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# Assessment of the probiotic isolates' features found in breast milk

**Elham Shamsaie[1], Shamsozoha Abolmaali[2], Shakiba Darvish Alipour Astaneh[1*]**

[1] *Department of Biotechnology, Faculty of New Sciences and Technologies, Semnan University, Semnan, Iran*
[2] *Department of Biology, Faculty of Basic Science, Semnan University, Semnan, Iran*

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**Abstract**

The probiotics in breast milk can help children restore optimal growth. This study aimed to separate and identify probiotic microorganisms in human milk and evaluate vitamin B production by these microorganisms. Biochemical tests were used to identify nine aerobic and 81 microaerophilic isolates from 34 breast milk samples. Probiotic traits were characterized for auto-aggregation, co-aggregation, hydrophobicity, acidity tolerance, lysozyme, bile salts, and papain treatment. Furthermore, in some of the probiotic isolates, the production of the vitamin B group was detected by thin-layer chromatography. Breastmilk samples were collected from 34 healthy women with an average age of 27.5 ± 6.5 years. Eighty-one colonies were isolated under microaerophilic conditions based on different morphological characteristics. Fungi, gram-positive, and gram-negative bacteria composed 36%, 34%, and 30% of the isolates, respectively. Two isolates with strong probiotic potential were molecularly examined and named *Enterococcus faecalis* (SUBC 156) and *E. faecalis* (SUBC 169). The highest resistance to acidity (18%) and bile salt (-1) was displayed by SUBC169, while SUBC156 survived lysozyme treatment and had a hydrophobicity of 56%. SUBC153 and SUBC156 produced vitamin B6 in a milk-containing MRS medium. Breast milk is recommended because of its probiotic qualities. Identifying those strains may confirm the actual beneficial effect of probiotics in infant nutrition.

## 1. Introduction

Breastfeeding *infants and young children consuming* breast milk receive ideal nutrition for the baby's health (Damaceno et al., 2017). Breast milk comprises three stages: colostrum, transition, and mature milk. Colostrum is rich in small amounts of immunoglobulin A, lactoferrin, leukocytes, and epidermal growth factor. Transition milk is produced between 5 to 14 days after birth, and mature human milk formula is complete after 4 to 6 weeks (Duraisamy et al., 2022). Breast milk has long been considered a sterile medium whose primary function is safety and nutrition. The health benefits of its consumption are related to bioactive molecules such as immunoglobulin, lactoferrin, and lysozyme (Damaceno et al., 2023), which also includes a significant number of microorganisms. Along with the mother's skin, mouth, and vaginal

*Corresponding author: Shakiba Darvish Alipour Astaneh. Department of Biotechnology, Faculty of New Sciences and Technologies, Semnan University, Semnan, Iran
E-mail address: Darvishalipour@semnan.ac.ir
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canal flora, the presence of $10^3$ CFU/ml bacteria in human milk plays an important role in the growth of the infant's gut microbiota. The human milk microbiota can colonize the neonatal gastrointestinal tract and aid the immune system's complete development (D'Alessandro et al., 2022).

It is currently unclear where this microbiota originates. One hypothesis is that the microbiota of the baby's mouth comes from the mother's skin; however, there is a significant similarity between the mother's milk and the microbiota of the baby's mouth. This may be due to the microbiota of the mother's gut entering the mammary gland through the entero-mammary pathway and consequently being transferred to the baby during breastfeeding. Colostrum bacteria affect the composition of the human milk microbiota (Damaceno et al., 2023). The infant's gut flora is influenced by the diversity of the breast milk microbiome, which is affected by factors such as maternal habits, milk components, diet, mode of delivery, and gestational age. In addition, breast milk is an important source of probiotics, including Bifidobacteria, Lactococcus, Lactobacil lus, Streptococci, Enterococci, and Micrococcus (Duraisamy et al., 2022). The milk microbiome differs among mothers due to various factors, such as genetics, lifestyle, dietary habits, antibiotic use, breastfeeding time, body mass index (BMI), and mode of delivery. The microbiome of human milk can be considered a natural symbiosis that contains probiotics and prebiotics (human milk oligosaccharides, or HMO) in its structure (D'Alessandro et al., 2022).

Oligosaccharides and microbiota are the two main components of milk that affect the infant's gut microbiota. They are controlled by two main forces - dispersal (colonization of new bacterial species) and selection (acquiring an environment suitable for survival). These processes can lead to an altered gut microbiota, potentially resulting in an unfavorable final composition, which can be one of the underlying mechanisms of susceptibility to various chronic diseases, including allergies and asthma (Poinsot et al., 2020). This study aims to isolate probiotics with functional antimicrobial potential from collected breast milk samples. Specific functional parameters, such as hydrophobicity and self-aggregation, were studied in these isolates and evaluated for their antagonistic activity against indicator species often associated with intestinal infections.

## 2. Materials and Methods

### 2.1. Designing the study

Human milk samples were obtained from 34 healthy women between October 2014 and May 2015. The volunteers were enlisted from the Mahdieh Hospital (Tehran, Iran), Amirul-Mominin Hospital (Semnan, Iran), and the Health House (Qom, Iran). Every individual signed an informed consent form.

### 2.2. Human milk sampling

The mothers' ages were between 21 and 32 years, and the children's age varied from one day to two years. Before sampling, the breast skin was disinfected with 70% ethanol. The following *maternal* characteristics were measured, including the type of delivery (vaginal or cesarean), antibiotic usage, age, and number of previous pregnancies.

### 2.3. Isolation of bacteria from human milk

The samples were kept in a refrigerator at 4-8 ℃ until use. The collected milk samples were diluted using phosphate-buffered saline (PBS). A volume of 100µl of serial dilutions $10^{-5}$ and $10^{-6}$ were cultured on MRS agar and incubated in a candle chamber at 37 °C for 24 to 72 h. The colonies with different morphologies were isolated and grown under anaerobic conditions. Biochemical tests such as catalase, Gram staining, oxidase, motility, indole production in SIM medium, and nitrate reduction were evaluated according to the standard methods of Bergey's Manual of Systematic Bacteriology (Vos et al., 2011). Glycerol stocks (50% v/v) were prepared and stored at - 8 ℃.

### 2.4. Study of probiotic characteristics

### 2.4.1. Hemolytic activity

The isolates were tested for hemolytic activity on 5% sheep blood agar after incubation for 24-48 h at 37 °C. β-hemolysis in *Pseudomonas aeruginosa*, *Staphylococcus aureus*, γ-hemolysis in *Streptococcus faecalis*, and α-hemolysis in *Streptococcus sanguinis* were used as controls.

### 2.4.2. Gelatinase activity

Each isolate was inoculated on MRS-Broth containing 12% gelatin and incubated for 15 to 20 days at 37 °C. Then, the gelatin tubes were chilled for 45 minutes in the refrigerator (at 4 °C). *B. subtilis* was chosen as the positive control, and *S. aureus* was the negative control.

### 2.4.3. Antibiotic sensitivity pattern

Isolates 0.5 McFarland was inoculated on MRS agar. Antibiotic disks (penicillin 10 mcg, gentamicin 10 mcg, ampicillin 10 mcg, chloramphenicol 30 mcg, erythromycin 15 mcg, and tetracycline 30 mcg) were placed on the medium and incubated for 48 h at 37 °C. Each halo diameter was measured according to the clinical and laboratory standard (CLSI). *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *S. flexneri* (ATCC 13833), and *S. typhimurium* were used as controls ($p < 0.05$).

### 2.4.4. Antibacterial activity

Antibacterial activity of 200 µl of cell-free supernatant of isolates (CFS) (pH 7 and without adjustment pH 4.2) against *S. typhimurium*, *P. aeruginosa* (ATCC 27853), *P. vulgaris*, *S. aureus*, *E. coli*, *K. pneumoniae* (ATCC 13883), Clinical *P. aeruginosa*, and Clinical *K. pneumoniae.* Clinical *E. coli* was evaluated using disk diffusion agar and cross streak tests ($p < 0.05$).

### 2.4.5. Auto-Aggregation assay

The overnight cultures of isolates were harvested at 8,000 g, 10 min, washed two times with PBS (pH 7.2), and resuspended in the PBS. The bacterial suspensions were incubated at 37 °C for 0, 2.5, and 5 hours without agitation. 900 µl of PBS was added to the 100 µl of suspension surface, and absorbance was measured at 600nm. The amount of auto-aggregation is measured by Formula 1 (Yuksekdag & Aslim, 2010; Zakaria Gomaa, 2013) ($p < 0.05$).

Formula1: $[(OD1-OD2)/ (OD1)] \times 100$

$OD_1$: Absorption of the isolate at time = 0 / $OD_2$: Absorption of the isolate at time = 2.5, 5 hours

### 2.4.6. Co-Aggregation assay

Equal volumes of isolates and indicators (4 ml), including *P. aeruginosa*, *S. typhimurium*, *E. coli*, *S. dysentery*, and *S. aureus*, were mixed and incubated without shaking at 37 °C. The $A_{600nm}$ of the mixtures were assayed at different times (0, 5 h) according to Formula 2 ($A_y$: Absorption of indicator bacteria/ $A_X$: Absorption of the isolate) of the adhesion of the isolates to the indicators (Khalil et al., 2018; Kos et al., 2003) ($p < 0.05$).

**Formula2***:* $\left[\frac{\left(\frac{Ax+Ay}{2}\right)-A(x+y)}{Ax+\frac{Ay}{2}}\right] \times 100$

### 2.4.7. Study of acidic conditions and bile salts tolerance

The overnight cultures of the isolates were pelleted at 10,000 g, 10 min, and 4 °C. The pellets were washed twice with PBS (pH 7.2) and divided into 1ml aliquots. These aliquots were resuspended in sterile PBS with pH values of 3.0 and 7.0 for 0, 1, and 3 hours. 100 µl of $10^{-5}$ and $10^{-6}$ dilutions were inoculated on MRS and incubated at 37 °C.

A volume of 100µl of suspension ($10^8$ CFU /ml) of the isolates, 800µl of PBS (pH 8.2), and 100µl of 0.5% Ox-bile were incubated at 37 °C for 4h. The survival was measured according to Formula 3 (Gilliland & Walker, 1990; Liong & Shah, 2005) ($p < 0.05$).

**Formula 3**: $\frac{[(T4-T0)control - (T4-T0)treat]}{(T4-T0)control}$

T4: OD of the isolate after 4 hours / $T_0$: OD of the isolate at t=0 / treat: OD of the isolate in the presence of bile salt/ control: OD of the isolate in the presence of PBS

### 2.4.8. Resistance against papain and lysozyme

The bacteria were collected at 10,000 g, 10 min, and 4 °C, then washed with PBS (two times) and resuspended in PBS. A volume of 100 µl of the suspensions, 800 µl of PBS, and 100 µl of 0.03% papain were incubated for 20 min at 37 °C. Then, $10^{-6}$ dilutions of suspension were cultured on the MRS agar for counting and incubated for 24- 48 hours at 37 °C. PBS was used as a control sample (Yildirim & Johnson, 1998).

The isolates were incubated in peptone water at 37 °C for 14–18 hours, and 100 µl of dilution $10^{-6}$ was inoculated on the MRS agar containing 0.5% lysozyme. After 24 hours, the resistance of isolates to lysozyme was determined by calculating the number of colonies on each plate (Duraisamy et al., 2022).

### 2.4.9. Surface hydrophobicity

Paraxylene was added to the suspension (160µl) and vortexed for 2 minutes. The isolates were incubated for 15 min at room temperature (Abdulla, 2014). The isolate's hydrophobicity was calculated using Formula 4: (Pessoa et al., 2017).

**Formula 4**: $((A_0 - A_1) / A_0) \times 100$

$A_0$: Absorption at the first/ $A_1$: Absorption of the underlying liquid after 15 minutes

### 2.4.10. Biofilm formation

A suspension of each isolate (5 µl) was added to 200 µl of MRS and BHI broth in each well of a 96-well plate and incubated for 72 h, 37 °C. The culture was drained, and the wells were washed twice with PBS; 200 µl of crystal violet (1%) was used to stain the wells for 30 min and rinsed twice with 200 µl of washing buffer, then 200 µl of 30% glacial acetic acid was added to each well. Finally, the absorbance of wells was measured at 570 nm (Maldonado et al., 2018; Pérez Ibarreche et al., 2014; Söderling et al., 2011) ($p < 0.05$).

## 2.5. Molecular identification of isolates

The 16 srRNA region was amplified using 27 F forward and 1492 R reverse primers (Frank et al., 2008). The PCR reaction was initiated at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 54 °C for 40 s, and extension at 72 °C for 90 s. A final extension step was programmed at 72 °C for 5 min. The PCR products were sequenced by the Genomin company using the Sanger chain termination method.

## 2.6. The potential of vitamin-B group production

The sterile milk (2ml) was added to 2 ml of overnight MRS broth of isolates and incubated at 37 °C for 24 hours, 5 days, and 7 days. The production potential of the vitamin B group was studied by thin-layer chromatography (LeBlanc et al., 2011). A volume of 4 ml of methanol was added to 1 ml of the supernatant of the isolates, vortexed for 1 min, and incubated at 70 °C for 30 min (water bath). The tubes were homogenized at intervals of 5 min. A Vit B complex vial from the Iran Hormone Company containing vitamins B1, B2, B3, B5, and B6 was used as the standard. To perform thin layer chromatography, a vial of vitamin B-complex was diluted with 37% methanol at a ratio of 1:15. The samples were analyzed in a volume of 20 µl and the standard in a volume of 10 µl. For the mobile phase, acetic acid, butanol, ammonium, chloroform, and deionized water were used in the ratio of 5:7:1:6:1. Finally, the profiles were analyzed with UV light (Cimpoiu & Hosu, 2007). The compound's movement speed ratio to solvent progress (Rf), Formula 5, includes all visible bands.

**Formula 5:** $$R_f = \frac{\textit{Distance from Baseline travelled by Solute}}{\textit{Distance from Baseline travelled by Solvent}}$$

### 2.7. Statistical analysis

The presented data correspond to the mean values of three replicates per experiment. The data were evaluated for the traits using analysis of variance and Duncan's multiple range test ($p < 0.05$) in SPSS (Statistical Package for the Social Sciences) version 22.

## 3. Results &Discussion

### 3.1. Primary identification of the isolates on the biochemical characterization

Probiotics are live microorganisms that help balance the gut microbiota and improve immune function. Breastmilk samples were collected from 34 healthy women. The average age was 27.5 ± 6.5 years. Nine strains with different morphological characteristics under microaerophilic and aerobic conditions were isolated from 81 colonies (two from the Amir al-Momenin Hospital in Semnan, six from the Mahdieh Hospital in Tehran, and 73 from the Qom Health Center). Since this study aimed to identify the lactic acid bacteria, isolation was performed based on the identification of these bacteria, and other isolates were excluded from the study.

Twenty-eight percent of the 81 isolates were gram-positive cocci, 22% gram-negative cocci, 6% gram-positive bacillus, 8% gram-negative bacillus, and 36% of the colonies were yeast. Seventy-eight percent of the isolates were catalase-positive, 34% were oxidase-positive, 84% were nitrate-negative, and 75% were gas-positive.

### 3.2. Study of probiotic features

#### 3.2.1. Hemolytic and gelatinase activity

Sixty percent of the isolates had no hemolytic activity or γ-hemolysis. Ninety-two percent of the isolates could not produce gelatinase enzyme, and 8% of the samples (SUBC107, SUBC121, and SUBC175) have gelatinase enzyme. The growth of isolates was observed at 10-40 °C. The gram-positive bacilli showed negative results for the indole, oxidase, and catalase tests and were selected for this study. Glycerol stocks of isolates were prepared (50% v/v) and kept at - 80 °C.

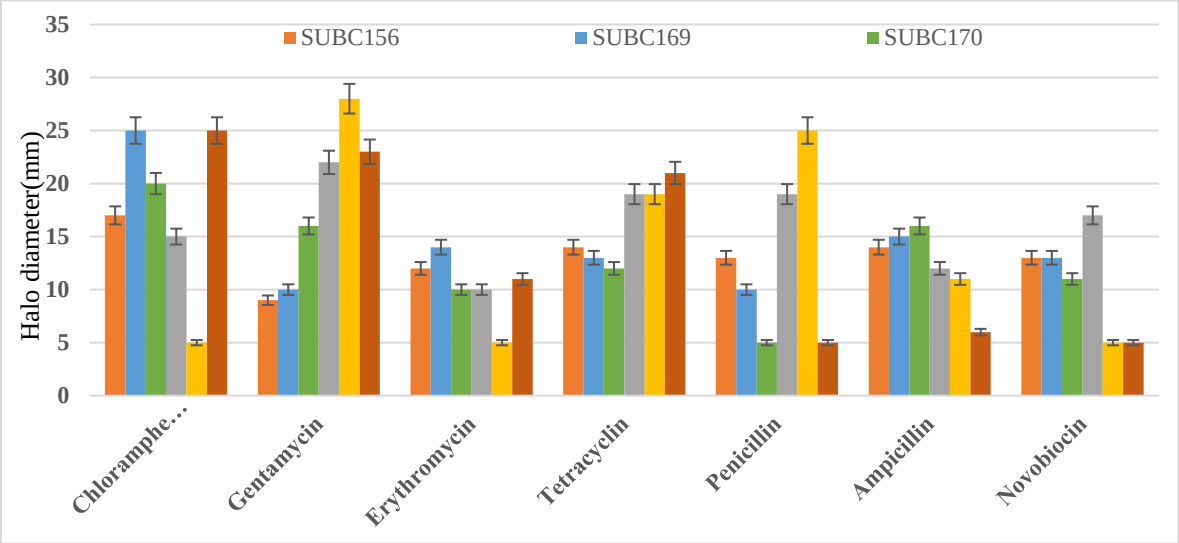
Damaceno et al. collected milk samples from 47 healthy women and cultured them on MRS agar under aerobic and anaerobic conditions (2017). They grew a total of 150 colonies on MRS with different morphological properties, and 85 colonies were gram-positive cocci and catalase-negative (Damaceno et al., 2017). Mahmoud et al. characterized the bacterial communities in Egyptian mothers and their young children throughout their first year of life while breastfeeding normally (2022). Only 41 gram-positive, catalase, oxidase-negative, and non-endospore-forming lactic acid bacterial isolates were selected from 100 bacterial isolates. Based on their microscopic examination, 14 isolates were classified as cocci-shaped bacteria, while 39 were classified as rod-shaped. All of the cocci isolates were positive for microaerophilic or facultative anaerobic conditions (Mahmoud et al., 2022).

#### 3.2.2. Antibiotic sensitivity pattern

Our results showed that SUBC153 and SUBC157 were resistant to the studied antibiotics. All isolates, with the exception of SUBC156, SUBC169, and SUBC170, were resistant to penicillin, tetracycline, chloramphenicol, ampicillin, erythromycin, and gentamicin SUB156, SUB169, and SUB170 are more susceptible to the studied antibiotics than other strains. Specifically, they are more sensitive to the antibiotics chloramphenicol and ampicillin compared to *S. aureus* and *S. flexenteri* ($p < 0.05$) (Fig. 1).

Anjum et al. (2022) used riboprinting to identify 18 *E. faecalis* from the milk of Pakistani mothers. They found that the isolates were susceptible to most clinically significant antibiotics except streptomycin. Reis et al. (2016) studied *E. thailandicus*, *E. durans*, and *E. faecalis*, the three most common species among the 33 lactic acid bacteria identified from 13 breast milk samples and found all isolates, except F2 and F1, were sensitive to chloramphenicol and imipenem.

**Figure1.** Antibiotic susceptibility profiles of the isolates. SUB156, SUB169, and SUB170 are more susceptible to the studied antibiotics than other strains. Specifically, they are more sensitive to the antibiotics chloramphenicol and ampicillin compared to S. aureus and S. flexenteri($p < 0.05$).




### 3.2.3. Antibacterial activity

The supernatant at pH 7 of the isolates did not show any antibacterial activity against the indicator bacteria. *S. aureus* and *S. dysentery* showed the highest sensitivity to the CFS (pH 4.2), while *E. coli*, *B. cereus*, and *P. aeruginosa* had the highest resistance. Two bacteria were sensitive to the SUBC153 supernatant at acidic conditions, and three bacteria were sensitive to the supernatant of the SUBC156, SUBC102, and SUBC117; SUBC118 has a broader spectrum of antagonistic activity against indicator bacteria than SUBC153 and SUBC169 (Table 1). The diameters of the halo against the indicator strains were in the range of 10–50 mm on average.

**Table 1.** Antibacterial Activity of Isolates Supernatants against the Indicator Strains (pH 4.2)

| Isolates | *K. pneumoniae* | *B.cereus* | *E. coli* | *S. aureus* | *P.vulgaris* | *P. aeruginosa* | *S. typhimurium* |
|---|---|---|---|---|---|---|---|
| SUBC102 | + | - | + | + | + | + | + |
| SUBC111 | - | - | - | + | + | - | - |
| SUBC117 | + | - | + | + | + | + | + |
| SUBC118 | + | - | + | + | + | + | + |
| SUBC151 | - | - | + | + | - | - | - |
| SUBC152 | - | - | + | + | + | + | + |
| SUBC153 | - | - | - | - | - | - | - |
| SUBC156 | + | - | - | + | - | - | + |
| SUBC157 | - | - | + | - | + | - | - |

Note: (+: halo - : non-halo)

**Table.1 Cont.**

| | *K. pneumoniae* | *B.cereus* | *E. coli* | *S. aureus* | *P.vulgaris* | *P. aeruginosa* | *S. typhimurium* |
|---|---|---|---|---|---|---|---|
| SUBC169 | - | - | - | - | - | - | - |
| SUBC170 | - | - | - | + | - | - | - |
| SUBC172 | - | - | - | - | + | - | - |
| SUBC173 | - | - | + | + | + | - | + |

Note: (+: halo -: non-halo)

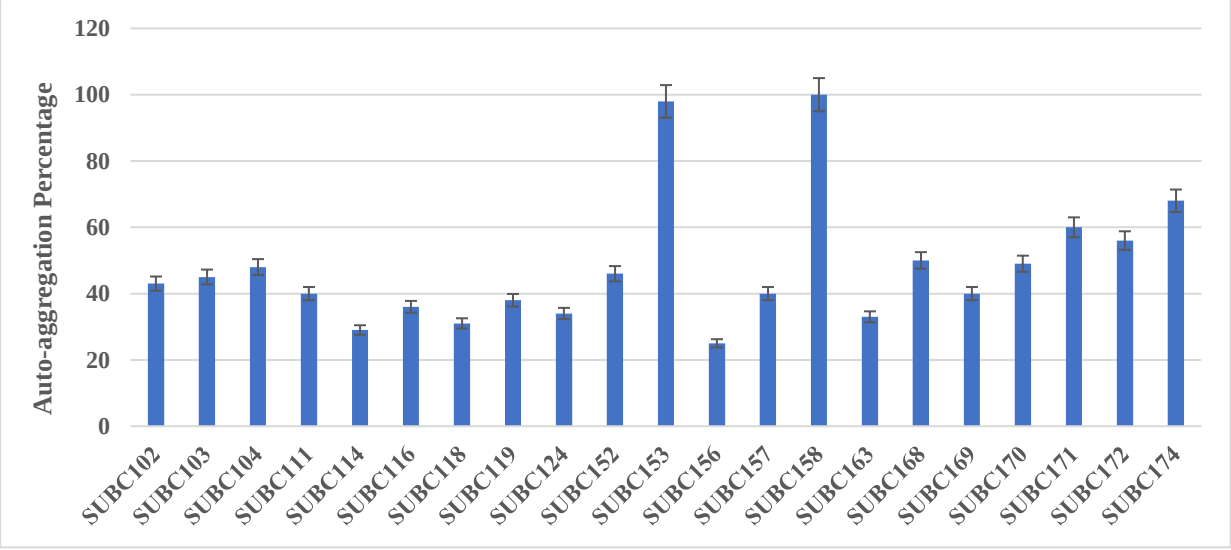
### 3.2.4. Auto-Aggregation and Co-Aggregation assay

Increasing turbidity decreases the intensity of the connection between the bacterial cells. The auto-aggregation abilities of the isolates ranged from 22% to 100% (Fig. 2). The lowest attachment of bacterial cells related to the SUBC114 is 29%, and the highest attachment of bacterial cells related to the SUBC166 is 99% ($p < 0.05$). SUBC114 has the lowest binding of bacterial cells.

The co-aggregation assay at lower turbidity showed that SUBC156 and SUBC169 could bind to four indicator bacteria, while SUBC153 and SUBC170 could bind to only one indicator bacterium. *B. cereus* showed the highest binding to the isolates, but *S. dysentery* did not bind to any of the isolates. Therefore, the selected strains were used for further studies.

Duraisamy et al. (2022) characterized the seven probiotics from human breast milk and showed a high co-aggregation percentage (62%) was recorded for *L. casei* BDUMBT13 and *L. gastricus* BDUMBT09 with *E. Faecalis.*

**Figure 2.** Auto-aggregation abilities of the potential probiotic isolates. The lowest attachment of bacterial cells related to the SUBC114 is 29% and the highest attachment of bacterial cells related to the SUBC166 is 99% ($p < 0.05$).




### 3.2.5. Resistance to acidic conditions and tolerance to bile Salts

The isolates' resistance was measured in acidic conditions. The number of isolates grown in acidic conditions decreased 100 times in SUBC153, SUBC156, and SUBC157, while SUBC169 showed a 10-fold decrease (Fig. 3). SUBC156,

SUBC157, and SUBC169 showed the highest resistance in acidic conditions ($p < 0.05$).

SUBC170 had the highest resistance to bile salt, and SUBC156 had the lowest. Fig. 4 accurately evaluates the isolates' resistance to bile salt.

**Figure 3.** Resistance of the isolates under acidic conditions. SUBC156, SUBC157, and SUBC169 showed the highest resistance in acidic condition ($p < 0.05$).

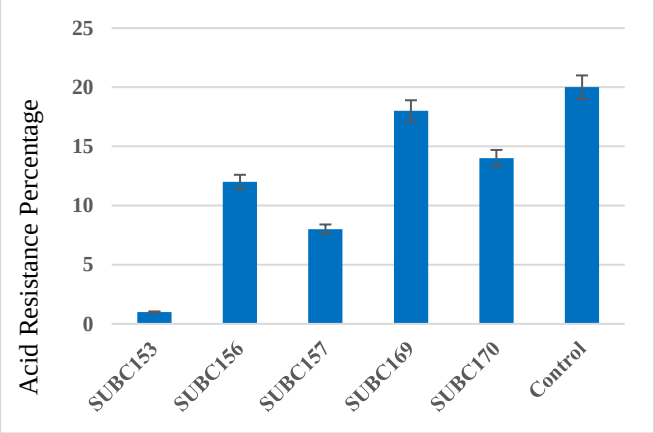
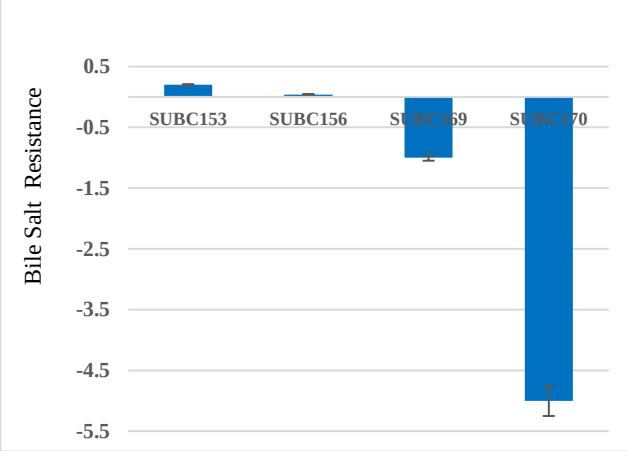



**Figure 4.** The resistance of the isolates to bile salt. The result of this test indicates that the values less than 0.4 are acceptable and are resistant to bile salts. Among the isolates, SUBC170 and SUB169 exhibit the highest resistance ($p < 0.05$).




### 3.2.6. Resistance against papain and lysozyme

The isolate SUBC166 had the highest resistance to papain, and SUBC153 and SUBC170 had the lowest resistance. The range of resistance among the isolates is between 5% and 43%. However,

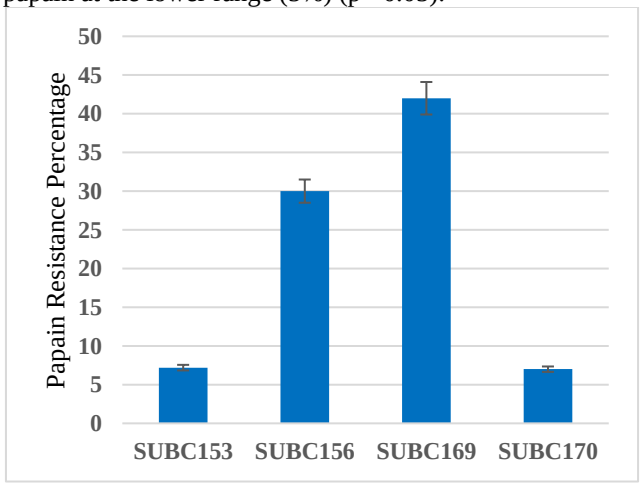
Damaceno et al. (2023) studied the survival of the probiotic isolates ranging from 70 to 100% at pH 2.0 and the bile concentration around 0.3%. The result of this test indicates that values less than 0.4 are acceptable and are resistant to bile salts. Among the isolates, SUBC170 and SUB169 exhibit the highest resistance ($p < 0.05$).

SUB153 and SUB170 exhibited resistance to papain at the lower range (5%) ($p < 0.05$) (Fig. 5).

Lysozyme treatments decreased the bacterial population after 24 h. SUBC156 had the highest resistance; the number of colonies in the control and culture medium containing lysozyme was equal. SUBC169, which grew less in the presence of the lysozyme, had the lowest resistance.

Alkalbani et al. (2018) described the bacterial, except for *E. faecalis* MF067467, decreasing slightly ($p > 0.05$) after heat and lysozyme treatments.

**Figure 5.** The resistance of the isolates to papain. The range of resistance among the isolates is between 5% and 43%. However, SUB153 and SUB170 exhibited resistance to papain at the lower range (5%) ($p < 0.05$).

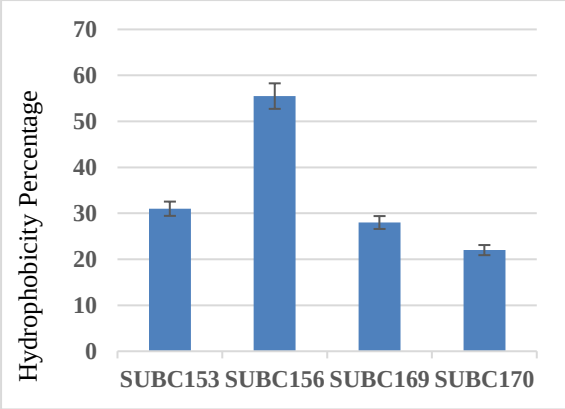



### 3.2.7. Surface hydrophobicity

The surface hydrophobicity of *SUBC156* and *SUBC170* was 56.0% and 22.0% respectively. The turbidity of the isolates was measured one hour after incubation with paraxylene, and the optical absorption was read at a wavelength of 600 nm. SUBC170 had the lowest surface hydrophobicity,

while *SUBC156* showed the highest ($p < 0.05$) (Fig. 6).

**Figure 6.** The cell surface hydrophobicity of the isolates. The turbidity of the isolates was measured one hour after incubation with paraxylene, and the optical absorption was read at a wavelength of 600 nm. The SUB156 showed the highest surface hydrophobicity ($p < 0.05$).




Bagci U. et al. similarly reported that the hydrophobicity of *E. faecium* was found for xylene and n-octane, between 35–56% and 37–47%, respectively (24). The additional hydrophilic activity of probiotic bacteria has been examined when values are less than 40% or comparable, while the hydrophobic feature is shown for such bacteria at more than 40% (Abdulla, 2014; Boris et al., 1998).

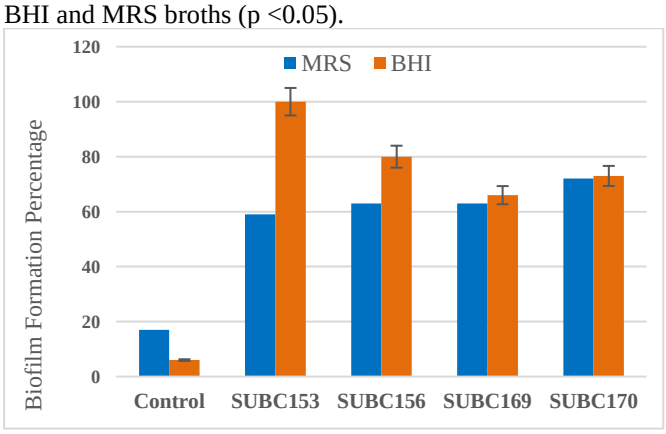
### 3.2.8. Biofilm formation

The ability to form biofilm in each isolate is shown in (Fig 7). SUB153 exhibits greater biofilm formation ability in BHI medium compared to MRS broth. However, the biofilm formation abilities of other strains are nearly equal in both BHI and MRS broths ($p < 0.05$). SUBC156, SUBC169, and SUBC170 showed no significant difference in biofilm production in the two culture media.

### 3.3. Molecular identification of isolates

The strains with higher probiotic potential were identified. The results showed that the isolates' sequences are 92-100% similar to the strains recorded in the GenBank. The sequences have been submitted to NCBI with the accession numbers ON076063 (SUBC156) for *Enterococcus fecalis* (100%), and ON076055 (SUBC169) for *Enterococcus fecalis* (92%), respectively.

**Figure 7.** Biofilm formation of the breast milk isolates. SUB153 exhibits greater biofilm formation ability in BHI medium compared to MRS broth. However, the biofilm formation abilities of other strains are nearly equal in both BHI and MRS broths ($p < 0.05$).




Heikkilä and Saris (2003) collected 40 breast milk samples from healthy lactating volunteers in Finland; the predominant bacterial species was *E. faecalis* in 7.5% of the samples. The lactic acid isolates from breastmilk, including *L. gasseri* CECT5714, *L. salivarius* CECT5713, and *L. fermentum* CECT5716, also another strain relevant to breast milk, *L. reuteri* ATCC55730, were obtained from breastmilk, although its source has not been disclosed yet (Olivares et al., 2006).

### 3.4. The production potential of the vitamin B group

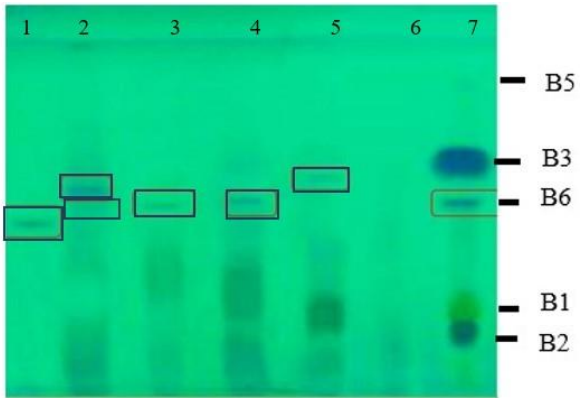
The isolate's supernatant was analysed for the production of vitamin B using thin-layer chromatography. The results showed that the bands of vitamins B2, B3, B5, and B6 could also be visualized as purple, and vitamin B1 is yellow. The Rf values are presented in Table 2. SUBC153 and SUBC156 exhibited Rf values similar to vitamin B6, while SUB169 showed Rf values comparable to vitamin B1 (Fig.8).

The popularity of probiotics has been on the rise lately. Ongoing research is being conducted to determine the therapeutic effects of probiotics on human health, such as gastrointestinal infections, traveler's diarrhea, antibiotic-associated diarrhea, and inflammatory bowel disease (Duraisamy et al., 2022). Mortality and chronic malnutrition can severely affect a child's physical growth. Consuming breast milk can help undernourished children achieve optimal growth and weight gain, increase cognitive function, and reduce the risk of associated diseases (Chou & Weimer, 1999).

**Table 2**. The Rf Value of Vitamins B1, B2, B3, B5, and B6

| Vitamin | Ds (cm) | $R_f$ |
|---|---|---|
| $B_1$ | 1.2 | 0.24 |
| $B_2$ | 0.6 | 0.12 |
| $B_3$ | 3.3 | 0.67 |
| $B_5$ | 4.6 | 0.93 |
| $B_6$ | 2.7 | 0.55 |

**Figure 8.** Thin Layer Chromatography of Vitamin B Isolates: 1; SUB157, 2; SUB156, 3; SUB153, 4; SUB166, 5; SUB169, 6: control-, ; 7 (B-Complex).




## 4. Conclusion

The two isolates of breast milk with higher probiotic compatibility were *Enterococcus faecalis* (SUBC 156) and *E. faecalis* (SUBC 169). The supernatant of SUBC156 affected *K. pneumoniae*, *S. aureus*, and *S. typhimurium* with the highest auto aggregation. Furthermore, SUBC156 and SUBC169 bound to four indicator bacteria with a 10-fold decrease in acid conditions. SUBC169 is resistant to bile salts, while both SUBC156 and SUBC169 isolates are resistant to papain. Additionally, SUBC156 exhibited the highest resistance to lysozyme. Both can form biofilm. Finally, SUBC156 can produce vitamin B6, while SUBC169 produces vitamin B1.

These probiotics in breast milk can help children restore optimal growth and have potential therapeutic roles, such as competitive elimination, production of antimicrobial compounds, and increased intestinal barriers. Developing new formulas with probiotic isolates is a promising strategy for infants.

## Conflict of interest

The authors declare that they have no competing interests.

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## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.



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## References

[1] Abdulla, A. (2014). Adhesion, Autoaggregation and Hydrophobicity of Six Lactobacillus Strains. *British Microbiology Research Journal*, *4*(4), 381–391. https://doi.org/10.9734/bmrj/2014/6462

[2] Anjum, J., Zaidi, A., Barrett, K., & Tariq, M. (2022). A potentially probiotic strain of *Enterococcus faecalis* from human milk that is avirulent, antibiotic sensitive, and

nonbreaching of the gut barrier. *Archives of Microbiology*, *204*(2), 158. https://doi.org/10.1007/s00203-022-02754-8

[3] Boris, S., Suárez, J. E., Vázquez, F., & Barbés, C. (1998). Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infection and Immunity*, *66*(5), 1985–1989. https://doi.org/10.1128/iai.66.5.1985-1989.1998

[4] Chou, L. S., & Weimer, B. (1999). Isolation and characterization of acid- and bile-tolerant isolates from strains of *Lactobacillus acidophilus*. *Journal of Dairy Science*, *82*(1), 23–31. https://doi.org/10.3168/jds.s0022-0302(99)75204-5

[5] Cimpoiu, C., & Hosu, A. (2007). Thin Layer Chromatography for the Analysis of Vitamins and Their Derivatives. *Journal of Liquid Chromatography & Related Technologies*, *30*(5-7), 701–728. https://doi.org/10.1080/10826070701191011

[6] Damaceno, Q. S., Souza, J. P., Nicoli, J. R., Paula, R. L., Assis, G. B., Figueiredo, H. C., Azevedo, V., & Martins, F. S. (2017). Evaluation of Potential Probiotics Isolated from Human Milk and Colostrum. *Probiotics and Antimicrobial Proteins*, *9*(4), 371–379. https://doi.org/10.1007/s12602-017-9270-1

[7] Duraisamy, S., Husain, F., Balakrishnan, S., Sathyan, A., Subramani, P., Chidambaram, P., Arokiyaraj, S., Al-Qahtani, W. H., Rajabathar, J., & Kumarasamy, A. (2022). Phenotypic Assessment of Probiotic and Bacteriocinogenic Efficacy of Indigenous LAB Strains from Human Breast Milk. *Current Issues in Molecular Biology*, *44*(2), 731–749. https://doi.org/10.3390/cimb44020051

[8] Frank, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., & Olsen, G. J. (2008). Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Applied and Environmental Microbiology*, *74*(8), 2461–2470. https://doi.org/10.1128/AEM.02272-07

[9] Gilliland, S. E., & Walker, D. K. (1990). Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *Journal of Dairy Science*, *73*(4), 905–911. https://doi.org/10.3168/jds.S0022-0302(90)78747-4

[10] Khalil, E. S., Abd Manap, M. Y., Mustafa, S., Alhelli, A. M., & Shokryazdan, P. (2018). Probiotic Properties of Exopolysaccharide-Producing Lactobacillus Strains Isolated from Tempoyak. *Molecules*, *23*(2), 398. https://doi.org/10.3390/molecules23020398

[11] Kos, B., Susković, J., Vuković, S., Simpraga, M., Frece, J., & Matosić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*, *94*(6), 981–987. https://doi.org/10.1046/j.1365-2672.2003.01915.x

[12] LeBlanc, J. G., Laiño, J. E., Del Valle, M. J., Vannini, V., van Sinderen, D., Taranto, M. P., Valdez, G. F. de, Giori, G. S. de, & Sesma, F. (2011). B-group vitamin production by lactic acid bacteria—current knowledge and potential applications. *Journal of Applied Microbiology*, *111*(6), 1297–1309. https://doi.org/10.1111/j.1365-2672.2011.05157.x

[13] Liong, M. T., & Shah, N. P. (2005). Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. *Journal of Dairy Science*, *88*(1), 55–66. https://doi.org/10.3168/jds.s0022-0302(05)72662-x

[14] Mahmoud, S. Y. M., Atallah, A. A., Badr, O. A., Moustafa, M. M. A., Esmael, A., Ebrahim, N., Aljeldah, M., Al Shammari, B., Alsafari, I. A., & Mohamed, S. A. (2022). Bioprospecting for Novel Probiotic Strains from Human Milk and Infants: Molecular, Biochemical, and Ultrastructural Evidence. *Biology*, *11*(10), 1405. https://doi.org/10.3390/biology11101405

[15] Maldonado, N. C., Ficoseco, C. A., Mansilla, F. I., Melián, C., Hébert, E. M., Vignolo, G. M., & Nader-Macías, M. E. F. (2018). Identification, characterization and selection of autochthonous lactic acid bacteria as probiotic for feedlot cattle. *Livestock Science*, *212*, 99–110. https://doi.org/10.1016/j.livsci.2018.04.003

[16] Nadya Salem Sultan Saeed Al Kalbani. (2018). Probiotic Characterization of Lactic Acid Bacteria (Lab) Isolated from Dried Emirati Fish and the Health Promoting Benefits of Fermented Fish Sausages by Selected Isolates [Master of Science (MS)]. College of Food and Agriculture.

[17] Olivares, M., Díaz-Ropero, M. P., Martín, R., Rodríguez, J. M., & Xaus, J. (2006). Antimicrobial potential of four Lactobacillus strains isolated from breast milk. *Journal of Applied Microbiology*, *101*(1), 72–79. https://doi.org/10.1111/j.1365-2672.2006.02981.x

[18] P, Vos, G Garrity, D Jones, NR Krieg, W Ludwig, FA Rainey, KH Schleifer, WB Whitman. (2011). *Bergey's manual of systematic bacteriology: The Firmicutes* (Vol. 3). https://books.google.com/books?hl=en&lr=&id=0-VqgLiCPFcC&oi=fnd&pg=PR1&dq=Bergey%27s+manual%C2%AE+of+systematic+bacteriology+lactobacillus&ots=kJxbz_Iu5A&sig=XHe-jedMpDCm0hD9TU5fSDryamM

[19] Ibarreche, M.P., Castellano, P., & Vignolo, G. (2014). Evaluation of anti-Listeria meat borne Lactobacillus for biofilm formation on selected abiotic surfaces. *Meat Science*, *96*(1), 295–303. https://doi.org/10.1016/j.meatsci.2013.07.010

[20] Pessoa, W. F. B., Melgaço, A. C. C., Almeida, M. E. de, Ramos, L. P., Rezende, R. P., & Romano, C. C. (2017). In Vitro Activity of Lactobacilli with Probiotic Potential Isolated from Cocoa Fermentation against *Gardnerella vaginalis*. *BioMed Research International*, *2017*(1), 3264194. https://doi.org/10.1155/2017/3264194

[21] Reis, N. A., Saraiva, M. A. F., Duarte, E. A. A., Carvalho, E. A. de, Vieira, B. B., & Evangelista-Barreto, N. S. (2016). Probiotic properties of lactic acid bacteria isolated from human milk. *Journal of Applied Microbiology*, *121*(3), 811–820. https://doi.org/10.1111/jam.13173

[22] Söderling, E. M., Marttinen, A. M., & Haukioja, A. L. (2011). Probiotic lactobacilli interfere with *Streptococcus mutans* biofilm formation in vitro. *Current Microbiology*, *62*(2), 618–622. https://doi.org/10.1007/s00284-010-9752-9

[23] Yildirim, Z., & Johnson, M. G. (1998). Detection and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* R isolated from radish. *Letters in Applied Microbiology*, *26*(4), 297–304. https://doi.org/10.1046/j.1472-765X.1998.00335.x

[24] Yuksekdag, Z., & Aslim, B. (2010). Assessment of potential probiotic- and starter properties of Pediococcus spp. Isolated from Turkish-type fermented sausages (sucuk). *Journal of Microbiology and Biotechnology*, *20*(1), 161–168. https://doi.org/10.4014/jmb.0904.04019

[25] Zakaria Gomaa, E. (2013). Antimicrobial and anti-adhesive properties of biosurfactant produced by lactobacilli isolates, biofilm formation and aggregation ability. *The Journal of General and Applied Microbiology*, *59*(6), 425–436. https://doi.org/10.2323/jgam.59.425