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New generation of functional yogurts fermented with probiotic lactic acid bacteria isolated from human milk

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ABSTRACT

Background: The cultures used in the production of "bio-yoghurts" typically consist of a blend of traditional yogurt starters, such as *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, along with probiotics like *L. acidophilus*, *L. casei*, *L. reuteri*, and *Bifidobacterium* spp. These probiotics, although beneficial, face challenges thriving in milk due to its short fermentation time (4–5 hours). The rapid acidification caused by the starter’s results in probiotic levels dropping below the "therapeutic minimum" during the intended refrigerated shelf life of the product. A potential solution to this issue lies in leveraging lactic acid bacteria isolated from the human milk which possess both robust probiotic characteristics and starter properties. Incorporating such strains in yogurt starter could address the acidity imbalance and contribute to maintaining optimal probiotic levels throughout the refrigerated shelf life of the product.

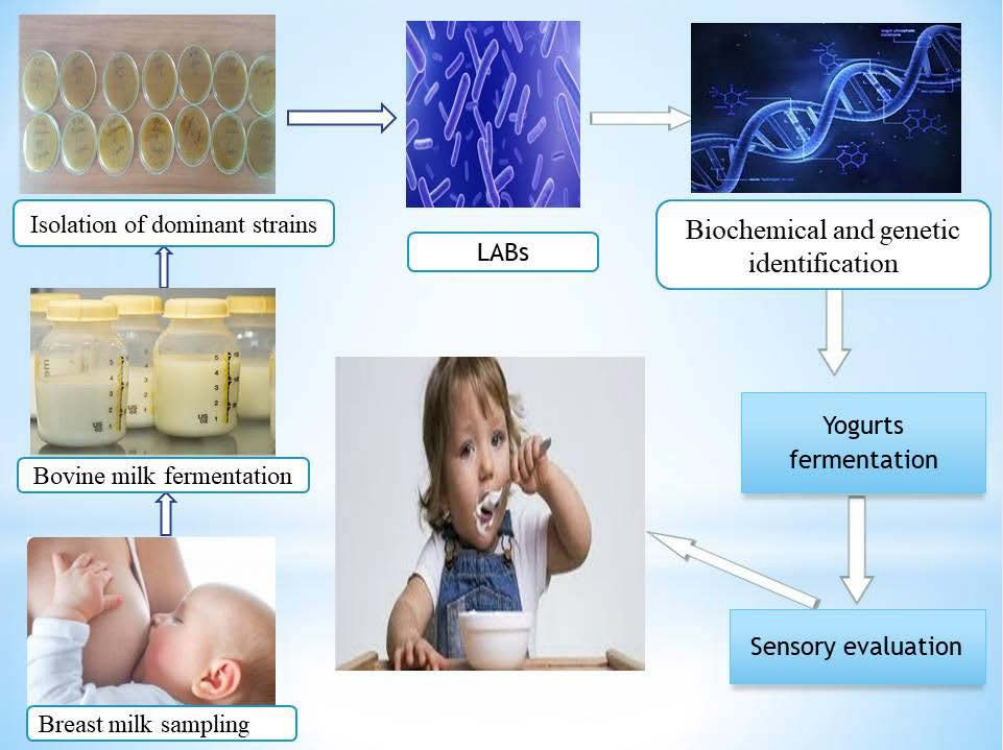
Objective: This research aims to develop a new generation of sustainable functional yoghurts suitable for consumers of all ages using as starters predominant probiotic lactic acid bacteria isolated from the breast milk of healthy women.

Results: This study marks the first implementation of the critical dilution culture method in sterile cow milk for the selection of predominant lactic acid bacteria (LABs) from women's breast milk. Remarkably, all samples yielded LAB capable of independently fermenting milk. Although breast milk is a good medium for the growth of residential lactic acid bacteria, it never coagulates due to low concentration of caseins. The isolated strains, identified as belonging to the species *L. delbrueckii* subsp. *lactis*, *L. fermentum*, *L. casei* subsp. *sakei*, *Streptococcus thermophiles* and *Lactococcus lactis*,

exhibited probiotic and adaptive properties essential for gut colonization in humans. Utilizing symbiotic LAB starters were created functional yogurts with heightened technological, physicochemical, and sensory characteristics.

Conclusions: Yogurts fermented using human milk LABs are safe and can be recommended as a functional dairy food, for pregnant women as well as for pre-term and full-term infants as a substitute/supplement for mother's milk. Moreover, these LABs can be included in infant formulas or used in pre-treatment of infant formula milk to improve its nutritional value and safety.

Key words: Breast milk, lactic acid bacteria, probiotic, milk fermentation, functional yogurt



INTRODUCTION

A woman's transient microflora associated with breast milk emerges in the third trimester of pregnancy, persists throughout the lactation period, and ceases post-lactation. The composition of women's breast milk microflora exhibits significant variability based on factors such as diet, cuisine, geographical location, climatic conditions, and lifestyle [1-3]. The prevailing belief is that the majority of probiotic bacteria in the milk originate from the maternal intestinal microbiota, establishing a connection with the mammary gland epithelium through an internal route. Bifido and lactic acid bacteria traversing the intestinal tract play a crucial role in safeguarding infants against infections and contribute to the development of the immune system [4-6].

Two decades ago, both in Spain and Finland, lactic acid bacteria with probiotic potential were independently isolated and characterized from breast milk [7-8]. Their research highlighted the protective role of microbial components in breast milk against infections for both the mother and the infant [3]. Notably, identified lactic acid bacteria, including *L. gasseri* CECT 571423, *L. salivarius* CECT 571324, and *L. fermentum* CECT 571623, exhibited probiotic properties comparable to many commercial strains isolated from the intestinal tract [9]. Subsequent studies analyzing breast milk from numerous women have revealed a diversity of probiotics, particularly lactobacilli. The species composition of lactobacilli in breast milk varies among countries, cities, and rural communities [1, 3, 10].

Presently, numerous companies manufacture capsules, tablets, and sachets containing probiotics isolated from breast milk, specifically designed for women in the third trimester of pregnancy and infants. While fermented milks are considered excellent vehicles for delivering probiotics to consumers in terms of safety, the inclusion of probiotic bacteria in fermented dairy products enhances their value as superior therapeutic functional foods [3, 8, 11]. However, issues such as the lack of acid tolerance, insufficient viability, and survival of these bacteria in fermented milk pose challenges in commercial food products. The viability of probiotic bacteria in fermented dairy products, whether added before fermentation, simultaneously with conventional starter cultures, or after fermentation to cooled (4°C) products before packaging, depends on factors such as final acidity, growth promoters and inhibitors, strains used, interaction between species present, culture conditions, level of inoculation, and storage temperature
[12]. The selection of a probiotic strain that aligns with food processing technology is crucial. Our early studies showed that breast milk samples collected aseptically had the ability to coagulate cow's milk, suggesting on the presence of starter cultures in breast milk microbiom [13].

This research aims to develop a new generation of sustainable functional yoghurts suitable for consumers of all ages using as starters predominant lactic acid bacteria isolated from the breast milk of healthy women. Furthermore, the aim is to incorporate these yogurts into the diets of pregnant women and infants, thereby contributing to the enhancement of nutritional value and potential health benefits for these specific demographic groups.

MATERIALS AND METHODS

Media: LAPTg agar and broth for cultivation of rod-shaped and coccoid lactic acid bacteria, consisting of 10 g/l yeast extract, 15 g/l Bacto peptone, 10 g/l Bacto tryptone, 10 g/l glucose, 1 g/l Tween 80. Muller-Hinton agar (Liofilchem, Italy), Nutrient Broth (NB) (Condalab, Spain), Dextrose Agar (SDA) (HiMedia, India), skimmed milk, methylene blue, H_2O_2, bile powder (Micromaster), Gram Staining Set (Ghatran, IIR), Nessler's reagent (Himedia), L-arginine (Sigma), NaCl, NaOH, oxidize discs (Himedia), sterile filter paper discs, 6 mm (Sigma), antibiotic discs (Liofilchem, USA). API 50 CH (BIOMÉRIEUX, France).

Test culture: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* MDC10002: The test cultures were sourced from the Microbial Depository Center (MDC), SPC "Armbiotechnology," NAS of RA. These cultures were maintained on agar media at 4°C and subjected to subculturing on a monthly basis.

Breast milk sampling: Breast milk samples were collected aseptically from healthy volunteers, with rigorous adherence to aseptic protocols. Prior to collection, hands, breast areola, and nipple were thoroughly washed

with soap and water, followed by cleansing with a 0.1% solution of chlorhexidine gluconate. To prevent potential skin contamination, approximately 0.5 ml of milk was intentionally discarded, after which 2-3 ml of milk was manually expressed into a sterile test tube. To maintain the integrity of the collected samples during transportation, they were promptly placed in a container with ice. Upon arrival at the laboratory, the analysis of the breast milk samples commenced without delay.

Isolation of predominant starter LABs from breast milk and total cell count: To isolate predominant lactic acid bacteria (LABs) from breast milk microbiota, a critical dilution assay was employed. A tenfold serial dilution of breast milk samples was prepared in cow's skimmed milk. Subsequently, 0.1 ml aliquots of these dilutions were plated on MRS agar. All tubes and plates were then incubated at 37°C until milk coagulation and colony formation occurred. The total microbial count was determined by counting the colonies (CFU/ml). The predominant LABs, capable of fermenting milk, were isolated from colonies that developed on the agar plates inoculated from the last coagulated milk dilution. Randomly selected colonies were transferred using a microbiological loop into 2 ml sterile milk and incubated at 37°C to confirm their fermentation ability.

Cell morphology assay: The cell morphology assay involved the examination of cells under a microscope with a magnification of 1000, utilizing the OMAX M83EZ-C50S microscope.

Determination of LAB stability to 0.1% methylene blue: A medium was prepared by combining skim milk with 0.1% methylene blue. To assess stability, 0.1 ml of an overnight culture was added to 2 ml of the medium. The mixture was then incubated at 37°C for 48 hours. The evaluation criteria involved observing any discoloration of the medium and monitoring the coagulation of the milk.

Resistance to bile salt: To evaluate the stability of lactic acid bacteria (LABs) to bile, milk formulations containing 2%, 3%, and 4% bile were prepared. Individual colonies of the strains under investigation were inoculated into the bile-infused milk and incubated at 37°C for 48 hours. The assessment of stability involved examining the formation of milk coagulum during the incubation period.

NaCl tolerance assay: 0.1 ml aliquots of overnight cultures of the test strains were introduced into 2 ml of LAPTg broth containing varying concentrations of NaCl (2%, 4%, and 6.5%). The mixtures were then incubated at 37°C for a duration of 48 hours. The growth of the strains were assessed based on the observation of turbidity in the broth, indicating bacterial proliferation.

API 50 CH Test: The carbohydrate-utilizing profiles of isolated lactic acid bacteria (LABs) were identified using the API 50 CH BioMerieux micro test system following the manufacturer's instructions. A pure bacterial culture was prepared and inoculated into individual wells on the test strip. Each well contained a specific substrate that reacted with enzymes produced by the bacterial strain. The inoculated strip was then incubated for a predetermined period, during which the bacteria metabolized the substrates, causing various chemical reactions. After incubation, the results were recorded based on observable changes in color, gas production, or other reactions in the wells. These reactions were then compared to a database of known reactions and interpreted using a web API.

Real time Polymerase Chain Reaction (RT-PCR): RT-PCR identification of lactobacilli was carried out in Laboratory of Standard Dialog LLC, Armenia

Potentiometric and titratable acidity: Potentiometric acidity was assessed using a Checker pH-meter (HANNA Instruments Inc., USA). Titratable acidity of fermented milk samples was determined according to Thorner (°Th). In this method, one unit of °Th corresponds to 9 mg of lactic acid in 100 ml of the sample. The titratable acidity provides information about the total amount of acid present in the fermented milk, measured in terms of lactic acid content.

Dynamic viscosity test: Kinematic viscosity of yogurts was measured using a Hoppler ball drop viscometer designed for the swift evaluation of the kinematic viscosity of fluids. In this test, the time it takes for a sphere to slide in the fluid placed within an inclined cylindrical tube is measured. The viscosity of the sample is related to the time the sphere requires to travel a known distance. This specific test is referred to as a Dynamic viscosity test, and it is suitable for measuring the viscosity of both Newtonian and non-Newtonian liquids. The results were expressed in the international standardized unit (mPa • s).

Antibiotic susceptibility assay: The susceptibility of lactic acid bacteria (LABs) to antibiotics was assessed using the disk diffusion method in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines [14]. In this method, filter paper disks (6 mm) impregnated with a standardized amount of antibiotic were placed onto Mueller-Hinton agar previously inoculated with 10^7 CFU of LAB. All agar plates were then incubated overnight at 37 °C for 24 hours. The zones of inhibition observed around the antibiotic disks were interpreted as either resistant or sensitive, based on the interpretative chart specified by the Kirby–Bauer method [14-15].

Antimicrobial activity of LAB cell-free supernatants: Briefly, cell-free supernatants (CFS) of LAB cultures were prepared, Overnight cultures grown in LAPTg were subjected to centrifugation (6000×g at 4 °C for 30 min) to remove cells, and the resulting CFS was filter-sterilized using a 0.45-µm Milipore filter. The sterile CFS was stored in tubes at 4 °C until use. The antimicrobial activity of CFS was assessed through the agar disk diffusion method. Paper disks soaked with CFS were placed on Muller-Hinton agar inoculated with test cultures and then transferred to a 37°C incubator. The following day, the growth inhibition zones of the test cultures were examined, providing insights into the antimicrobial properties of the lactic acid bacteria.

Microbial Adhesion to Solvents Test (MATS): Microbial Adhesion to Solvents (MATS) was assessed using the method outlined by Rosenberg et al. (1980) with some modifications [16]. LAB from overnight grown cultures were harvested by centrifugation at 5000 g for 15 minutes, washed twice, and then resuspended in 0.1 M KNO_3 (pH 6.2) to achieve a concentration of approximately 10^8 CFU/ml. The absorbance of the cell suspension was measured at 600 nm (A0). One milliliter of solvent was introduced to 3 ml of the cell suspension. After a 10-minute preincubation at room temperature, the two-phase system was mixed by vortexing for 2 minutes. Following 20 minutes of incubation at room temperature, the aqueous phase was removed, and its absorbance (A1) at 600 nm was measured. The percentage of bacterial adhesion to the solvent was then calculated using the formula: $(1 - A_1/A_0) \times 100$

Yogurt preparation: Milk was pasteurized at 95°C for 5 minutes to ensure safety and eliminate unwanted microorganisms then cooled to 45°C and inoculated with 3% concentration of starter lactic culture. The mixture was blended then poured into 200 g clear plastic cups and incubated at 37°C. The coagulation of the milk was monitored visually and by observing pH changes during the incubation period until a pH of 4.6 was attained. Once the desired pH was achieved, yogurt samples were stored at 4°C to allow for proper setting.

Sensory analysis: The yogurt samples were evaluated by a trained panel to assess characteristics such as texture, flavor, and overall quality. A total of 12 panelists, comprising staff from the laboratory of lactic acid bacteria at the S&P Center of "Armbiotechnology," were engaged in sensory evaluation. These panelists received instructions on how to conduct the sensory assessment. To minimize bias, the order of yogurt sampling was randomized for each panelist, and water was provided for mouth rinsing between tasting samples. Panelists were instructed to rate their liking of seven predefined characteristic properties, which included: Appearance: Evaluating color and syneresis (separation of whey from the yogurt).

Flavor: Assessing aroma and the balance between acid and sweet taste. Texture: Considering firmness, creaminess, and homogeneity. A hedonic scaling system was used, ranging from 1 (indicating extreme dislike) to 10 (indicating extreme liking). This allowed panelists to express their preferences for each characteristic property.

Experimental design and statistical analysis: The experiments were meticulously designed and executed with 3-5 repetitions to ensure robust data collection. The collected data underwent statistical analysis for reliability assessment, utilizing the Student's t-test. The statistical computations were performed using the R Project for Statistical Computing version R 3.1.1 program.

RESULTS AND DISCUSSION

Isolation and identification of LABs: To isolate predominant lactic acid bacteria from human milk, which could potentially serve as starters for cow's milk, a method involving serial 10-fold critical dilutions in cow milk was employed. Subsequently, 0.1 ml from each dilution was spread-plated onto LAPTg agar, followed by incubation at 37 °C. An intact breast milk sample served as a negative control (Table 1). This isolation process aims to identify lactic acid bacteria from human milk that demonstrate the potential to be effective starters for cow's milk products. The amount of bacteria in breast milk was determined immediately and after sampling and after overnight cultivation at 37 °C using pour plate technique (Tab. 1).

Table 1. Cow milk coagulation and plate counts of LAB in the breast milk samples

BM Sample	Breast milk dilutions in cow milk and coagulation					LAB *in situ* growth in breast milk samples	
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	IT	FT
DH1	+	+	+	+*	-	1.3×10^{-5}	3.7×10^{-11}
DH2	+	+	+	+*	-	3.8×10^{-4}	3.7×10^{-11}
DH3	+	+	+	+*	-	3.8×10^{-4}	3.7×10^{-11}
DH4	+	+	+	+*	-	3.8×10^{-4}	3.7×10^{-11}
DH5	+	+	+*	-	-	3.8×10^{-4}	3.7×10^{-11}

*- Milk coagulation on the second day of incubation
IT-Initial titer of human milk resident bacteria
FT- Final titer of human milk resident bacteria after overnight cultivation

The counting of coagulated cow's milk samples revealed that breast milk contains lactic acid bacteria in the range 10^4 - 10^5 CFU/ml. The bacteria causing the coagulation in the last samples were deemed predominant in human milk. Throughout the entire cultivation period of human milk, the titer of resident bacteria increased to over 10^{11} CFU/ml, yet without causing coagulation. However, when a dilution up to 10^{-10} was made from overnight cultivated breast milk, it induced coagulation in cow's milk. Colonies grown on LAPTg agar from the last dilutions of curdled milk were considered predominant, and these were isolated for further study. Gram staining and morphometric assessment of colonies and cells of lactic acid bacteria isolated from breast milk were performed, as outlined in Table 2.

Table 2. Gram staining and morphometric characteristics of breast milk microorganisms

Strain	Gram staining	Colony morphology		Cell morphology	
		Appearance	Diameter, mm	Appearance	Size, µm
DH1	+	Creamy	2.0-2.2	Streptococci	0.7-0.9
DH2	+	Creamy	1.8-2.0	Cocci couples, tetrads, and short chains	0.5-1.5
DH3	+	Light brown	1.2-1.5	Rod-shaped	0.5-0.8 × 2-9
DH4	+	Light brown	1.0-1.5	Rod-shaped	0.5-0.9 × 1.3-1.8
DH5	+	Light brown	1.2-1.6	Rod-shaped	0.7-1.1 × 2-4

Microscopic examination revealed the presence of 3 rod-shaped and 2 coccoid types of lactic acid bacteria (LAB), as outlined in Table 2. Colonies formed on MRS agar exhibited variations in appearance, color, and size. Cocci formed colonies with a diameter ranging from 1.8 to 2.2 mm, while rods formed light brown colonies with diameters of 1-1.6 mm. To further characterize these LAB isolates, several parameters were studied, and the results are presented in Table 3. The investigations included assessing the growth temperature range, oxidase and catalase activity, the ability to form ammonia from arginine, and the capacity to grow in milk with 0.1% methylene blue. These analyses contribute to a comprehensive understanding of the physiological and biochemical characteristics of the isolated lactic acid bacteria.

Table 3. Growth temperature range**,** catalase activities, ability to produce ammonia from arginine and tolerance to 0.1% methylene blue of LABs

LAB	Growth in temperatures, °C			Catalase & Oxidase Activity		Formation of NH_3 from arginine	Growth in milk with 0.1% methylene blue
	min	opt	max	-	-	-	-
DH1	10	45	50	-	-	-	-
DH2	10	37	45	-	-	+	-
DH3	25	45	50	-	-	-	-
DH4	15	45	50	-	-	-	+
DH5	15	45	50	-	-	-	-

(+) growth, (-) no growth

Based on the data presented in Tables 2 and 3, it is observed that all coccoid and rod-shaped lactic acid bacteria isolated from breast milk exhibit properties characteristic of lactic acid bacteria. Specifically, they are gram-positive, catalase-negative, tolerant to 0.1% methylene blue, and capable of producing NH3 from arginine. To more precisely identify the species of these lactic acid bacteria, they were subjected to API CH 50 and RT-PCR testing. The data are shown in Figure 1 and detailed in Table 4.

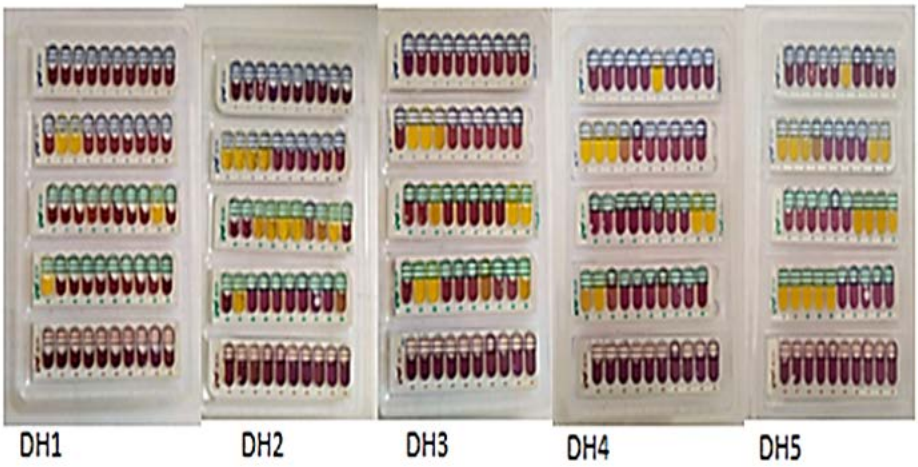


Figure 1. Breast milk LABs identification by API 50 CH.test.

Table 4. LABs identification by RT PCR and API 50 CH test.

Strain	API 50 CH, %	RT PCR, %	Species
DH1	100	99/100	*Streptococcus thermophilus*
DH2	100	99/100	*Lactococcus lactis*
DH3	99	97/99	*Lactobacillus delbrueckii* subsp. lactis
DH4	98	97/99	*Lactobacillus fermentum*
DH5	99	99/100	*Lactobacillus casei* subsp. *sakei*

Based on the results of API 50 CH and RT-PCR tests, the LABs isolated from breast milk DH1, DH2, DH3, DH4, and DH5 with high percentages of similarity belongs to the species *Streptococcus thermophiles* (100 and 99/100), *Lactococcus lactis* (100 and 99/100), *Lactobacillus delbrueckii* subsp. *lactis* (99 and 97/99), *Lactobacillus fermentum* (98 and 97/99), and *Lactobacillus casei* spp *sakei* (99 and 99/100), respectively.

Adaptive and probiotic properties of LABs isolated from breast milk: In the general selection and definition of probiotic bacteria, several criteria are commonly considered. These include: (a) it should be of human origin; (b) it must withstand transit through the gastrointestinal tract, that is show acid and bile tolerance; (c) ability to adhere to intestinal mucosa; (d) colonization potential in the human gastrointestinal tract; (e) production of antimicrobial substances; and (f) should demonstrate efficacy and safety, or be associated with other health benefits. The isolated lactic acid bacteria (LABs) were evaluated against some of these requirements for consideration as probiotics, as presented in Table 5.

Table 5. Bile, NaCl and pH tolerance and adhesion (MATS) of LABs

LAB	Growth with bile, %			NaCl tolerance, %			Growth in pH		MATS, %
	1	2	4	2.0	4.0	6.5	2.0	9.0	
DH1	+	+	+	+	+	+	-	+	42.32
DH2	+	+	-	+	+	-	-	+	53.52
DH3	+	+	+	+	+	+	+	+	72.84
DH4	+	+	+	+	+	+	+	-	58.36
DH5	+	+	-	+	+	-	+	+	66.28

(+) growth, (-) no growth. Adhesion properties was evaluated by the MATS test, where xylene was used as solvent.

The data presented in Table 5 reveals distinctive characteristics among the tested strains of lactic acid bacteria (LABs) isolated from human milk. The strains exhibit differences in their growth temperature range. All LABs except DH2 strain wers resistant to 4% bile. Strains DH2 and DH5 were sensitive to 6.5% NaCl, while coccoid strains were sensitive to low pH. All LABs from human milk demonstrated high adhesive properties. Notably, strains *St. thermophilus* DH1 and *L. lactis* DH2 exhibited previously undescribed hydrophobicity for cocci, measuring 42.32% and 53.52%, respectively. These values were significantly superior to known strains (*L. lactis* ATCC 19435 and *L. lactis* HV219) isolated from other sources. Hydrophobicity of Rod-Shaped LABs: (*L. delbrueckii*, *L. fermentum*, and *L. casei*) showed comparatively higher hydrophobicity, measuring 72.84%, 58.36%, and 66.28%, respectively.

Antimicrobial activity: The study investigated the antibacterial effects of probiotic lactic acid bacteria (LABs) against test microorganisms by assessing the antimicrobial activity of supernatants from overnight cultures. The results, as presented in Figure 2, were recorded on both the first and second days of cultivation.

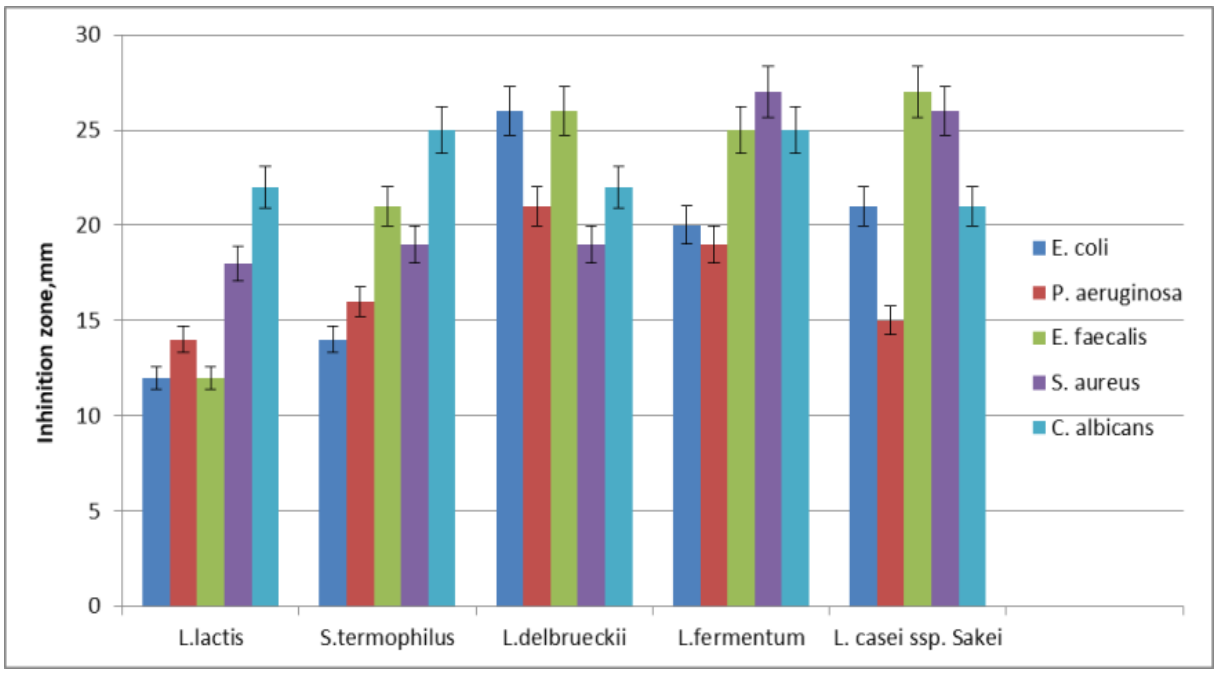


Fig. 2 Pathogens inhibition by LAB strains overnight cultures CFS

The study results indicate that all tested lactic acid bacteria exhibited a substantial antagonistic effect by inhibiting the growth of all pathogenic strains. Importantly, there was no significant difference observed between the inhibition diameters recorded on the first and second days ($p > 0.05$). This suggests that the antagonistic activity of the lactic acid bacteria remained consistent over the cultivation period. To ensure the safety of the isolated LABs, it is imperative to investigate their sensitivity to antibiotics.

This step is crucial to mitigate the potential risk of horizontal transfer of antibiotic resistance genes to commensal microorganisms within the human microbiome. Assessing antibiotic sensitivity is a standard practice in probiotic research to ensure that the use of these bacteria does not contribute to the spread of antibiotic resistance, maintaining their safety for consumption and potential therapeutic applications. Data demonstrating the sensitivity of LAB to antibiotics are presented in Fig. 3.

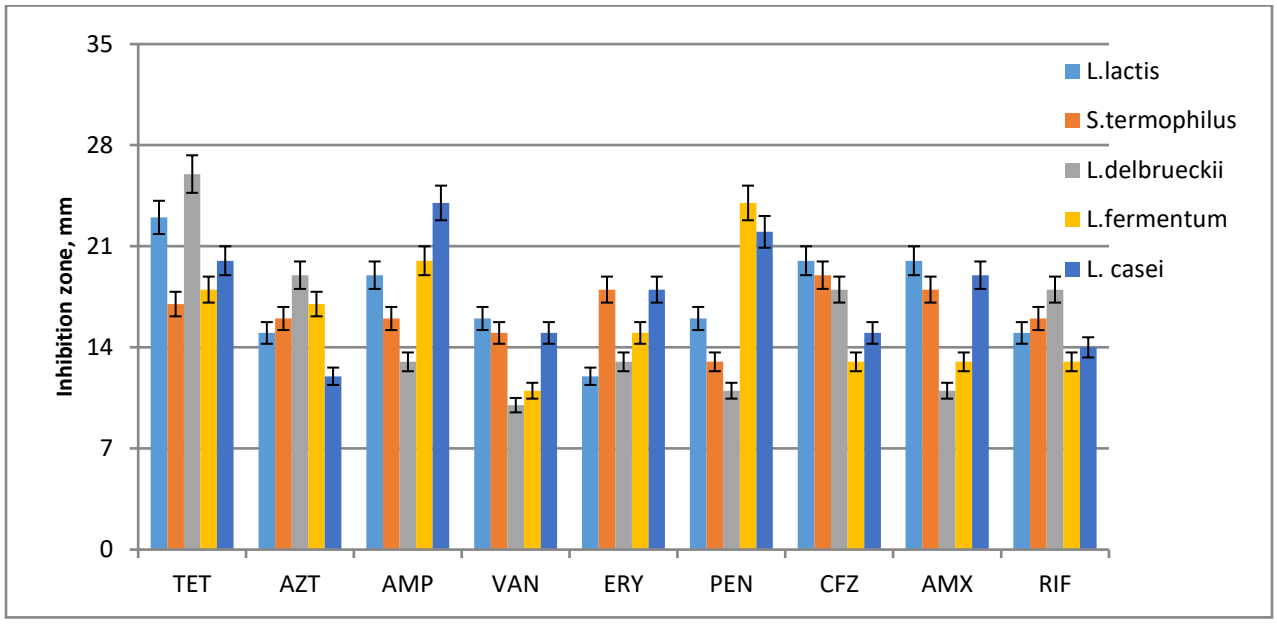


Fig.3 Antibiotic susceptibility of Lactic Acid Bacteria. TET: Tetracycline (30µg), AZT: Azitromicin (15 µg); AMP: Ampicillin (10 µg); VAN: Vancomycin (30 µg); ERY: Erythromycin (15 µg); PEN: Penicillin (10 µg); CFZ: Cefazolone (30 µg); AMX Amoxacillin (10 µg); RIF: Rifampicin (30 µg).

The findings indicate that despite the intrinsic resistance of lactic acid bacteria (LAB) to many antibiotics, the selected strains demonstrated sensitivity to the doses included in the disks, implying their safety.

Consequently, based on the comprehensive data presented, including tolerance to bile, NaCl, and extreme pH values, high hydrophobicity, utilization of maltose along with lactose, antibacterial activity, and sensitivity to antibiotics, it can be concluded that all strains isolated from breast milk meet the requirements to be considered probiotics.

Functional yogurt-like products fermented using LABs isolated from of breast milk: The conventional starter bacteria typically lack the ability to survive the passage through the intestinal tract, and therefore, they are not considered probiotics. In this study, the coagulation rate of milk by single lactic acid bacteria (LABs) was

investigated at a temperature of 37°C. Additionally, titratable acidity, measured by Thorner (°Th), was determined after 6 hours of maturation of curds at 4°C. The results are presented in Table 6. The coagulation rate and titratable acidity are essential parameters in assessing the fermentation and curd formation abilities of LABs. The data in Table 6 provide insights into the performance of the LABs in terms of their ability to ferment milk and contribute to the desired characteristics of dairy products.

Table 6. Milk coagulation rate by LABs and titrable acidity of fermented products

LAB	Milk coagulation rate, h						Titrable acidity, °Th
	4	5	6	7	8	14	
S. thermophilus DH1	-	-	-	-	-	+	61±2.5
L.lactis DH2	-	-	-	-	-	+	72±3.1
L. delbrueckii DH3	-	-	-	-	+	+	77±2.8
L. fermentum DH4	-	-	-	+	+	+	91±1.6
L.casei DH5	-	-	-	-	+	+	94±3.7

(+) coagulation, (-) no coagulation

The results presented in Table 6 indicate that all lactic acid bacteria (LABs) isolated from breast milk exhibited proper acidification and coagulation ability. Their coagulation times and acidity values ranged from 7 to 12 hours and 50 to 98°Th, respectively. Among rod-shaped lactobacilli, the strain *L. delbrueckii* DH3 demonstrated a faster coagulation property within 6 hours. Among coccoid LABs, the best coagulation rate was observed with *L. thermophilus* DH1. All LABs demonstrated continuous subculture stability.

Consumers generally prefer yogurts with mild acidity, therefore microbial cultures with mild acid production ability are typically selected to achieve yogurts with mild acidity and pH stability during shelf-life. Notably, in terms of acidity, both strains DH3 and DH1 exhibited the lowest acidity levels at 77 and 61°Th, respectively, resulting in mild fermented yogurt-like drinks. Therefore, it was concluded that the combination of *L. delbrueckii* DH-3 and *S. thermophilus* DH-1 would result in better sensory properties of yogurt (Tab.7).

Table 7. Microbiological and physical properties of yogurts fermented by sole and mixed cultures. The sensory properties of functional yogurt fermented by a 50/50 combination of *L. thermophilus* DH1 and *L. delbrueckii* DH3 starter cultures were evaluated for color, aroma, taste, and thickness. The results are presented in Table 8.

LAB	Titre, CFU/ml	pH	Acidity, °Th	Milk coagulation rate, h	Viscosity, mPa · sec
L.thermophilus DH1	4×10^{10}	5.0	60	18	32.1±0.4
L.delbrueckii DH3	7×10^{10}	4.5	65	6	62.3±0.4
Mix culture	3×10^{11}	4.2	72	6	81.2±0.2

Table 8. Affective scores obtained for functional yogurt

Descriptor	HMM yogurt	Plain Yogurt
Appearance	7.52±1.27	7.17±1.32
Odor	6.41±1.26	6.36±1.36
Taste	7.63±1.59	6.80±1.51
Texture	8.31±1.72	6.56±1.51
Overall acceptance	7.37±1.56	6.35±1.26

Mean ± standard deviation, HMM yogurt - Yogurt fermented by human milk microbes

The strains *L. thermophilus* DH1 and *L. delbrueckii* DH3 incorporated into the created starter blends, exhibit synbiotic properties. The resulting fermentation yields high-quality, low-fat functional yogurt (Tab. 8), making it a recommended functional food, particularly for women in the third trimester of pregnancy and for innate and premature babies as a substitute for mother's milk or a supplement to infant formula.

DISCUSSION

Human breast milk comprises several predominant bacterial species such as streptococci, micrococci, lactobacilli, lactococci, and bifidobacteria [17-20] and its intake favours the predominance of lactobacilli and bifidobacteria in the infant intestinal microbiota. The statement about the intestinal microbiota of full-term, vaginally delivered breast-fed infants being considered the gold standard aligns with scientific consensus. Breast milk is known to provide essential nutrients and bioactive compounds, including beneficial bacteria, to infants. The microbiota in breast-fed infants is often characterized by a predominance of beneficial bacteria, such as lactobacilli and bifidobacteria, contributing to the overall health of the infant. [11, 19-20]. It has only recently become accepted that breast milk constitutes an interesting source of probiotic LAB and bifidobacteria for inclusion in infant formulas and foods targeted to both pre-term and full-term infants [17, 21]. Human breast milk contains several predominant bacterial species, such as streptococci, micrococci, lactobacilli, lactococci, and bifidobacteria therefore, during breastfeeding, lactobacilli and bifidobacteria predominate in the baby's intestinal microbiota [22-24].

We have demonstrated that breast milk lactic acid bacteria possess high antimicrobial and antifungal activity is noteworthy. This aligns with existing literature and reinforces the potential of lactobacilli isolated from breast milk as a viable alternative to commonly prescribed antibiotics for the treatment of infectious mastitis during lactation [22-25]. The antimicrobial and antifungal properties of breast milk LABs suggest their potential role in supporting maternal and infant health. This natural defense mechanism may contribute to the prevention and treatment of infections, particularly in the context of lactation. The idea of utilizing these naturally occurring LABs as an alternative to antibiotics aligns with the growing interest in probiotics and their diverse health-promoting properties.

This research not only adds to the understanding of the beneficial aspects of breast milk but also highlights the potential applications of breast milk-derived LABs in the development of alternative therapeutic approaches for certain health conditions.

Lactobacillus species, such as *L.fermentum, L.gasseri*, *L. reuteri*, *L. casei*, *L. delbrueckii,* and *L. salivarius*, as well as lactococci like *L lactis*, are commonly identified in breast milk. These microbes exist in a balanced state in mother's milk, with a total count reaching up to 10^{4-5} GFU/ml [10, 13, 26-27]. The amounts

of LABs in the breast milk of Armenian healthy women vary between 10^4 - 10^5 CFU/ml, which coincides with the data presented in the literature. This microbial quantity is sufficient for effective colonization of the infant gut. An average 800 ml daily milk intake by an infant is equal to the ingestion of approximately 10^6 microbes [28-29].

Examinations of breast milk samples from hundreds of women across various countries have unveiled a noteworthy abundance of probiotics, primarily lactobacilli and bifidobacteria. Nevertheless, it is crucial to acknowledge that the specific species of these probiotic bacteria in breast milk exhibit variation among women from different countries [4-6, 10]. In our earlier report it was demonstrated that amount of microorganisms in breast milk of a healthy woman depends on the feeding rate [13].

Research has shown that breast milk is a selective and suitable environment for the growth of lactic acid bacteria. Thus, in samples of intact breast milk cultured ex vivo, the amount of predominant LAB in breast milk exceeded 10^{11} CFU/ml (Table 1).

All LABs isolated from human milk possessed tolerance to bile, NaCl, and extreme pH values, high hydrophobicity, antibacterial activity, and sensitivity to antibiotics. These distinct characteristics contribute to the uniqueness of each LAB strain and can influence their potential applications, particularly in probiotic formulations where adhesion, salt tolerance, and hydrophobicity are crucial factors. The ability of probiotic bacteria to proliferate and colonize in the intestine is crucial, and one important factor is their capacity to utilize maltose, a byproduct of starch metabolism commonly found in the intestine [31-35, 37]. The API CH 50 test revealed also that strains isolated from breast milk demonstrated the ability to utilize maltose. This finding is significant because it suggests that the lactic acid bacteria from human milk may possess specific metabolic capabilities that enhance their adaptability and potential beneficial effects in the gastrointestinal environment. The ability to utilize maltose can contribute to the successful establishment and survival of these bacteria in the intestinal tract, supporting their role as potential probiotic candidates.

Historically, fermented dairy foods have been recognized for their health-promoting and therapeutic effects. The market for probiotic dairy products is experiencing annual growth, driven by the increasing demand for these products due to the health benefits associated with probiotic bacteria originating from milk products. Bioactive compounds found in fermented dairy products also contribute to this demand, along with the potential prevention of lactose intolerance. Consequently, the development of such products stands as a key research priority in food design and presents a challenge for the dairy industry.

It is noteworthy that conventional starter bacteria lack the ability to survive passage through the intestinal tract, rendering them unsuitable as probiotics. Interestingly, all lactic acid bacteria isolated from breast milk simultaneously possessed starter and probiotic properties [5, 13, 36]. There is still no explanation for this phenomenon. Perhaps there is a selection mechanism for such bacteria in the mammary glands. Lactic acid bacteria isolated from breast milk alone or in combination are powerful starters for the production of functional yogurts. The bioyogurt we created based on probiotic LAB fully complies with the definitions of functional products given in the literature [38-39]. Strains of *L. thermophiles* DH1 and *L. delbrueckii* DH3 included in a combination starter showed biocompatibility and synergism in the fermentation of high-quality functional low-fat yogurt. These starters can be used also in preprocessing of milk serve as base in production of infant formulas.

CONCLUSIONS

The critical dilution method applied to cow's milk cultures proves effective in isolating predominant lactic acid bacteria from human breast milk. The microbiota of breast milk typically harbors potent starter cultures capable of fermenting cow's milk. Despite the robust growth of these resident starters in breast milk, coagulation does not occur due to the low content of caseins. The LAB (lactic acid bacteria) starter cultures isolated belong to the species *L. delbrueckii*, *L. fermentum*, *L. casei* ssp. *sakei*, *S. thermophilus,* and *L. lactis*.

Yogurt prepared using the most active synbiotic starter cultures is recommended as a functional food, especially for women in the third trimester of pregnancy, newborns, and premature babies. It serves as a viable substitute for mother's milk and an additive to infant formula. The specific strains identified contribute to the development of a product with potential health benefits.

List of Abbreviations: LAB: Lactic acid bacteria, HMM: Human milk microbes, HBM: Human breast milk, MDC: Microbial Depository Center, CFU: Colony forming unit, ATCC: American Type Culture
Collection, GIT: Gastrointestinal tract.

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