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Functional and Probiotic Characterization of Newly Isolated Strains from Infant Feces and Breast Milk

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Abstract: Infant feces and breast milk are sources of lactic acid bacteria with interesting functional and technological properties. Therefore, we focused on the isolation and characterization of new potential probiotic strains from infant feces and breast milk. Nine strains were identified based on 16S rRNA sequences of *Lacticaseibacillus rhamnosus*, *Limosilactobacillus reuteri*, and *Lactobacillus gasseri*. Subsequently, these isolates were evaluated for their safety (antibiotic resistance, hemolytic, and enzymatic activity) and functional properties (gastrointestinal condition tolerance, hydrophobicity, auto-aggregation, adhesion to Caco-2/HT-29 cell lines, antimicrobial and antioxidant properties, and the production of exopolysaccharides). All the isolates were non-hemolytic and resistant to streptomycin, kanamycin, and vancomycin. They also demonstrated antimicrobial activity against five selected pathogens (*Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Salmonella* sp., and *Enterococcus faecalis*) and the production of exopolysaccharides. Isolates *Lacticaseibacillus rhamnosus* S1F and S6C together with *Lactobacillus gasseri* M8C showed the highest ability to survive 3.0 g/L of bile salts and a good adhesion property, which is important for gastrointestinal tract (GIT) colonization or the inhibition of pathogens. Following in vivo characterization, three strains S1F and S6C from infant feces, and M6C from breast milk, showed probiotic properties with potential applications in the food industry.

Keywords: probiotics; *Lactobacillaceae*; adherence; antimicrobial activity

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1. Introduction

Evidence of the relationship between microbial composition and an individual's physical and psychological state has recently been growing [1–3]. Thus, the consumption of foods that bring health benefits is becoming increasingly common among the public. International market competition and increasing public interest in healthy lifestyles challenge innovations in the probiotic bacteria research sector. In addition to the commonly used microorganisms, efforts are being made to identify new strains of probiotic bacteria that positively affect human health. As our understanding of the intricate relationship between the microbiota and human health continues to evolve, harnessing the probiotic potential of the family *Lactobacillaceae* is a significant step toward personalized medicine and improved health outcomes for infants and beyond [4].

In recent years, potential probiotic microorganisms, such as lactic acid bacteria, bifidobacteria, and yeasts, have been isolated from different sources, including dairy and non-dairy fermented foods or the gastrointestinal tract of humans, animals, and insects [5–8].

However, the selection of new probiotics requires a systematic approach to characterize these candidates. According to the FAO/WHO [9], probiotics must be assessed as safe, must survive the inhospitable conditions of the gastrointestinal tract (low pH and bile salts), and have good functional and technological properties, such as antimicrobial activity against pathogens or adherence to the epithelial surface.

The most common sources of potentially beneficial microorganisms are fermented dairy products and other fermented foods, such as cabbage and other vegetables. However, many studies have addressed the isolation and characterization of novel potential probiotic strains from breast milk [10–12] or from the stools of adults and infants [13,14]. Breast milk provides a suitable environment for health-promoting microbial species because of its prebiotic milk oligosaccharide content [15]. Therefore, it is a source of lactic acid bacteria with remarkable properties [2]. Bacteria isolated from stool have been attributed to properties such as high resistance to bile salts, low pH, and lysozyme tolerance, as well as their ability to adhere to the intestinal epithelium [16]. Therefore, this study focused on the isolation of new strains from the genera *Lactobacillus*, *Lacticaseibacillus*, and *Limosilactobacillus* from infant feces and breast milk and investigated their probiotic potential. First, the isolates were evaluated for safety parameters (antibiotic resistance, hemolytic activity, and enzyme activity). Subsequently, functional attributes, such as resistance to gastrointestinal conditions, adhesion capabilities, antimicrobial and antioxidant activities, and exopolysaccharide production, were evaluated using in vitro methods.

2. Materials and Methods

2.1. Isolation and Identification

Samples of infant feces and breast milk were obtained from six volunteer donors (mothers and their children; Prague, Czech Republic). Voluntary donors signed informed consent forms. The fecal samples were sourced from healthy six to ten weeks old, breast-fed infants. Breast milk samples were collected in sterile tubes, and fecal samples were collected in tubes containing Wilkins Chalgren broth (Merck, Darmstadt, Germany). Strains from family *Lactobacillaceae* were isolated using two different media: Wilkins-Chalgren agar (Merck, Darmstadt, Germany) enriched with glacial acetic acid 0.1% (*v*/*v*; Sigma Aldrich, Prague, Czech Republic); and MRS agar, pH 5.7 (De Man, Rogosa and Sharp) (Merck, Germany). The cultivation was carried out anaerobically (37 °C, 72 h). First, the newly isolated strains were tested to determine bacterial cell morphology, and Gram staining was performed using an Eclipse E200 microscope (Nikon Europe B.V., Prague, Czech Republic). Subsequently, the molecular identification of the isolated bacteria was performed based on the alignment of the 16S SSU rRNA gene sequences using the BLAST alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 25 August 2021). Bacterial DNA was extracted using a DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The 16S SSU rRNA gene was amplified using the fD1 (5′-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3′) and rP2 (5′-CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T-3′) primers [17]. PCR products were separated on a 1% agarose gel (SeaKem® LE; Lonza, Rockland, ME, USA), stained with GelRed® (Biotium, Fremont, QC, Canada) at 70 V for 60 min, and visualized using the GENE GENIUS Bio Imaging System (Syngene, Frederick, MD, USA). All PCR products were treated with 2 µL of ExoSAP-ITTM according to the manufacturer's instructions (ThermoFisher Scientific, Baltic UAB, Vilnius, Lithuania), and sequenced by Eurofins Genomics Germany GmbH (Ebersberg, Germany) using fD1. The pure cultures of identified isolates were preserved in MRS broth (pH 5.7) with the addition of 20% glycerol (Sigma Aldrich, Czech Republic) at −20 °C.

2.2. Hemolytic Activity

The hemolytic activity of the isolates was determined using the method described by Sui et al. [18], with minor modifications. Bacterial cultures in the exponential growth phase were streaked onto Columbia blood agar plates (Merck, Darmstadt, Germany) enriched

with 5% sheep blood. The plates were incubated anaerobically for 48 h at 37 °C. Thereafter, the hemolysis zones were observed.

2.3. Antibiotic Susceptibility

The antibiotic resistance of the nine potential probiotic strains was assessed using the disc diffusion method [19]. Tested antibiotics were selected according to the European Food Safety Authority guidelines (EFSA; [20]): gentamicin (30 µg), erythromycin (15 µg), clindamycin (2 µg), streptomycin (25 µg), quinupristin/dalfopristin (15 µg), kanamycin (30 µg), chloramphenicol (30 µg), vancomycin (5 µg), ampicillin (10 µg), and tetracycline (30 µg; Oxoid, Hampshire, UK). The MRS agar plates with tested antibiotics, and strains were cultivated anaerobically at 37 °C for 24 h. Thereafter, the diameter of the inhibition zone was measured, and the resistance or sensitivity of strains to the antibiotics was evaluated such as resistant (R) when the inhibition zone had a diameter of <14 mm, intermediate (I) =15–19 mm, and sensitive (S) > 20 mm.

2.4. Antimicrobial Activity

The antimicrobial activity of the tested isolates against five pathogenic bacteria (*Bacillus cereus* CCM 869, *Escherichia coli* CCM 3954, *Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 3958, and *Salmonella enterica* serotype Typhimurium CCM 7205) obtained from the Czech Collection of Microorganisms (Brno, Czech Republic) was determined using a microtiter plate assay following the methods described by Kostelac et al. [21], with slight modifications. Briefly, the acid supernatants were separated by centrifugation (5000× *g*, 5 min) from the medium cultivated at 37 °C for 24 h. Subsequently, 20 µL of the pathogen suspension at a density of 3 McF, 20 µL BHI (brain heart infusion, Merck), and 160 µL supernatant was placed into the microtiter plate ($A_{s,0}$) and incubated for 48 h at 37 °C. After 48 h, the absorbance was measured at 620 nm using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland). A mixture of 20 µL pathogen suspension and 180 µL BHI (A_c) was used as the control. The measurements were made in triplicates. The inhibition level was determined using the following equation:

$$\text{inhibition } (\%) = \left(1 - \frac{A_{S,48} - A_{S,0}}{A_{C,48} - A_{C,0}}\right) \cdot 100$$

2.5. Hydrophobicity

The hydrophobicity was determined using the partially modified MATH method described by Krausová et al. [22]. The cells were harvested by centrifugation (5 min, 5000× *g*), washed twice with phosphate saline buffer (pH 7.0) and resuspended to an optical density of 0.50–0.60 at 620 nm (H_0) (Onda-V10 PLUS, Giorgio Bormac, Capri, Italy). Thereafter, 5 mL of bacterial suspension was mixed with 150 µL hexane (Merck, Darmstadt, Germany) and vortexed for 1 min. After 30 min at room temperature, the hydrophobicity of the aqueous phase was measured spectrophotometrically at 620 nm (H_t). Hydrophobicity was calculated using the following formula:

$$\text{Hydrophobicity } (\%) = ((H_0 - H_t)/H_0) \times 100.$$

2.6. Auto-Aggregation

The auto-aggregation of potential probiotic strains was evaluated using the method described by Reuben et al. [5], with some modifications. In brief, overnight-growth cultures of each strain (18 h, 37 °C) were centrifuged (7 min, 5000× *g*), washed twice, and resuspended in PBS to an optical density of 0.50–0.60 at 600 nm (A_0). The samples were incubated at 37 °C for 7 h. The absorbance of the suspension was measured after 2, 4, and 7 h of incubation and auto-aggregation was calculated as follows:

$$\text{auto-aggregation } (\%) = (1 - (A_h/A_0)) \times 100.$$

2.7. Adherence Assay

The adherence of isolates from the genera *Limosilactobacillus*, *Lactobacillus*, and *Lacticaseibacillus* to mixed human colon adenocarcinoma cell lines Caco-2 (ATCC HTB-37) and HT-29 (ATCC HTB 38; American Type Culture Collection, Rockville, MD, USA) was tested according to the method described by Musilova et al. [23]. A mixture of Caco-2 and HT-29 cells (9:1) prepared as described above was seeded in 24-well plates at a final concentration of 4×10^4 cells/mL. The isolates were cultivated overnight, washed thrice with PBS, and added to the seeded well plates at a density of 10^7 CFU/mL. Subsequent incubation was performed for 90 min at 37 °C. After incubation, the cells were detached from the wells. The remaining suspensions containing viable adherent bacterial strains were diluted and cultivated on MRS agar (Merck, Darmstadt, Germany) at 37 °C for 3 d under anaerobic conditions. The adherence of the isolates to HT-29/Caco-2 cells was evaluated as the percentage of adhered cells compared to the total cell count.

2.8. Scavenging of DPPH Radical

The DPPH (1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazyl) radical scavenging assay was performed according to the method described by Hyrslova et al. [24].

2.9. Resistance to Gastrointestinal Tract (GIT) Condition

The simulation of human gastric juice was performed according to the method described by Kostelac et al. [21], with slight modifications. Briefly, bacterial cells from the tested strains were harvested by centrifugation (7 min, 5500× *g*), washed twice with PBS (pH 7.0), and resuspended in the simulated gastric juice (SFG). SFG (pH 8.0) was prepared using pancreatin (1 g/L) (Sigma-Aldrich, Prague, Czech Republic) and bile salts (1.5 and 3.0 g/L) (Sigma-Aldrich, Czech Republic). The ability to survive under GIT conditions was evaluated by cell viability (%) using the ratio of cell counts (MRS agar, 37 °C, 72 h) at the beginning and after 4 h. The measurements were made in triplicates.

2.10. Production of Exopolysachaccharides

To assess the production of exopolysaccharides, the following basal medium was used: MRS-0 agar (10 g/L tryptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L K_2HPO_4, 1 mL/L Tween 80, 2 g/L ammonium citrate, 5 g/L sodium acetate, 0.2 g/L $MgSO_4 \cdot 7\ H_2O$, 0.04 g/L $MnSO_4 \cdot 4\ H_2O$, and 12 g/L agar) supplemented with 40 g/l carbohydrates. Glucose (G), lactose (L), and sucrose (S) were used as carbohydrate sources in this study. After sterilization (121 °C, 15 min), Kongo red solution (0.8 g/L) was added to the agar medium. Separated and washed bacterial suspensions were streaked onto plates with the prepared agar media and overlaid with agar. Incubation was performed at 37 °C for 72 h. The ability of bacterial isolants to produce exopolysaccharides was demonstrated by the black coloring of the center of the colonies (Figure 1).

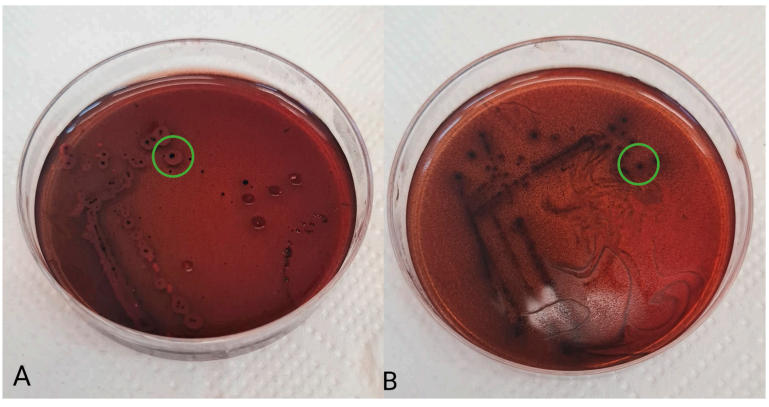


Figure 1. Production of exopolysaccharides (green circle) by *Lactobacillus reuteri* 2SA (**A**) and *Lacticaseibacillus rhamnosus* S6C (**B**).

2.11. Statistical Analysis

ANOVA with a post hoc Scheffé-test for multiple comparisons was used to evaluate the results, with statistical significance set at $p < 0.05$. For the comparison of only two datasets, a two-sample t-test ($p < 0.05$) was used instead of ANOVA. A Real Statistics Resource Pack software (Release 7.2; Charles Zaiontz) was used to perform the tests.

3. Results

3.1. Identification and Safety Assessment

Isolates from infant feces and breast milk, as shown in Table 1, belong to the new nomenclature of the genus *Lactobacillus* to three genera: *Lactobacillus*, *Limosilactobacillus*, and *Lacticaseibacillus* [25]. Specifically, *Lactobacillus gasseri*, *Lacticaseibacillus rhamnosus*, and *Limosilactobacillus reuteri* strains were identified using 16S rRNA gene sequencing. Potential probiotics must possess non-hemolytic properties to be safe for use. We confirmed that all the isolates tested in this study complied with this requirement. The resistance of these strains to commonly used antibiotics ($n = 10$) was evaluated. All the strains were shown to be resistant to streptomycin, kanamycin, and vancomycin. However, the results showed that the tested isolates were intermediately sensitive or sensitive to the other tested antibiotics, with the exception of gentamycin (Table 2). Three strains of *Lacticasebacillus rhamnosus* (S4F, S6C, and M8A) were resistant to all four abovementioned antibiotics.

Table 1. List of isolated strains from genera *Lactobacillus*, *Limosilactobacillus*, and *Lacticaseibacillus*.

Isolates		Origin	No. Accession	Gram Staining	Cell Morphology
Lacticaseibacillus rhamnosus 2	S1E	Infant feces	OR636880	+	rod
Limosilactobacillus reuteri 1	S2A	Infant feces	OR636881	+	rod
Limosilactobacillus reuteri 2	S2C	Infant feces	OR636882	+	rod
Lacticaseibacillus rhamnosus 1	S4F	Infant feces	OR636883	+	rod
Lacticaseibacillus rhamnosus 1	M6B	Breast milk	OR636884	+	rod
Lactobacillus gasseri 1	M6D	Breast milk	OR636885	+	rod
Lacticaseibacillus rhamnosus 3	S6C	Infant feces	OR636886	+	rod
Lacticaseibacillus rhamnosus 2	M8A	Breast milk	OR636887	+	rod
Lactobacillus gasseri 1	M8C	Breast milk	OR636888	+	rod

M—breast milk; S—infant feces; + —Gram-positive bacteria.

Table 2. Antibiotic resistance profile of tested strains.

Isolates		CN	E	CLI	S	Q/D	K	C	VA	AMP	TE
Lacticaseibacillus rhamnosus 2	S1E	I	S	S	R	S	R	S	R	I	I
Limosilactobacillus reuteri 1	S2A	I	S	S	R	I	R	I	R	I	I
Limosilactobacillus reuteri 2	S2C	I	S	S	R	S	R	S	R	I	I
Lacticaseibacillus rhamnosus 1	S4F	R	S	S	R	S	R	S	R	I	I
Lacticaseibacillus rhamnosus 1	M6B	I	S	I	R	I	R	S	R	I	I
Lactobacillus gasseri 1	M6D	I	S	S	R	S	R	S	R	I	I
Lacticaseibacillus rhamnosus 3	S6C	R	S	S	R	S	R	S	R	I	I
Lacticaseibacillus rhamnosus 2	M8A	R	S	S	R	S	R	S	R	S	S
Lactobacillus gasseri 1	M8C	I	S	S	R	S	R	S	R	I	S

Resistant (R) > 14 mm, intermediate (I) = 15–19 mm, sensitive (S) > 20 mm; CN—gentamycin (30 µg), E—erythromycin (15 µg), CLI—klindamycin (2 µg), S—streptomycin (25 µg), Q/D—quinupristin/dalfopristin (15 µg), K—kanamycin (30 µg), C—chloramfenikol (30 µg), VA—vankomycin (5 µg), AMP—ampicilin (10 µg), TE—tetracyklin (30 µg).

The extracellular enzyme activity was monitored using a commercial API ZYM kit (Table 3). All tested isolates were positive for esterase (C4), lipase-esterase (C8), leucinary-lamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α- and β-galactosidase, and β-glucosidase. Eight of the nine strains exhibited valine arylamidase activity. Seven strains tested positive for the enzyme α-glucosidase. Alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were not detected in any of the isolates.

Table 3. Enzymatic activity.

Enzyme	Isolates								
	S1E	**S2A**	**S2C**	**S4F**	**M6B**	**M6D**	**S6C**	**M8A**	**M8C**
Alkaline phosphatase	−	−	−	−	−	−	−	−	−
Esterase (C4)	+	+	+	+	+	+	+	+	+
Lipase esterase (C8)	+	+	+	+	+	+	+	+	+
Lipase (C14)	−	−	−	−	−	−	−	−	−
Leucin arylamidase	+	+	+	+	+	+	+	+	+
Valin arylamidase	+	−	+	+	+	+	+	+	+
Cystine arylamidase	+	−	+	+	+	+	+	−	+
Trypsin	−	−	−	−	−	−	−	−	−
α-chymotrypsin	−	−	−	−	−	−	−	−	−
Acid phosphatase	+	+	+	+	+	+	+	+	+
Naphtol-AS-BI-fosfohydrolase	+	+	+	+	+	+	+	+	+
α-galaktosidase	+	+	+	+	+	+	+	+	+
β-galaktosidase	+	+	+	+	+	+	+	+	+
β-glukuronidase	−	−	−	−	−	−	−	−	−
α-glukosidase	+	+	+	+	−	+	−	+	+
β-glukosidase	+	+	+	+	+	+	+	+	+
N-acetyl-β-glukosaminidase	−	−	−	−	−	−	−	−	−
α-mannosidase	−	−	−	−	−	−	−	−	−
α-fucosidase	−	−	−	−	−	−	−	−	−

+ Positive, − negative.

3.2. Hydrophobicity of Cell Surface and Aggregation Potential

The MATH method was used to assess the cell surface hydrophobicity of potential probiotic isolates. The tested strains displayed significantly different degrees of affinity for hexane (Table 4). Hydrophobicity was the highest in the *Lactobacillus gasseri* M6C strain (57.1%), followed by *Lacticaseibacillus rhamnosus* M8A (50.8%), and S1E (46.1%). The mean values obtained were also significantly different between the origins and genera.

Auto-aggregation is an important probiotic ability that helps reduce pathogen colonization. In this study, the auto-aggregation of the tested isolates was observed at 2, 4, and 7 h (Figure 2). The auto-aggregation abilities of isolates had a variable range between 5.8 and 28.5%, depending on incubation time and strain. *Lacticaseibacillus rhamnosus* S1E and S6C showed the strongest percentages of auto-aggregation (28.5% and 27.2%) after 7 h.

Table 4. Probiotic properties of tested isolates.

Isolates		Hydrophobicity (%)	DPPH (%)	Adherence (%)	Bile Salt Tolerance (%)	
					1.5 g/L	3.0 g/L
Lacticaseibacillus rhamnosus 2	S1E	46.1 ± 7.5 ^{a}	34.9 ± 4.1 ^{a}	0.5 ± 0.2 ^{a}	89.8 ± 1.5 ^{a}	89.6 ± 1.0 ^{a}
Limosilactobacillus reuteri 1	S2A	15.4 ± 5.3 ^{bd}	39.3 ± 9.2 ^{a}	3.8 ± 0.6 ^{b}	71.7 ± 3.0 ^{b}	41.5 ± 2.0 ^{b}
Limosilactobacillus reuteri 2	S2C	9.8 ± 4.6 ^{b}	40.4 ± 4.3 ^{a}	3.9 ± 1.0 ^{b}	67.2 ± 3.5 ^{b}	32.0 ± 0.5 ^{c}
Lacticaseibacillus rhamnosus 1	S4F	31.3 ± 5.2 ^{eh}	69.3 ± 2.5 ^{b}	0.3 ± 0.0 ^{a}	87.8 ± 0.5 ^{a}	85.4 ± 1.5 ^{a}
Lacticaseibacillus rhamnosus 1	M6B	12.3 ± 3.8 ^{eg}	52.1 ± 4.7^{c}	0.4 ± 0.1 ^{a}	78.9 ± 1.5 ^{c}	49.1 ± 3.0 ^{d}
Lactobacillus gasseri 1	M6D	31.2 ± 5.8 ^{dgh}	42.6 ± 4.6 ^{a}	0.4 ± 0.02 ^{a}	78.6 ± 1.0 ^{c}	64.5 ± 3.0 ^{e}
Lacticaseibacillus rhamnosus 3	S6C	27.8 ± 2.3 ^{b}	69.3 ± 2.1 ^{c}	0.4 ± 0.01 ^{a}	84.6 ± 7.5 ^{a}	69.9 ± 1.5 ^{e}
Lacticaseibacillus rhamnosus 2	M8A	50.8 ± 5.5 ^{af}	20.4 ± 1.6 ^{d}	0.8 ± 0.2 ^{a}	95.5 ± 4.0 ^{d}	48.4 ± 4.0 ^{d}
Lactobacillus gasseri 1	M8C	57.1 ± 8.9 ^{cf}	18.4 ± 7.6 ^{d}	6.0 ± 0.3 ^{c}	72.2 ± 4.0 ^{c}	69.0 ± 1.5 ^{e}

Values represent mean ± SD; data in each column with different superscripts ($^{a–h}$) are significantly different ($p < 0.05$).

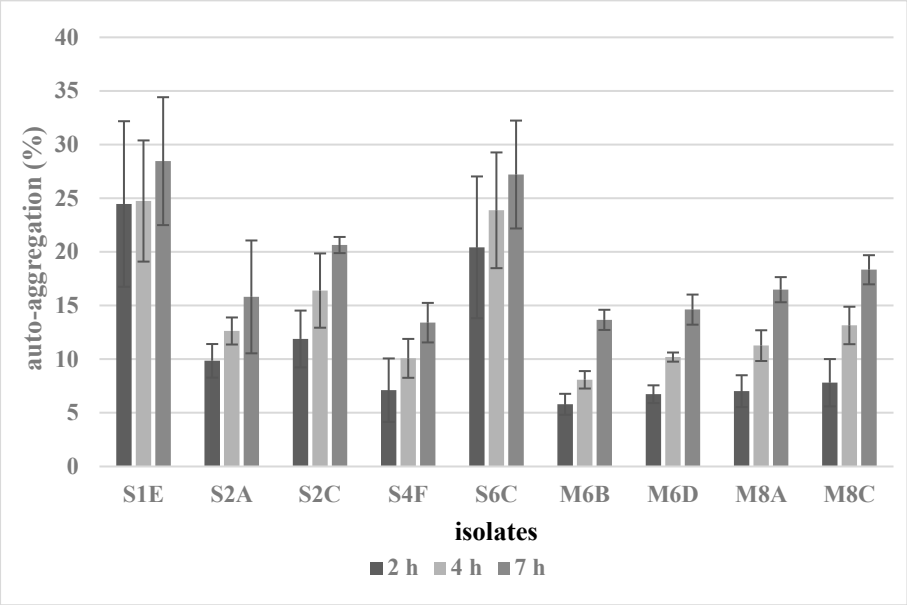


Figure 2. Auto-aggregation of selected isolates.

3.3. Adherence Assay

Caco-2 and HT-29 cell lines at a 9:1 ratio were used as models of the intestinal epithelium for in vitro determination of the adherence of isolates from the genera *Lactobacillus*, *Lacticaseibacillus*, and *Limosilactobacillus*. *Lactobacillus gasseri* M8C showed the strongest adherence capacity (6.0%) compared to the other tested isolates. Both *Limosilactobacillus reuteri* S2A and S2C strains isolated from infant feces also proved good adhesion capacities of >3.5%.

3.4. Antioxidant Activity of the Tested Isolates

The antioxidant capacity of the potential probiotic isolates was evaluated using DPPH radical scavenging activity. The DPPH radical scavenging activity of these strains ranged from 18.4 to 69.3%, as shown in Table 4. Mainly, two strains of *Lacticaseibacillus rhamnosus*, S4F and S6C, isolated from infant feces exhibited considerably higher DPPH activity (69.3%) than the other isolates.

3.5. Bile Salt Tolerance

The effects SFG with pancreatin and 1.5 or 3.0 g/L bile salts are presented in Table 4. Two strains of *Lacticaseibacillus rhamnosus* S1E and S4F, significantly showed the highest viability

(>85%) after 4 h incubation in simulated gastric juice containing both concentrations of bile salts. Remaining strains proved a good bile salt tolerance for the lower concentration (>67%) but the higher concentration led to a decrease in cell viability of approximately 10–30%.

3.6. Production of Exopolysaccharides

To determine the production of exopolysaccharides by the tested strains, MRS agar enriched with Congo red, glucose, sucrose, or lactose was used. All isolates produced exopolysaccharides on all three agar types (Table 5). Nevertheless, the highest production was observed on MRS agar supplemented with glucose.

Table 5. Production of exopolysaccharides by isolates from breast milk and infant feces.

Isolates		Production of Exopolysaccharides		
		Glucose	Lactose	Sucrose
Lacticaseibacillus rhamnosus 2	S1E	+	+	+
Limosilactobacillus reuteri 1	S2A	+	+	+
Limosilactobacillus reuteri 2	S2C	+	+	+
Lacticaseibacillus rhamnosus 1	S4F	+	+	+
Lacticaseibacillus rhamnosus 1	M6B	+	+	+
Lactobacillus gasseri 1	M6D	+	+	+
Lacticaseibacillus rhamnosus 3	S6C	+	+	+
Lacticaseibacillus rhamnosus 2	M8A	+	+	+
Lactobacillus gasseri 1	M8C	+	+	+

+, Positive.

3.7. Antimicrobial Activity

Antimicrobial activity was determined in the supernatants of the isolates against *Bacillus cereus* CCM 869, *Escherichia coli* CCM 3954, *Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 3958, and *Salmonella enterica* serotype Typhimurium CCM 7205, which are known to cause various infectious diseases. Acidic supernatants inhibited the growth of most of the tested pathogens (>80%), as is shown in Figures 3–7. One exception was *Lactobacillus gasseri* M6D, for which the acidic supernatant showed the lowest antimicrobial activity. The neutralization and heat treatment of the supernatants decreased their antimicrobial activities, mainly against *B. cereus*, *E. coli*, and *Salmonella enterica* serotype Typhimurium.

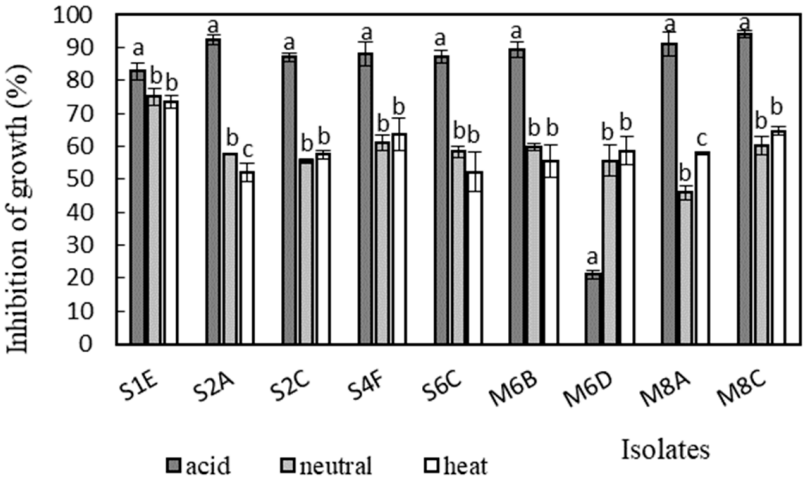


Figure 3. Antimicrobial activity of isolates against *B. cereus* CCM 869. Data with different superscripts ($^{a–c}$) are significantly different ($p < 0.05$).

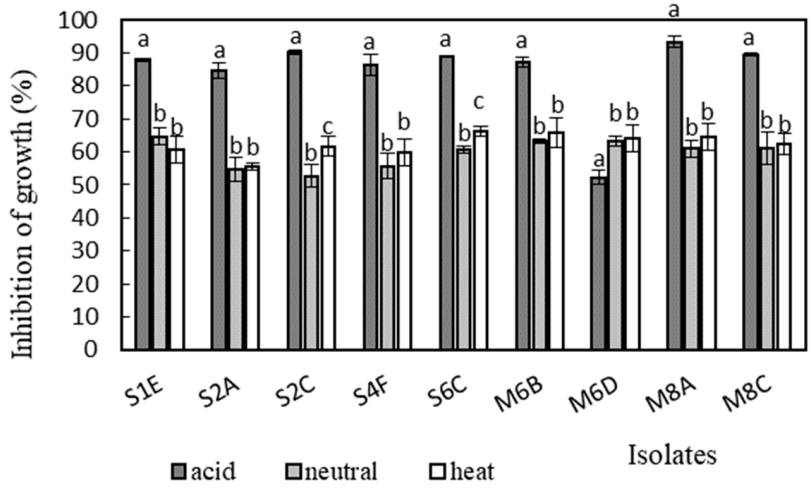


Figure 4. Antimicrobial activity of isolates against *E. coli* CCM 3954. Data with different superscripts ($^{a–c}$) are significantly different ($p < 0.05$).

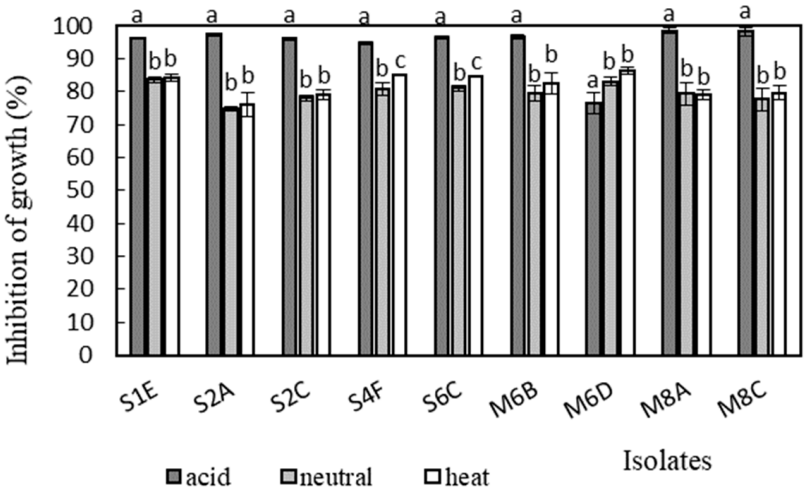


Figure 5. Antimicrobial activity of isolates against *E. faecalis* CCM 4224. Data with different superscripts ($^{a–c}$) are significantly different ($p < 0.05$).

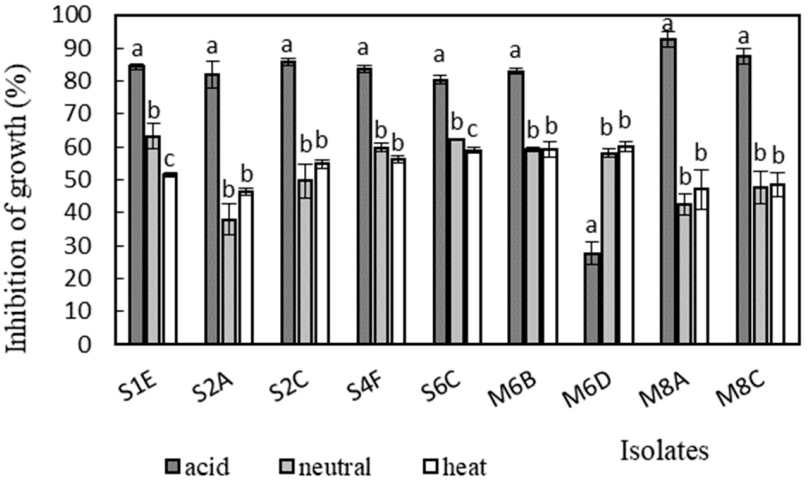


Figure 6. Antimicrobial activity of isolates against *Salmonella enterica* serotype Typhimurium CCM 7205. Data with different superscripts ($^{a–c}$) are significantly different ($p < 0.05$).

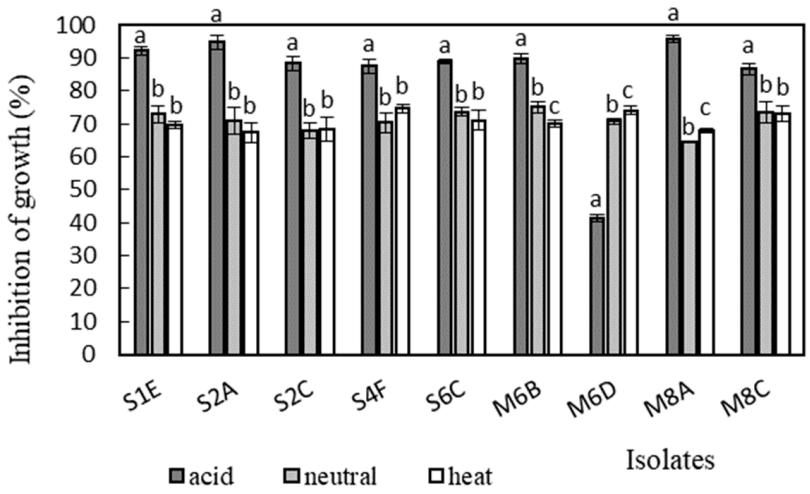


Figure 7. Antimicrobial activity of isolates against *S. aureus* CCM 3958. Data with different superscripts ($^{a–c}$) are significantly different ($p < 0.05$).

4. Discussion

In recent years, potential probiotic microorganisms, such as lactic acid bacteria, bifidobacteria, and yeasts, have been isolated from different sources, including dairy and non-dairy fermented foods, and the gastrointestinal tract of humans, animals, and insects [5–8]. Nevertheless, the selection of new probiotics requires a systematic approach to characterize these candidates. According to the FAO/WHO [9], probiotics must be assessed as safe, survive in the inhospitable conditions of the gastrointestinal tract (low pH and bile salts), and have good functional and technological properties, such as antimicrobial activity against pathogens or adherence to the epithelial surface. In this study, nine strains of the genera *Lactobacillus*, *Lacticaseibacillus*, and *Limosilactobacillus* were isolated from breast milk and infant feces, and identified by 16S rRNA sequencing using the NCBI database. Subsequently, these strains were characterized for safety (resistance to ATB and hemolytic enzymatic activity) and functional properties, such as resistance to the harsh conditions of the digestive tract, adhesion abilities (hydrophobicity, auto-aggregation, and adherence to the co-culture of Caco-2 and HT-29 cell lines), antimicrobial and antioxidant activities, and the production of exopolysaccharides.

Currently, antibiotics are used on a large scale, and their resistance is a serious problem. For this reason, both the EFSA and WHO/FAO issued regulations for testing the antibiotic profiles of probiotic and potentially probiotic microorganisms [9,20,26]. Evaluating the antibiotic resistance of potential probiotic microorganisms is important to limit the gene transfer for antibiotic resistance between common microbiota and pathogens [12]. All tested isolates were evaluated as resistant to the aminoglycoside antibiotics streptomycin and kanamycin, and the glycopeptide antibiotic vancomycin. Vancomycin is a glycopeptide antibiotic that inhibits cell wall synthesis [27]. However, vancomycin resistance genes are chromosomally coded; therefore, there is no risk of transfer as in the case of plasmids. Lactobacilli are generally vancomycin-resistant and naturally resistant to aminoglycoside antibiotics. Gentamicin is also an aminoglycoside antibiotic; however, only three strains in this study were resistant to gentamicin. Similar results have been reported in previous studies [28–30]. Sensitivity to gentamicin can be explained by its ability to cross the bacterial membrane more efficiently than the other aminoglycoside antibiotics (such as streptomycin or kanamycin) owing to differences in the chemical structures of antibiotics and their different interactions with bacterial membranes [31]. However, the tested isolates were sensitive to the remaining therapeutically important antibiotics. In the present study, no hemolytic activity was observed in any of the tested isolates. These results are in agreement with studies that observed hemolytic activity in lactobacilli isolated from the stool of infants [12], children [32], and adults [33], or from breast milk [2]. Probiotic microorganisms

must also be examined for the production of appropriate enzymes to prevent the synthesis of toxic compounds and ensure host safety [34]. None of the isolates showed the activity of β-glucuronidase, which is a bacterial carcinogenic enzyme [34]. From a safety perspective, all tested isolates showed favorable properties.

One of the crucial criteria for selecting a potential probiotic strain is its ability to survive the inhospitable conditions of the human GIT (low pH and high levels of bile salts). Tested strains were exposed to simulated gastric juice containing pancreatin and two different concentrations of bile salts (1.5 and 3.0%) for 4 h. The concentration of bile salts in the human intestine can vary depending on the type of food intake and the secretion of pancreatic enzymes [35]. However, its average concentration ranges from 0.1 to 0.5% [36]. *Lacticaseibacillus rhamnosus* S1E and S4F, isolated from infant feces, showed the highest viability (>85%) after exposure to both concentrations of bile salts. Resistance to bile salts can influence a wide range of factors, such as bacterial cell wall structure, transcription, and the expression of proteins or BSH enzymes. Ali et al. [37] evaluated the probiotic properties of *Limosilactobacillus reuteri* PSC102 isolated from pig feces. This strain exhibited tolerance toward low pH and bile salts and did not produce harmful enzymes or possess hemolytic activity, similar to the strains used in this study. The strain *L. rhamnosus* WEI 30 and *Lcb. rhamnosus* WEI 41, isolated from piglets were also characterized by high resistance (>80%) to bile salts (3.0%) [38].

Adherence to the intestinal epithelium is important for the interaction between probiotic microorganisms and the host and influences the time of the potential beneficial effect of probiotics [39]. The adhesion properties of the newly isolated strains were evaluated by testing the hydrophobicity of the cell surface, auto-aggregation, and adherence to a co-culture of Caco-2 and HT-29 cells. Our results revealed significant differences in the hydrophobicity of the tested isolates. These differences were also observed within species and genera. This is also confirmed by the results described in the study of de Souza et al. [40], in which the hydrophobicity of different strains of *Lacticaseibacillus casei* ranged from 9.66 to 69.36%, similar to this work, and for *L. fermentum* strains, the hydrophobicity values ranged from 0.30 to 68.81%. Similar differences in the hydrophobicity of newly isolated probiotic strains have been reported by Krausová et al. [22]. Generally, these results indicate that the hydrophobicity of the cell surface is a unique property of each strain. De Souza et al. [40] reported that bacteria with higher hydrophobicity can adhere better to epithelial cells and significantly influence the microbial composition in the intestine. This claim is in contradiction to another study by Todorov/Dicks [41], which determined a high percentage of hydrophobicity (75–80%) of *Lacticaseibacillus rhamnosus* ST461BZ and ST462BZ; however, these strains proved a lower ability to adhere to HT-29 cell lines. In the present study, the strain *Lactobacillus gasseri* M6C, isolated from breast milk, showed the highest ability to adhere to Caco-2/HT-29 cells and the highest cell surface hydrophobicity to hexane. Nevertheless, other strains (*Limosilactobacillus reuteri* S2A and S2C) with higher adherence abilities had the lowest hydrophobicity. These results indicate that hydrophobic interactions may facilitate the adhesion process through initial interactions with the intestinal surface; however, they are not a precondition for high bacterial adherence. In addition to hydrophobicity, auto-aggregation may also be correlated with the ability to adhere. A correlation between these properties has been described in studies by MacKenzie et al. [42] and Krausová et al. [22], in which *Limosilactobacillus reuteri* strains showed high auto-aggregation and good adherence. In contrast, the correlation between auto-aggregation and adhesion capacity was refuted by Chaffanel et al. [43]. No association between aggregation and adhesion capacity was observed for any of the 14 *Streptococcus salivarius* strains tested. The results of this study suggest that the processes of auto-aggregation and adherence are probably mediated by distinct mechanisms involving different surface molecules. In this study, the auto-aggregation ability of isolates had a variable range between 5.8 and 28.5%, depending on time and strain. *Lacticaseibacillus rhamnosus* S1E and S6C showed the strongest percentages of auto-aggregation (28.5% and 27.2%) after 7 h.

Lactic acid bacteria produce a wide range of antimicrobial compounds including organic acids, bacteriocins, and hydrogen peroxide [44]. The mentioned production of organic acids (acetic acid, lactic acid, 3-phenyl lactic acid, 4-hydroxyphenyl lactic acid, and benzoic acid) leads to a decrease in pH, which can inhibit the growth of pathogens [21,45]. In this study, acidic supernatants inhibited the growth of the five tested pathogens (*B. cereus*, *E. coli*, *E. faecalis*, *S. aureus*, and *Salmonella enterica* serotype Typhimurium) probably because of the presence of organic acids and a low pH. The neutralization of these supernatants caused a significant decrease in their antimicrobial activity, similar to the results of other studies [21,46,47]. The heat treatment of neutralized supernatants did not affect their antimicrobial activity compared to neutralized supernatants. This indicates the presence of thermostable compounds with antimicrobial activity, such as thermostable bacteriocins. The thermostable bacteriocins (gassericin and plantaricin) produced by *Lactobacillus gasseri* and *Lactiplatibacillus plantarum* have been described previously [48,49].

Antioxidant activity is an important property by which cells protect themselves from oxidative stress [50]. Recently, LABs have been studied for their ability to actively participate in the reduction in reactive oxygen species (ROS) levels [51,52]. The DPPH radical scavenging method was used to determine the antioxidant capacity in our study. The two strains of *Lacticaseibacillus rhamnosus* S4F and S6C, isolated from infant feces, showed the highest antioxidant activity (69.3%). Kim et al. [8] also determined the antioxidant activities of strains of the genera *Lactobacillus* and *Lacticaseibacillus* that only reached values of 21.6 and 27.5%, respectively [8]. In another study by Oh et al. [53], the values of antioxidant activities of *Lacticaseibacillus rhamnosus* and *Lactobacillus gasseri* isolated from infant feces did not exceed 40% [53]. On the contrary, another study described antioxidant activity in the range of 76–88% for *Lacticaseibacillus rhamnosus* obtained from breast milk [16]. These results suggest that antioxidant activity is highly strain-specific and does not depend on the origin of the strain. In the future, it will be important to test the antioxidant activity of the isolates using other methods, such as scavenging of hydroxyl radicals or ABTS radical methods.

EPSs produced by lactic acid bacteria are generally considered GRASs and may represent a new generation of natural, healthy, and prebiotic food compounds. Owing to their unique physicochemical properties, they can replace many substances used to improve the sensory properties. Because of their ability to retain large amounts of water and form gel-like and viscous structures, EPS play an important role in the food, pharmaceutical, and cosmetic industries. However, their disadvantages include small quantities and difficult isolation and purification [54,55]. Some studies have described the positive health effects of EPS, such as effective cholesterol-lowering, antioxidant, anticancer, immunomodulatory, and prebiotic activities [56]. In the present study, EPS production was observed in all nine isolates in a medium containing all the tested substrates (glucose, lactose, and sucrose). The production of EPS from different carbohydrate sources has been observed in previous studies by Badel et al. [54], Oleksy-Sobczak et al. [57], and Rendueles et al. [58].

5. Conclusions

In conclusion, this study focused on the isolation and investigation of potential probiotic microorganisms from breast milk and infant feces. Nine strains were identified by 16S rRNA sequencing *Lactobacillus gasseri*, *Lacticaseibacillus rhamnosus*, and *Limosilactobacillus reuteri.* All tested isolates were non-hemolytic and sensitive to therapeutically important antibiotics. The acidic supernatants of the isolates inhibited selected pathogenic microorganisms (*B. cereus*, *E. coli*, *S. aureus*, *Salmonella*, and *E. faecalis*). Additionally, partial inhibition was observed after treatment with the neutralized and heated supernatants. However, a more complex characterization of the mechanisms of antimicrobial activities of the tested strains, such as the production of organic acids or bacteriocins, remains an area that needs further investigation and may be considered a limitation of this study. The evaluation of the results showed that the two strains, *Lacticaseibacillus rhamnosus* S1F and S6C, isolated from infant feces, together with *Lactobacillus gasseri* M8C, isolated from breast milk, showed

the highest ability to survive in the presence of high concentrations of bile salts and a good adhesion property, which is important for the colonization of the GIT or the inhibition of pathogens. The three isolates mentioned here could be considered potential probiotics with future applications in the food industry. Nevertheless, other in vitro property tests need to be performed to confirm their probiotic status.

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