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Effects of *Mentha piperita* L. and *Stevia rebaudiana* extracts on the growth of lactic acid bacteria and their potential for functional food development

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ABSTRACT

Background: A wide variety of prebiotic and probiotic formulations are currently employed in modern medicine to manage gastrointestinal disorders. *Mentha piperita* L. (peppermint) is well known for its anti-inflammatory, antipyretic, antihypertensive, and anti-allergenic properties. Due to its distinctive aroma and bioactive profile, it is widely used in traditional medicine, the food industry, and cosmetics/perfumery. *Stevia rebaudiana* is another valuable plant that contains steviol glycosides, widely recognized as natural, non-caloric sweeteners. These compounds not only enhance food palatability but also support the nutritional needs of individuals with dietary restrictions, including those managing diabetes and/or obesity.

Objective: The research aimed to evaluate the effects of these plant extracts on the growth of *Lactobacillus acidophilus* Er 317/402 and *Lactobacillus delbrueckii* subsp. *bulgaricus* B7—two probiotic strains with well-established health benefits.

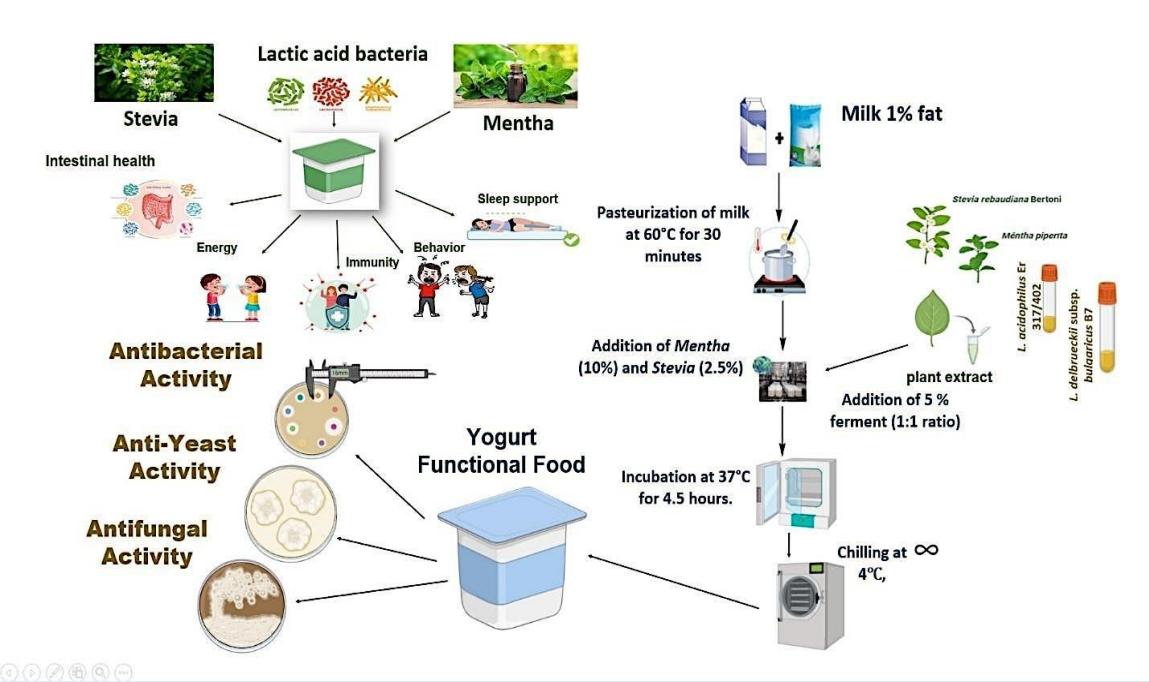
Results: Both aqueous and ethanolic *M. piperita* extracts showed no inhibitory effects on LAB, indicating compatibility

with plant bioactives. The two strains exhibited mutualistic interaction in co-culture, supporting their combined use. The optimal formulation included 10% *M. piperita*, 2.5% *S. rebaudiana*, and 5% inoculum of both strains, with fermentation optimized at 37 °C for 4.5 hours in 1% fat raw milk.

Novelty: This study is among the first to explore the combined impact of *M. piperita* and *S. rebaudiana* extracts on probiotic lactic acid bacteria (LAB) within a dairy matrix. Furthermore, it uniquely integrates a honeybee gut-derived *Lactobacillus acidophilus* strain with a standard probiotic, demonstrating their synergistic potential in developing functional dairy products enriched with bioactive plant compounds. This dual innovation—using both underexplored plant-microbe interactions and a novel probiotic source—provides new perspectives for designing nutritionally enhanced and shelf-stable fermented foods.

Conclusion: The final product exhibited antibacterial, anti-yeast, and antifungal activity, suggesting improved shelf life and safety— key traits for functional foods. This is among the first studies to use LAB from the honeybee gut with plant extracts in dairy fermentation. The synergy between a novel bee-derived strain and a standard probiotic not only produced a nutrient- and bioactive-rich product but also demonstrates the commercial potential in enhancing shelf life and health benefits.

Keywords: lactic acid bacteria, pre- and probiotics, *Mentha piperita* L., *Stevia rebaudiana,* functional food



Graphical abstract: Effects of *Mentha piperita* L. and *Stevia rebaudiana* extracts on the growth of lactic acid bacteria and their potential for functional food development

INTRODUCTION

Microecological imbalances in the gastrointestinal tract are often linked to various health conditions. Growing evidence shows that disturbances in the intestinal microbiota—such as dysbiosis or bacterial overgrowth—may contribute to the onset and progression of many diseases. Although not classified as independent nosological entities, these conditions pose significant challenges in modern medical practice [1]. To manage them, a wide range of probiotic formulations is commonly used. Recently, however, interest has shifted toward both prebiotic and probiotic preparations. Prebiotics are natural compounds that promote the growth and activity of beneficial gut microbes. While substances like lactulose and pectin are well-studied, many plant-based compounds remain underexplored and may hold promise as prebiotics [2].

LAB are well-established probiotic microorganisms known for their diverse beneficial properties, including antimicrobial, proteolytic, antioxidant, lipolytic, and immunostimulatory activities [3]. Incorporating plant-based extracts into dairy products necessitates more than just demonstrating their functional benefits—it also requires thorough safety validation. As emphasized by Son and Martirosyan [4], obtaining GRAS (Generally Recognized As Safe) status involves comprehensive scientific documentation, including detailed analysis of composition and potential toxicity, which is crucial for regulatory approval in the functional food industry.

Among these, *Lactobacillus acidophilus* Er 317/402 is a notable strain that was originally isolated from the meconium of a healthy newborn by Dr. Yerzinkyan in 1963 and has been used in Armenia as a probiotic ever since. This strain has demonstrated strong antagonistic activity against a variety of pathogenic microorganisms, including clinical isolates of *Clostridium difficile* [5], as well as *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Klebsiella* species. Moreover, it expresses colonization factor antigen (CFA) type I adhesion molecules on its cell surface, facilitating effective adhesion to intestinal epithelial cells [6].

Mentha piperita, a member of the Lamiaceae family, is widely recognized for its medicinal properties, primarily attributed to its rich content of essential oils. Traditionally, it has been used for its anti-inflammatory, antipyretic, antihypertensive, anti-allergenic, and aromatic effects [7].

Mentha piperita (peppermint) produces a volatile essential oil rich in bioactive compounds, including menthol (38.3–69.1%), menthone (0.4–20.9%), menthofuran (up to 15%), menthyl acetate (3.5–4.5%), and iso-menthone (0.8–8.8%). It also contains bitter substances, caffeic acid, flavonoids (12%), polymerized polyphenols (19%), carotenes, tocopherols, betaine, choline, and tannins [7]. Its chemical composition may vary with climate, geography, and processing conditions.

Peppermint oil and its components are widely used in the food, pharmaceutical, and cosmetic industries. Menthol, the main active compound, is found in products like toothpaste, mouth fresheners, cough medicines, perfumes, gum, and tobacco. Peppermint oil and menthol show moderate anti-inflammatory [8] and antibacterial activity against both Gram-positive and Gram-negative bacteria [7]. They also exhibit antiviral and antifungal properties; aqueous peppermint extracts are effective against influenza, herpes simplex, and other viruses [9]. Additionally, peppermint oil may help reduce benzopyrene-induced lung carcinogenesis and mutagenicity [10].

Stevia rebaudiana Bertoni, a perennial herb from the Asteraceae family native to northeastern Paraguay, is valued for its natural sweetness and low-calorie content. Its sweetness comes from steviol glycosides like stevioside, rebaudioside A, and others [11]. Stevia leaves also contain phenolics, tannins, alkaloids, and flavonoids, which provide antioxidant, antiparasitic, antiviral, anti-inflammatory, and antiproliferative effects. These

antioxidants help preserve food by preventing lipid oxidation, reducing rancidity, and extending shelf life [12].

The development of low-calorie food products with reduced sugar content has become a pressing priority in the modern food industry. Incorporating zero-calorie, high-sweetness plant extracts like *S. rebaudiana* into functional foods supports new scientific models that highlight both biochemical effectiveness and optimal timing. Martirosyan and Stratton [13] suggest that synchronizing the intake of bioactive compounds with the body's circadian rhythms—through their Quantum and Tempus theory—can enhance absorption and health outcomes, offering a promising approach for the use of natural sweeteners in food systems.

Increasing evidence suggests that artificial sweeteners like aspartame, saccharin, sucralose, and acesulfame potassium may pose health risks, including weight gain and type 2 diabetes [14]. This has led to growing interest in natural alternatives. *S. rebaudiana* is a promising low-calorie sweetener with high sweetness potency. It is approved by the Codex Alimentarius Commission, and JECFA has set safety guidelines and acceptable daily intake levels for steviol glycosides, supporting their global use [13].

The objective of this study was to evaluate the effects of *M. piperita* L. and *S. rebaudiana* Bertoni extracts on the growth and activity of LAB, with the ultimate goal of developing novel functional food products.

MATERIAL AND METHODS.

The objects of research: The following bacterial strains, used as the test organisms, are maintained at the Department of Biochemistry, Microbiology, and Biotechnology, Faculty of Biology, Yerevan State University (YSU): *Lactobacillus rhamnosus* R-2002 (accession number: KY054594) in the National Center for Biotechnology Information (NCBI) gene library and licensed to the Microbial Depository Center (UNC) (WDM803) at the "Armbiotechnology" Scientific Production Center, National Academy of Sciences of the Republic of Armenia (RA), with MDC number 9661;

Lactobacillus delbrueckii subsp. *lactis* INRA-2010-4.2, represented by MDC 9632; *Lactobacillus delbrueckii* subsp. *bulgaricus* INRA-2010-5.2, represented by MDC 9633; *Streptococcus thermophilus* VKPM B-3386; *Enterococcus durans* (Provided by the National Agricultural Research Institute, Nantes, France, INRA); *Lactobacillus delbrueckii* subsp. *bulgaricus* B7 (accession number MK494928) in the NCBI gene library; and *Lactobacillus delbrueckii* subsp. *bulgaricus* (RIN-2003-Ls), *Lactobacillus rhamnosus* 21.1, *Lactobacillus acidophilus* JM-2012 strains (isolated from Armenian dairy products). The control strain used in this study was *Lactobacillus acidophilus* ER-317/402 (licensed to the Microbial Depository Center (MDC) of "Armbiotechnology" Scientific Production Center, National Academy of Sciences of the (RA), under the designation WDM803 and under the number MDC9602), the most commonly utilized probiotic strain in Armenia. All strains were stored under refrigerated conditions at +4°C and were subcultured every 30 days to maintain their viability. PRO-Seq data of 16srRNA were deposited into the Gene Expression Omnibus database under accession numbers MK494928. The data are available at the National Center for Biotechnology Information [15]

Leaves of *M. piperita* L. were collected from the Melikgyugh settlement in the Aragatsotn region (40°40'10"N, 44°21'27"E, elevation: approximately 900 meters) in July and August. *S. rebaudiana* Bertoni was cultivated in hydroponic plant vessels (1 m^2), in the Ararat Valley (elevation: 850-900 meters above sea level). This region is characterized by a predominantly dry climate, with an average annual temperature ranging from 11.0°C to 11.8°C, relative humidity of approximately 40%, and annual precipitation between 200 mm and 300 mm. In the hydroponic system, the plants were provided with the nutrient solution recommended by Babakhanyan et al. [16]. The growing medium consisted of red and black volcanic slags, as well as gravel particles with diameters

ranging from 3 to 15 mm. Prior to use, the filler material was sterilized using a 0.05% $KMnO_4$ solution [16]. The dried leaves of *S. rebaudiana* were provided by the Institute of Hydroponic Problems after G.S. Davtyan. The stevia leaves were dried in a well- ventilated room, away from direct sunlight, for 3 days at an air temperature of 37°C.

Preparation of plant extracts: Distilled water and 96% ethanol were used to prepare the plant extract solutions. For the *M. piperita* aqueous extract, 100 g of washed *Mentha piperita* L. leaves were crushed in 100 mL of sterile distilled water and incubated at +4°C for 12 to 18 hours. The resulting mixture was then filtered through gauze and sterilized at 115°C for 15 minutes. For the ethanol extract, an equal amount of 96% ethyl alcohol was used as a solvent and incubated under the same conditions. After the complete evaporation of ethanol using a centrifugal evaporator (Labconco 7810010 Centrifugal Evaporator), the resulting mixture was transferred to a sterile mortar and kept under sterile conditions until all ethanol evaporated. After complete evaporation, 100 mL of sterile distilled water was added to the remaining extract to maintain the equal volume of extracts.

For the preparation of the *Stevia rebaudiana* Bertoni extract, 10 g of dried, crushed *S. rebaudiana* leaves were added to 100 mL of sterilized distilled water and stored in the refrigerator at +4°C for 12 to 18 hours. The mixture was then filtered using gauze and sterilized at 115°C for 15 minutes.

Both prepared solutions were stored in the refrigerator at +4°C and used as needed.

Since distilled water was used as the primary solvent for the plant extracts, sterile distilled water served as the negative control in all experiments. No positive control (e.g., antibiotics or synthetic antimicrobials) was included, as the tested substances were natural plant extracts, which are not directly comparable to pure chemical agents. The aim of this study was to develop a functional food product based on a natural mixture of plant extracts and probiotic bacteria.

Therefore, comparing these natural formulations to synthetic compounds was not considered appropriate, and a positive control was intentionally omitted.

Investigation of the Antibacterial Activity of *M. piperita* L. and *S. rebaudiana* Bertoni Extracts: The antibacterial properties of the plant extracts were evaluated using the well diffusion method on agar plates. Wells were created in the solid nutrient medium using a sterile punch. The LAB strains were cultured in milk for 24 hours. A total of 10 LAB strains, as previously mentioned, were used as test organisms. To prepare an overnight culture, the test organisms were first inoculated into a liquid growth medium using a sterile needle and then incubated at 37°C for 24 hours.

The following day, 0.1 mL of the overnight culture was transferred to a sterilized Petri dish and mixed with an MRS agar (0.9% agar) medium that had been thawed and cooled to 45–50°C. Wells were then created in the solid medium using a sterile punch, and 0.1 mL of the alcoholic or aqueous extracts—freshly prepared from *M. piperita* and *S. rebaudiana* leaves before each experiment—was added to the wells. The Petri dishes were incubated at 37°C for 24 hours under optimal conditions for microbial growth. After incubation, the diameter of the inhibition zones was measured to assess antibacterial activity.

Bacterial growth was monitored in MRS broth over a 27-hour period at 37°C using a spectrophotometer (Bioevopeak SP-LUV752P, China) at a wavelength of 595 nm. pH measurements were taken using a K-766 Knick pH meter (Germany).

Development of a Novel Dairy Product Using Plant Extracts: The first stage in developing a new dairy product involves fermenting pasteurized milk with starter strains *L. acidophilus* Er 317/402 and *L. delbrueckii* subsp. *bulgaricus* B7, either separately or as a 1:1 mixture. For

fermentation, 5% of the starter strains were added, along with 5%, 10%, or 15% of either aqueous or alcoholic extracts of *M. piperita*, or 1.5%, 2.5%, or 5% of *S. rebaudiana* extracts. In the case of using the mixture of starter strains, 2.5% of each strain was added. Subsequently, the fermentation duration and organoleptic properties were evaluated.

A minimum of 30 participants, including students, assessed the organoleptic properties using a questionnaire that described 10 specific attributes: product appearance, color, aroma, taste, viscosity, acidity, aftertaste, density, composition, and structure. For comparison, the traditional Armenian functional product (milk fermented with *L. acidophilus* Er 317/402 strain) available on the Armenian market was used as a control.

Evaluation of antibacterial, anti-yeast and antifungal activities in a novel dairy product: The Well diffusion method was applied to view antibacterial activity. Briefly, agar plates were seeded with the target indicator microorganisms, and 6 mm wells were punched into the agar. A volume of 100 µL of each test sample was aseptically dispensed into the wells. Plates were incubated under appropriate conditions depending on the indicator strain.

Control formulations were prepared using the same procedure. They excluded *Mentha piperita* and *Stevia rebaudiana* extracts and added 2.5% (w/v) sugar to match the sweetness level provided by stevia in the experimental samples. The diameters of inhibition zones were measured after incubation to evaluate antimicrobial efficacy. All tests were performed in triplicate.

The anti-yeast activity against *Candida albicans* ATCC 10291 and *Saccharomyces cerevisiae* YK-12 strains was evaluated using a method modified from Afzali et al. [17]. Initially, the dairy product was incubated in modified MRS medium (composition: peptone – 10 g/L, beef extract – 10 g/L, yeast extract – 5 g/L, glucose – 20 g/L, ammonium citrate – 2 g/L, magnesium sulfate – 0.05 g/L, Tween 80 – 0.8%, agar – 1.5%, sterilization: 121°C for 20 minutes) at 37°C for 48 hours.

Following the incubation, the plates were overlaid with 7 mL of Sabouraud nutrient medium containing 0.9% agar, which had been pre-thawed and cooled to 50°C. The yeast suspension, previously cultured in liquid Sabouraud medium at 37°C for 24 hours, was serially diluted to 10^6, and 0.5 mL from each dilution was applied to the Sabouraud medium, evenly spread across the surface and incubated at 37°C for another 48 hours. A reduction in the CFU/mL of yeast relative to the control suggests the presence of anti-yeast activity. The control setup was identical, except it did not include any dairy product. The antifungal activity of the dairy product was assessed using the agar diffusion method.

All experiments were conducted with modified MRS medium, that supported the growth of both fungi (molds) and LAB. Different species of molds were utilized as test organisms: *Mucor plumbeus*, *Fusarium oxysporum*, *Cladosporium herbarum* (isolated from spoiled food and provided by the Biopolymers Interaction Assemble, Function and Interaction of Proteins Laboratory (FIPL), INRA) [18], *Aspergillus flavus*, *Penicillium aurantioviolaceum*, *Penicillium candidum*, *Trichoderma viride, Trichoderma harzianum* (isolated from spoiled food and provided by Dr. K. Grigoryan and Dr. S. Badalyan, Yerevan State University, Yerevan, Armenia) and *Ascosphaera apis* (isolated from dead honeybees by us, Yerevan State University, Yerevan, Armenia). By using the agar diffusion method, 250 µL of overnight dairy product culture was added to small (7 mL) Petri dishes, which were then topped with modified MRS agar. After 48 hours of the dairy product cultivation, a fungal spore suspension (with a concentration of 10^4 spores per mL) was carefully applied dropwise to the surface of the medium. The fungal spore suspensions were prepared following the method outlined by Bazukyan et al. [19]. Antifungal activity was indicated by the absence of fungal or mold growth on the surface of the medium.

Statistical analysis: Results are presented as mean values with standard deviations, calculated from three independent experimental replicates. Data analysis was performed using Statgraphics software (Statpoint Technologies, Inc., Warrenton, VA, USA). Statistical significance was evaluated using R version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria), with a threshold of $P < 0.05$. Additionally, a two-way ANOVA followed by Tukey's post hoc test was applied using GraphPad Prism version 8.0.2 (GraphPad Software, USA) to further assess differences between groups.

RESULTS

The goal of this study was to develop a new functional food incorporating *M. piperita* plant extract, recognized for its antibacterial properties. To achieve this, LAB strains were screened in order to identify those unaffected by the extract. For this experiment, LAB strains maintained at the Department of Biochemistry, Microbiology, and Biotechnology, Yerevan State University, Faculty of Biosciences, were utilized. Figure 1 illustrates the antibacterial activity of *M. piperita* leaf extracts against ten different LAB strains.

Figure 1. Antibacterial effect of *M. piperita* extracts against LAB

On each Petri dish, "1" represents the water extract, and "2" represents the ethanol extract. 1. *Lactobacillus delbrueckii* subsp. *bulgaricus* INRA-2010-5.2, 2. *Lactobacillus rhamnosus* 21.1, 3. *Streptococcus thermophilus* VKPM B-3386, 4. *Lactobacillus delbrueckii* subsp. *bulgaricus* B7, 5. *Enterococcus durans*, 6. *Lactobacillus acidophilus* ER-317/402, 7. *Lactobacillus delbrueckii* subsp. *lactis* INRA-2010-4.2, 8. *Lactobacillus rhamnosus* R-2002, 9. *Lactobacillus delbrueckii* subsp. *bulgaricus* (RIN-2003-Ls), 10. *Lactobacillus acidophilus* JM-2012. 0.1mL of water was used as a control (data not shown)

The image demonstrates that *M. piperita* extracts had no inhibitory effect on the six strains studied. However, growth of *Lactobacillus delbrueckii* subsp. *lactis* INRA-2010-4.2, *Streptococcus thermophilus* VKPM B-

3386, and *Lactobacillus delbrueckii* subsp. *bulgaricus* (RIN-2003-Ls) was suppressed by the extracts. Diameters of the inhibition zones where LAB growth was absent are shown in Table 1.

Since the *M. piperita* extract inhibited the growth of certain bacteria, and *Enterococcus durans* required 48hours for growth, no further experiments were conducted with these strains. Instead, experiments were conducted with *Lactobacillus rhamnosus* R-2002, *Lactobacillus delbrueckii* subsp. *bulgaricus* B7, *Lactobacillus rhamnosus* 21.1, *Lactobacillus acidophilus* JM-2012, *Lactobacillus acidophilus* ER-317/402, and *Lactobacillus delbrueckii* subsp. *bulgaricus* INRA-2010-5.2 strains, cultured for 27 hours with varying extract concentrations.

Table 1. Antibacterial effect of *M. piperita* extracts against LAB.

LAB	Zones where LAB growth was inhibited, Ømm	
	water	ethanol
***Lactobacillus delbrueckii* subsp. *lactis* INRA-2010-4.2**		10*±0.2
***Streptococcus thermophilus* VKPM B-3386**	10±0.5	14±1
***Lactobacillus delbrueckii* subsp. *bulgaricus* (RIN-2003-Ls)**	10±0.3	12±0.2

* All results represent the mean values ± SD from three independent experiments. P<0.0001

From Figure 2, it is evident that the 1% and 5% *M. piperita* leaf extracts did not inhibit the growth of *Lactobacillus acidophilus* ER-317/402 and *Lactobacillus delbrueckii* subsp. *bulgaricus* B7. Therefore, all subsequent experiments were conducted with these two strains.

To create the starter culture mixture, the mutual antibacterial effects of the two strains are depicted (Figure 3).

L. acidophilus Er 317/402 and *L. delbrueckii* subsp. *bulgaricus* B7 strains, individually and in a 1:1 mixture, were used to prepare milk products in the initial stage. The CFU of each culture was no less than 10^8 per 1 mL. A 5% concentration of water or alcoholic extracts, prepared from *M. piperita* leaves, was added to the pasteurized milk, along with a 5% starter culture. After 5–6 hours of cultivation at 37°C, the pH reached 4.5 ± 0.3, indicating low amounts of synthesized lactic acid; therefore, the effect of acidity was disregarded in all subsequent experiments. Organoleptic evaluation by 20 students revealed that the product prepared with the alcohol extract of *M. piperita* did not have the characteristic mint aroma and taste. Consequently, all subsequent experiments were carried out using only the water extract of *M. piperita*.

Furthermore, varying concentrations of the water extract were tested to optimize the food product. After organoleptic assessment, it was determined that although a 15% extract concentration imparted a stronger taste and aroma, a 10% concentration was preferable both economically and in terms of providing a pleasant color to the product. To enhance the taste properties of the food, sucrose syrup and an aqueous solution of dried *S. rebaudiana* Bertoni leaves were also evaluated. It is worth noting that previous studies by the department demonstrated a stimulating effect of stevia extracts on LAB growth and acidification. Based on these findings, the optimal concentration of the stevia solution for the food product was determined to be 2.5%.

The antibacterial activity of the novel dairy product containing *M. piperita* extracts was evaluated using the well diffusion method. The inhibition zones were measured against a range of Gram-positive and Gram-negative bacterial strains.

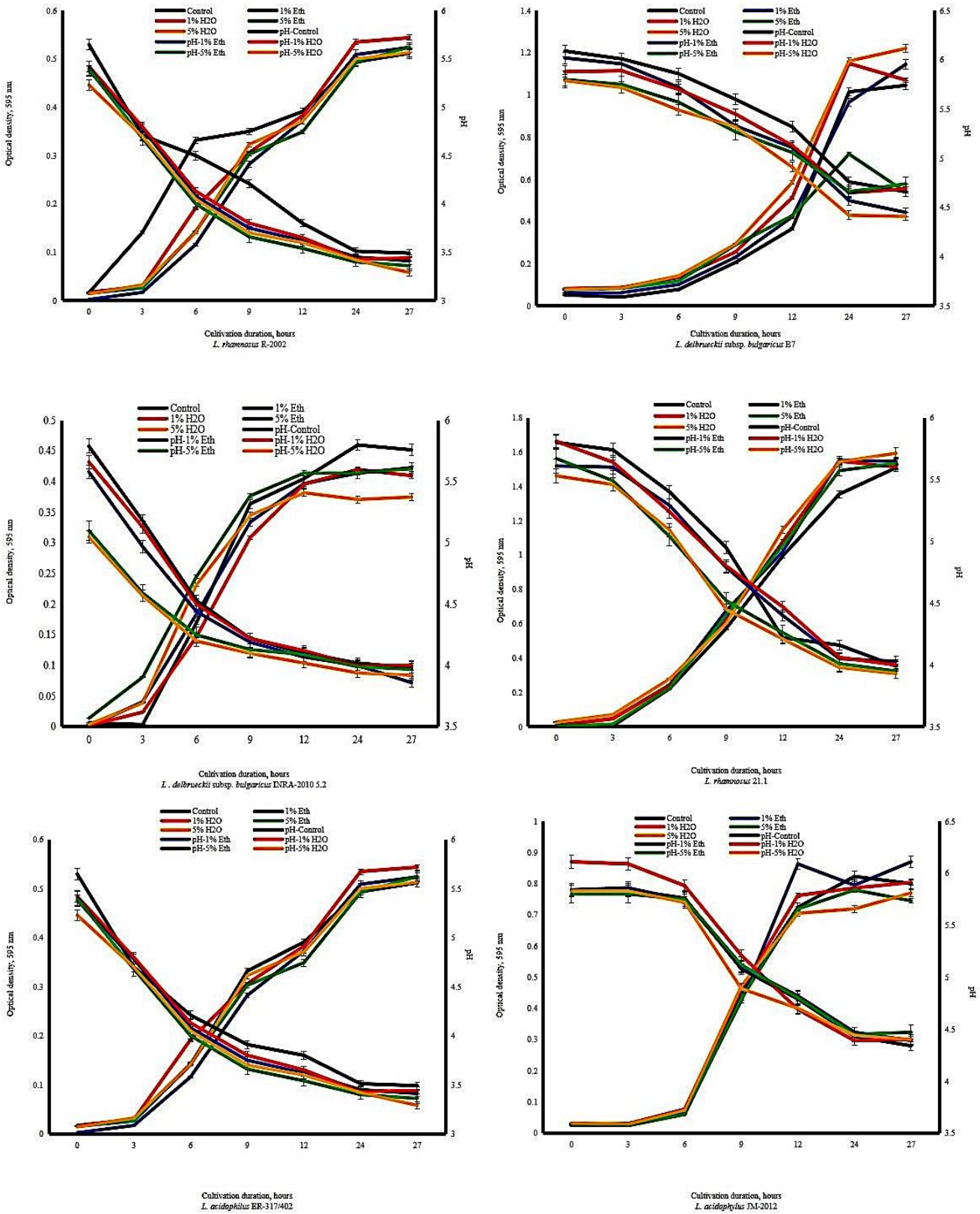


Figure 2. Growth and acid production of LAB in the presence of *M. piperita* extracts.

The figure illustrates the effect of aqueous and ethanolic peppermint extracts on the growth and acid production of the six studied strains. The x-axis shows the growth duration, while the left y-axis represents optical density measured at 590 nm, and the right y-axis indicates the pH measured potentiometrically.

Figure 3. The antibacterial effect of *Lactobacillus acidophilus* ER-317/402 and *Lactobacillus delbrueckii* subsp. *bulgaricus* B7 against each other.

On the left is a Petri dish with a lawn of *Lactobacillus acidophilus* ER-317/402, into which the culture fluid of *Lactobacillus delbrueckii* subsp. *bulgaricus* B7 was added after 24 hours of cultivation in 10% skim milk.

On the right is a Petri dish with a lawn of *Lactobacillus delbrueckii* subsp. *bulgaricus* B7, into which the culture fluid of *Lactobacillus acidophilus* ER-317/402 was added after 24 hours of cultivation in 10% skim milk.

The results demonstrated varying degrees of antimicrobial activity, with some strains showing significant inhibition, while others showed less or little inhibition. The antibacterial activity was assessed by measuring the diameter of the inhibition zones in millimeters. The figure 4 presents the results of the antibacterial activity of the final product.

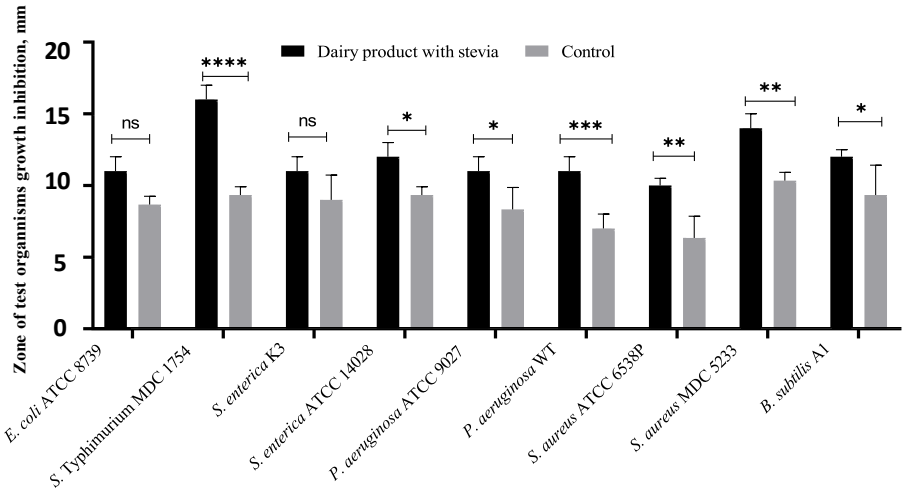


Figure 4. The antibacterial activity of the final product. P<0.0001

The anti-yeast activity of the novel dairy product was evaluated by assessing the reduction in yeast colony-forming units (CFUs) of *C. albicans* ATCC 10291 and *S. cerevisiae* YK-12. The results showed a notable reduction in the CFU count of *C. albicans* and complete inhibition of *S. cerevisiae* when exposed to the product containing *Mentha piperita* extracts. For *C. albicans*, the CFU count was reduced to 5×10^5, compared to the control group, which showed a CFU count of 2×10^6. For *S. cerevisiae*, complete inhibition was observed, with no CFUs detected in the sample treated with the dairy product containing the extract, compared to the control, which had a CFU count of 5×10^6.

The anti-mold activity of the product with inhibited growth of all test molds (Figure 5).

Figure 5. Anti-mold activity of the final product. From left to right: controls (left side of each plate) where molds were grown without treatment, and experimental samples (right side) where the fermented product was applied. **A)** Top to bottom: *Penicillium candidum*, *Trichoderma viride*, *Penicillium aurantioviolaceum, Fusarium oxysporum*, *Cladosporium herbarum*. **B)** Top to bottom: *Aspergillus flavus*, *Mucor plumbeus*, *Trichoderma harzianum*, *Ascosphaera apis*. As it can be seen the product inhibited the growth of all test molds.

DISCUSSION

The genus *Mentha*, encompassing multiple species, is widely recognized for its potent antibacterial properties. According to Muharran and coauthors [20], *Mentha piperita* leaf extracts at concentrations of 10%, 25%, and 50% demonstrated strong antibacterial activity against *Lactobacillus acidophilus*. In contrast, our study showed that neither the aqueous nor the ethanolic extracts of *Mentha piperita* L. inhibited *L. acidophilus* 317/402 or *L. delbrueckii* subsp. *bulgaricus* B7 strains.

Our research recommended combining *L. acidophilus* Er 317/402 with *L. delbrueckii* subsp. *bulgaricus* B7, a strain originally isolated from the honeybee gut. While LAB are known to inhibit members of the same genus, our study observed a neutral interaction between these two strains, indicating their compatibility, thus supporting their potential as starter cultures in functional food development.

Our findings revealed that newly created dairy products showed significantly high antibacterial activity against a broad spectrum of test bacteria.

Previous research studies have demonstrated that strain 317/402 has strong antagonistic activity against a broad range of pathogens, including clinical isolates of *Streptococcus* spp., *Staphylococcus aureus*, *E. coli*, *Salmonella* spp., *Shigella* spp., *Klebsiella*, *Proteus*, *Peptostreptococcus*, *Pseudomonas.* In our experiments, the fermented product derived from the co-culture of the

two strains, enriched with *Mentha* and *Stevia* extracts, exhibited 20–30% greater antibacterial activity against a broad spectrum of bacteria compared to the product fermented solely by strain 317/402.

Bazukyan et al. [19] reported that *L. acidophilus* 317/402 completely suppressed *Candida guilliermondii* H17 but had no impact on *C. albicans* 301. The obtained results are in agreement with their data, as the extract completely inhibited *S. cerevisiae* YK-12 and partially inhibited *C. albicans* ATCC 10291. This suggests that the tested combination of the strains may not be suitable for use in the baking process, where *S. cerevisiae* serves a key functional role.

Bazukyan et al. [19] showed that *L. acidophilus* 317/402 was limited to delayed spore germination of *Cladosporium* species. Experimental evidence suggests that the final product effectively inhibits the growth of nine distinct mold species.

Further evidence revealed that certain metal ions can enhance the resistance of *Lactobacillus acidophilus* to bacteriophage attacks, particularly those affecting gram-positive bacteria, while some vitamins may reduce this protective effect [21]. The strain also demonstrated antioxidant activity, with its effectiveness influenced by the method of cell drying. This probiotic strain is included in commercial products that combine it with plant-derived compounds to support digestion, immunity, and circulation. In veterinary applications, formulations that include this strain along with specific herbal extracts have shown improved effects on gut microbiota, including a reduction in harmful bacteria and an increase in beneficial LAB [21].

Building on these findings, future research may explore how optimizing the balance of metal ions and minimizing vitamin-induced inhibition could further enhance the fermented product's resilience and probiotic functionality. Additionally, improving preservation techniques to maintain its antioxidant activity, and combining it with carefully selected plant-based compounds, could result in more potent, multifunctional probiotic formulations suitable for both human and veterinary use. These strategies may significantly increase the product's value as a component in next-generation functional foods.

CONCLUSION

This study successfully developed a novel functional dairy product incorporating *Mentha piperita* leaf extract, leveraging its antibacterial properties. Screening of various LAB strains identified *Lactobacillus acidophilus* Er 317/402 and *Lactobacillus delbrueckii* subsp. *bulgaricus* B7 as compatible starters unaffected by the extract, exhibiting mutualistic interactions. Fermentation trials demonstrated that the water extract of *M. piperita* at 10% concentration provided an optimal balance of sensory quality and economic feasibility. Supplementation with 2.5% stevia extract further enhanced the product's taste while supporting LAB growth.

The final dairy formulation exhibited significant antimicrobial activity against a broad spectrum of bacterial pathogens, yeasts, and molds. Notably, it markedly reduced *Candida albicans* viability and completely inhibited *Saccharomyces cerevisiae*, alongside strong anti-mold effects. The synergistic use of a novel bee-derived strain (*L. delbrueckii* subsp. *bulgaricus* B7) and a standard probiotic strain (*L. acidophilus* Er 317/402) as starter cultures not only produced a nutrient-enriched, bioactive-rich product but also reveals their potential for application in commercial dairy production to extend shelf life and improve health benefits. These results underscore the potential of combining *M. piperita* extracts with selected LAB strains to create functional foods with enhanced safety and health-promoting properties.

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Data availability: All data generated or analyzed during this study are included in this published article. PRO-Seq data of 16srRNA were deposited into the Gene Expression Omnibus database under accession numbers MK494928. The data are available at the following URL https://www.ncbi.nlm.nih.gov/nuccore/MK494928.1/

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