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Bioactivity of Organic Fermented Soymilk as Next-Generation Prebiotic/Probiotics Mixture

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Abstract: Fermented soymilk (soymilk yogurt) was made by fermenting soymilk with five probiotic bacterial strains (*Lactobacillus plantarum* ATCC 14917, *Lactobacillus casei* DSM 20011, *Lactobacillus acidophilus* ATCC 20552, *Lactococcus thermophilus* DSM 20259, and *Bifidobacterium longum* B41409) that were used as monocultures and combined with them as consortia cultures. Seven pathogenic strains, *E. coli* O157H7, *S. aureus* As4, *S. typhimurium* As3, *S. shigae* As2, *L. monocytogenes* As1, *P. aeruginosa* ATCC 27853, and *B. cereus* Dsmz 345, were used to study the antibacterial activity of fermented soymilk by agar well diffusion assay. Results indicated that Gram-negative pathogenesis was more sensitive to probiotic cultures than Gram-positive pathogenesis. *E. coli* O15H7, *S. typhimirium* As3, and *Shigella shigae* As2 were more sensitive to probiotic cultures, presenting inhibition zone diameters (IZA) ranging from 10 to 20 mm, 12 to 16 mm, and 10 to 16 mm, respectively. At the same time, *P. aeruginosa* Atcc 27853 showed the lowest (IZA), ranging from 3 mm to 8 mm. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined at various concentrations of soymilk fermented by T1, T4, and T5, ranging from 0.031 mg/mL to 1 mg/mL against pathogenic bacterial strains. The sensory properties of FSM were evaluated, and sensory analysis during soymilk fermentation showed significant improvement. The effect of shelf life (storage period) on FSM quality and properties was evaluated; during shelf life (storage period), FSM saved its properties and quality after 28 days of cold storage. Finally, it was stated that the soymilk yogurt can be used as a substitute for buffalo and cow milk for therapeutic feeding in the future.

Keywords: fermented soymilk; probiotic bacteria; soybean; pathogenic bacteria; antibacterial; minimum inhibitory concentration (MIC); minimum bactericidal concentration (MBC); storage period

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1. Introduction

Probiotics are viable mono or co-cultures of bacteria or yeast that, when administrated, and ingested by human beings or animals in adequate quantity, improve the properties of the indigenous flora, and provide health benefits to the host [1]. Probiotics have active live cultures such as *Lactobacilli*, *Lactococci*, and Bifidobacterium, among other bacteria, and some yeast strains such as the genus *Saccharomyces* may also be used in probiotic products [2,3].

Probiotics are potentially beneficial microbiota playing a vital role in various fields, including digestion improvement, intestinal health, inhibition of the pathogenic microbes

in the intestinal tract, immune system enhancement, cofactors and vitamins production, tumors and cancers protection, and help in the reduction of blood pressure and prevent it in some cases [4].

Due to its high protein and phytochemical content, Soymilk is an excellent promising dairy milk substitute for health-conscious consumers [5]. Moreover, soymilk is a rich source of bioactive phenolic compounds with potential health benefits related to cardiovascular diseases, menopausal symptoms, osteoporosis, and breast and prostate cancers [6]. Soymilk is a good food base for dairy milk substitution for those lactose-intolerant or allergic to milk proteins because it contains all amino acids essential to human nutrition. It is also one of the richest sources of isoflavones. Even though soymilk is a perfect nutrient supplement, it lacks popularity due to the beany flavor and the flatulence it causes [7]. However, fermentation is now the best way to decrease soymilk's beany taste and flatulence, promoting soy milk's functional properties by increasing bioactive components and reducing antinutritional components. Fermentation reduces the beany flavor caused by lipoxygenase activity, hence increasing the acceptability of soymilk [8]. Fermentation also reduces the oligosaccharides content, namely verbascose, stachyose, and raffinose, which cause flatulence [9]. In addition, fermentation improves the bioavailability of soy minerals, vitamins, protein, and isoflavones [10]. Fermentation of soymilk products by probiotic bacteria will enhance soymilk's health beneficial properties and commercial values and create more alternative soy products to the delight of consumers [11].

Fermented soymilk is easily digestible and has antioxidant properties that prevent and protect from cancer [12]. This may be due to the improvement in β-galactosidase activity converting isoflavone glycosides to aglycones; the latter is the bioactive form known for its health benefits [13]. Therefore, this study aims to evaluate the antimicrobial and antioxidant activities of fermented soymilk yogurt.

2. Materials and Methods

2.1. Samples Collection

Soybean seeds (*Glycine max*) were collected from the market in Cairo, Egypt, and were used to prepare soymilk.

2.2. Soymilk Preparation

Soybeans were washed and soaked overnight in distilled water at 5 °C. After water separation, the soybeans were blended at 1:5 *w/v* with distilled water. The resultant slurry was then filtered through a double-layered cheese cloth and sterilized for 15 min at 121 °C [3,14]. Soymilk with 2.13% fat, 2.10% protein, 2.43% carbohydrates, and energy content of 37.29 Kcal/100 has been recorded in a previous study [14].

2.3. Bacteria Used and Standard Inoculum Preparation

2.3.1. Preparation of Standard Fermented Bacterial Inoculum and Inoculation of Soymilk

Probiotic bacteria of *Lactiplantibacillus plantarum* ATCC 14917, *Lacticaseibacillus casei* DSM 20011, *Lactobacillus acidophilus* ATCC 20552, *Lactococcus thermophilus* DSM 20259, and *Bifidobacterium longum* B41409 (Food Technology Research Institute, Agriculture Research Center in Giza, Egypt) were used to ferment soymilk in the previous study [14]. Standard bacterial inoculums were prepared following Shah's method [15]. Briefly, the tested probiotic bacteria were inoculated in 50 mL of de Man, Rogosa, and Sharpe (MRS) broth medium [16] except *Lc. Thermophilus* DSM 20259 which was inoculated in M17 broth medium [17] with a stock culture loop. Inoculated flasks were incubated for 48 h at 37 °C under static conditions. For the fermentation process study, these flasks were used as standard inoculum (1 mL contained 2.3×10^7 CFU/ mL). Soymilk was inoculated as a single culture with a 5% (*v/v*) inoculum size for each probiotic bacterial culture. Co-cultures (di and tri-cultures) have also been inoculated with different inoculum sizes of 5 mL. The inoculated soymilk was incubated at 37 °C for 8 h and then stored at 4 °C in an airtight container before use. Single cultures were T1 (*L. plantarum* ATCC 14917), T2 (*Lc. thermophilus* DSM

20259), and T3 (*B. longum* B41409); di-cultures being T4 (*L. planturum* + *Lc. thermophilus)*; tri-cultures being T5 *(L. planturum* + *Lc. thermophilus* + *B. longum)*, T6 (*L. planturum* + *L. casei* + *Lc. thermophilus)*, T7 (*L. casei* + *L. acidophilus* + *Lc. thermophilus)*, and T8 (*L. acidophilus* + *Lc. thermophilus* + *B. longum*) [16].

2.3.2. Preparation of Standard Pathogenic Bacterial Inoculum

Seven pathogenic bacterial strains were obtained from the Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt, and were used to test the inhibitory activities of yogurt. These bacteria included *Escherichia coli* O157H7, *Shigella shag* As2, *Salmonella typhimirium* As3, *Staphylococcus aureus* As4, *Bacillus cereus* Dsmz345, *Pseudomonas aeruginosa* Atoc27853, and *Listeria monocytogenes* As1. A loopful of new bacterial cultures was transferred into a freshly prepared nutrient broth and standardized to 0.5 McFarland turbidity. The 0.5 McFarland turbidity standard was prepared by adding 0.05 mL of 1.18% barium chloride dihydrate ($BaCl_2$ $2H_2O$) with 9.95 mL of 1% sulfuric acid (H_2SO_4), then measuring the optical density measurement at 625 nm wavelength and absorption readings were fixed to be within the range of 0.08–0.10. The standardized inoculum has a concentration of 1.2×10^8 CFU/mL. The disk-diffusion agar technique detected the antagonistic effects of probiotic bacteria in fermented soymilk against some pathogenic bacterial strains [17]

2.4. Antibacterial Activity

The disk-diffusion agar technique was used to detect the antagonistic effects of probiotic bacteria-fermented soymilk against pathogenic bacterial strains. A 100 µL of pathogenic bacterial suspension was spread on an agar plate with a glass rod, allowing the medium's surface to dry for 5 min. A 7 mm in diameter sterile filter paper (Whatman NO. 1) disk saturated with 20 µL of the tested soymilk fermented by probiotic bacterial cultures suspension was placed on the surface of the inoculated plate. These plates were incubated at 37 °C for 24 h. After incubation, the antimicrobial activity is expressed as an inhibition zone [17].

2.4.1. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The procedure defined by the Clinical and Laboratory Standard Institute was used to perform the MIC assay. Minimum inhibitory concentration (MIC) value of three treatments of fermented soymilk cultures against seven tested pathogenic bacterial cultures after 24 h of incubation was recorded. The fermented soymilk cultures were diluted with a two-fold dilution of the tested suspension of the soymilk fermented by probiotic bacterial cultures by serial dilution. As previously mentioned, the sterile filter paper disk (Whatman NO. 1) saturated with these dilutions ranging from 1 to 1/32 with concentrations of 0.5, 0.25, 0.125, 0.0625, and 0.0312 was attached to the surface of the inoculated plate and then incubated at 37 °C for 24 h. Naked eye examination revealed that the lowest concentration (highest dilution) of the tested agent that prevented microbial growth was described as MIC [17].

To determine the minimum bactericidal concentration (MBC), the plates evaluated with no growth from the MIC assay were subcultured into nutrient agar and incubated for 48 h at 37 °C. The lowest concentration on the nutrient medium showed no change indicating the MBC value [17].

2.4.2. Evaluation of the Bacteriostatic and Bactericidal Effect

The ratio of MBC/MIC that can describe the action of fermented soymilk as antibacterial activity on test bacteria was calculated. When the ratio = 1 or 2, it means bactericidal effect, and when the balance is $\geq$4 or 16, it means bacteriostatic effects.

2.5. Quantitative Analysis of Antioxidants by Measuring a Di Phenyl Picrylhydrazyl (DPPH) Free

According to Zhao et al. Field, the scavenging activity of DPPH free radicals was measured [18]. An aliquot of 1 mL of DPPH (0.1 mM) solution in ethanol and 0.5 mL of antioxidant extract (water or solvent extracts) were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the decrease in absorbance at 517 nm using the spectrophotometer model (Unico S2100 series UV/Vis), and the DPPH radical scavenging was calculated according to the following equation,

$$\text{DPPH scavenging effect (\%)} = (\text{A0} - \text{A1/A0}) \times 100$$

where A0 = The absorbance of control. A1 = The absorbance of sample.

2.6. Sensory Properties Evaluation of Fermented Soymilk

The sensory attributes of the yogurt samples were evaluated organoleptically [19] for appearance, aroma, taste, mouth feel, and general acceptability. Twenty members of academic and graduate student panelists conducted the test. The panelists were asked to score each attribute on a 9-point hedonic scale ranging from 1 (dislike immensely) to 9 (like extremely).

2.7. Shelf Life of Fermented Soymilk

After fermentation of the soymilk samples by the test strains of probiotic bacteria, the soymilk yogurts formed were cooled in the refrigerator at 4° C and stored for different periods (0, 1, 7, 14, 21, and 28 days). During the storage period (shelf life) of soymilk yogurts, the changes in pH value, total acidity, and the number of viable cells were detected periodically.

2.8. Analytical Methods

2.8.1. pH Value

The pH of fermented soymilk was measured with a calibrated digital pH meter (model Adwa 1000).

2.8.2. Determination of Total Acidity

The titratable acidity of the sample was measured by taking 10 mL of weighed sample in a conical flask, and adding 3 drops of phenolphthalein indicator and titrating with 0.1 mL of sodium hydroxide (NaOH) until a pink color appeared. The titer value was recorded and was expressed as a percentage of the lactic and acetic acid [20].

2.8.3. Determination of Total Phenolic Content, Free Amino Acids, Saponin, and Isoflavone

- Preparation of solvent extracts

The extraction method was according to our previous work [21]. Nonfermented and fermented soymilk was freeze-dried using a freeze drier (FD-IC-5D, Bo Medical Experimental Instrument Co., Ltd.), and fermented soymilk that was freeze-dried was extracted using ultrasound (100 W) with water and an ethanol solvent 80% (1:10, *w/v*) while being kept at 25 °C for 6 h. The supernatants were obtained after the extracts were centrifuged at 10,000× *g* for 10 min at 4 °C. The extracts were frozen and kept at 80 °C.

- Determination of total phenolic contents

As described by Chen et al. Field [22], the total phenolic content of samples was analyzed with a few minor adjustments. The samples were freeze-dried and dissolved in 0.1 mL of DMSO (Merck). A total of 1.0 mL of Folin-Ciocalteu phenol reagent (Sigma-Aldrich Co.), and 1.9 mL of deionized water were added to the solution. Then, 5.0 mL of 20% Na_2CO_3 was added to the mixture and left to react for 20 min at room temperature

and in the dark. The samples' absorbance was then measured at 735 nm. The sample's total phenolic content was compared to a prepared gallic acid solution standard curve.

- The free amino acid content measurement:

The free amino acid concentrations of fermented and unfermented soymilk were measured as described by Xing et al. [23]. Before analysis, fermented and unfermented soymilk pH was adjusted to 4.6. To extract supernatants, the samples were centrifuged at 3000× *g* for 30 min at 4 °C. Fifty microliters of the supernatants were mixed with 2 mL of O-phthalaldehyde and incubated at room temperature for 2 min. At 340 nm, the absorbance of the samples was measured. The results were expressed as milligrams of Leucine extract from a standard curve constructed with Leucine standard.

- Quantification of total saponin

The saponin quantification in the soymilk was determined as described by the method [24] with slight modification. Briefly, the freeze-dried samples were dissolved in MeOH at 80%. Aliquots of the samples (0.1 mL) for each tube, 0.1 mL vanillin reagent (8%, 0.25 mL) were added, and sulfuric acid (72% *v/v*, 2.5 mL) was added slowly on the inner side of the wall. The mixtures were given a 20-min soak in 60 °C water before being allowed to stand in ice-cold water for 5 min. The amount of saponin in the mixture was determined by measuring the mixture's absorbance at 544 nm and using a standard curve created using purified soy saponin.

- HPLC analysis of the soy isoflavone

The soy isoflavone contents were determined according to [21]. The soymilk and fermented soymilk ethanol extracts were redissolved in 80% methanol. Before being evaluated by HPLC, the materials were filtered through a 0.22 µ-pore-size polyvinylidene fluoride filter (Teknokroma, Barcelona, Spain). Waters 2695 Alliance, a Waters 2998 PDA detector, and a C18 column (Optimapak, 4.5 250 mm, 5 m) made up the analytical HPLC. About 20 µL of the samples was injected using the autoinjector, and isoflavone was determined by measuring the extraction at 260 nm.

2.9. Statistical Analysis

The data were represented as mean ± standard deviation (SD). The statistical analysis was performed using SPSS (version 20.0) based on Duncan's Multiple Range Test at $p \leq 0.05$ [25]. All studies were carried out in triplicate.

3. Results and Discussion

3.1. Antibacterial Effect of Fermented Soymilk Products against Pathogenic Bacterial Strains

Results showed (Figure 1) that all tested probiotic cultures had more significant ($p \leq 0.05$) antibacterial activity against *E. coli* O15H7, *S. typhimirium* As3, and *S. shigae As2*, which gave inhibition zone diameter (IZD) ranging from 10 to 20 mm, 12 to 16 mm, and 10 to 16 mm, respectively. The significant ($p \leq 0.05$) probiotic cultures appeared to have high antibacterial activity against the tested pathogens being, T5, T4, and T1.

The co-culture of *L. plantarum* + *L. thermophilus* + *B. longum* (T5) fermented soymilk presented the highest IZD toward *E. coli* O15H7 (22 mm), *S. shigae* As2 (17 mm), *S. typhimirium* As3 (16 mm), and *Staph. aureus* As4 (15 mm), *L. monocytogenes* (13 mm), and *B. cereus* Dsmz345 (12 mm), while the lowest IZA was observed by *P. aeruginosa* ATCC27853 (8 mm). The inhibition of the growth of pathogens may be through the generation of antimicrobial compounds, antimicrobial peptides, and organic acids during fermentation [26]. Some previous reports support that specific strains of Lactobacillus species such as *L. plantarum* and *L. rhamnosus* can produce some antimicrobial compounds [27]. Similar to our results, [28] also documented the antibacterial effect of soy products against Gram-positive pathogenic bacteria. Moreover, Mishra et al. [29] found the antibacterial activity of flavored fermented soy milk against *L. monocytogenes*, *B. subtilis*, and Staph. Aureus, *S. typhi*, and *E. coli* strains. Antimicrobial activities of the cell-free supernatant of soy milk fermented by *L. helveticus*

were also found against *B. subtilis* and *E. coli* strains [13]. The soymilk fermented products show low activity against *P. aeruginosa* ATCC27853, *L. monocytogenes* As1, and *B. cereus* Dsmz345 strains [30]. The results of the effect of fermented soymilk products as an antimicrobial efficiency observed that the most efficient strains were shown by T1 (*L. plantarum)*, T4 (*L. plantarum* + *Lc. thermophiles*), and T5 (*L. plantarum* ATCC14917 + *Lc. thermophilus DSM20259* + *B. longum* B41409). So, these strains were chosen for the subsequent studies.

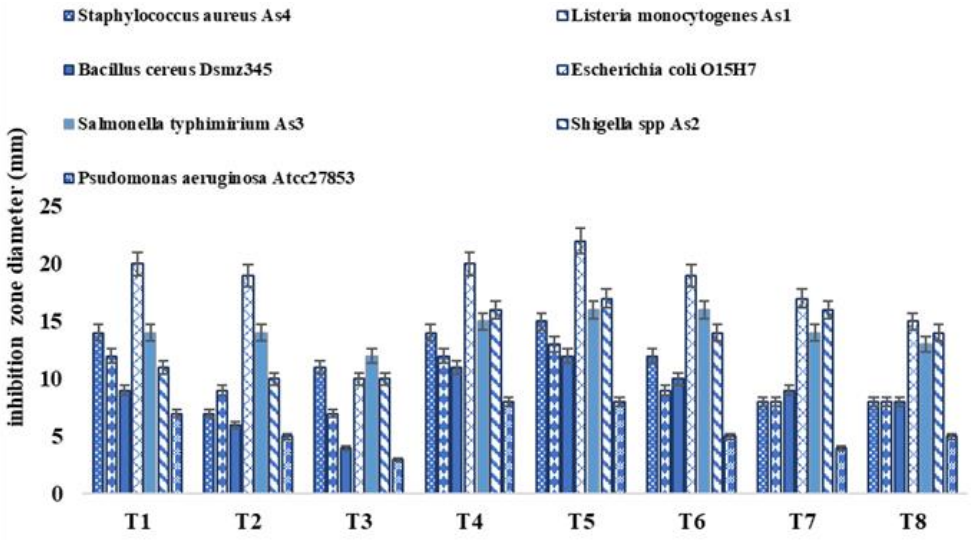

Figure 1. Antimicrobial activity of fermented soymilk against pathogenic bacteria. T1 *(L. plantarum* ATCC14917), T2 (*Lc. thermophilus* DSM20259), T3 (*B. longum* B41409), T4 (*L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259), T5 *(L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 + *B. longum)*, T6 (*L. plantarum* ATCC14917 + *L. casei* DSM20011 + *Lc. thermophiles* DSM20259), T7 (*L. casei* DSM20011 + *L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259), and T8 (*L. acidophilus* ATCC20552 + *Lc. thermophilus* DSM20259 + *B. longum* B41409). Standard division bars resented standard division.

3.2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Fermented Soymilk Products

The MIC values of the tested fermented soymilk cultures were found at dilution ranged between 3.72 and 2.11 CFU/mL (0.25 and 0.125 dilutions), respectively (Table 1). Gram-negative bacteria of *E. coli*, *shigella* sp., and *S. typhi* were observed to be susceptible to all fermented soymilk cultures treatments (T1, T4, and T5) at a MIC value of 2.11 CFU/mL (0.125 dilutions) expect *P. aeruginosa* which was sensitive at a MIC value of 3.72 CFU/mL (0.25 dilution). Whereas Gram-positive bacteria of *Staph. aureus*, *B. cereus*, and *L. monocytogenes* appeared sensitive to T1 (*L. planetarium* ATCC14917) at a MIC value of 3.17 CFU/mL (0.25 dilution) and T4 (*L. plantarum* ATCC14917 + *L. thermophiles* DSM20259) and T5 (*L. plantarum* + *Lc. thermophilus* DSM20259 + *B. longum* B41409) at MIC value of 2.11 CFU/mL (0.125 dilutions).

Minimum bactericidal effects with different values were observed in all tested fermented soymilk samples. Table 1 shows that MBC values at dilutions ranged between 1 and 0.125 (9.98 and 3.72 CFU/mL). In the case of Gram-negative bacterial pathogenic bacteria, *E. coli*, *S. shigae*, and *S. typhi* appeared sensitive to T1, T4, and T5 at MBC of 9.98 and 2.11 CFU/mL (0.25 and 0.125 dilutions), respectively. In the case of Gram-positive bacterial pathogenic, *Staph. aureus* and *L. monocytegene* were sensitive to T1 and T4 at MBC of 9.47 and 2.11 CFU/mL (1.0 and 0.5 dilutions), respectively. At the same time, *B. cereus* had sensitivity to T1 and T4, and T5 at 9.98 and 2.11 CFU/mL (1.0 and 0.25 dilutions) of MBC, respectively.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fermented soymilk product.

	Probiotic Strain	**T1**								**T4**								**T5**							
Parameters	**Pathogenic Bacterial Strains**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Spectrum Activity (%)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Spectrum Activity (%)**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Spectrum Activity (%)**
MIC	1	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100
	0.5	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100
	0.25	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100
	0.125	+	+	+	-	-	-	+	43	-	-	-	-	-	-	+	85	-	-	-	-	-	-	+	85
	0.0625	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0
	0.0312	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0
MIC value		0.25	0.25	0.25	0.125	0.125	0.125	0.25		0.125	0.125	0.125	0.125	0.125	0.125	0.25		0.125	0.125	0.125	0.125	0.125	0.125	0.25	
MBC	1	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100	-	-	-	-	-	-	-	100
	0.5	+	-	+	-	-	-	+	57	+	-	-	-	-	-	+	72	-	-	-	-	-	-	+	85
	0.25	+	+	+	-	-	-	+	43	+	+	-	-	-	-	+	57	+	-	-	-	-	-	+	72
	0.125	+	+	+	+	+	+	+	0	+	+	+	-	-	-	+	43	+	-	+	-	-	-	+	43
	0.0625	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0
	0.0312	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	0
MBC value		1	0.5	1	0.25	0.25	0.25	1		1	0.5	0.25	0.125	0.125	0.125	1		0.50	0.25	0.25	0.125	0.125	0.125	1	
MBC/MIC Ratio		4	2	4	2	2	2	4		8	4	2	1	1	1	4		4	2	2	1	1	1	4	
* Effect		-	+	-	+	+	+	-		-	-	+	+	+	+	-		-	+	+	+	+	+	-	

1 = *Staph. aureus* As4, 2 = *L. monocytogenes* As1, 3 = *B. cereus* Dsmz345, 4 = *E. coli* O15H7, 5 = *S. typhimirium* As3, 6 = *S. shigae* As2, 7 = *P. aeruginosa* ATCC27853. * Bactericidal (+) = ≤ 2 and bacteriostatic (-) effect = ≥ 4.

3.3. Antibacterial Mode of Action

Results represented in Table 1 revealed that the tested fermented cultures had a bactericidal effect with an MBC / MIC ratio ≤ 2 against four pathogenic strains out of seven strains in the case of T1 (*L. planetarium* ATCC14917) and T4 (*L. plantarum* ATCC14917 + *Lc. thermophiles* DSM20259), and in case of T5 treatment for the three strains *L. plantarum, Lc. thermophilus* DSM20259 and *B.longum* B41409.

Moreover, all fermented cultures also presented bacteriostatic effect (MBC / MIC ratio ≥ 4) against both *Staph. aureus* As4, *P. aeruginosa* ATCC27853, *B. cereus* DSMZ345, and *L. monocytogenes* As1 in the case of T1 and T4, respectively.

Furthermore, it could be stated that the fermenter culture of T5 (*L. plantarum* ATCC14917 + *Lc. thermophilus* DSM20259 + *B.longum* B41409) was favored compared to T1 (*L. planterium* ATCC14917) and T4 (*L. plantarum* + *L. thermophiles*) which killed five pathogenic strains out of the seven strains, which are *B. cereus* DSMZ345, *L. monocytogenes* As1, *E. coli* O15H7, *S. shigae* As2, and *S. typhimurium* As3; so, the fermenter culture of T5 was chosen for further studies.

3.4. The Total Phenolic, Antioxidant, Aglycone Isoflavones, Free Amino Acids, and Saponin Contents in the Soymilk Fermented by Consortia Probiotic Bacterial Cultures L. plantarum + Lc. thermophilus + B. longum Probiotic Bacterial Strains

Table 2 clearly shows that the total phenolic content of the soymilk fermented by probiotic bacterial cultures significantly ($p < 0.05$) increased up to 43.5 mg/mL with the prolonging of fermentation time as well as the aglycone isoflavones, daidzein, and genistein, concentrations in the fermented soymilk significantly ($p < 0.05$) increased to 0.75 and 1.5 mg/mL. In contrast, the concentrations of the glucosides isoflavones (daidzin and genistin) in the fermented soymilk decreased significantly to 0.5 and 0.35 mg/mL. The increase in aglycone isoflavones could be due to the bioconversion of glucosides isoflavones by probiotic bacterial cultures; with the prolonging of fermentation time, the saponin contents of the ethanol extracts and water extracts of the probiotic bacterial cultures fermented soymilk reduced. These consequences might be caused by increased β-glucosidase activity in the probiotic bacterial culture-fermented soymilk.

Table 2. The soymilk fermented probiotic bacterial strains contain total phenolic, antioxidant, aglycone isoflavones, free amino acids, and saponin.

Parameters		Treatment	
		Control	Fermented Soymilk
Total phenol content (mg/mL)	Ethanol extract	39.70 ± 0.02^{aB}	43.75 ± 0.12^{aA}
	Water extract	25.13 ± 0.43^{bB}	31.87 ± 0.30^{bA}
Inhibition of DPPH (%)	Ethanol extract	1.00 ± 0.05^{aB}	80.0 ± 0.10^{aA}
	Water extract	1.00 ± 0.07^{aB}	79.93 ± 0.1^{bA}
Saponin contents (mg/mL)	Ethanol extract	3.1 ± 0.01^{aA}	2.5 ± 0.03^{aB}
	Water extract	0.9 ± 0.01^{bA}	0.5 ± 0.05^{bB}
Isoflavones concentration (mg/mL)	Diadzin	1.5 ± 0.01^{bA}	0.50 ± 0.07^{cB}
	Genistin	2.50 ± 0.02^{aA}	0.39 ± 0.01^{dB}
	Daidzein	0.02 ± 0.07^{dB}	0.75 ± 0.08^{bA}
	Genistein	0.4 ± 0.03^{cB}	1.5 ± 0.02^{aA}
Free amino acids (mg/mL)		0.27 ± 0.01^{B}	0.5 ± 0.09^{A}

Data are represented as mean ± SE ($n = 3$). $^{a, b, c}$ Values with small letters in the same column having different superscripts are the significant differences at ($p < 0.05$) between extraction methods. $^{A, B}$ Values with capital letters in the same row having different superscripts are the significant differences at $p < 0.05$ between treatments (control and fermented soymilk).

The free amino acid contents of the probiotic bacterial cultures fermented soymilk increased significantly ($p < 0.05$), prolonging the fermentation time. A higher amino nitrogen content implied a higher degree of protein hydrolysis and higher amino acid and peptide contents in the sample.

These results support the claims stated by Rekha and Vijayalakshmi [31] that some *Lactobacillus* strains generate the enzyme β-glucosidase, which catalyzes the conversion of isoflavone glucosides in soymilk to aglycone isoflavone. The planned ring of the isoflavone and the sugar moieties are conjugated by the β-1-6 glycosidic bond, broken down by the -glucosidase produced by the lactic acid bacteria field [32]. The biological process may be improved by the fermentation-induced breakdown of isoflavone glycosides into sugar moieties and bioactive isoflavone aglycones.

The activity of soymilk *Lactobacillus casei* 16 β-glucosidase activity may also reduce the levels of saponin in the fermented soymilk. Steroid and triterpenoid saponins' sugar side chains can be broken by β-glucosidase, reducing the compounds' water solubility. In addition, [22] reported that the number of peptides and amino acids increased in black soybeans fermented by *Aspergillus awamori*. Moreover, [21] found that the β-glucosidase activities of the probiotic bacterial cultures-fermented soymilk increased significantly during the fermentation time ranging from 0 to 8 h. The increases in total phenolic content and aglycone isoflavone levels may be due to probiotic bacterial cultures β-glucosidase enzymes catalyzing the release of phenolics during fermentation. Yan et al. discovered that the *B. longum* β-glucosidase BIBG3 catalyzed the hydrolysis of saponin with a higher level of efficiency [27].

Fermentation of soymilk with micro-organisms improves the biological activities of soymilk. The proteolytic enzyme produced by microorganisms hydrolyzes the intact protein of soy into different oligopeptides and free amino acids during fermentation. Protein degradation into oligopeptides is a good source of bioactive peptides. Several lactic acid bacteria (LAB) have been reported to possess proteases, which generate bioactive peptides during the fermentation [33].

Soymilk fermented products with probiotic bacterial cultures for 8 h resulted in significantly higher antioxidant activity in DPPH radical-scavenging (Table 2). The results also exhibited that the treatments extracted by ethanol were favored as antioxidant activity, showing that extraction by water and ethanol presented an antioxidant activity of 79.9 and 80%, respectively, which significantly increased, by 1.00-fold more than those removed by water. Soymilk fermented with lactobacilli possessed excellent DPPH radical-scavenging activity of over 50% as opposed to unfermented soymilk yield [34]. The antioxidant activity increases after fermentation with probiotic bacterial strains compared to unfermented ones. It may be due to the increase in isoflavones in the aglycone form during fermentation. It is known that isoflavones protect cells from the damaging effects of free radicals [35]. It was suggested that aglycones (mainly daidzein and genistein) generated through the enzymatic action of probiotic bacterial strains were able to act as suitable hydrogen donors that could effectively scavenge DPPH radicals in the soymilk [36].

3.5. Sensory Properties Evaluation of Fermented Soymilk

Because of the presence of hexanal and pentanal, soymilk had limited consumer acceptance due to an unpleasant or "beany" aftertaste. These aldehydes are formed mainly by the hydro peroxidation of polyunsaturated fatty acids catalyzed by lipoxygenase. The development of fermented soymilk was aimed at reducing the beany flavor. Fermentation of soy milk offers a chance to vary the sensory features of soy-based foods. It gives a peculiar aroma due to lactic acid production, which dramatically contributes to the flavor of products. It will decrease the levels of volatiles that cause the natural beany flavor in soy products [37]. Sensory evaluation is an essential critical tool to process this all. Sensory evaluation of soy milk is vital to understanding the consumer perception of a value-added product. Soy milk was evaluated using a 9-point hedonic scale at four intervals for parameters, i.e., appearance, appearance, aroma, flavor, taste, and overall acceptability.

A graphical representation in Figure 2 depicts the results. It shows a higher score for all sensory parameters in fermented soy milk. In any food product, appearance is the main attribute influencing the consumer's opinion and perceptions of taste, flavor, and acceptance. The results showed that fermentation helped increase the flavor score compared to nonfermented soy milk. It has been stated that lactic acid increases the nutritional value of fermented products by engendering flavor and structure. It is found that a significant enhancement in the odor and taste is due to the use of probiotics in the fermentation of soy milk compared with unfermented soy milk. This may be due to organic acids and flavoring agents produced by probiotic bacteria in soy milk. LAB can influence the metabolism of carbohydrates and proteins, improving fermented products' nutritional and sensory quality. Moreover, soy milk fermentation improves the final product's sensory quality by metabolizing n-hexanal and n-pentanal, which causes the beany flavor in soy milk by probiotic bacteria, into lactic acid and diacetyl. Fermentation also decreases the activity of galactooligosaccharides that improves the digestibility of fermented soy milk.

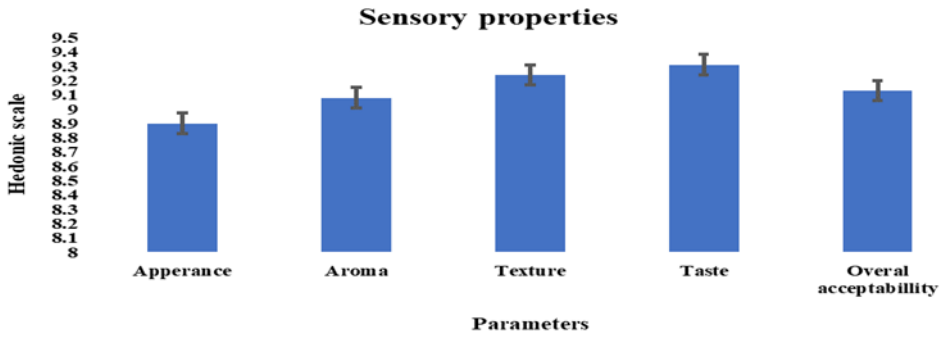

Figure 2. Sensory properties evaluation of fermented soymilk.

3.6. Shelf Life (Storage) Period of Fermented Soymilk

Viability of Probiotic Bacterial Cultures and Change in pH and Total Acidity during Shelf Life of Fermented Soymilk

To maintain the beneficiary effect of probiotic products, it is important to demonstrate the viability of bacteria throughout the product's shelf life; the final population of the probiotic organisms in the fermented product at the end of the shelf life. Fermented soymilk showed considerable stability during all shelf life. Results in Figure 3 show the viable cells count of fermented soymilk. The initial viability of fermented soymilk was 9.98 CFU/mL; it was 9.77 CFU/mL after 7 days, 9.55 CFU/mL after 14 days, 8.47 CFU/mL after 21 days, and 8.41 CFU/mL at the end of the storage period (28 days). The viable cells count in the final product above the probiotic minimum (10^6 CFU/mL), which would be stable during 28-day excellent storage, is the requirement for probiotic foods [38].

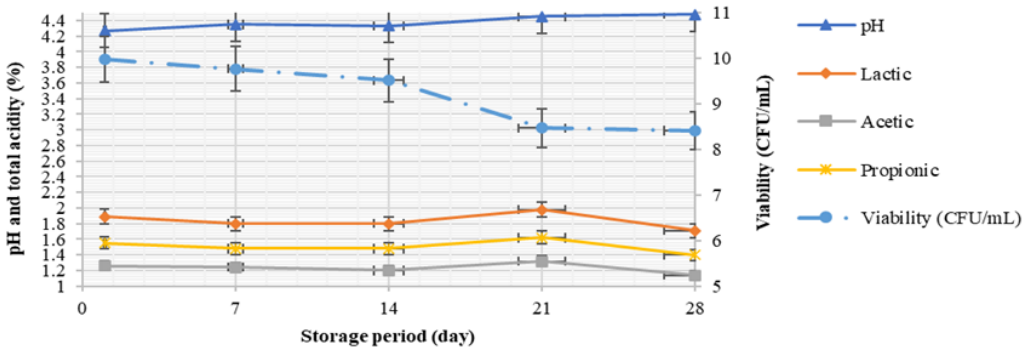

Figure 3. Viability of fermented soymilk during shelf life (storage period) for 28 days at 4 *degrees*. Standard error bars resented standard error.

Data in Figure 3 show the pH values of fermented soymilk during the refrigerated storage period. The initial pH value for the freshly fermented soymilk was 4.27. The pH of fermented soymilk increased and decreased slightly during storage; the pH was 4.35 on the 7th day, 4.33 on the 14th day, 4.45 on the 21st day, and 4.48 at the end of storage (the 28th day). At the same time, the titratable acidity values of fermented soymilk during refrigerated storage are also shown in Figure 3. The initial acidity of the freshly fermented soymilk on the first day was 1.89% for lactic acid, 1.55% for propionic acid, and 1.26% for acetic acid, respectively. While during storage for 7 and 14 days, the acidity of fermented soymilk appeared slightly decreased, and the acidity of lactic acid, propionic acid, and acetic acid were 1.8%, 1.48%, and ranged from 1.20 to 1.24%, respectively. While at 21 days of the storage period, the acidity of lactic acid, propionic acid, and acetic acid increased up to 1.98%, 1.62%, and 1.32%, respectively, and decreased after this period at 28 days to 1.71%, 1.40%, and 1.14%, respectively.

The changes in titratable acidity occurred to a greater or lesser degree, depending on the product's chemical composition, especially the viable fermentable sugars, the cold storage temperature, and time. Moreover, protein content can influence the acidity of dairy products, as proteins act as a buffer due to the large number of groups that can reversibly interact with protons. Probiotic bacteria (LAB) viability during cold storage is fundamental for producing organic acids (mainly lactic acid) and pH value, which determine microbiological stability and avoid food-borne pathogens, allowing longer shelf life to fermented products than to traditional products and promoting the probiotic properties. Higher product acidity can protect the product from developing spoilage microorganisms, increasing shelf life and not changing the product's sensory or technological characteristics [39].

4. Conclusions

In the current investigation, fermented soymilk (yogurt) containing probiotic bacterial strains as a single or consorting culture showed excellent efficacy for suppressing food-borne pathogens of Gram-positive and -negative bacteria with MIC values varying from 0.031 mg/mL to 1.00 mg/mL. This product had antioxidant effects and seemed to taste good and be favored by consumers. In frigid temperatures, soymilk yogurt's shelf life was increased to 28 days. Therefore, using soymilk yogurt as a replacement for cow's milk can be a viable option. In the upcoming trials, therapeutic nutrition will be used to study the soymilk yogurt's antibacterial and antioxidant activities.

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