yoghurt package. Then, after opening the package, the general appearance of the yoghurt was evaluated among others by dipping a spoon and observing how the yoghurt looks on the spoon. After that, the consistency of the yoghurt samples was evaluated in the mouth as well as their taste and flavor, body and overall sensory quality. The intensity of sensory attributes was evaluated by an expert panel using a 10-point unstructured linear scale (c.u.—contractual units) with extremes ranging from 0 (low perception) to 10 (high perception).

Expert Panel

The Quantitative Descriptive Profile assessment was carried out by a panel of 10 trained experts who met the requirements of ISO 8586:2012 [31]. They were research and teaching staff from the Institute of Human Nutrition Sciences, women aged between 35 and 53, with experience in profile assessment and yoghurt evaluation.

Study Conditions

The study was conducted by a panel of experts in an accredited sensory laboratory (accreditation number AB 564) meeting the requirements of ISO 8589:2007/AMD 1:2014 [32]. Evaluation of yoghurts took place in individual test booths equipped with the ANALSENS computer system (Cogitos, Sopot, Poland), which enables test planning, product evaluation and data collection. Lighting, temperature and humidity were controlled during evaluation. The evaluations were carried out in two sessions with a break of 3 h during the day.

Preparation and Presentation of Samples

Samples for evaluations were prepared in cylindrical, transparent, plastic containers coded with three-digit codes generated by the computer system (height, 50 mm; ø, 50 mm; volume, 100 mL). The yoghurt samples prepared in this way were randomly placed on a tray and given at 7 °C to experts who evaluated them directly from the containers. To neutralize the mouthfeel between samples, still mineral water was used.

2.2.2. Instrumental Analysis

Yield Stress

The yoghurt's yield stress (Pa) was analyzed by the rheometer (DV3T, Brookfield, Middleboro, MA, USA), using a vane four knife spindles V74 with a torque range HA that was constantly share with the rate 0.1 s^{-1}. The presented values are the averages of six replicates. The yield stress values were analyzed using the dedicated software (PG Flash, Brookfield, Middleboro, MA, USA).

Textural Properties

The firmness (N) and adhesiveness (Ns) of yoghurts were measured using TA.XT Plus (Stable Microsystems, Surrey, UK) with a 5 kg load cell. The device was equipped with a cylindrical container with a 0.5-cm diameter (P/0.5R) probe. The probe was penetrating the yoghurt sample for 8 cm distance, with the speed of 1.0 mm/s, and the trigger force used was 0.01 N.

Physical Stability—CSA Method

The physical stability of yoghurts has been presented as a space and time related transmission profiles using LUMiSizer 6120-75 (L.U.M. GmbH, Berlin, Germany). The applied measuring setting were: wavelength 870 nm, volume 1.8 mL of dispersion; light factor: 1; 1500 rpm; experiment time, 15 h 10 min; interval time 210 s. The instability analysis that allowed to calculate the instability index was performed using the SepView 6.0; LUM (Berlin, Germany) software. The trait was quantified by dividing the sample clarification of at a given separation time by the maximum sample clarification. It is set that the instability index can take values in the range from 0 to 1, in this calculation 0 indicates a stable system whereas 1 an unstable system [33].

Color Parameters

The L^*, a^*, and b^* color parameters (CIEL*a*b*) of yoghurts were analyzed with a Minolta CR-200 colorimeter (Minolta, Osaka, Japan; source of light D65, a measuring hole of 8 mm) at the surface of yoghurt. To determine color differences, total color differences were determined between yoghurts with tea added and the control sample without tea and inulin, and between yoghurts with green tea and inulin added and the control sample with inulin added. The total color difference (ΔE) was calculated [34]:

$$\Delta E = \sqrt{\left(L_C^* - L_G^*\right)^2 + \left(a_C^* - a_G^*\right)^2 + \left(b_C^* - b_G^*\right)^2}$$

where $L_c^*,\ a_c^*, b_c^*$ and $L_G^*,\ a_G^*, b_G^*$ refers to the color parameters of compared yoghurts C is a control sample and G is a yoghurt with green tea addition.

Depending on the ΔE calculated values the color difference between the yoghurts can be estimated as not noticeable for the observer ($0 < \Delta E < 1$), noticeable but only by experienced observer ($1 < \Delta E < 2$), noticeable by unexperienced observer ($2 < \Delta E < 3.5$), clear difference in color is noticed ($3.5 < \Delta E < 5$) and observer notices two different colors ($5 < \Delta E$) [34].

2.2.3. Statistical Analysis

The obtained results of sensory and instrumental analysis presented on the tables and figures are the mean values with the standard deviation (±SD), data were statistically analyzed using Statistica 13.3 (TIBICO Software Inc., Palo Alto, CA, USA). To determine the significance differences in the intensiveness of the different sensory characteristics as well as the differences between the average values of yield stress, firmness, adhesiveness, instability index, and color parameters of yoghurts a one-way ANOVA analysis of variance were used. Significant differences in the intensity of sensory attributes were verified by Fisher's post hoc NIR test at the significance level of $p \leq 0.05$, while significant differences between yoghurts in instrumental assessment by Tukey's test at the significance level of $\alpha = 0.05$. In addition, using the built-in statistical package of XLSTATS software, Principal Components Analysis (PCA) was performed to investigate similarities and differences in the sensory quality profile of the samples.

3. Results

3.1. Sensory Evaluation

3.1.1. Quantitative Descriptive Profile Analysis

The use of infused green tea as well as inulin in yoghurt production significantly influenced the sensory quality of yoghurt. Details of the research results obtained are presented in Table S2 (Supplementary Material).

Odor

The sensory profile of the odor of six types of yoghurts is shown in Figure 1.

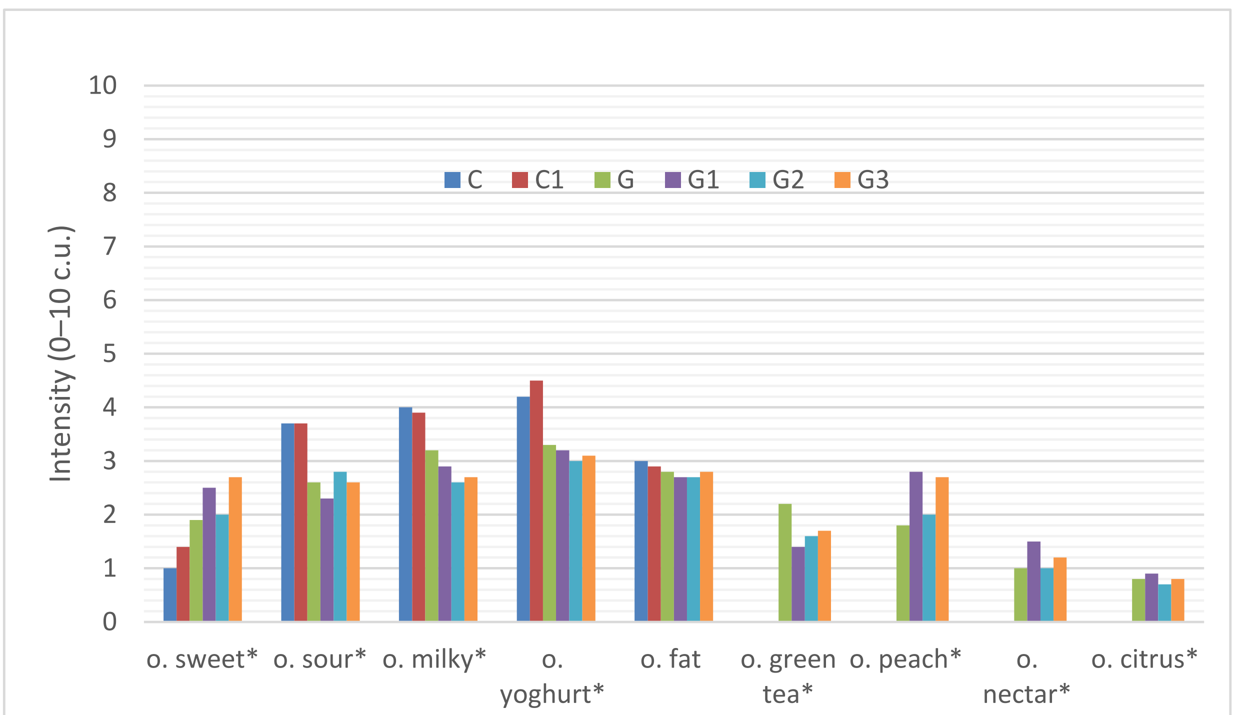

Figure 1. Sensory profile of the odor of yoghurts C, C1, G, G1, G2. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3) (o- odor; * significantly differed at $p \leq 0.05$).

We can note that the addition of inulin and the use of infused green tea in the yoghurt production process significantly influenced the aroma profile of the yoghurts. The control (C) yoghurt was characterized by an intense yoghurt (4.2 c.u.), milky (4.0 c.u.), sour (3.7 c.u.), fatty (3.0 c.u.) and slightly sweet smell (1.0 c.u). The control yoghurt with inulin had a similar sensory quality of yoghurt (4.5 c.u.), milky (3.9 c.u.), sour (3.7 c.u.), fatty (2.9 c.u.) smell and was slightly sweeter in smell (1.4 c.u.) than the control sample, but in terms of yoghurt, milk, sour and fatty and sweetness smell the control yoghurt and the control sample with inulin did not differ statistically significantly from each other.

The use of infused green tea in the yoghurt changed the smell of the yoghurt. It was characterized by a light green tea infusion (2.2 c.u.) with a delicate peach (1.8 c.u.), nectar (1.0 c.u.) and citrus (0.8 c.u.) smell, which differed significantly from the control yoghurt (C) and the control with inulin (C1). Yoghurt with green tea was also significantly more intensely sweet (1.9 c.u.) in aroma than the control sample without tea and significantly less sour (2.6 c.u.), milky (3.2 c.u.) and yoghurt-like (3.3 c.u.) in odor.

On the other hand, the addition of inulin at different levels (G1, G2 and G3) to green tea-infused yoghurt did not significantly change the odor of green tea infused yoghurt (G). With the addition of inulin, the sweet and peach smell increased (G3-2.7 c.u.

and 2.7 c.u., respectively) and the green tea smell decreased (1.7 c.u.), but there were no significant differences.

Appearance Perceived Visually

The appearance of the six yoghurts was also changed by the use of infused green tea and the addition of inulin (Figure 2).

Figure 2. Sensory profile of the appearance of yoghurts C, C1, G, G1, G2. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3) (* significantly differed at $p \leq 0.05$).

The control sample (C) was white (0.8 c.u.) with a shiny surface (7.3 c.u.) and whey presence (3.1 c.u.). It was characterized by a visually perceptible smoothness (7.5 c.u.), adhesion to the spoon (6.5 c.u.) and filling of the spoon (7.3 c.u.) after scooping the yoghurt with a spoon as well as uniformity of consistency (7.5 c.u.). The addition of inulin to natural yoghurt had a slight effect on reducing whey flow (2.6 c.u.) and increasing adhesiveness (7.0 c.u.), but these differences were not statistically significant. Both natural (C) and natural yoghurt with inulin (C1) did not differ statistically significantly in appearance.

There was a statistically significant difference in the color and flow of whey in the yoghurt with green tea added. Yoghurt with green tea had a significantly higher whey flow compared to natural yoghurts (C and C1), as well as a significantly darker creamy-grey color (4.3 c.u.). The other appearance parameters of yoghurt did not change significantly. However, the addition of inulin to green tea yoghurt significantly increased the whey flow in this yoghurt, to 7.8 c.u. in green tea yoghurt with inulin added at 9% (G3), and the darkening of the yoghurt sample (G2—5.8 c.u.; G3—5.6 c.u.).

Consistency Perceived in the Mouth

The sensory profile of six types of yoghurts consistency perceived in the mouth is shown in Figure 3.

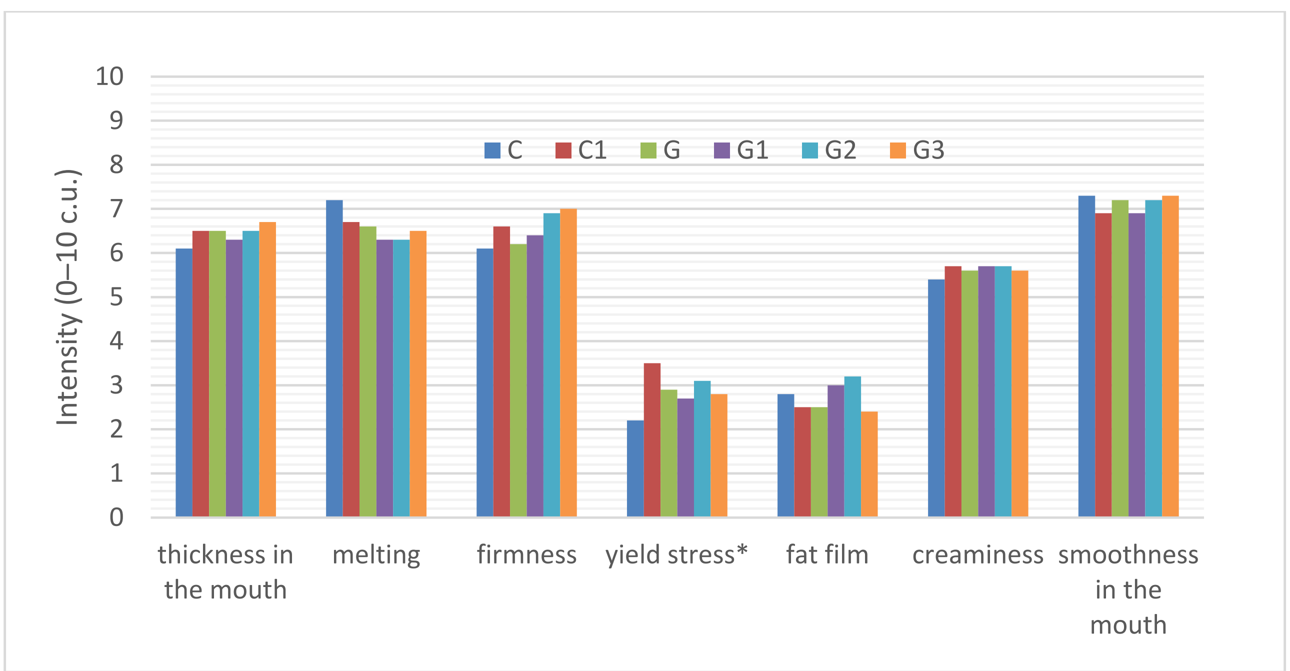

Figure 3. Sensory profile of the consistency of yoghurts C, C1, G, G1, G2. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3) (* significantly differed at $p \leq 0.05$).

In the case of all the yoghurts analyzed, we can see that they were characterized by a similar consistency assessed by spreading the yoghurt samples in the mouth. All the yoghurts were thick, compact, smooth, creamy, well-melting in the mouth, slightly viscous, with a perceptible fat film. Addition of inulin had a statistically significant effect on yoghurt viscosity, causing an increase in viscosity of the control yoghurt with inulin (C1—3.5 c.u.) compared to the control yoghurt (C—2.2 c.u.). The addition of infused green tea to the yoghurt (G) slightly increased the viscosity of the yoghurt (2.9 c.u.) but there was no statistically significant difference. The addition of inulin at different levels to the yoghurt with infused green tea did not significantly change the yoghurt viscosity.

Flavor/Overall Quality

The taste and flavor of the six yoghurts was changed by the use of infused green tea and the addition of inulin (Figure 4).

The control (C) yoghurt was characterized by an intense yoghurt (5.1 c.u.), milky flavor (3.9 c.u.), sour taste (4.4 c.u.), as well as slightly sweet taste (1.9 c.u) and quark flavor (2.2 c.u.), with a lightly perceptible astringent (0.8 c.u) and bitter taste (0.4 c.u). The addition of inulin to natural yoghurt significantly influenced sweet and sour taste perception. The sweet taste (4.0 c.u.) was more pronounced in the yoghurt with inulin (C1) and the sour taste was less pronounced (3.2 c.u.) than in the control natural yoghurt (C). The addition of inulin had no statistically significant effect on the other taste/flavor characteristics of the natural yoghurt.

The use of infused green tea also had a significant effect on the flavor profile of the yoghurt. The yoghurt with green tea became significantly more bitter (1.9 c.u.) and astringent (2.4 c.u.) in taste. In this yoghurt, the taste was typical for green tea (2.7 c.u.) and slight peach (0.9 c.u.) and nectar (0.5 c.u.) flavors were also significantly perceptible compared to natural yoghurt (C). Additionally, green tea yoghurt was significantly less sweet (0.8 c.u.), milky (2.3 c.u.), yoghurt-like (2.9 c.u) and quark (2.1 c.u) in taste. The addition of inulin at different levels to the green tea infused yoghurt significantly increased the sweet taste (G3—2.5 c.u.) and peach flavor (G3—1.9 c.u.) in the yoghurt, especially with

the highest level of inulin and decreased the sour taste (G3—3.4 c.u.). The remaining tastes were perceived at similar levels regardless of the amount of inulin added to the yoghurt infused with green tea.

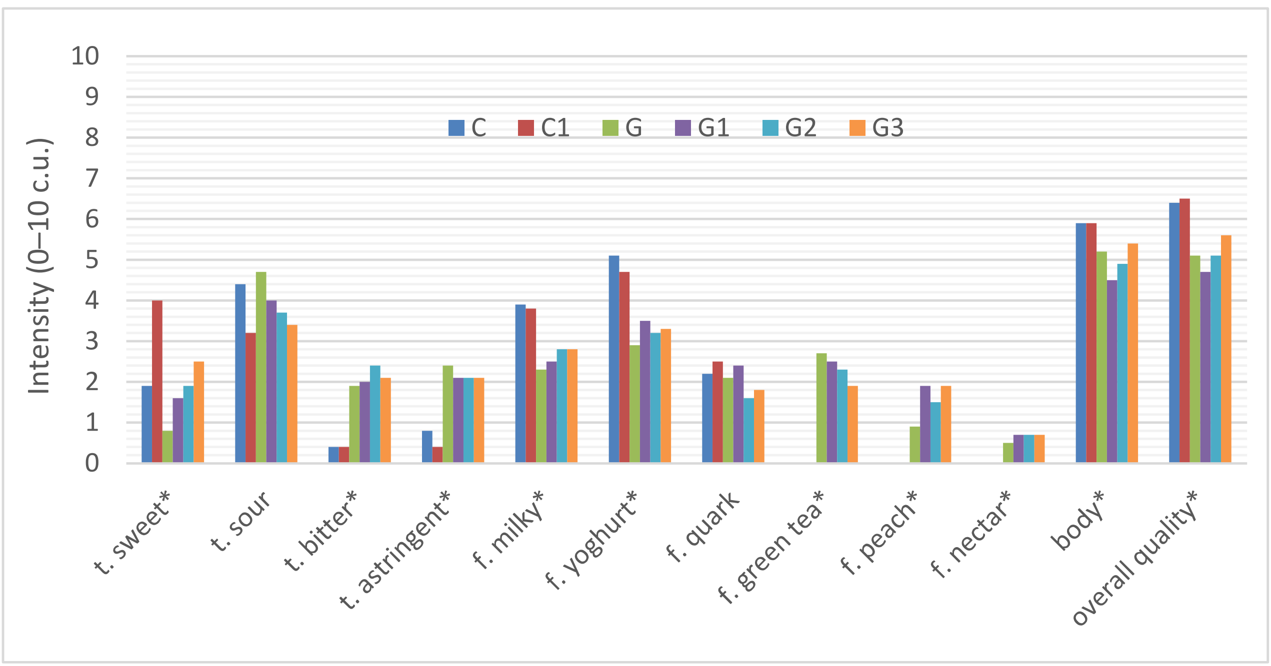

Figure 4. Sensory profile of the taste/flavor/overall quality of yoghurts C, C1, G, G1, G2. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3) (t—taste, f—flavor; * significantly differed at $p \leq 0.05$).

The addition of inulin to the natural yoghurt, although increased the sweet taste and decreased the bitter taste, had no significant effect on the body and overall sensory quality of the natural yoghurt. Both yoghurts, natural (C) and natural with inulin (C1), had a similar body (C—5.9 c.u.; C1—5.9 c.u.) and overall quality (C—6.4 c.u.; C1—6.5 c.u.).

However, significant changes in the overall quality of yoghurt resulted from the use of infused green tea in yoghurt production. The addition of green tea significantly reduced the sensory quality of the yoghurt (5.1 c.u.) and only the addition of inulin at the highest level raised the overall sensory quality of the yoghurt to 5.6 c.u.

3.1.2. Principal Component Analysis

Principal Component Analysis (PCA) was also performed for all yoghurt samples (C, C1, G, G1, G2, G3). In Figure 5, out of the 35 evaluated factors, only those factors are presented which, according to the statistical evaluation, significantly differentiated ($p \leq 0.05$) the samples. In the Principal Component Analysis of the six yoghurt types analyzed, the variability of the samples was attributed to the principal component (PC1), which accounted for 83.62% of the total variability, and was assigned to viscosity (yield stress) and a second component (PC2) which accounted for 7.82% of the total variation was assigned to overall quality.

The positioning of the tested samples on the PCA graph indicates differences in their quality. The samples formed two expressive clusters. The first cluster contained samples of natural yoghurt (C) and natural yoghurt with inulin (C1), while the second cluster on the opposite side contained samples of yoghurts with infused green tea (G) and yoghurt with green tea and inulin at three different levels (G1, G2, G3).

Natural yoghurt (C) and natural yoghurt with inulin (C1) were characterized by a milky and yoghurt-like taste and smell and by a sour smell. These characteristics were positively correlated with the body and overall quality. Similar correlations were observed in the QDP analysis, where control yoghurt and natural yoghurt with inulin were characterized by high body and overall quality. On the opposite side of the PCA graph were yoghurt samples with infused green tea and with added inulin, that correlated negatively with the overall quality.

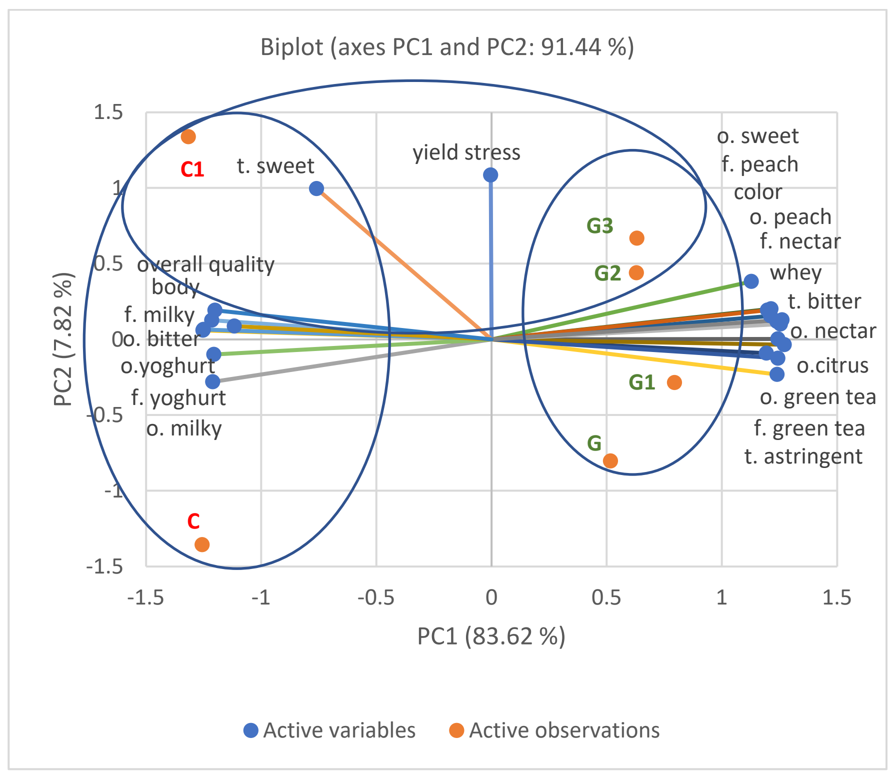

Figure 5. Principal Component Analysis (PCA) of the yoghurt samples: C, C1, G, G1, G2, G3. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3) (o—odor, t—taste, f—flavor). Attributes that significantly statistically differentiated the samples are on PCA ($p \leq 0.05$).

Yoghurt samples with infused tea and with inulin added to it (G, G1, G2, G3), were correlated with green tea flavor and odor, astringent and bitter taste, peach and nectar flavor and odor, as well as citrus flavor, more intense color and whey flow. As can be seen from the QDP evaluation, characteristics such as astringent and bitter taste as well as taste and smell typical for green tea and dark color and intensive whey flow had a negative influence on the overall quality of the evaluated samples of green tea-infused yoghurt and with inulin added to it, which corresponds with the PCA results.

In addition, on the PCA graph we can distinguish another cluster which shows the influence of inulin on the quality of analyzed yoghurts. Natural yoghurt with inulin added at the level of 6% (C1) as well as yoghurt with infused green tea and inulin added at the levels of 6% (G2) and 9% (G3) were positively correlated with higher yoghurt sweetness in taste and smell as well as with higher viscosity and peach taste and smell. On the other side of the PCA graph are samples of natural yoghurt without inulin and infused with green tea and yoghurt with green tea and the lowest inulin content of 3%, which were characterized by lower viscosity (yield stress) and lower sweetness. These relationships are also reflected in the results of the QDP analysis.

3.2. Instrumental Analysis

To complete the sensory evaluation of yoghurts, an instrumental analysis was also performed, the results of which are presented in Tables 1 and 2 and Figures 6, 7 and S1.

The results obtained in the measurement of the yield stress of the tested yoghurts showed that addition of inulin regardless of green tea infusion had a significant effect on this parameter.

The highest yield stress was noticed for the C1, G2 and G3 samples that contained 6 or 9% of inulin. Additionally, the green tea infusion resulted in increasing of the yield stress from 127.2 Pa obtained for the control sample to 154.9 Pa for the sample with green tea. The same tendencies were found in the firmness measurement. The greatest impact on this parameter had addition of inulin. The difference between the control samples C and C1 were significant and reached the level of 0.319 N. The highest force needed for breaking the yoghurt structure (1.302 N) was noticed for the highest tested inulin concentration (9%), whereas the lowest one (0.817 N) for the control yoghurt C. The yoghurt's infusion did not influence the firmness of the samples. The green tea addition resulted in significant growth (more than doubled) of the adhesiveness, as compared to the control sample C1, however the inulin addition in the highest concentration diminished the green tea impact, and the adhesiveness of G3 yoghurt was similar to the control sample with inulin.

The sensory evaluated color was compared with the instrumental color measurement. The obtained results are presented in the Figure S1 and in Table 2.

Table 1. The physical properties of yoghurts. The abbreviations in the table refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3).

Sample	Yield Stress [Pa]	Texture	
		Firmness [N]	Adhesiveness [Ns]
C	127.2 ^{a} ± 6.7	0.817 ^{a} ± 0.111	−0.048 ^{b} ± 0.007
C1	182.3 ^{d} ± 7.7	1.136 ^{bc} ± 0.068	−0.078 ^{ab} ± 0.009
G	154.9 ^{bc} ± 9.7	0.950 ^{ab} ± 0.105	−0.104 ^{a} ± 0.011
G1	149.5 ^{ab} ± 16.5	0.923 ^{ab} ± 0.115	−0.101 ^{a} ± 0.010
G2	176.8 ^{cd} ± 7.7	1.058 ^{b} ± 0.036	−0.105 ^{a} ± 0.028
G3	189.9 ^{d} ± 7.2	1.302 ^{c} ± 0.058	−0.087 ^{ab} ± 0.013

Values are mean ± SD (n = 3), a, b, c, d—values followed by the same letter within a column do not differ significantly according to Tukey's test ($p < 0.05$).

Table 2. Influence of the green tea addition on color parameters and the total color difference parameter of yoghurts. The abbreviations in the table refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3).

Sample	Color Parameters				Instability Index
	L^*	a^*	b^*	ΔE	
C	89.80 ^{c} ± 0.59	−1.31 ^{a} ± 0.06	9.52 ^{a} ± 0.22	-	0.517 ^{a} ± 0.022
C1	90.47 ^{c} ± 0.13	−1.20 ^{a} ± 0.04	10.17 ^{a} ± 0.15	0.97 ± 0.43	0.618 ^{b} ± 0.018
G	84.42 ^{b} ± 0.19	0.78 ^{b} ± 0.21	15.69 ^{b} ± 0.77	8.47 ± 0.52	0.705 ^{c} ± 0.021
G1	85.41 ^{b} ± 0.47	0.90 ^{b} ± 0.25	15.91 ^{b} ± 0.30	1.40 ± 0.59	0.702 ^{c} ± 0.025
G2	82.35 ^{a} ± 0.10	0.97 ^{b} ± 0.05	15.15 ^{b} ± 0.07	2.24 ± 0.39	0.706 ^{c} ± 0.012
G3	82.35 ^{a} ± 0.62	1.01 ^{b} ± 0.44	15.45 ^{b} ± 0.73	2.47 ± 0.82	0.725 ^{c} ± 0.030

Values are mean ± SD (n = 3), a, b, c—values followed by the same letter within a column do not differ significantly according to Tukey's test ($p < 0.05$).

The biggest difference, which can be detected by not experienced observer, were discovered after green tea addition (ΔE = 8.47). The green tea addition resulted in darkening

of the samples. Generally, the inulin addition did not improve the lightness of the yoghurts. The control yoghurt C and control yoghurt with inulin C1 were also not significantly differ.

The significant difference was also noticed for evaluation a^* color parameters. All tested green tea-infused yoghurts were characterized by significantly higher a^* parameter, what is more the a^* values of green tea yoghurts were positive what indicates that their color changed towards red color. The addition of inulin did not influence this parameter. Similar observations were made for the b^* parameter. Significantly higher values of b^* parameter was obtained for samples with green tea addition (b^* for C—9.52, and for G—15.69) whereas the inulin addition did not influence this parameter. There were no differences between samples C and C1, but also there were no differences between yoghurt with green tea addition and different concentration of inulin (3–9%).

The stability of yoghurts was examined with the multi-sample analytical centrifuge based on the space-time resolved extinction profiles technology (STEP). The start transmission profiles of tested yoghurts were over 80% regardless of green tea addition and inulin concertation (Figure 6).

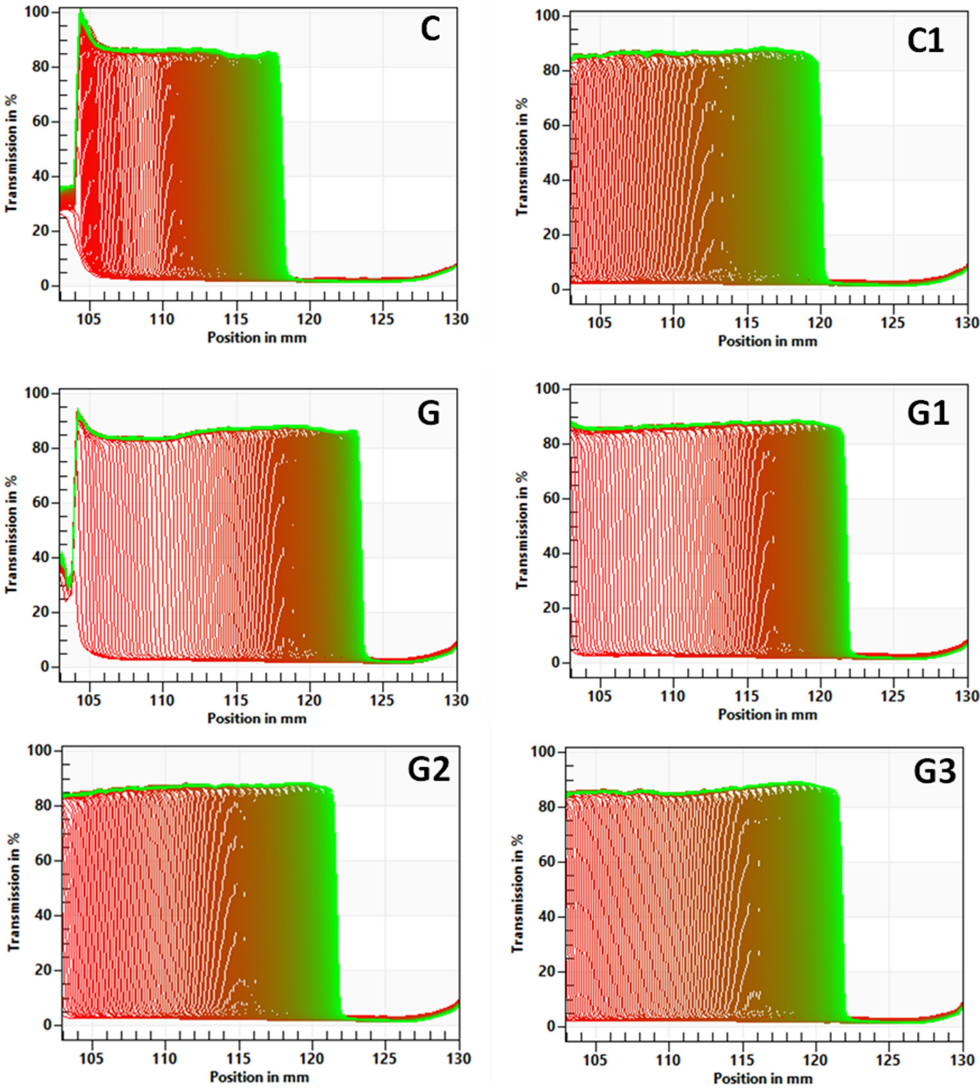

Figure 6. Influence of green tea and inulin addition on stability of set yoghurts indicated as transmission profiles presented enabling LUMiSizer® analysis. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3).

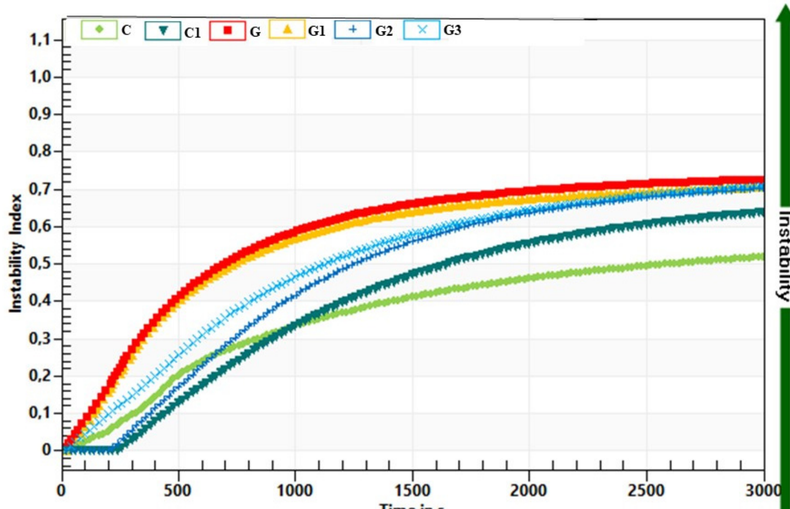

Figure 7. Influence of green tea and inulin addition yoghurt instability index. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3).

The analysis of transmission profiles showed that on the top-part of the products the syneresis was detectable. The most stable yoghurts according to the instability index analysis were control samples, without the addition of green tea (Table 2).

The instability index for yoghurt without green tea and inulin addition (C) was 0.517, whereas after inulin addition (C1) was 0.618. The green tea addition increased the instability index to 0.702–0.725. The separation of the fluid layer (syneresis) of yoghurts with green tea was the fastest at the beginning of the measurement, and then the greatest differences between the samples were also visible (Figure 7).

At the end of the measurement (after 15 h), the samples had similar instability index values regardless of the inulin addition.

4. Discussion

The use of infused green tea in the yoghurt production process significantly influenced its sensory quality and textural properties. The same applies to the addition of inulin to the evaluated yoghurts. From previous studies we can see that the sensory quality and texture properties of the tea-infused yoghurt will depend significantly on the type of tea used [2]. Green tea is characterized by bitter and astringence taste, and floral, grassy or burn leaf flavor [35], which significantly reduced the overall sensory quality of the yoghurt infused with green tea [2]. The use of green tea in yoghurt production also significantly affected the appearance of the yoghurt, which is also confirmed in this study. The yoghurt was characterized by a large whey flow and a dark creamy color, which is also reflected in the instrumental analysis. In a study by Bulut et al. [19] on yoghurt with extracts of various plants and green tea, among others, the effect of green tea on the acceptability of yoghurt and on texture parameters was observed. Although a detailed sensory evaluation of the yoghurts with experts and the QDP method supported by the statistical analysis was not carried out in this study, the hedonic evaluation of yoghurts with green tea used by the investigators indicated the lower appearance of yoghurt with green tea extract than control one. Additionally, the lower overall score of the yoghurt with green tea extract compared to the other samples evaluated could, according to the evaluators, be attributed to the undesirable garlic and iron taste present in it. Such extraneous flavors were not perceived by the experts in the samples of yoghurt with infused green tea [2]. The study by Bulut and co-authors [19] used the addition of green tea extract to the yoghurt, while our methodology used infusion with green tea leaves, which may also have influenced the results obtained. Similar to Bulut et al. [19], a hedonic evaluation of yoghurts with

green tea extract was conducted by Shokery et al. [16], who evaluated the acceptability of appearance, color, smell, flavor and overall acceptability of the yoghurt with green tea extract. This study found that yoghurt with green tea extract was rated significantly lower in appearance, odor, taste and overall acceptability than the control yoghurt, while still its acceptability ratings oscillated quite high between 5.9 and 7.2, while the control yoghurt ranged from 8.1 to 9.6 on a scale of 0–10, depending on the attribute assessed and the storage period. Acceptability ratings of appearance, taste, texture of yoghurts with green tea extract and its overall acceptability using a five-point hedonic scale were also studied by Rahmani and co-authors [20]. The results obtained by the researchers indicated that the addition of green tea extract worsened its taste, appearance, texture and overall acceptability compared to the control sample without tea, but still the yoghurt was above the acceptable level.

In our study, the addition of infused green tea to yoghurt significantly worsened the overall sensory quality of the yoghurt compared to the control sample, especially for the quality of taste and smell of the yoghurt. In order to improve the sensory quality of the infused green tea yoghurt in our study, we decided to verify the effect of the addition of inulin on its quality.

The results show that the addition of inulin to green tea infused yoghurt increased the perception of sweet smell and taste and peach taste in yoghurt and decreased the perception of sour taste. This increased the body and overall quality of the green tea-infused yoghurt to a level similar to the overall quality and body of the control yoghurt (there was no statistically significant difference between the plain yoghurt and the green tea yoghurt with 9% inulin). This was best seen with inulin addition at the highest level (9%).

The lack of similar detailed studies on expert evaluation of sensory quality of yoghurts with infused tea and the inulin addition makes the comparison of results difficult, but on the other hand points to a new research area that we have addressed in this study. A little more research was conducted on the evaluation of textural properties of yoghurts with green tea and yoghurt with inulin, therefore we refer to them more extensively in the discussion.

The yoghurt is characterized by semisolid texture which is built from creation of a three-dimensional network mostly of milk proteins but also with the polysaccharides and fats. It is known that the main factor responsible for milk gelatinization is the reduction of the high negative net charge on casein micelles as a result of acid release from microbial activity. Thanks to the fermentation, casein micelles and denatured whey proteins, aggregate into structures through hydrophobic and electrostatic bonds, building the yoghurt structure [36]. The yoghurts tested in our experiments are set types, that form a dispersion system consisting of small particles that are responsible for the formation of yield stress. This parameter is defined as the initial force required to initiate the yoghurt to flow [37]. Both the addition of green tea and inulin into yoghurts influenced the yield stress. For the sensory evaluation, only the addition of inulin at a level of 6% increased the yield stress of the control yoghurt and with green tea.

The green tea affected the yield stress probably by the presence of polyphenolic compounds, which are able to interact with milk proteins and in consequence increasing the yield stress [38]. The same results obtained Najgebauer-Lejko et al. [39], who tested the influence of the addition of green tea water extract to yoghurt and Dönmez et al. [40] who tested effect of green tea powder addition on yoghurt structure. On the other hand, the inulin addition caused the increase of a total solids of yoghurts and probably molecules of inulin dispersed among the casein micelles interfering protein matrix formation [41]. Similar results as obtained in our work—discovering the positive influence of the inulin on the rheological parameters including yield stress—was found by Guggisberg et al. [28]. Authors have investigated the addition of 1–4% inulin into the yoghurts and discovered that yield stress values generally increased with rising levels of inulin, so it increased similar to the increase of total solids. On contrast Paseephol et al. [27] discovered that the addition of inulin (at the level of 4%) to yoghurt altered the rheological and textural

properties of the product. The inulin-containing yoghurts showed a low magnitude of yield stress value and firmness than yoghurts did without inulin.

The firmness of yoghurt is directly dependent on its total solids [36], and that is why it increase with the addition of inulin and in direct proportion to its concentration. Inulin has a gelling property. Inulin gels are composed of a tridimensional network of insoluble submicron crystalline particles that immobilized large amounts of water [42]. What is more, inulin is a water-binding agent, which is why in yoghurt it might act as a thickener by combining with the protein aggregates [25]. The network of inulin gel is an additional structure to the protein network, what results in obtaining yoghurts that requires greater strength to destroy their structure [27]. The influence of the inulin on the yoghurt firmness depends also on the chain length and the degree of inulin polymerization. In the work the long-chain inulin was used, which is why the yoghurts with inulin were characterized by higher firmness values, but on the surface of yoghurt the syneresis was observed (Figure 6) in sensory and instrumental analysis. Similar observations were made by Paseephol et al. [27].

The yoghurts with green tea were characterized by higher adhesiveness values, but no significant differences were observed in the sensory evaluation. Bulut et al. [19] fund that green tea addition increased significantly the adhesiveness of yoghurt. This effect was probably caused by the presence in green tea yoghurts polyphenolic compounds, which are known to interact with milk proteins [38]. The protein-polyphenol associates hydrogen bonds consolidated gel network, as it was stated by Harbourne et al. [43], for the acidified milk gels, fortified with gallic or tannic acid.

The instrumentally measured color parameters of the yoghurts leads to the observation that lightness (L^* color parameter) was significantly affected by the green tea presents. All probes with green tea were significantly darker. This was evident in both sensory and instrumental tests. Similar founding of decreasing the L^* values after addition the plant extract into yoghurts was made by Shokery et al. [16]. Authors had investigated the color changes of set-type yoghurts enriched with the extracts of green tea and moringa leaves. Additionally, Bulut et al. [19] found out that the addition of green tea extracts at 0.5% (w/v) level to yoghurts slightly lowered L^* values. For the darker color of green tea yoghurts, pigments such as chlorophyll and carotenoids as well as catechins degradation products are responsible [44]. The green tea addition also influenced the a^* parameter, all green tea yoghurts were characterized by the positive value of this parameter. This change is caused probably by phenolic compounds of tea, that are easily degraded by oxidative changes and form colorless or brown-colored compounds [45]. The same shifting towards positive values of a^* parameter was reported by Bulut et al. [19] and Najgebauer-Lejko et al. [46]. The green tea infusion also changed the b^* color component significantly. The color of the yoghurts with green tea was significantly changed towards yellowish. For this change tea colorants such as chlorophyl and its degradation as well as polyphenol autoxidation are also responsible [47].

The spontaneous whey separation of the yoghurts can result from the unstable gel network. Responsible for its formation might be the rearrangements of the gel matrix or damage to the weak gel network [48] caused by the interaction of the lactic acid produced during fermentation. Observed in our work the deterioration of stability of yoghurts after addition of green tea is a phenomenon commonly described in the literature [46,49,50]. On the other hand, Dönmez et al. [40], who tested the green tea powders addition into yoghurts reported that green tea increased the stability and caused the reduction of whey separation. The differences between the stability may be due to the different yogurt production method used compared to our experiment. Authors have explained the phenome by formation of interactions between polyphenols from tea and milk proteins. The differences between the effects of tea on the stability of the gel structure may result primarily from the differences in pH of the tested systems. According to the literature, this pH will have the greatest influence on the formation and maintenance of a stable yoghurt gel network [51]. In our experiment the addition of inulin into green tea-infused yoghurt did not change the

stability. What is more, its addition to the control yoghurt without green tea also resulted in lowering the stability of the yoghurt. The effect of lower stability can be detected sensorially by evaluation of way separation, it was observed that green tea caused a greater whey separation in yoghurt, not leveled by inulin addition.

According to literature data, inulin should act as a water holding agent and increase the stability by limiting the occurrence of syneresis [52]. Inulin being a polydisperse polysaccharide should strengthening the network and improving the whey binding capacity of the yoghurts. However, in our work we obtained different results. Additionally, Guven et al. [24] discovered that addition of inulin in ranges 1–3% did not influence the yoghurt stability. On the other hand, the deterioration of stability after inulin addition was reported by Moghadam et al. [53], where the authors explain that the results are caused by occurrence of bacterial enzymatic activity (proteolytic) that influence on the casein network. Additionally, Arango et al. [54] reported that the spontaneous syneresis increased with the inulin content in low fat yoghurts.

The reason why the inulin did not stabilize the structure is explained by the fact that the gel structure of inulin becomes stronger and coarser with larger pores, increasing permeability and syneresis. The reason why in our experiment the inulin did not work as a stabilizer might be additionally connected with the fact that inulin properties depend on many factors such as inulin molecular weight and size, interaction with solvent, pH, temperature and process conditions [55].

5. Conclusions

The use of green tea infusion and inulin in yoghurt production has significantly changed the characteristics of yoghurt. Based on QDP analysis, it was found that the use of infused green tea in yoghurt production resulted in a significant increase in the perception of green tea flavor, bitterness, astringency, dark color of yoghurt and whey presence, while the perception of milky, yoghurt and sweetness decreased, which significantly worsened the overall sensory quality. On the other hand, the addition of inulin to the green tea yoghurt, especially at the level of 9%, significantly increased the perception of sweet, pleasant peach flavor and aroma and improved the firmness of the yoghurt while reducing the perception of sour taste, which improved the sensory quality of the yoghurt. The addition of green tea and inulin also affected physical parameters, which were measured instrumentally. Both additives changed the stability of the yoghurts, causing deterioration and separation of whey. Green tea significantly changed the color of the yoghurts by reducing the lightness. Green tea had a positive effect on the yield stress, the mean values increased after its addition, which was also enhanced by inulin at the highest concentration of 9%. The use of infused green tea in yoghurt production makes it necessary to use ingredients that will neutralize its adverse effects on sensory quality and physical parameters of yoghurt, and such an additive can be inulin at a concentration of 9%.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11040566/s1, Table S1: Table of sensory attributes and their definitions for the profiling of yoghurts, Table S2: Results of sensory evaluation of yoghurts by panel of experts (n = 20). The abbreviations in the table refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3) (* significantly differed at $p \leq 0.05$), Figure S1: Color parameters in yoghurts: C, C1, G, G1, G2, G3. The abbreviations in the figure refer to the control sample (C), control yoghurt with 6% inulin (C1), yoghurt with green tea (G), yoghurt with green tea and 3% inulin (G1), yoghurt with green tea and 6% inulin (G2), yoghurt with green tea and 9% inulin (G3).

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Article

Effect of the Addition of Whole and Milled Flaxseed on the Quality Characteristics of Yogurt

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Abstract: The present study aimed to analyze the effect of the addition of whole and milled flaxseed on the quality characteristics of yogurt. In the first stage of the research, the optimal dose of flaxseed was determined. In the second stage of the research, it was assessed whether the selected qualities of yogurt were affected by the form of flaxseed (whole or milled) and the time of addition (before or after fermentation). The yogurts obtained were stored at 5 °C for 21 days, and the changes in active acidity, apparent viscosity, syneresis, and the number of yogurt bacteria were determined. The results of the second stage of the study were subjected to two-way analysis of variance (ANOVA) ($p < 0.05$). The study showed that the addition of milled flaxseed to yogurts in the amount of 1% was optimal. Time and form of flaxseed supplementation significantly influenced the changes in active acidity, apparent viscosity, and syneresis in the tested yogurts. The addition of flaxseed did not significantly change the content of yogurt bacteria. The results indicate that to achieve increased apparent viscosity and reduced syneresis, it is more advantageous to use milled flaxseed rather than whole flaxseed.

Keywords: functional food; milk fermentation; flaxseed; active acidity; yogurt bacteria; apparent viscosity; syneresis; bioactive compounds

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1. Introduction

Consumers' awareness of the importance of a healthy diet is growing and, therefore, they often prefer products containing functional foods [1,2]. Functional foods can be defined as foods or food ingredients that can provide physiological benefits and help in the prevention and/or treatment of disease [3]. The dairy industry is increasingly using various types of functional ingredients as additives to traditional products such as yogurt. This allows not only a new range of products to be created but also their properties to be modified by limiting the use of food additives [4,5]. Flaxseed is an interesting additive which not only affects the nutritional value of the product but also improves its structure by influencing its rheological properties [3].

Yogurt is one of the most popular fermented dairy products worldwide that has great consumer acceptability because of its health benefits [6]. The basic ingredients of yogurt are milk and live yogurt bacteria cultures [7]. The active strains of bacteria are mainly *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* [8,9]. Consumption of yogurt has a positive effect on health as it contributes to the prevention of gastrointestinal infections, lowering of serum cholesterol level, and antimutagenic activity [6]. Lactic acid bacteria (LAB) help to increase the absorption of nutrients, participate in the synthesis of vitamins, and reduce the development of undesirable microflora in the intestine [7,8,10]. Because of these known health benefits of yogurt, the consumer demand for yogurt has

increased. Yogurts are now manufactured in several styles and varieties with different fat contents, flavors, and textures [6].

During the production of yogurt, it is important to obtain its appropriate quality characteristics, which include acidity, flavor characteristics, clot structure, and degree of syneresis. The quality characteristics of yogurt can be controlled and improved using stabilizers, e.g., starch, guar gum, carboxymethyl cellulose, carrageenan, and pectin [11–13]. The use of stabilizers allows us to modify rheological properties (mainly viscosity) and reduce the level of syneresis [13,14]. The "clean label" trend popular among consumers encourages producers to use natural plant materials for stabilization purposes, the presence of which in the product does not raise any controversies or health concerns [15].

Flaxseed (*Linum usitatissimum* L.), also known as linseed, is one of the most important oilseed crops for industrial applications [16,17]. It produces small, flat seeds whose color range from golden yellow to reddish brown [18]. It is used as food and animal feed and to produce fiber. As a food, flaxseed is used in the form of whole seeds, flour, or as oil [19,20]. The pro-health effect after consuming flaxseed depends on the type of product and form of consumption of the seed [21]. Flaxseed is a rich source of proteins (10–30% of the content), fat (40% of the content), and fiber (28% of the content) [16,22–25]. Food fortification with flaxseed components has been proven to offer many health benefits [26,27]. Flaxseed is considered as a functional food because of the high content of bioactive ingredients such as α-linolenic acid (ALA), lignans, phenolic compounds, and soluble fiber [18,20,28]. Previous studies have proved that flaxseed has a potential in disease prevention, particularly cardiovascular disease (CVD), obesity, osteoporosis, rheumatoid arthritis, and breast and colon cancer, and can boost immunity [24,25,27,29]. Lignans are a phytoestrogen that helps in decreasing cell proliferation and can prevent cancer [16,17,20]. ALA is beneficial for infant brain development and for reducing blood lipid levels, and it has anti-inflammatory, anticoagulant, and antiarrhythmic properties [3,18]. The soluble fiber fraction contains mucilaginous substances (5–8% of the soluble fraction), which are characterized by high water absorption and functional properties like those of Arabic gum. Consuming soluble fiber helps to prevent constipation, lowers the risk of colon and rectal cancer, lowers total cholesterol level, and regulates blood sugar fluctuations [24–27]. Flaxseed also contains antinutritional components, including cyanogenic glycosides, cadmium, and trypsin inhibitors. Their presence should be reduced or eliminated as much as possible with mechanical and thermal processing [20–22].

Because of the high content of proteins, fiber, and mucous substances, flaxseeds are used for producing new food products [25]. To date, flaxseed has been used in different forms for the production of various dietary products such as baked cereal products, fiber bars, meat fillers, bread, muffins, and spaghetti [3]. The enrichment of food products with flaxseed affects the sensory characteristics, nutritional value, and rheological properties of the products [30]. In the technology of dairy products, the most important component of flaxseed is soluble fiber, which may have a stabilizing effect. The key issues when using various stabilizers in dairy formulations are improvement of their physical properties, including syneresis, gelling, thickening capacity, stability, and rheological properties [31,32]. Soluble dietary fiber-enriched dairy products may serve as functional foods, as dietary fiber helps to improve sensory characteristics, shelf life, and structural properties (i.e., viscosity, texture, and water and oil holding capacity) of dairy foods [26,27].

Flaxseed is emerging as one of the nutritive and functional ingredients in food products. [25] It increases the availability of healthy food and enhances the nutrient profile of foods by reducing salt, sugar, and saturated fat content as well as by increasing the content of bioactive compounds. Markets for healthy foods are growing worldwide, and in the future, flaxseed can be used as an ingredient of functional foods and nutraceuticals, which are foods with health-promoting or disease preventive properties [29,33]. The functional and health-promoting properties of flaxseed can enable the food processing industry to create a large variety of foods that contain this plant, thus offering the public an increased opportunity to incorporate flaxseed into their everyday diet. In the coming years, it is

expected that flaxseed will become more economically important for farmers, food processers, and retailers in food market [34]. Therefore, the present study aimed to analyze the effect of the addition of whole and milled flaxseed on the quality characteristics of yogurt. The influence of the addition of flaxseed in various forms and at various stages of yogurt production on the selected quality characteristics of the product during the storage period was investigated.

2. Materials and Methods

2.1. Materials

The research materials were ultra-high temperature (UHT)-treated milk (Mlekovita, Wysokie Mazowieckie, Poland, 2.0% fat content) for producing yogurts; gold flaxseed (Agnex, Białystok, Poland), milled flaxseed (Agnex, Białystok, Poland), and freeze-dried yogurt culture YC-X16 (Chr. Hansen, Warsaw, Poland, containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*).

2.2. Methods

The research was performed in two stages. In the first stage (pilot study), the optimal dose of flaxseed was determined, the addition of which met the physicochemical and rheological parameters of the yogurt. At this stage, milled flaxseed in the amount of 1%, 2%, or 3% relative to the amount of milk was added to the UHT-treated milk, except for the control sample, and the whole mixture was pasteurized at 80 °C for 5 min. All yogurt variants (0, 1%, 2% and 3% flaxseed supplement) were prepared in nine samples in jars of 160 mL each. The samples were then cooled to 39 °C and inoculated with 0.004% yogurt bacteria starter culture. The inoculated and mixed solutions were incubated at 37 °C for 5 h. After the incubation period, the yogurts were cooled and stored at 5 °C for 24 h. After this time, the pH, apparent viscosity, and syneresis of the obtained yogurts were analyzed.

In the second stage of the research (main research), four types of yogurt were prepared with the addition of an optimized dose of flaxseed, which was determined in the first stage of the research. All yogurt variants were prepared in 12 samples in jars of 160 mL each. All samples were prepared by inoculating UHT-treated milk at 39 °C with yogurt bacteria starter culture at the dose of 0.004%. The fermentation process was carried out at 37 °C for 5 h, and the samples were then cooled to 5 °C. Sterilized (121 °C/5 s) whole or milled flaxseed were added to the samples before or after the fermentation process to obtain the following yogurt variants:

- Variant 1: yogurt with the addition of milled flaxseed added before fermentation;
- Variant 2: yogurt with the addition of whole flaxseed added before fermentation;
- Variant 3: yogurt with the addition of milled flaxseed added after fermentation;
- Variant 4: yogurt with the addition of whole flaxseeds added after fermentation;
- Control sample: yogurt without the addition of whole or milled flaxseed.

The yogurts obtained were stored at 5 °C for 21 days. In the prepared samples, the changes in active acidity, apparent viscosity, syneresis, and the number of yogurt bacteria during the storage period were determined.

2.2.1. Active Acidity (pH) Analysis

The active acidity (pH) analysis was performed using the Elmetron CPO-505 pH meter (Elmetron, Zabrze, Poland). The device was properly calibrated using the buffer solutions according to the manufacturer's instructions. The analyses were performed by immersing the pH meter electrode in the obtained yogurts. Measurements were made in 3 repetitions according to the following measurement points:

For pilot studies (first stage of research):

- after 3 h of incubation at 37 °C.
- after 24 h of storage at 5 °C.

For main research (second stage of research):

- after 24 h of storage at 5 °C.
- after 7 days of storage at 5 °C.
- after 14 days of storage at 5 °C.
- after 21 days of storage at 5 °C.

2.2.2. Apparent Viscosity Analysis

The apparent viscosity of the yogurts was determined using a Brookfield RV-D-II + pro viscometer (Brookfield Engineering Laboratories INC., Middleboro, MA, USA) coupled with a computer software (Brookfield Engineering Laboratories INC., Middleboro, MA, USA). Spindle no. 04 was used for the assessment. The rotation speed was 10 rpm. The spindle was completely immersed in the mixed samples. The analysis was performed at 5 °C. Measurements were taken in three repetitions according to the following measurement points:

For pilot studies (first stage of research):

- after 24 h of storage at 5 °C.

For main research (second stage of research):

- after 24 h of storage at 5 °C.
- after 7 days of storage at 5 °C.
- after 14 days of storage at 5 °C.
- after 21 days of storage at 5 °C.

2.2.3. Syneresis Analysis

Syneresis was determined using the MPW-350R centrifuge (MPW MED. INSTRUMENTS, Warsaw, Poland). The samples were mixed, weighed to 40 g, and centrifuged at 4 °C and 16,125× *g* for 20 min. After completion of centrifugation, the separated whey was decanted and weighed. The value of syneresis *S* [%] was calculated according to Formula (1):

$$S = A/B \times 100\%, \quad (1)$$

where:

A—mass of whey separated during centrifugation [g]

B—yogurt mass before centrifugation [g]

Measurements were made in three repetitions according to the following measurement points:

For pilot studies (first stage of research):

- after 24 h of storage at 5 °C.

For main research (second stage of research):

- after 24 h of storage at 5 °C.
- after 7 days of storage at 5 °C.
- after 14 days of storage at 5 °C.
- after 21 days of storage at 5 °C.

2.2.4. Number of Yogurt Bacteria Analysis

Microbiological analysis was performed in Petri dishes. The M17 agar medium (Merck, Darmstadt, Germany) was used to determine the number of *S. thermophilus*, while MRS (De Man, Rogosa and Sharpe) agar medium (Merck, Germany) was used to determine the number of *L. delbrueckii* subsp. *bulgaricus*. The media were prepared and sterilized (M17 agar: 121 °C, 15 min; MRS agar: 117 °C, 15 min) several days prior to inoculation. Petri dishes with media were incubated at 37 °C for 2 days to dry them and then stored at 5 °C until analysis.

The drop plate method was used to determine the number of yogurt bacteria cells. A dilution series of test samples was prepared ranging from 10^{-1} to 10^{-6}. The drop in inoculation was carried out for dilutions ranging from 10^{-6} to 10^{-3} by placing 20 µL of each

dilution in appropriately marked zones of the plates. The M17 agar plates were incubated at 37 °C for 48 h under aerobic conditions, while the MRS agar plates were incubated at 37 °C for 72 h under anaerobic conditions. Anaerobic cultures were obtained in Anaerocult containers (Merck, Germany). After incubation, the grown colonies were counted and converted to CFU/mL. The result was expressed as the logarithm of the total cell count of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The incubation was performed in 3 replications according to the following measurement points:

- after 24 h of storage at 5 °C.
- after 7 days of storage at 5 °C.
- after 14 days of storage at 5 °C.
- after 21 days of storage at 5 °C.

2.2.5. Statistical Analysis

The results of the first stage of the study were subjected to one-way analysis of variance (ANOVA), and the results of the second stage of the study were subjected to two-way ANOVA using the software Statistica 13.1 (StatSoft, Kraków, Poland). The two-way ANOVA allowed us to determine the effect of the storage period and the form of flaxseed supplementation and their interaction on the studied quality characteristics of yogurt. The significance of the differences was analyzed by Tukey's test at $\alpha = 0.05$. Results are presented as mean and standard deviation (SD).

3. Results

3.1. Pilot Study Results

In the first stage of the research, four variants of yogurt were prepared to determine the optimal dose of flaxseed supplement. Milled flaxseed was used in three doses (1%, 2%, and 3%), which were added before yogurt fermentation. The type and dose of the supplement are important in the parameters of finished products. These parameters include active acidity, apparent viscosity, and syneresis. The addition of flaxseed to yogurt at levels of 1%, 2% and 3% was selected based on the study by Kumar et al. [35], which investigated the effect of the addition of flaxseed oil, flaxseed flour and fruits for the sensory, physico-chemical, and fatty acid profile of yogurt. The study showed that fruit yogurt (20% fruit and sugar mixture) with the incorporation of flaxseed oil up to 2% and flaxseed flour up to 1% in combination can be used for the preparation of fruit yogurt with acceptable sensory attributes. The scores drastically reduced for yogurt samples wherein 2% flaxseed flour was incorporated. The results indicate that too high a dose of flaxseed addition to yogurt may have a negative impact on the sensory acceptability of the product.

To determine the effect of the addition of various doses of milled flaxseed on acidity, the pH of the obtained yogurts was analyzed after 3 h of incubation at 37 °C and 24 h of storage at 5 °C (Table 1). The results obtained revealed that the addition of milled flaxseed, regardless of the dose used, did not significantly affect the pH of the yogurts obtained as compared to that of the control sample, both after 3 h of incubation and 24 h of storage.

Table 1. Assessment of the quality parameters of yogurts with various additives of milled flaxseed.

Yogurt	pH after 3 h of Incubation	pH after 24 h of Storage	Apparent Viscosity [mPa × s]	Syneresis [%]
flaxseed 0%	4.76 ± 0.01^{a}	4.36 ± 0.32^{a}	6312.00 ± 9.85^{a}	29.90 ± 2.31^{a}
flaxseed 1%	4.43 ± 0.26^{a}	4.25 ± 0.32^{a}	9498.00 ± 79.90^{b}	26.10 ± 4.43^{a}
flaxseed 2%	4.51 ± 0.71^{a}	4.32 ± 0.94^{a}	1308.00 ± 17.08^{c}	51.10 ± 7.63^{b}
flaxseed 3%	4.61 ± 0.80^{a}	4.43 ± 0.58^{a}	4408.00 ± 68.53^{d}	43.10 ± 2.00^{b}

Table shows mean values ± standard deviations; a, b, c, d-mean values in columns denoted by different letters differ significantly ($p \leq 0.05$).

The apparent viscosity analysis showed that the addition of milled flaxseed to yogurts in the amount of 1% significantly increased the apparent viscosity in the tested yogurts

as compared to that of the control sample (Table 1). The addition of milled flaxseed in the amount of 2% and 3% significantly reduced the viscosity of the tested yogurts as compared to that of the control sample. With an increase in the amount of the added flaxseed, the apparent viscosity in the tested yogurts decreased significantly.

The analysis of syneresis showed that the addition of milled flaxseed to yogurts in the amount of 1% did not significantly affect the syneresis in the tested yogurts as compared to that in the control sample (Table 1). For higher doses of flaxseed (2% and 3%), a significant increase in the syneresis was observed in the tested yogurts as compared to that in the control sample.

The optimal dose of milled flaxseed supplement was 1% relative to the amount of milk. The addition of flaxseed in this amount allowed yogurts to be obtained with the lowest degree of whey leakage (syneresis) and the highest apparent viscosity. These parameters allowed a product to be obtained with properties that were almost like those of basic yogurt without additives (control sample).

3.2. Main Research Results

3.2.1. Active Acidity (pH) Analysis

During the refrigerated storage of yogurts, changes in active acidity may occur, which affect the organoleptic properties of the product. It is desirable to maintain the acidity of the product during the storage period at a level close to the initial acidity. In the yogurts tested, changes in active acidity during storage were examined by determining it after the fermentation process, and after 7, 14 and 21 days of storage (Table 2).

Table 2. Changes in quality characteristics during the storage period of the tested yogurts.

Quality Characteristics	Type of Sample	Storage Time [day]			
		1	7	14	21
pH	Control sample	4.42 ± 0.05^a	4.34 ± 0.04^b	4.27 ± 0.09^b	4.20 ± 0.06^c
	Variant 1	4.49 ± 0.08^a	4.38 ± 0.09^b	4.27 ± 0.02^c	4.22 ± 0.07^c
	Variant 2	4.44 ± 0.10^a	4.36 ± 0.02^b	4.29 ± 0.09^b	4.22 ± 0.06^c
	Variant 3	4.57 ± 0.10^a	4.47 ± 0.06^b	4.32 ± 0.01^c	4.31 ± 0.06^c
	Variant 4	4.47 ± 0.09^a	4.37 ± 0.08^b	4.28 ± 0.03^b	4.19 ± 0.06^c
apparent viscosity [mPa×s]	Control sample	1950.00 ± 29.02^a	2580.00 ± 7.00^b	2742.00 ± 14.64^c	2782.00 ± 31.43^c
	Variant 1	4764.00 ± 24.84^a	5064.00 ± 7.55^b	4596.00 ± 21.57^c	5332.00 ± 13.05^d
	Variant 2	1124.00 ± 16.65^a	1682.00 ± 9.94^b	2522.00 ± 26.65^c	2294.00 ± 22.94^d
	Variant 3	6624.00 ± 5.13^a	5126.00 ± 22.87^b	5470.00 ± 18.93^c	5456.00 ± 21.22^c
	Variant 4	4684.00 ± 6.66^a	5086.00 ± 17.58^b	4702.00 ± 15.27^a	4202.00 ± 25.01^c
syneresis [%]	Control sample	40.60 ± 9.46^a	50.10 ± 9.05^a	52.40 ± 10.91^a	44.70 ± 11.55^a
	Variant 1	36.40 ± 9.37^a	43.60 ± 6.41^a	47.90 ± 5.62^a	42.10 ± 11.19^a
	Variant 2	26.20 ± 4.91^a	44.20 ± 9.60^b	58.70 ± 5.64^b	46.40 ± 5.72^b
	Variant 3	20.50 ± 5.76^a	25.60 ± 5.68^a	24.10 ± 6.93^a	17.00 ± 3.00^a
	Variant 4	20.10 ± 5.43^a	50.10 ± 5.98^b	52.40 ± 5.25^b	44.70 ± 6.64^b
number of yogurt bacteria [log CFU/mL]	Control sample	8.90 ± 0.21^a	9.00 ± 0.42^a	9.00 ± 0.13^a	9.00 ± 0.53^a
	Variant 1	8.90 ± 0.44^a	8.70 ± 0.45^a	9.00 ± 0.46^a	9.10 ± 0.99^a
	Variant 2	9.00 ± 0.78^a	9.00 ± 0.44^a	9.00 ± 0.45^a	8.80 ± 0.53^a
	Variant 3	8.80 ± 0.62^a	8.70 ± 0.26^a	8.70 ± 0.52^a	8.80 ± 0.62^a
	Variant 4	8.90 ± 1.01^a	8.80 ± 0.61^a	8.60 ± 0.26^a	8.60 ± 0.61^a

Control sample—yogurt without the addition of whole or milled flaxseed; Variant 1—yogurt with milled flaxseed added before fermentation; Variant 2—yogurt with whole flaxseed added before fermentation; Variant 3—yogurt with milled flaxseed added after fermentation; Variant 4—yogurt with whole flaxseed added after fermentation. Table shows mean values ± standard deviations; a, b, c, d-mean values in rows denoted by different letters differ significantly ($p \leq 0.05$).

The analysis showed that time of storage ($p < 0.001$) and form of flaxseed supplementation ($p = 0.006$) significantly influenced the active acidity in the tested yogurts. There was no significant interaction effect of the storage time × form of flaxseed supplementation for active acidity of the tested yogurts ($p = 0.959$). In all samples, the acidity changed

significantly after 7 days of storage. There was a general tendency of decrease in the pH of the tested yogurts, both in the control sample and in the variants with the addition of milled and whole flaxseed. A significantly lower decrease in pH as compared to other samples was demonstrated for the addition of milled flaxseed added after the fermentation process.

3.2.2. Apparent Viscosity Analysis

To assess whether the addition of flaxseed affects the natural stabilization of the yogurts obtained, their apparent viscosity was analyzed. In the tested yogurts, changes in apparent viscosity during storage were analyzed by determining the viscosity after the fermentation process and after 7, 14, and 21 days of storage (Table 2). The conducted analysis showed that time of storage ($p < 0.001$) and form of flaxseed supplementation ($p < 0.001$) significantly influenced the changes in apparent viscosity in the tested yogurts. There was a significant interaction effect of the storage time × form of flaxseed supplementation for apparent viscosity of the tested yogurts ($p < 0.001$). The changes in the apparent viscosity of the yoghurts were already apparent after 7 days of storage. The ANOVA results showed a statistically significant increase in viscosity for yogurt with milled flaxseed added before fermentation and for yogurts with milled and whole flaxseed added after fermentation as compared to that for the control sample. The highest viscosity was shown by yogurt with milled flaxseed added after fermentation, while the lowest one was shown by yogurt with whole flaxseed added before fermentation.

3.2.3. Syneresis Analysis

To determine whether the addition of flaxseed as a natural stabilizer would improve the quality of the tested yogurts, syneresis was assessed, which indicates the degree of leakage of whey from the yogurt. The changes in syneresis in the yogurts during the storage period were determined after the fermentation process and after 7, 14, and 21 days of storage (Table 2). The analysis showed that time of storage ($p < 0.001$) and form of flaxseed supplementation ($p < 0.001$) significantly influenced the apparent viscosity in the tested yogurts. There was significant interaction effect of the storage time × form of flaxseed supplementation for syneresis of the tested yogurts ($p < 0.038$). The analysis of variance showed that time of storage had a significant effect on syneresis only for yogurts with the addition of whole flaxseed, both before fermentation (Variant 2) and after fermentation (Variant 4). For these yogurts, syneresis was significantly increased after 7 days of refrigerated storage. In the remaining samples, syneresis was at a similar level during the entire storage period.

The analysis conducted showed that the form of adding flaxseed to yogurts significantly influenced their syneresis. The highest ability to retain whey (the lowest syneresis) was shown by yogurt with milled flaxseed added after fermentation. The lowest ability to retain whey (the highest syneresis) was shown by yogurt without the addition of flaxseed (control sample) and by yogurt with whole flaxseed added before fermentation.

3.2.4. Analysis of Number of Yogurt Bacteria

In the yogurts tested, the number of selected LAB was analyzed to determine whether their number is typical for yogurts available on the market. According to the International Dairy Federation [36], the number of viable LAB cells in yogurt should not be less than 7 log CFU/mL. The changes in the number of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* cells in the tested yogurts during the storage period were determined after the fermentation process and after 7, 14 and 21 days of storage (Table 2). Before fermentation, the number of yogurt bacteria in the tested samples was 8.5 log CFU/mL, and after the fermentation process, the total number of yogurt bacteria of the obtained yogurts was 8.6–9.1 log CFU/mL.

The analysis showed that time of storage ($p < 0.929$) and form of flaxseed supplementation ($p < 0.734$) did not significantly affect the changes in the number of yogurt bacteria in the tested yogurts. There was no significant interaction effect of the storage time × form of

flaxseed supplementation for the number of yogurt bacteria of the tested yogurts (p = 0.969). After fermentation and during 21 days of storage, the number of tested LAB in all types of yogurts was at a similar level, i.e., all obtained yogurts had a normative number of viable cells ($\geq 10^6$ CFU/mL or g). The analysis showed no statistically significant differences between the number of yogurt bacteria in the tested yogurts. This value was similar in all the tested samples, which indicates that the addition of flaxseed did not significantly change the content of yogurt bacteria.

The mean number of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* cells for all the tested yogurts was 8.66 and 8.4 log CFU/mL, respectively, and did not differ significantly during the entire storage period. Therefore, the tested yogurts showed microbial stability in terms of count of lactobacilli and streptococci during the entire storage process.

4. Discussion

The pilot study showed that the addition of milled flaxseed to yogurts in the amount of 1% relative to the amount of milk was favorable. The use of this dose allowed yogurt to be obtained with the lowest syneresis, which indicates the amount of whey leakage. A similar tendency was found for the addition of selected hydrocolloids to yogurt [37]. The lowest syneresis was obtained in yogurts with the addition of carrageenan, locust bean gum, guar gum, and xanthan gum (0.01%), and syneresis became higher with the increase in their concentration. This effect may be because the casein micelles were surrounded by the stabilizer molecules, which reduced the interactions between the casein micelles. The use of a 1% dose of milled flaxseed in the pilot study also allowed yogurts to be obtained with the highest apparent viscosity. The presence of natural additives in dairy products, which have a positive effect on their apparent viscosity, also influences their better acceptance among consumers [38].

Similar results were obtained by Ismail et al. [39], who studied the influence of milk supplemented with different percentages of flaxseed oil (0.0%, 0.5%, 1.0%, 1.5% and 2.0%) on the physicochemical, microbiological, and organoleptic characteristics of yogurt during storage at 6 °C for 14 days. Yogurt samples supplemented with a different percentage of flaxseed oil showed higher curd tension and whey syneresis than the control sample. The supplementation of milk with 1.0% flaxseed oil was found to be most acceptable.

Mousavi et al. [40] produced prebiotic yogurt supplemented with powdered flaxseed (0–4%) and investigated its texture and sensory properties; however, they obtained a different conclusion. The addition of flaxseed to yogurt samples increased the hardness, gumminess, chewiness, cohesiveness, and springiness values of the produced yogurt samples. The addition of 2.63% flaxseed into yogurt samples enables functional food to be produced with satisfactory texture and sensory characteristics. The differences may be due to the origin of the tested flaxseed (Poland vs. Iran), as the composition of flaxseed can vary with genetics, growth environment, seed processing, and analytical method [18].

In the main study, an optimized dose of flaxseed (1%) was used, and it was assessed whether the selected qualities of yogurt were affected by the form of flaxseed (whole or milled) and by the time of addition (before or after fermentation). The study conducted showed that time significantly influenced the active acidity, apparent viscosity, and syneresis in the tested yogurts. Active acidity (pH) decreased significantly after 7 days of storage. The study showed that the form of flaxseed supplement significantly influences the pH of the tested yoghurts. A significantly lower decrease in pH as compared to other samples was demonstrated for the addition of milled flaxseed added after the fermentation process. However, for all samples, the acidity did not decrease below 4.19 during the entire storage period, which is within the normal yogurt pH range of 4.0 to 4.5 [9]. Similar results were obtained by Jeong et al. [41], who studied the physicochemical characteristics of bioactive kefir containing different concentrations of flaxseed. The pH of this bioactive kefir decreased with increasing incubation time, which lasted for 48 h. The final pH value was 4.50, 4.52, and 4.51, respectively, for kefir with the addition of 1%, 2%, and 3% flaxseed. However, these parameters were not affected by the amount of added flaxseed.

The significant increase in the viscosity of the tested yogurts with time was demonstrated for yogurt containing milled flaxseed added before fermentation and for yogurt containing milled and whole flaxseed added after fermentation in relation to the control sample. The quality of the raw material, the type of structure-forming additives, the type of microorganisms, and the fermentation conditions affect the rheological properties of the yogurt. It is necessary to obtain the desired viscosity of the yogurt that remains throughout its shelf life [42]. The highest viscosity was possessed by yogurt with milled flaxseed added after fermentation, and the lowest by yogurt with whole flaxseed added before fermentation. It can therefore be concluded that the addition of milled flaxseed has a positive effect on the apparent viscosity of the obtained yogurts, regardless of whether it is added before or after the fermentation process. To obtain yogurt with the addition of whole flaxseed with increased apparent viscosity, it is necessary to add whole flaxseed after the fermentation process. Similar results were obtained by Marand et al. [43], who tested yogurt samples enriched with flaxseed powder. The results showed that the addition of flaxseed powder significantly increased water holding capacity and the viscosity of yogurts.

Time significantly affects the increase in syneresis for yogurts with whole flaxseed added before or after fermentation. In other samples, syneresis remained at a constant level during the entire period of storage. The highest ability to retain whey (the lowest degree of syneresis) was found in yogurt with milled flaxseed added after fermentation. The lowest ability to retain whey (the highest degree of syneresis) was found in yogurt without flaxseed (control sample) and by yogurt with whole flaxseed added before fermentation. Therefore, it can be concluded that to reduce syneresis in yogurts, the addition of milled flaxseed (both before and after fermentation) is more advantageous, because it did not significantly affect the increase in the leakage of whey from the products.

Leakage of whey can limit the shelf life of yogurts. To prevent this leakage, it is common to use stabilizers such as pectin, carrageenan, guar gum, starch, or locust bean gum. The addition of stabilizers also serves to reduce the calorie count of yogurt by limiting the fat content and makes the yogurt economically cheaper by reducing the consumption of skim milk powder [34,44]. Reduced syneresis in yogurts with milled flaxseed may result from the presence of mucilage polysaccharides, which can bind water and are released during the grinding process [45]. Arabshahi-Delouee et al. [46] studied the effect of the addition of flaxseed mucilage on the physicochemical and sensorial properties of semi-fat (1.5% fat) set yogurt. Yogurt samples were incorporated with flaxseed mucilage at the levels of 0.00%, 0.10%, 0.15%, and 0.20% and analyzed periodically during storage at 5 °C (1, 7, and 15 days). The pH value of all the samples significantly decreased; viscosity, consistency, and water holding capacity increased; and the syneresis value decreased with the increase in the amount of flaxseed mucilage and storage time.

Time and form of flaxseed supplementation did not significantly affect the changes in the number of yogurt bacteria in the tested yogurts. No significant differences were observed between the number of yogurt bacteria in the tested yogurts, which indicates that the addition of flaxseed did not significantly change the content of yogurt bacteria. The average number of yogurt bacteria of the species *S. thermophilus* for all the tested yogurts was 8.6 log CFU/mL and that of *L. delbrueckii* subsp. *bulgaricus* was 8.4 log CFU/mL, and these values did not differ significantly throughout the storage period. Mihoubi et al. [47] used two types of milk powder for manufacturing yogurt: skimmed milk powder at 15% (*w*/*v*) and whole milk powder at 13.7% (*w*/*v*), which were fermented and stored at 4 °C for 28 days. The addition of ground flaxseed decreased the pH values during fermentation and refrigerated storage. In the yogurts obtained, the average values of LAB CFU counts for the control sample were significantly increased (*S. thermophilus*: 9.2 log CFU/mL; *L. bulgaricus*: 8.7 log CFU/mL at the 14th day of storage); however, a reduction was noted during the last two weeks of storage (*S. thermophilus*: 8.1 log CFU/mL; *L. bulgaricus*: 6.9 log CFU/mL). This effect was attributed to *L. delbrueckii* subsp. *bulgaricus*, which produces lactic acid during cold storage. This process is known as post-acidification in the dairy industry. Lactic acid produced during cold storage causes loss of bacterial viability.

In the tested yogurts, slight differences were noted between the amount of *Lactobacillus* and *Streptococcus*, with a slight dominance of *S. thermophilus*. These differences were not more than 1 log unit. The pH value showed that the acidity did not drop below 4.0. This may be due to the smaller population of *L. delbrueckii* subsp. *bulgaricus*, which has a greater acidifying capacity than lactic streptococci. This bacterial species can lower the pH value to 3.8. *S. thermophilus* is not resistant to high acidity. Its development is inhibited at pH 4.1 [48]. A comparable relationship was found in studies that analyzed the microflora of yogurts following the addition of amaranth seeds and oat grains [36]. The tested yogurts showed a lower level of *L. delbrueckii* subsp. *bulgaricus* than that of *S. thermophilus*. As noted for yogurts with the addition of flaxseed, throughout the cold storage period, yogurts with the addition of amaranth and oats showed a high level of characteristic microflora in accordance with the FIL/IDF (Fédération Internationale de Laitiere/International Dairy Federation) guidelines. The present study showed that the addition of flaxseed has a positive effect on the selected quality characteristics of yogurt. The results indicate that to achieve increased apparent viscosity and to reduce syneresis, it is more advantageous to use milled flaxseed rather than whole flaxseed.

The 1% addition of flaxseed allows yogurts to be obtained with favorable technological parameters. However, such an addition is probably insufficient to provide a therapeutic effect, e.g., for the prevention of cardiac diseases. Previous studies indicate that a daily intake of about 20–40 g of flaxseed has a positive effect on health by improving the lipid profile and acting against breast cancer [49,50]. Yogurts are usually available on the market in packages weighing 150–250 g. Yogurt with this weight containing 1% of flaxseed will allow 1.5–2.5 g of this ingredient in one package. This amount of flaxseed has a positive effect on the selected physico-chemical properties of the obtained yogurts and can be just one of the sources of this component during the day. Increasing the dose of flaxseed in yogurt could require the use of additional stabilizers in its production, which would make it impossible to obtain a "clean label" product. Future research should focus on obtaining yogurts with a dose of linseed close to the therapeutic dose, which also have good physico-chemical properties. The presented study focused mainly on the effect of the addition of different doses of flaxseed on the physico-chemical properties of yogurt—mainly its stability. However, there is a need for further research to determine the impact of the storage process on the chemical composition of flaxseed. The impact of the storage process on the level and bioavailability of selected beneficial compounds of flaxseed, such as ALA and soluble fiber, should also be investigated. This will allow the potential impact of the consumption of yogurt with the addition of flaxseed on the improvement of human health to be determined.

5. Conclusions

The pilot study showed that the optimal dose of flaxseed added to the tested yogurts was 1% relative to the amount of milk. The main study showed that the time and form of flaxseed supplementation significantly influenced the active acidity, apparent viscosity, and syneresis in the tested yogurts. Active acidity (pH) decreased significantly after the 7 days of storage, and a significantly lower decrease in pH as compared to other samples was demonstrated for the addition of milled flaxseed added after the fermentation process. The highest viscosity was found in yogurt with milled flaxseed added after fermentation, and the lowest viscosity was found in yogurt with whole flaxseed added before fermentation. Therefore, it can be concluded that the addition of milled flaxseed has a positive effect on the apparent viscosity of the yogurts obtained, regardless of whether it is added before or after the fermentation process. Time significantly affects the increase in syneresis for yogurts with added whole flaxseed—both before or after fermentation. Therefore, it can be concluded that to reduce syneresis in yogurts, the addition of milled flaxseed (both before and after fermentation) is more advantageous, because it does not significantly affect the increase in the leakage of whey from the products. No significant differences were observed between the number of yogurt bacteria in the tested yogurts, which indicates

that the addition of flaxseed in any form did not significantly change the content of yogurt bacteria.

The present study shows that the addition of flaxseed has a positive effect on the selected quality characteristics of yogurt. The results indicate that to achieve increased apparent viscosity and to reduce syneresis, it is more advantageous to use milled flaxseed rather than whole flaxseed. The functional and health-promoting properties of flaxseed can help the food processing industry to create a greater variety of foods that contain this plant.

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Review

Fate of Bioactive Compounds during Lactic Acid Fermentation of Fruits and Vegetables

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Abstract: Consumption of lactic acid fermented fruits and vegetables has been correlated with a series of health benefits. Some of them have been attributed to the probiotic potential of lactic acid microbiota, while others to its metabolic potential and the production of bioactive compounds. The factors that affect the latter have been in the epicenter of intensive research over the last decade. The production of bioactive peptides, vitamins (especially of the B-complex), gamma-aminobutyric acid, as well as phenolic and organosulfur compounds during lactic acid fermentation of fruits and vegetables has attracted specific attention. On the other hand, the production of biogenic amines has also been intensively studied due to the adverse health effects caused by their consumption. The data that are currently available indicate that the production of these compounds is a strain-dependent characteristic that may also be affected by the raw materials used as well as the fermentation conditions. The aim of the present review paper is to collect all data referring to the production of the aforementioned compounds and to present and discuss them in a concise and comprehensive way.

Keywords: vitamins; GABA; phenolic compounds; organosulfur compounds; bioactive peptides; biogenic amines

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1. Introduction

Lactic acid fermentation has been applied for centuries on substrates of plant and animal origin. The seasonal and geographical diversity of the raw materials results in a great variability of products. The qualitative and quantitative composition of the micro ecosystem that is developed during fermentation; the biotic and abiotic factors that direct it, along with the physicochemical changes of the substrate itself, have been in the epicenter of intensive research for many decades. Nowadays, the interest in lactic acid fermentation has been re-fueled, and its value has again been praised due to the health benefits that their consumption may confer. Indeed, a series of health benefits, including anti-allergic, anti-hypertensive, anti-inflammatory, anti-diarrheal, anti-infection, and anti-aging, as well as prevention and control of chronic diseases such as cardiovascular diseases, type 2 diabetes, obesity, and cancer, has been associated with the consumption of lactic acid fermented commodities. These health benefits have been attributed to the lactic acid bacteria that drive the fermentation as well as to the bioactive compounds that are present in the final product [1–8]. Their presence depends upon the occurrence of the necessary precursor molecules in the raw materials and the capacity of the lactic acid bacteria strains to carry out the required biotransformations.

Lactic acid fermentation of fruits and vegetables is no exception. Indeed, the suitability of fermented fruits and vegetables as probiotic carriers has been adequately exhibited [9–13].

In addition, specific health benefits have been associated with the consumption of specific products, such as the antioxidant, anti-obesity, anti-cancer, anti-hypertensive, and immunomodulatory potential of kimchi [14].

The biotic and abiotic factors that affect the production of vitamins (especially of the B-complex), gamma-aminobutyric acid, bioactive peptides, as well as phenolic and organosulfur compounds during lactic acid fermentation of fruits and vegetables have attracted specific attention over the last decade. In addition, the production of biogenic amines has also been intensively studied due to the adverse health effects that are caused by their consumption. The aim of the present review paper is to collect all relevant information and to present and discuss them in a concise and comprehensive way.

2. Vitamins

The role of vitamins in human life and well-being is very important; they facilitate metabolic reactions, including energy-yielding ones, as well as many physiological processes. Depending on their chemical nature, they may be distinguished into water-soluble (B-complex, C) and fat-soluble (A, D, E, K) vitamins. They are considered essential micronutrients since the human body is not able to synthesize the majority of them. Thus, adequate dietary supply is necessary to prevent deficiency. Biofortification, i.e., the utilization of microorganisms capable of producing them, has been proposed as a strategy to improve the vitamin content of certain commodities. This approach is particularly valuable in the case of fermented fruits and vegetables.

The vitamin content of fruits and vegetables has been extensively studied. Fruits are recommended as sources of vitamin C; they also contain vitamin K and carotenoids, and leafy vegetables contain vitamin C, folate, and carotenoids [15]. More specifically, cucumbers and Chinese cabbage contain vitamins C, B1, B2, B11, B3, B6, A, E, and K, with Chinese cabbage appearing to contain more per 100 g. Olives contain vitamins B1, B3, B6, A, E, and K; black olives also contain vitamin C, while green olives also contain vitamin B11. Green olives appear to contain quantitatively more vitamins than black olives, with the exception of vitamin K, where they both contain 1.4 mg/100 g. Vitamins B12 and D seem to be absent from cucumbers, Chinese cabbage, and olives (data from fdc.nal.usda.gov, accessed on 29 July 2021).

Vitamin production by lactic acid bacteria has been in the epicenter of intensive research over the last decade, particularly vitamins of the B-complex and, more specifically, vitamins B2 (riboflavin), B9 (folate), and B12 (cobalamin). Vitamin production seems to be a strain-dependent property. Strains of *Enterococcus faecium*, *Lactococcus lactis* subsp. *lactis*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Limosilactobacillus fermentum*, *Lacticaseibacillus rhamnosus*, *Lm. mucosae*, and *Leuconostoc mesenteroides* have been reported as riboflavin producers [16–23]. Extracellular folate production has been reported for strains of *Streptococcus thermophilus*, *Lb. amylovorus*, *Lp. plantarum*, *Latilactobacillus sakei,* and *Lc. lactis*. [24–28], while cobalamin production has been verified for strains belonging to the lactic acid bacteria species *E. faecium*, *E. faecalis*, *La. casei*, *Furfurilactobacillus rossiae*, *Lm. reuteri*, *Lp. plantarum*, *Loigolactobacillus coryniformis*, *Lm. Fermentum,* and *La. rhamnosus* [29–35]. The capacity of lactic acid bacteria strains to produce vitamin B1 (thiamine), B3 (niacin), as well as K2 has also been reported [36–38].

Although fruits and vegetables and, especially, green vegetables have been recognized as the main sources of folates for humans [39] and certain fruits and vegetables and, especially, dark green vegetables are very good sources of riboflavin [40], the fate of vitamins during lactic acid fermentation has only been marginally studied. Jagerstad et al. [41] reported that folate production takes place during lactic acid fermentation, depending on the starter culture. More accurately, the starter culture, consisting of a mixture of *Lp. plantarum*, *Lc. lactis/cremoris* and *Leuconostoc* sp. strains, was able to produce up to 125 μg/kg 5-CH3-H4 folate during fermentation of a mixture of beetroots, turnips, and onions and 110 μg/kg during fermentation of a mixture of roots consisting of carrots, turnips, parsnips, celeriacs, and onions. Thompson et al. [42] used four *Lp. plantarum* strains

to ferment cauliflower, white beans, and their 50:50 mixture and reported a statistically significant increase in riboflavin and folate content. More accurately, after fermentation of the latter at 30 °C for 44 h, riboflavin increased to 75.64–91.60 μg/100 g fresh weight from the 42.83 μg/100 g fresh weight of the unfermented control; folate increased to 48.74–58.82 μg/100 g fresh weight from the 36.84 μg/100 g fresh weight of the unfermented control. In addition, *Lp. plantarum* strain 299 was able to produce vitamin B12, increasing its concentration to 0.048 μg/100 g fresh weight from the 0.029 μg/100 g fresh weight of the unfermented control.

3. Gamma-Aminobutyric Acid

The occurrence of gamma-aminobutyric acid (GABA) in plants, microorganisms, and vertebrates has been adequately exhibited. In plants and humans, GABA is mostly associated with signaling functions. Indeed, its role in plant growth and stress response has been established [43–45]. In humans, it acts as the major inhibitory neurotransmitter in the central nervous system. The latter has played a decisive role in the ongoing trend of enriching food with this molecule; however, Hepsomali et al. [46] mentioned that although GABA oral intake resulted in various responses [47–49], it is still unknown whether brain GABA concentration is increased. On the other hand, it seems to have a different role in microorganisms; it has been associated with resistance to acidic conditions [50] as well as spore germination, at least in *Neurospora crassa* [51] and *Bacillus megaterium* [52]. In lactic acid bacteria, GABA production has been reported as a strain-dependent characteristic. It takes place mostly through L-glutamate decarboxylation since it also contributes to acid resistance through proton consumption [53]. L-glutamate supply may be exogenous through the glutamate/GABA antiporter or endogenous through the activity of glutamate synthase on α-ketoglutaric acid. Then, GABA may be transported extracellularly through the aforementioned antiporter or degraded to succinic acid through GABA aminotransferase and succinate semialdehyde dehydrogenase (Figure 1) Among others, strains belonging to *E. durans*, *La. paracasei*, *La. rhamnosus*, *Lp. plantarum*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lc. lactis* subsp. *lactis*, *Le. buchneri*, *Leu. mesenteroides*, *Leu. pseudomesenteroides*, *Lv. brevis*, *S. salivarius* subsp. *thermophilus*, and *Weissella cibaria* have been reported as GABA-producers [54–56].

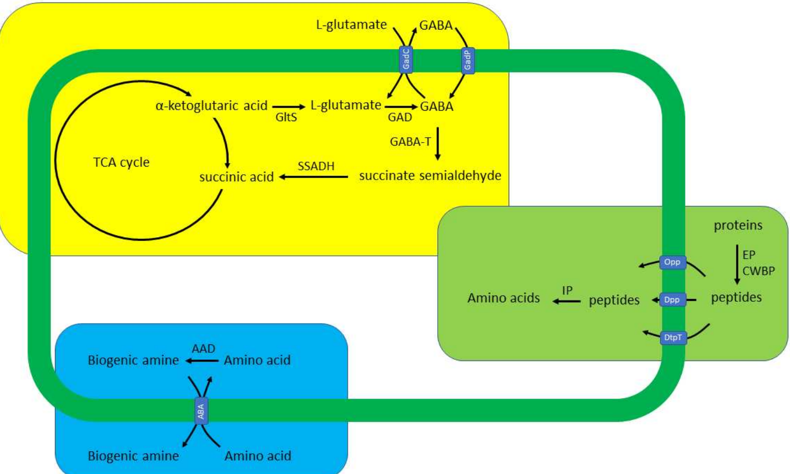

Figure 1. Production of GABA (**yellow box**), bioactive peptides (**green box**), and biogenic amines (**blue box**) by LAB. AAD: amino acid decarboxylase; ABA: amino acid/biogenic amine antiporter; CWBP: cell-wall-bound proteinases; Dpp: peptide (2–9 amino acids) ABC transporter; DtpT: ion linked peptide (2–3 amino acids) transporter; EP: extracellular proteinases; GABA-T: GABA aminotransferase; GAD: glutamate decarboxylase; GltS: glutamate synthase; IP: intracellular peptidases; Opp: oligopeptide (4–18 amino acids) permease; SSADH: succinate semialdehyde dehydrogenase.

The amount of GABA synthesized by a plant depends upon several factors, such as variety, type, and severity of biotic and abiotic stresses; however, its occurrence has been

characterized as ubiquitous [57]. Indeed, GABA amount may range from the 0.007 mg/g dry weight in an epicarp/mesocarp mixture of apples and the 0.019 mg/g dry weight of chestnuts to the 1.86 mg/g dry weight of mulberries and the 174.30 mg/g fresh weight of *Vitis vinifera* L. cultivar Pinot Noir [58–61]. Regarding the raw materials mostly used as substrates for lactic acid fermentation, the occurrence of GABA has also been reported. In olives and in extra virgin olive oil, the amount of GABA was cultivar-dependent [62,63]. In the latter case, its amount was less than 0.00014 mg/g. Leaves and roots of Chinese cabbage were reported to contain 4.69 and 7.02 μmol/g dry weight, respectively, accounting for the 8% and 26.86% of total free amino acids, respectively [64]. Finally, fresh cucumbers were reported to contain 105 mg/kg GABA [65]. Current evidence shows that lactic acid fermentation may increase GABA content. Indeed, spontaneously fermented cucumbers were reported to contain 150 mg/kg GABA, with the majority of it being formed during the first day of fermentation [65]. Notably, GABA concentration remained stable throughout the 6-month storage period at 28 °C. In the case of spontaneously fermented olives, GABA was formed only upon monosodium glutamate addition [66]. The amount of GABA formed was proportional to the amount of monosodium glutamate added and irrespective of the osmotic dehydration of olives, which was applied as a pre-fermentation treatment. GABA production was also reported during spontaneous kimchi fermentation [67]. In that study, GABA production took place within the first 25 days of storage at 4 °C, reaching approximately 4 mM; this amount of GABA remained stable until the end of storage (120 days). Analysis of the microecosystem identified strains of *Leuconostoc* spp. and *Lt. sakei* as the GABA producers. Seok et al. [68], Cho et al. [69], and Lee et al. [70] studied GABA production during kimchi fermentation inoculated with GABA producing strains. Seok et al. [68] used *Lactobacillus* sp. strain OPK 2–59 and 5 g monosodium glutamate and managed to produce 18 mg/100 g GABA, a notable increase from the initial amount of 2.84–4.06 mg/100 g. Interestingly, rapid GABA production was observed after the 9th day of storage. In the kimchi produced by the addition of either the GABA-producing strain or monosodium glutamate, the GABA amount at the end of storage (21 d) was less than 6 mg/100 g. Cho et al. [69] analyzed commercially available kimchi and Mukeunjee kimchi products and reported that the GABA content ranged from 1.9 to 12.9 mg/100 g and from 18.2 to 99.0 mg/100 g, respectively. Then, a GABA-producing *Le. buchneri* strain was employed as a starter culture, resulting in kimchi with 61.7 mg/100 g GABA, which was significantly higher than the 8.1 mg/100 g of the spontaneously fermented one. Notably, the sensory scores of the products were comparable. Lee et al. [70] prepared kimchi with the addition of *Lv. zymae* strain GU240 as a starter culture and evaluated the effect of L-glutamic acid, monosodium glutamate, and kelp extract as GABA precursors. Storage took place at −1 °C for 20 weeks. Monosodium glutamate was the most effective GABA precursor. The most rapid increase was observed between weeks 2 and 4, and the maximum GABA concentration reached 120.3 mg/100 g in week 8. Then, it was reduced to the final amount of 95.6 mg/100 g. The GABA content of the kimchi that was prepared without the addition of starter or precursor, as well as the kimchi prepared with only the addition of a starter, was 47 mg/100 g. The addition of kelp extract resulted in the accumulation of 55 mg/100 g GABA, and the addition of L-glutamate resulted in 62.5 mg/100 g. In all cases, maximum GABA concentration was observed in weeks 8 and 10, which was then reduced until the end of storage (week 20).

4. Bioactive Peptides

Bioactive peptides are short peptides that, upon release from the parent protein molecule, exert a biological function. Decryption from the parent protein molecule may take place during gastrointestinal digestion or due to the proteolytic activity of microorganisms, such as the LAB that direct a fermentation process. Their occurrence depends on the activity of extracellular and cell envelope proteinases, as well as the peptide transportation capacity into the cell, towards their complete hydrolysis to amino acids [71] (Figure 1). A wide range of biological activities has been described for such peptides, including anti-

diabetic, antioxidant, anti-microbial, anti-thrombotic, hypocholesteromic, hypotensive, mineral-binding, opioid, and anti-opioid.

The liberation of bioactive peptides through lactic acid fermentation of protein-rich substrates, such as milk and soy, has been extensively studied. Data on the bioactivity of the peptides released by the lactic acid fermentation of meat, fish, grains, and legumes are also available. Thus, the decryption of such peptides through the application of LAB, such as *E. faecalis*, *Lp. plantarum*, *Lb. helveticus*, *La. casei*, *La. rhamnosus*, *Companilactobacillus farciminis*, *Fructilactobacillus sanfranciscensis*, *Lc. lactis*, *Lb. delbrueckii* subsp. *lactis*, and *Pediococcus acidilactici* single strains [35,72–81], or microbial consortia [82–90], has been reported.

In general, fruits and vegetables are not rich in protein; however, the occurrence of bioactive peptides in some of them has been reported (recently reviewed by Sosalagere et al. [91]). Cucumbers, Chinese cabbage, and green and black olives contain 0.65%, 1.5%, 1.03%, and 0.84% protein, respectively (https://fdc.nal.usda.gov/, accessed on 21 August 2021). In olive seeds, the occurrence of the peptide LLPSY exhibited significant anti-proliferative capacity on prostate cancer cells (PC-3) and breast cancer cells (MDA-MB-468) [92]. Occurrence of bioactive peptides in cucumbers that were raw, acidified, spontaneously fermented, or fermented with the addition of *Lp. pentosus* strain LA0445 was assessed by Fideler et al. [93]. Five peptides with potential anti-hypertensive activity were detected, namely, IPP, LPP, VPP, KP, and RY. KP was present in all cases; the amount in the fermented ones was significantly higher than the rest. Acidified cucumbers also contained KY, a peptide that was not detected in spontaneously fermented ones. The cucumbers that were fermented with the addition of the starter culture contained all five peptides [93].

5. Phenolic Compounds

The occurrence of phenolic compounds in plants has been extensively assessed. They are the third-largest group of secondary metabolites, after terpenes and alkaloids; they hold a very important physiological role as they participate in processes such as photosynthesis, respiration, and cell development. Regarding the total phenolic content (TPC) of the fruits and vegetables mostly used as a substrate of lactic acid fermentation, it seems to be rather low; it has been reported to vary between 0.58–1.42 mg GAE/g fresh weight for Chinese cabbage, 0.17 mg GAE/g fresh weight for cucumbers, and 82.29–287.29 mg GAE/100 g for olives [94,95]. Their amount depends upon factors associated with the plant type and variety, cultivation conditions, processing, and storage [96,97]. The interest in phenolic compounds is fueled by the correlation that has been achieved between them and antioxidant capacity as well as the prevention of chronic diseases and inflammation [98].

Based on the fact that lactic-acid-fermented fruits and vegetables consist of two phases, namely, a solid and a liquid one, Ciniviz and Yildiz [99] studied the TPC of both juice and pulp portions of 30 kinds of lactic acid fermented fruits and vegetables. In all cases but two, namely, wild pears pickle and sour grapes pickle, the amount of TPC in juice was higher than the respective in the pulp portion. In the latter, TPC ranged from below detection limit in carrots pickle and white cabbage pickle to 135.39 μg GAE/mg in pinecone pickle, while in the juice portion, it ranged from 16.94 μg GAE/mg in tomatoes pickle to 235.19 μg GAE/mg in pinecone pickle. The most common phenolic acid seemed to be sinapic acid, which was detected in all juice and pulp samples at concentrations ranging from 135.91 mg/L in sour grapes pickle to 236.32 mg/L in sweet long green pepper pickle and from 104.25 mg/kg in white cucumber pickle to 107.43 mg/kg in unripe melon pickle, respectively. Vanillic acid, caffeic acid, and chlorogenic acid were present in all juice samples, ranging from 0.08 mg/L in white cabbage pickle to 31.81 mg/L in carrot pickle, from 30.06 mg/L in unripe melon pickle and chard pickle to 74.61 mg/L in hot pepper pickle, and from 62.21 mg/L in cauliflower pickle to 200.30 mg/L in rock samphire pickle, respectively. 4-hydroxybenzoic acid and p-coumaric acid were not detected in any sample.

The fate of phenolic compounds during lactic acid fermentation has only been marginally studied. The mode by which lactic acid fermentation may increase the TPC of the raw materials is either through the lysis of the cell wall of the plant cells with concomi-

tant facilitation of their release from the vacuole, in which they are mainly localized, or by enzymatic conversion of their glycosides into their aglycone form [100]. The latter may take place through β-glycosidase activity, which several lactic acid bacteria strains have exhibited [101,102]. Indeed, several *Lp. plantarum* strains have been reported to hydrolyze oleuropein, which is the main phenolic glucoside of olives [103]. More accurately, an initial action of β-glycosidase, followed by an esterolytic activity on the aglycone moiety, has been reported to produce olenoic acid and hydroxytyrosol [100]. Moreover, through the production of phenolic acid decarboxylases, some *Lp. plantarum* strains may decarboxylate phenolic acids [104,105].

A wide range of phenolic compounds have been reported to occur in the brine or the flesh of fermented olives, including apigenin, apigenin-7-O-glucoside, caffeic acid, p-coumaric acid, cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, ferulic acid, p-hydroxybenzoic acid, hydroxytyrosol, luteolin, luteolin-4-O-glucoside, luteolin-7-O-glucoside, protocatechuic acid, pyrocathecol, rutin, tyrosol, vanillic acid, vanillin, and verbascoside [106–110]. Their fate during fermentation depends upon the cultivar, the addition and type of starter culture, brine composition, as well as fermentation temperature and time [107–109]. Indeed, proper arrangement of the aforementioned conditions may result in the complete decomposition of oleuropein and an increase in hydroxytyrosol, tyrosol, p-coumaric acid, vanillic acid, caffeic acid, verbascoside, and ferulic acid [107,109,110]. Initial concentration increase has also been reported for apigenin-7-O-glucoside, luteolin-4-O-glucoside, and luteolin-7-O-glucoside, which was followed by a decrease until the end of fermentation [110].

In the case of kimchi, Park et al. [111] reported that over-ripened kimchi contained more TPC than short-term fermented ones. Park et al. [112] reported that the TPC of mustard kimchi increased during the first two months to 482.4 mg GAE/g extract powder but then decreased during the third month to 475.3 mg GAE/g extract powder, which had no statistically significant difference from the control. Regarding the specific phenolic compounds assessed, the amount of caffeic acid increased throughout the three months of fermentation; the amount of naringin, catechin gallate, and epigallocatechin gallate initially increased, but after three months of fermentation, their amount was less than that of the control. The amount of chlorogenic acid and epicatechin gallate decreased throughout the three-month fermentation compared to the control; p-coumaric acid and gallocatechin gallate were only detected after one month of fermentation, and catechin was only detected after one and two months of fermentation. Epicatechin and rutin were present in the control, and their amount increased after two months of fermentation. However, they were not detected after three months of fermentation. Finally, gallic acid and epigallocatechin were not detected to the control and throughout fermentation. Oh et al. [113] studied the TPC of Dolsan leaf mustard kimchi and reported that the TPC of leaves decreased during the first 21 days of fermentation but then increased to the initial amount of ca. 100 mg GAE/100 g. On the contrary, the TPC of stems gradually increased from the initial ca. 40 mg GAE/100 g to a final of ca. 110 mg GAE/100 g. A novel insight was provided by Jung et al. [114]. In that study, the increase of TPC over the 24-day fermentation of kimchi made of young Chinese cabbage was reported. However, the initial TPC and the TPC at the end of fermentation were determined at 83.2 and 102.5 mg GAE/100 g, respectively, when the young Chinese cabbage was cultivated using nature-friendly composts. These amounts of TPC were higher than 63.2 and 98.2 mg GAE/100 g, respectively, when the young Chinese cabbage was cultivated using general composts and higher than 57.9 and 81.0 mg GAE/100 g, respectively, when the young Chinese cabbage was cultivated using chemical fertilizers.

Ciska et al. [115] and Kapusta-Duch et al. [116] studied the TPC of sauerkraut. The first study reported that sauerkraut extract contained 8.25 mg/g TPC while white cabbage contained 5.72 mg/g. In white cabbage, only esterified phenolic acids were detected, with sinapic acid being the most prevalent (278 μg/g). On the contrary, apart from esterified phenolic acids, their glycosides were also detected in the sauerkraut extract. As in the previous

case, sinapic acid was the prevalent one, with 20 μg/g being quantified as esterified acid and 84 μg/g as its glycoside. Kapusta-Duch et al. [116] assessed the effect of package type, namely, low-density polyethylene and metalized polyethylene terephthalate with polyethylene bags, on the TPC content during four months of chilled storage of white sauerkraut. It was revealed that package type had no effect on the TPC levels as in both cases, the reduction was at ca. 12% and 20% after three and four months of storage, respectively.

The fate of phenolic compounds has also been assessed in less known regional lactic acid fermented fruit and vegetable products, such as African nightshade leaves and kiwi fruit. In the first case, the effect of fermentation that was carried out at 37 °C for 3 days on the phenolic profile of the product was strain-dependent [117]. For example, fermentation with *Lp. plantarum* strain 75 resulted in an increase of the amount of gallic acid, vanillic acid, 2,5 dihydroxybenzoic acid, p-coumaric acid, and ellagic acid, as well as the flavonoids assessed, namely, catechin, quercetin, and luteolin. On the contrary, fermentation with *Leu. pseudomesenteroides* strain 56 resulted in an increase of the amount of ellagic acid and quercetin and a decrease in gallic acid, caffeic acid, vanillic acid, 2,5 dihydroxybenzoic acid, p-coumaric acid, ferulic acid, and catechin. The effect of fermentation at 37 °C for 28 h by *Lp. plantarum* on the phenolic profile of kiwifruit pulp was studied by Zhou et al. [118]. The TPC increased after the 21st hour of fermentation. The amount of protocatechuic acid, esculetin, and p-coumaric acid was increased due to the fermentation, while the amount of gallic acid, chlorogenic acid, catechin, and epicatechin was decreased.

6. Organosulfur Compounds

Vegetables of the family Brassicaceae are very rich in organosulfur compounds in general and glucosinolates in particular. Among others, this family includes all types of cabbage and mustard greens, which are very important raw materials for lactic acid fermentation.

Glucosinolates are secondary metabolites, the stability of which depend upon their contact with myrosinase, a β-thioglucosidase that catalyzes its decomposition. In intact plant cells, they are spatially separated; however, upon conditions that compromise plant tissue integrity, such as infection by herbivores and phytopathogenic microorganisms, the substrate and the enzyme are mixed, leading initially to the formation of the unstable thiohydroximate-O-sulfonate and β-D-glucose. The fate of the former depends on the nature of the side chain present in the glucosinolates molecule as well as the environmental conditions. Especially regarding the latter, neutral pH favors the formation of isothiocyanates while acidic pH in the presence of ferrous ions and epithiospecifier protein favors the formation of nitriles [119–121]. The physiological role of glucosinolates and their breakdown products, especially isothiocyanates, against biotic stresses has been verified [122]. Their concentration depends upon plant species, variety, and tissue, as well as environmental conditions and agricultural practices [123]. Although this response may indicate a possible role in abiotic stresses as well, this has not been yet clarified [122].

The interest on glucosinolates and their breakdown products results from their biological activity; many of them have exhibited anti-bacterial, anti-fungal, and anti-proliferative activity against human cancer cells [124]. The biotransformations of glucosinolates during lactic acid fermentation of *Brassica* vegetables have been studied to some extent. In general, fermentation seems to facilitate glucosinolates decomposition and an increase of the concentration of the breakdown products, the type and concentration of which are related to the glucosinolate type and concentration in the raw material as well as the capacity of the microbial strains that drive the fermentation.

Glucosinolate decomposition during fermentation has been exhibited in the case of sauerkraut [125–128] and has been primarily attributed to the shredding of the cabbage that precedes fermentation and secondarily to hydrolysis by lactic acid bacteria [129,130]. Interestingly, the capacity of LAB to produce nitriles instead of reduced glucosinolates, which were produced by Enterobacteriaceae, was highlighted by Mullaney et al. [129].

Ciska and Pathak [131] reported that glucobrassicin and sinigrin were the most abundant glucosinolates in the shredded cabbage used for fermentation. Ascorbigen, indole-3-

carbinol, and indole-3-acetonitrile were identified as degradation products of the former, while allyl isothiocyanate, allyl cyanide, and 1-cyano-2,3-epithiopropane were identified as degradation products of the latter.

Penas et al. [132] highlighted the importance of the starter culture, cabbage cultivar, and fermentation conditions on the volatile glucosinolate breakdown products. Iberin, iberin nitrile, allyl isothiocyanate, sulforaphane, and allyl cyanide were detected, with the latter being the most abundant, ranging from 65 to 75 μmol/100 g DM. Ascorbigen has been reported as the most abundant glucosinolate degradation product in sauerkraut [126,128,131,133]. Ciska and Pathak [131] reported that ascorbigen concentration could be as high as 14 μmol/100 g. Palani et al. [126] quantified ascorbigen at the end of fermentation at 13 μmol/100 g FW. Indole-3-acetonitrile was also present at the end of fermentation at 4.52 μmol/100 g FW. The concentration of both compounds decreased during storage at 4 °C. These results concur with the ones presented by Penas et al. [133] but only as far as the decrease of ascorbigen concentration is concerned; the concentration of indole-3-carbinol and indole-3-acetonitrile was stable throughout three-month storage at 4 °C. Ascorbigen was also reported by Ciska et al. [128] as the main glucobrassicin breakdown product in sauerkraut, which, at the end of fermentation, reached 9.59 μmol/100 g. The concentration of indole-3-acetonitrile and 3,3′-diindolylmethane also increased during fermentation to 0.036 and 0.0099 μmol/100 g, respectively. After 17 weeks of storage at 5 °C, the concentration of ascorbigen decreased to 8.59 μmol/100 g, but the respective of indole-3-acetonitrile and 3,3′-diindolylmethane increased to 0.057 and 0.0187 μmol/100 g, respectively. Regarding the decomposition products of aliphatic and aryl glucosinolates, an increase in the concentrations of allyl isothiocyanate, but-3-enyl isothiocyanate, 3-(methylthio) propyl isothiocyanate, 1-cyano-3-(methylthio) propane, 4-(methylthio) butyl isothiocyanate, 3-(methylsulfinyl) propyl isothiocyanate, 1-cyano-3-(methylsulfinyl) propane, 4-(methylsulfinyl) butyl isothiocyanate, and 2-phenethyl isothiocyanate was reported at the end of fermentation. Isothiocyanates increased during the first days of fermentation, reaching their peak on the 4th day and then decreasing. Allyl isothiocyanate was the most abundant breakdown product, with 2.848 μmol/100 g, followed by 3-(methylsulfinyl) propyl isothiocyanate, with 2.453 μmol/100 g. The concentration of all compounds decreased after 17 weeks of storage at 5 °C, with the exception of 1-cyano-3-(methylthio) propane, which increased [128].

The significance of starter cultures in the fate of glucosinolates during the fermentation of broccoli puree and juice was highlighted by Cai et al. [134], Ye et al. [135], and Xu et al. [136]. Ye et al. [135] studied the effect of lactic acid fermentation of autoclaved broccoli puree using five *Lp. plantarum* and 2 *Leu. mesenteroides* strains on the glucosinolate content. In general, a total of 10 glucosinolates have been detected in broccoli florets, namely, glucoalyssin, glucobrassicanapin, glucobrassicin, 4-hydroxy glucobrassicin, 4-methoxy glucobrassicin, glucoerucin, glucoiberin, glucoraphanin, neoglucobrassicin, and progoitrin [137–139]. A strain-dependent increase in the concentration of glucoraphanin, glucoiberin, and progoitrin to 29.0–236.5, 16.1–56.2, and 24.5–65.9 μg/g, respectively, from the initial trace levels, was reported. Notably, the maximum amounts were achieved by *Lp. plantarum* strain F1. Xu et al. [136] reported an increase of glucoraphanin, a decrease of gluconapin, glucoerucin, 4-hydroxy-glucobrassicin, and neoglucobrassicin, and no statistically significant change in glucobrassicin and 4-methoxy-glucobrassicin when juice made of broccoli florets was fermented with two *P. pentosaceus* strains at 37 °C for 36 h, in a strain-dependent manner. Finally, Cai et al. [134] reported that the preheating of broccoli florets at 65 °C for 3 min increased the concentration of sulforaphane, a glucoraphanin decomposition product, from the initial 806 to 3536 μmol/kg DW. Fermentation by a mixture of *Lp. plantarum* and *Leu. mesenteroides* strains at 30 °C for 15 h further enhanced sulforaphane concentration to 13,121.3 μmol/kg DW, most likely by facilitating the release and accessibility of glucoraphanin for decomposition.

Endogenous myrosinase inactivation and concomitant sinigrin retention after lactic acid fermentation of Indian mustard leaves at 22 °C for 7 d were assessed by Nugrahedi et al. [140].

Although oven heat treatment at 35 °C for 2.5 h and microwave treatment at 180 W for 4.5 min effectively reduced myrosinase activity, complete inactivation was achieved by microwave treatment at 900 W for 2 min, leading to the production of sayur asin with the sinigrin concentration of 11.4 μmol/10 g d.m. Mustard leaves are also the basic ingredient for the production of mustard leaf kimchi. Oh et al. [113] studied the fate of glucosinolates during the fermentation of mustard leaf kimchi at 0 °C for 35 d. Sinigrin, gluconapin, glucobrassicin, and glucoraphanin were detected at day 0 in both mustard leaves and stems; gluconasturtiin was only detected in leaves, while glucoiberin was only detected in stems. Reduction of the total amount of glucosinolates was evident throughout fermentation in both leaves and stems, which is mainly assigned to the reduction of sinigrin concentration, which was the most abundant glucosinolate; it was quantified at 21.43 and 22.47 mg/100 g at day 0 and 12.5 and 10.4 mg/100 g at day 35 in leaves and stems, respectively.

7. Biogenic Amines

Biogenic amines are compounds formed through the amination and transamination of aldehydes and ketones or the decarboxylation of amino acids. Their physiological role is very important. In plants, the role of polyamines in cell division [141], root growth [142–145], and vegetative propagation [146,147], as well as flower and fruit development [148–154], has been exhibited. Moreover, their role in abiotic and biotic stress responses has also been claimed [155–168]. Similarly, the contribution of cadaverine and dopamine to signaling stress response as well as plant growth and development has been reported [169,170]. In addition, tyramine and tryptamine are produced as defensive substances against aggressors [171] and serve as precursors for the production of alkaloids [172] and melatonin [173], respectively.

Regarding microbial physiology, the role of biogenic amines in gene expression [174,175], protection against oxidative stress [176–179], biofilm formation [180,181], signaling [182,183], and virulence [184,185] has been indicated. From a fermentation perspective, the most important role seems to be the response mechanism against acid stress. This mechanism involves a membrane antiport, which couples amino acid uptake with biogenic amine excretion, and intracellular amino acid decarboxylases, which decarboxylate the inserted amino acid with simultaneous proton consumption (Figure 1). Then, the amine is excreted, and ATP synthesis through proton motive force is directed [186,187]. Such mechanisms have been reported for histidine/histamine, lysine/cadaverine, ornithine/putrescine, and tyrosine/tyramine [186,188,189].

Based on the above, the occurrence of biogenic amines in plant tissues seems justified even without microbial infection and proliferation. Indeed, several studies have reported their presence in nonfermented fruits, vegetables, nuts, legumes, and cereals (reviewed by Sanchez-Perez et al. [190]). Putrescine seems to be commonly occurring and may be accompanied by tyramine, cadaverine, spermine, spermidine, and even histamine [190,191]. This is also the case for white cabbage, Chinese cabbage, and cucumbers, which are commonly used as raw materials for lactic acid fermentation [192–198]. On the other hand, the occurrence of biogenic amines has not been reported in the flesh of fresh olives at any ripeness stage [199].

In Table 1, the outcome of studies on the quantitative determination of biogenic amines in fermented fruits and vegetables is summarized. Kimchi seems to be the most studied product, most likely due to the variety of raw materials employed, which results in a large diversity of products [14]. Regarding the mean values that have been reported, the highest were 16.81 mg/kg for agmatine [200], 14.3 and 49.8 mg/100 g for cadaverine and histamine, respectively [201], 4.4 mg/kg for phenylethylamine [202], 334.64 mg/kg for putrescine [203], 31.30 mg/kg for spermine [204], and 24.6, 32.3 and 78.0 mg/kg for spermidine, tryptamine and tyramine, respectively [205]. Regarding the highest amounts, agmatine was reported at 86.0 mg/kg [200], phenylethylamine and tyramine at 15.75 and 181 mg/kg, respectively [204], putrescine at 982.32 mg/kg [203], while for cadaverine, histamine, spermidine, spermine, and tryptamine were reported at 155, 535, 8.8, 12.1, and 11.4 mg/100 g, respectively [201].

Table 1. Occurrence of biogenic amines in fermented fruits and vegetables.

Product.	N	AGM	CAD	HIS	PHE	PUT	SPD	SPM	TRP	TYR
Kimchi types										
Kkakdugi kimchi [1]	5		27.28 (54.44) [<0.1–124.60]	55.94 (44.45) [18.75–127.78]	3.61 (6.55) [<0.1–15.24]	334.64 (427.97) [10.85–982.32]	9.40 (6.68) [<0.1–16.76]	1.03 (1.31) [<0.1–3.10]	<0.1	25.42 (29.59) [2.97–76.95]
Chonggak kimchi [1]	5		64.08 (65.51) [2.00–148.50]	58.73 (46.02) [8.24–131.20]	0.78 (1.23) [<0.1–2.80]	269.07 (349.93) [3.89–853.70]	9.06 (2.99) [6.10–14.00]	6.23 (8.79) [<0.1–20.74]	9.02 (9.86) [<0.1–23.70]	8.49 (6.80) [0.79–18.70]
Pa kimchi [2]	13		44.07 (42.85) [<0.1–123.29]	155.85 (139.26) [8.67–386.03]	1.77 (2.04) [<0.1–5.97]	78.79 (79.00) [<0.1–158.33]	9.91 (4.89) [2.32–18.74]	21.75 (8.94) [<0.1–33.84]	6.99 (5.74) [<0.1–14.92]	66.88 (74.91) [<0.1–181.10]
Gat kimchi [2]	13		20.5 (18.52) [2.12–48.60]	58.44 (75.77) [3.30–232.10]	3.44 (4.30) [<0.1–15.75]	134.96 (220.53) [1.89–720.82]	20.31 (6.35) [12.26–28.49]	31.30 (22.35) [<0.1–58.57]	11.22 (8.23) [<0.1–26.74]	76.15 (65.91) [1.28–149.77]
Cabbage kimchi (Korean) [3]	10		15.2 [3.6–44.9]	50.0 [3.4–142.3]	3.0 [nd–6.8]	69.7 [15.1–44.9]	12.0 [7.8–16.5]	2.4 [1.2–3.7]	12.3 [2.3–22.6]	49.4 [9.7–118.2]
Cabbage kimchi (Chinese) [3]	10		12.5 [3.7–31.0]	2.7 [0.6–8.5]	4.4 [2.1–6.7]	70.6 [16.0–240.4]	11.9 [7.7–15.2]	2.1 [nd–3.7]	12.1 [2.4–20.0]	35.1 [10.7–76.0]
Baechu kimchi [4]	14		18.0 (18.6) [nd–45.0]			64.6 (73.1) [nd–245.9]	7.8 (5.8) [nd–14.9]		15.0 (16.1) [tr–43.9]	44.0 (35.8) [tr–103.6]
Kkakduki [4]	5		31.0 (26.8) [nd–56.2]			30.3 (21.7) [nd–51.6]	6.7 (10.3) [nd–21.8]		14.2 (6.1) [5.5–18.6]	4.8 (5.6) [nd–10.8]
Chonggak kimchi [4]	3		28.6 (49.5) [nd–85.7]			10.7 (10.2) [nd–20.3]	nd		7.3 (6.9) [2.3–15.2]	45.4 (21.9) [20.2–58.1]
Matkimchi [4]	4		30.5 (35.3) [nd–64.2]			72.1 (27.7) [40.2–104.6]	5.2 (4.5) [nd–10.8]		32.3 (26.9) [nd–60.5]	78.0 (22.0) [54.3–105.1]
Ripened Baechu kimchi [4]	4		55.7 (18.9) [28.0–63.3]			110.3 (44.4) [57.2–154.6]	24.6 (34.0) [tr–74.8]		5.5 (6.7) [nd–13.6]	46.6 (39.6) [nd–95.6]
Baek kimchi [4]	3		18.5 (7.1) [11.5–25.6]			20.7 (18.9) [1.9–39.6]	0.8 (0.9) [nd–1.7]		tr	36.4 (28.6) [7.8–64.9]
Super market kimchi [5]	20	<0.1	14.3 (9.2) [<0.1–155]	49.8 (32.5) [<0.1–535]	<0.1	2.06 (1.33) [<0.1–7.3]	0.65 (0.51) [<0.1–8.8]	1.96 (1.31) [<0.1–12.1]	1.0 (1.05) [<0.1–11.4]	0.46 (0.48) [<0.1–4.2]
Retail market kimchi [5]	17	<0.1	1.59 (1.44) [<0.1–4.8]	5.59 (4.57) [<0.1–18.6]	<0.1	0.67 (0.79) [<0.1–5.1]	0.48 (0.52) [<0.1–8.2]	<0.1	<0.1	0.40 (0.65) [<0.1–3.5]

Table 1. *Cont.*

Product.	N	AGM	CAD	HIS	PHE	PUT	SPD	SPM	TRP	TYR
Cabbage kimchi [6]	20		8.3 [0.9–39.8]	6.3 [nd–21.8]	0.5 [nd–2.0]	47.6 [2.3–148.6]	2.9 [nd–6.7]	1.1 [nd–5.1]	11.6 [nd–74.8]	8.3 [1.1–27.9]
Kimchi [7]	ud	16.81 (30.81) [<0.14–86.00]	63.51 (69.81) [1.13–193.00]	18.53 (28.07) [<0.09–74.94]	2.59 (1.27) [0.94–4.50]	208.70 (186.90) [2.25–475.06]	10.35 (4.49) [5.55–18.25]	1.38 (0.68) [0.56–2.38]	4.75 (9.41) [<0.29–24.88]	59.11 (44.70) [1.25–98.31]
					Sauerkaut					
Czech [8]	53		64.8 (56.8) [1.9–293]	12.1 (31.6) [nd–229]		181 (108) [2.8–529]	8.2 (7.3) [nd–47.0]		4.6 (9.0) [nd–36.5]	235 (213) [nd–951]
Austrian [8]	10		43.4 (21.0) [19.3–77.4]	2.1 (2.4) [nd–8.0]		179 (80.2) [51.0–295]	6.5 (5.5) [nd–16.9]		2.4 (3.2) [nd–7.7]	130 (71.3) [14.0–214]
Household [8]	29		29.8 (23.0) [nd–82.7]	4.6 (6.8) [nd–32.4]		87.3 (72.2) [4.3–260]	10.2 (7.5) [nd–28.3]		4.7 (7.9) [nd–28.1]	117 (113) [nd–384]
Sterilized [8]	29		45.5 (40.1) [6.9–167]	4.9 (6.4) [nd–26.4]		132 (81.5) [18.4–359]	6.8 (4.0) [nd–15.2]		7.2 (10.2) [nd–37.5]	134 (90.4) [26.3–345]
Sauerkraut [9]			3.9	1.5	tr	9.2	0.5	0.2	nd	4.8
					Cucumbers					
Fermented cucumber brine [10]	1	3.19	45.11	3.07	1.83	61.70	21.16	9.77	7.37	5.24
Pickled cucumbers [11]	11		nd	nd		4.5 (5.0)		2.9 (4.2)		0.7 (0.8)
Cucumber [7]	ud	0.65 (1.05) [<0.14–2.88]	82.14 (44.03) [39.60–179.19]	31.54 (7.43) [18.50–40.85]	3.10 (1.64) [1.15–6.31]	171.30 (55.46) [103.13–286.88]	8.08 (3.77) [2.25–14.05]	1.28 (0.71) [0.56–2.65]	14.49 (5.82) [6.00–22.88]	62.24 (20.12) [28.38–86.75]
					Olives					
Fermented olive brine [10]	1	0.81	15.62	1.14	1.78	42.94			0.51	6.93
Olives [7]	ud	<0.14	2.54 (3.28) [<0.06–6.25]	1.71 (1.58) [<0.09–3.13]	0.23 (0.40) [<0.35–0.70]	17.13 (15.00) [5.75–34.13]	1.21 (0.85) [0.25–1.88]	1.58 (0.85) [0.60–2.13]	3.33 (5.77) [<0.29–10.00]	2.56 (1.29) [1.75–4.05]
Olives [12]	7		0.80 (0.00) [<0.4–0.8]	nd		5.00 (2.96) [<0.5–7.8]				nd

Table 1. *Cont.*

Product.	N	AGM	CAD	HIS	PHE	PUT	SPD	SPM	TRP	TYR
Various products										
Beetroot [7]	ud	0.48 (0.94) [<0.14–2.50]	5.45 (7.96) [0.10–20.50]	6.84 (12.84) [<0.09–31.25]	0.63 (0.96) [<0.35–2.25]	21.25 (33.98) [1.80–80.65]	2.31 (0.54) [1.35–3.00]	0.80 (0.84) [0.45–2.88]	2.46 (5.08) [<0.29–14.20]	16.76 (20.18) [1.20–47.80]
Broccoli [7]	ud	0.93 (1.85) [<0.14–3.70]	119.42 (127.40) [6.80–302.50]	36.86 (42.95) [<0.09–98.95]	2.40 (0.56) [1.88–3.06]	173.32 (121.1) [72.00–326.38]	15.52 (8.31) [9.38–27.13]	5.27 (4.77) [1.13–10.25]	0.69 (0.80) [<0.29–1.50]	93.04 (62.71) [47.25–181.88]
Brussel sprout [7]	ud	0.45 (0.78) [<0.14–1.35]	115.05 (174.71) [1.60–316.25]	37.39 (43.78) [1.31–86.10]	9.41 (4.20) [4.60–12.38]	252.58 (150.91) [114.31–413.56]	17.08 (7.97) [10.50–25.94]	2.97 (2.65) [<0.08–5.10]	10.59 (12.63) [<0.29–24.56]	166.58 (43.09) [119.50–204.06]
Carrot [7]	ud	0.90 (1.60) [<0.14–3.69]	12.13 (16.88) [<0.06–41.25]	7.03 (9.26) [<0.09–17.50]	1.88 (1.93) [<0.35–4.38]	64.66 (83.96) [4.25–186.63]	5.52 (1.60) [3.10–7.50]	1.67 (0.86) [0.44–2.38]	5.39 (8.90) [<0.29–20.50]	23.35 (31.95) [<0.07–61.19]
Cauliflower [7]	ud	0.52 (0.25) [0.38–0.80]	91.98 (110.97) [0.06–215.25]	32.22 (50.23) [0.94–90.15]	1.38 (2.40) [<0.35–4.15]	80.24 (69.17) [26.75–158.35]	21.27 (5.15) [17.44–27.13]	5.58 (0.84) [4.60–6.06]	21.58 (37.38) [<0.29–64.75]	46.23 (77.28) [0.31–135.45]
Celery [7]	ud	0.33 (0.58) [<0.14–1.00]	58.29 (20.18) [35.50–73.88]	25.33 (21.94) [<0.09–38.38]	2.13 (0.87) [1.63–3.13]	93.17 (19.77) [70.50–106.88]	6.73 (1.29) [5.38–7.94]	1.46 (0.69) [1.00–2.25]	1.17 (1.02) [<0.29–1.88]	51.69 (13.23) [36.44–60.13]
Champignon [7]	ud	6.73 (1.03) [5.60–8.15]	1.40 (3.07) [<0.06–6.90]	<0.09	0.52 (0.48) [<0.35–0.90]	1.93 (1.59) [0.45–4.45]	74.58 (18.71) [58.65–106.65]	2.10 (0.55) [1.40–2.65]	<0.29	38.56 (37.11) [0.50–85.20]
Fermented lupine brine [10]	1	0.05	0.40	0.67	nd	13.14	2.90	5.48		0.21
Garlic [7]	ud	1.75 (2.06) [<0.14–4.50]	8.46 (7.84) [<0.06–17.69]	3.04 (4.83) [<0.09–11.25]	0.69 (1.22) [<0.35–2.81]	67.65 (105.29) [4.25–249.44]	18.62 (9.21) [8.31–33.06]	6.44 (2.06) [3.94–9.40]	1.47 (2.51) [<0.29–5.80]	8.44 (7.75) [1.06–21.45]
Pepper [7]	ud	2.48 (0.50) [2.00–3.10]	0.08 (0.15) [<0.06–0.30]	<0.09	0.88 (0.09) [0.75–0.95]	9.29 (3.17) [6.45–13.80]	1.21 (0.14) [1.10–1.40]	0.99 (0.11) [0.90–1.15]	<0.29	18.98 (2.92) [15.65–22.75]
Pickled caperberries [11]	9		3.2 (3.1)	14.7 (17.2)		13.1 (8.5)		4.9 (4.4)		1.6 (2.6)
Pickled capers [11]	8		nd	8.2 (6.7)		2.3 (1.3)		2.3 (2.4)		0.2 (0.6)
Pumpkin [7]	ud	1.08 (1.29) [<0.14–2.75]	20.97 (0.76) [20.00–21.69]	29.58 (31.13) [2.88–73.94]	1.13 (1.44) [<0.35–3.00]	136.98 (54.51) [55.44–169.13]	8.68 (1.23) [7.06–9.75]	49.63 (34.89) [2.00–83.20]	4.88 (3.95) [<0.29–9.25]	62.61 (39.09) [20.06–111.69]
Radish [7]	ud	0.32 (0.55) [<0.14–0.95]	14.18 (20.27) [0.88–37.50]	22.37 (22.04) [<0.09–44.06]	1.77 (0.93) [0.75–2.56]	32.08 (22.78) [6.38–49.80]	6.40 (2.92) [4.40–9.75]	0.93 (0.62) [0.45–1.63]	10.25 (11.38) [<0.29–22.50]	22.50 (11.33) [15.25–35.56]

Table 1. *Cont.*

Product.	N	AGM	CAD	HIS	PHE	PUT	SPD	SPM	TRP	TYR
Red cabbage [7]	ud	3.76 (4.60) [0.85–9.06]	90.22 (61.65) [34.75–156.60]	32.00 (52.03) [0.44–92.05]	1.11 (1.56) [<0.35–2.90]	124.17 (140.70) [4.00–278.95]	10.75 (4.77) [6.25–15.75]	3.23 (0.64) [2.70–3.94]	9.90 (17.15) [<0.29–29.70]	59.65 (56.04) [0.19–111.50]
Sunchoke [7]	ud	<0.14	4.50 (3.48) [1.50–8.31]	0.46 (0.79) [<0.09–1.38]	0.67 (1.15) [<0.35–2.00]	28.90 (18.15) [16.69–49.75]	7.83 (2.07) [5.88–10.00]	3.50 (0.22) [3.38–3.75]	<0.29	0.58 (0.71) [<0.07–1.38]
Tomato [7]	ud	0.06 (0.11) [<0.14–0.19]	1.58 (1.00) [0.75–2.69]	1.65 (2.21) [0.15–4.19]	2.09 (2.47) [<0.35–4.81]	42.05 (29.97) [10.95–70.75]	3.79 (1.18) [2.50–4.81]	1.21 (1.02) [0.50–2.38]	1.21 (2.09) [<0.29–3.63]	8.34 (13.30) [0.45–23.69]
White cabbage [7]	ud	3.14 (3.05) [<0.14–8.05]	35.76 (45.14) [<0.06–125.44]	55.60 (21.14) [32.55–83.81]	1.92 (0.95) [0.90–3.69]	190.59 (163.47) [57.50–524.63]	9.08 (2.48) [5.81–11.85]	2.55 (1.78) [0.69–5.38]	11.27 (5.89) [3.10–17.19]	60.69 (29.30) [29.05–105.13]
White turnip [7]	ud	<0.14	4.57 (4.31) [1.13–10.70]	0.02 (0.03) [<0.09–0.06]	1.31 (2.63) [<0.35–5.25]	15.49 (12.96) [2.25–32.63]	6.38 (2.41) [4.31–9.69]	1.44 (1.37) [<0.08–3.00]	<0.29	16.26 (15.57) [<0.07–35.31]

The average amounts of the biogenic amines are given. Standard deviation is given in parenthesis, and the range is given in square brackets. Amounts are given in mg/kg unless otherwise stated. CAD: cadaverine; DOP: dopamine; HIS: histamine; NOR: noradrenaline; PHE: 2-phenylethylamine; PUT: putrescine; SER: serotonin; SPD: spermidine; SPM: spermine; TRP: tryptamine; TYR: tyramine; N.: number of samples examined; ud: undefined; nd: not detected; tr: traces. [1] Hornero-Mendez and Garrido-Fernandez [206] (in µg/mL); [2] Garcia-Garcia et al. [207]; [3] Moret et al. [194] (in mg/100 g fresh weight); [4] Jin et al. [203]; [5] Lee et al. [204]; [6] Cho et al. [202]; [7] Kang et al. [205]; [8] Tsai et al. [201] (in mg/100 g); [9] Shin et al. [208]; [10] Swider et al. [200]; [11] Kalac et al. [209]; [12] Tofalo et al. [210].

The level of biogenic amines in sauerkraut was lower than that of kimchi, with the exception of tyramine (Table 1). In the latter case, Kalac et al. [209] analyzed 53 samples of Czech sauerkraut and reported the mean amount at 235 mg/kg and the highest amount at 951 mg/kg. Reports on the biogenic amine content of fermented cucumbers and olives are generally lacking in the literature. Based on the available data (Table 1), fermented cucumbers seem to contain more biogenic amines than fermented olives but less than kimchi and sauerkraut. Regarding fermented olives, they seem to contain less biogenic amines than kimchi, sauerkraut, and fermented cucumbers. Regarding the rest of the fermented fruits and vegetables, the high amounts of agmatine (6.73 mg/kg) and spermidine (74.58 mg/kg) detected in champignon, of histamine (55.60 mg/kg) in white cabbage, of phenylethylamine (9.41 mg/kg), putrescine (252.58 mg/kg), and tyramine (166.58 mg/kg) in Brussels sprouts, of cadaverine (119.42 mg/kg) in broccoli, of spermine (49.63 mg/kg) in pumpkin, and of tryptamine (21.58 mg/kg) in cauliflower should be noticed (Table 1).

The increase in the amount of biogenic amines in fermented foods compared to that of raw materials has been correlated with the microbiota that drive the fermentation. Indeed, the capacity of lactic acid bacteria to decarboxylate amino acids has been adequately exhibited [211]. However, it should be noted that this is a strain-dependent property. Thus, the haphazard nature of the biogenic amine content of spontaneously fermented fruits and vegetables is indicated. On the other hand, qualitative and quantitative control of biogenic amine production is an option that is offered when lactic acid fermentation is performed with the addition of starter cultures.

In the case of sauerkraut, the effect of raw materials on the production of biogenic amines has been highlighted by Majcherczyk et al. [212] and by Satora et al. [213]. In the latter study, eight cabbage varieties were employed to make sauerkraut through spontaneous fermentation; statistically significant differences in tyramine, histamine, cadaverine, putrescine, and tryptamine content were reported. Interestingly, a positive correlation between biogenic amine production and yeast presence was reported. The contribution of yeasts in the accumulation of biogenic amines is already known in products of alcoholic fermentation, such as wine [214]. The addition of ingredients that have an organoleptic impact, such as onion and caraway, affected the accumulation of some biogenic amines; the most pronounced effect was the reduced amounts of cadaverine and tyramine at the end of the 14-day fermentation period [212]. On the other hand, at the end of the 12-month storage at 4 °C, the sauerkraut made at 18 °C, with the addition of onion, accumulated significantly less cadaverine and phenethylamine compared to the control [212]. During spontaneous sauerkraut fermentation, the accumulation of biogenic amines seems to be affected by the aforementioned parameters, along with fermentation temperature and time. Indeed, Rabie et al. [215] reported an accumulation of histamine, tyramine, putrescine, and cadaverine after 10 days of fermentation at 15 °C, while Majcherczyk and Surowka [212] reported accumulation of cadaverine, tryptamine, and tyramine during fermentation at 18 °C for 14 d and of putrescine and tryptamine during fermentation at 31 °C for 14 d. Similarly, accumulation during sauerkraut storage seems to be affected by the same parameters as above. Regarding the effect of cultivar, the accumulation pattern seems to be affected by the cabbage cultivar [216–218]; however, no details were provided on the capacity of the members of the microecosystem to perform amino acid decarboxylation. The addition of onion seemed to prohibit the accumulation of cadaverine and phenethylamine but only in sauerkraut fermented at 18 °C and not 31 °C [212]. The paramount effect of a lactic acid bacteria strain's decarboxylating capacity in the accumulation of biogenic amines has also been adequately exhibited. Indeed, statistically significant differences in the accumulation during storage were observed and assigned to the *Lp. plantarum* and *Leu. mesenteroides* strains that were used as inocula [219]. In addition, suppression of biogenic amine accumulation during fermentation and storage through inoculation with *Lp. plantarum*, *Lt. curvatus*, and *La. casei* was reported by Rabie et al. [215]. Interestingly, the importance of the interaction between the selected starter culture and the cabbage cultivar was highlighted by Kalac et al. [216] and Spicka et al. [220].

In the case of kimchi, the accumulation of biogenic amines during fermentation of four kimchi types, namely, Pa, Gat, Kkakdugi, and Chonggak, has been assessed. Lee et al. [204] prepared Pa and Gat kimchi and studied the effect of myeolchi-aekjeot, a fermented anchovy sauce, the addition of which has been correlated with increased biogenic amine content [202,221,222]; tyramine-producing *Lv. brevis* strains and *Lp. plantarum* strains were unable to produce biogenic amines. During fermentation of Pa and Gat kimchi, the amount of tryptamine and histamine was reduced. During Pa fermentation, accumulation of tyramine, putrescine, and cadaverine in all experimental cases was noted. Spermine was accumulated only in some cases, while β-phenylethylamine and spermidine amounts were stable throughout fermentation. During Gat fermentation, only the cadaverine amount remained unchanged throughout the fermentation, and the accumulation of tyramine, β-phenylethylamine, putrescine, and spermidine was recorded. Spermine was also accumulated but only in some cases. In general, the addition of myeolchi-aekjeot and the tyramine-producing *Lv. brevis* strain enhanced biogenic amine accumulation, with the exception of spermine. Jin et al. [203] prepared Kkakdugi and Chonggak kimchi and studied the effect of myeolchi-aekjeot and saeu-jeotgal, a fermented shrimp product, the utilization of which has also been correlated with increased biogenic amine levels [221] in tyramine-producing *Lv. brevis* strains and *Lp. plantarum* strains. During fermentation of both products, the histamine amount decreased and the spermidine amount increased. In the case of Kkakdugi, tyramine was accumulated only in the samples inoculated with *Lv. brevis*, the putrescine amount slightly increased only in the uninoculated sample and the one inoculated with *Lv. brevis* JCM 1170, and cadaverine accumulated in all samples with the exception of the one that did not contain myeolchi-aekjeot, saeu-jeotgal, and inoculum. The latter sample was the only one in which spermine content increased, while in the rest, it was decreased. In the case of Chonggak, cadaverine remained stable in all samples but seemed to increase in the sample that did not contain the fermented fish condiments and inoculum; the tyramine amount increased in all samples with the exception of the one inoculated with *Lp. plantarum*, and cadaverine was accumulated only in the sample prepared with the addition of the fish condiments but without inoculum. In the same sample, along with the samples inoculated with *Lv. brevis*, the amount of spermine decreased. Combining the studies of Lee et al. [204] and Jin et al. [203], it can be concluded that the accumulation of biogenic amines could not always be predicted through the addition of ingredients that have been correlated with increased biogenic amine content (fish condiments) of starter cultures with known capacities. This indicates the existence of additional parameters that, at least in some cases, may affect biogenic amine accumulation. Since biogenic amine accumulation and decomposition are strain-dependent properties, it can be hypothesized that the native microbiota, and especially the proportion of which that manages to participate in the developing microecosystem, may be this additional parameter.

In the case of fermented cucumbers, biogenic amine accumulation during fermentation was assessed by Alan [223]. In the latter study, gherkin fermentation was performed spontaneously or with the addition of *Lp. plantarum*, *Lp. pentosus,* or *Lp. paraplantarum* strains as starter cultures. Spermidine was not detected in any experimental case. On the contrary, putrescine, cadaverine, histamine, and tyramine were detected, and their amount was strain-dependent. More accurately, spontaneously fermented gherkins contained an equal amount of putrescine with the one started with *Lp. plantarum* strain 49, less than the one started with *Lp. plantarum* strain 51, and more than the ones started with *Lp. plantarum* strain 13, *Lp. pentosus* strain 2, and *Lp. paraplantarum* strain 16. Cadaverine and histamine were not accumulated in the gherkins started with all three *Lp. plantarum* strains, but larger amounts were detected in the gherkins started with *Lp. pentosus* strain 2 and *Lp. paraplantarum* strain 16 compared to the spontaneously fermented ones. Finally, an equal amount of tyramine was accumulated in the gherkins started with *Lp. plantarum* strain 13 comapred with spontaneously fermented ones and larger amounts in the ones started with *Lp. plantarum* strains 49 and 51, *Lp. pentosus* strain 2, and *Lp. paraplantarum* strain 16.

The fate of biogenic amines during the fermentation of olives of the Manzanilla cultivar was assessed by Garcia-Garcia et al. [224]. Putrescine, tryptamine, β-phenylethylamine, spermidine, spermine, histamine, and agmatine were not detected during storage at 15, 20, and 28 °C for 12 months. Only cadaverine and tyramine were accumulated. The former was produced only at 20 and 28 °C, and the production rate increased after 7 and 5 months, respectively. Washing was correlated with increased production. Tyramine production followed a similar trend, with the exception that accumulation also occurred during storage at 15 °C.

8. Conclusions

The functional potential of lactic acid fermented fruits and vegetables relies on the interplay between the quality of the raw materials and the capacity of the microbial consortium to carry out certain biotransformations. The former depends on the type and variety of the raw materials, climatic conditions, and agricultural practices, as well as the occurrence and conditions of processing and storage. On the other hand, the production of bioactive compounds by microorganisms is a strain-dependent characteristic that also depends on the fermentation temperature and time. Thus, optimization of the functional potential requires a thorough study of all the aforementioned parameters. Although a lot of information is available in some cases, e.g., the production of biogenic amines, the trophic relationships within the microecosystem are very complex, and, thus, further study is still necessary to enable practical recommendations.

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