Uttar Pradesh Journal of Zoology

Volume 45, Issue 10, Page 114-124, 2024; Article no.UPJOZ.3448
ISSN: 0256-971X (P)

Exploration and Characterization of Novel Probiotic Bacteria Isolated from Human Mother's Milk: Evaluation of Antibiotic Resistance and Antimicrobial Activity

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.56557/UPJOZ/2024/v45i104055

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://prh.mbimph.com/review-history/3448

Original Research Article

Received: 18/02/2024
Accepted: 24/04/2024
Published: 29/04/2024

ABSTRACT

The field of probiotics research has experienced a notable upsurge in interest in recent years, particularly in the exploration and characterization of novel probiotic bacteria that hold potential health benefits and disease prevention. This study aimed to isolate and identify new probiotic bacteria from human mother's milk and evaluate their probiotic potential. A total of 10 bacterial strains were isolated from mature human milk and subsequently identified through biochemical evaluation. Five of these strains were selected for further assessment, including investigations into survival ability, hemolytic activity, antibacterial effects, and antibiotic resistance. The results

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revealed that three out of the five selected strains demonstrated promising characteristics. These strains were identified as *Bacillus sp*. MM28, MM38, MM39, *Lactobacillus* MM18, and MM103. Notably, these five strains exhibited non-hemolytic activity, displayed effective antibacterial activity against selected bacterial pathogens, and demonstrated resistance to most of the tested antibiotics. The isolated bacterial strains exhibited favorable probiotic attributes, including antibiotic resistance and the ability to inhibit the growth of bacterial pathogens. Consequently, these strains hold potential as probiotics in both the pharmaceutical and food industries.

Keywords: Mother milk; probiotics; Lactobacillus; hemolysis; Bacillus.

1. INTRODUCTION

"Human breast milk is rich in essential nutrients such as carbohydrates, essential fatty acids, proteins, vitamins, and minerals for infants. This has led to its recognition as the gold standard for infant nutrition" [1,2,3,4,5] "Numerous studies have found that human breast milk contains a broad range of indigestible nutrients. While the infants do not directly utilize these nutrients, the probiotic bacteria which present in the mother milk play a crucial role in establishing the infants' native microflora due to their potent bioactive properties" [6,7].

"Over the years, more than 200 different species have been identified in human milk" [8] "It has been established that breast milk is a consistent source of commensal, mutualistic, and/or probiotic bacteria to the infant gut, including staphylococci, streptococci, bifidobacteria, and lactic acid bacteria" [9,10]. The genera Lactobacillus, Pediococcus, and Lactococcus are part of the lactic acid bacteria (LAB) group, and strains from these genera are often used extensively in food production and preservation, as well as probiotics for humans and animals" [11,12,13].

"LAB with probiotic activity are typically part of the enteric flora and are thought to play a beneficial role in the human gastrointestinal (GI) tract ecosystem. Probiotics are defined by the WHO and FAO's international scientific consensus in 2002 as live microorganisms that, when administered in sufficient quantities, confer health benefits to the consumer" [14] "It has been demonstrated that human milk from healthy women contains about 10^3-10^4 CFU/mL, making it a continuous source of potential commensal bacteria for the infant" [14,15] "Some of the lactic acid bacteria strains isolated from this biological fluid can inhibit the growth of a broad range of pathogenic bacteria through competitive exclusion and/or the production of antimicrobial compounds such as bacteriocins, organic acids, or hydrogen peroxide" [14,15,16] "Several strains originating from human milk have been used as probiotics" [17]. "For example, *Limosilacto bacillus fermentum* (formerly *Lactobacillus fermentum*) CECT5716, isolated from the breast milk of a Spanish mother, was approved for use in baby food by the National Health Commission of the People's Republic of China in 2016" [18,19,20]. The aim of this study was to isolate LAB strains from the breast milk of healthy women and evaluate their probiotic potential through a series of in vitro tests. These tests included the production of lactic acid, hemolytic activity, antibiotic resistance, and antibacterial activity.

2. MATERIALS AND METHODS

2.1 Collection of Milk Sample

The study utilized mother's milk, which the Cuddalore District Medical College and Hospital in Chidambaram generously donated. The selection of samples adhered to the ethical guidelines set by the same institution. A group of ten healthy mothers, who were 1 to 4 months postpartum and aged between 24 and 38 years, voluntarily participated in the study. The milk samples were collected in a sterile tube by manual expression using sterile gloves. Before sample collection, nipples and mammary areola were cleaned with soap and sterile water and soaked in chlorhexidine. The first drop (500 µl) was discarded. Skin sampling was performed briefly, a 4-cm^2 area of the upper outer quarter of each breast was gently rubbed using sterile cotton swabs soaked in ST solution (0.15 M NaCl with 0.1% Tween 20). The head of each swab was aseptically cut from the handle, placed into a falcon tube. The samples were collected in sterile carriers and stored on ice until delivery to the laboratory.

2.2 Isolation of Lactic Acid Bacteria

Pour plate technique was used to isolate the organisms. 1 ml aliquots of the samples were

plated into MRS (Man, Rogosa and Sharpe) agar (pH 6.2). The plates were incubated at 37 °C for 2-3 days under anaerobic conditions (in anaerobe jar using Oxoid anaerogen compact). After incubation, individual colonies were selected and transferred into sterile broth mediums. The isolates were purified by selecting colonies with streak plate technique.

2.3 Hemolytic Activity

Fresh bacterial cultures were applied to blood agar media, which contained 5-10% sheep blood (sourced from Zist Royesh Co, Iran), then incubated for 24 hours at a temperature of 37°C. Following incubation, the bacterial isolates were inspected for clear zones around the colonies. These clear zones indicate beta hemolysis, while greenish zones suggest alpha hemolysis. The absence of such zones, known as gamma hemolysis, indicates no hemolysis. Only colonies exhibiting gamma hemolysis were selected for further study, while those showing signs of beta or alpha hemolysis were disregarded [21].

2.4 Biochemical and Morphological Characterization

The process of morphological characterization involves the use of the Gram staining technique. Biochemical characterization was achieved through the catalase test and the analysis of carbohydrate fermentation profiles. Physiological tests assessed the ability to grow in the presence of NaCl, at concentrations of 3% and 4.5% (w/v), and at temperatures of 15°C and 45°C.

2.5 Antibacterial Activity of Isolated Strains Against Bacterial Pathogens

The pathogenic bacterial cultures *Haemophilus influenzae* (ATCC- 49247), Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 33591), *Escherichia coli* (ATCC 25922), *Streptococcus pneumoniae* (ATCC - 19615), *Klebsiella pneumoniae* (ATCC - 13883), were obtained from the American Type Culture Collection (ATCC). The acquired cultures were maintained in NA slants.

A test solution was prepared by dissolving cell-free supernatants in 5% Dimethyl Sulphoxide (DMSO) at a specific weight. To achieve the required concentration of 100 µg/20 µL, 500 µg was dissolved in 1 mL. This solution was freshly prepared before commencing the antibacterial experiments. Bacterial pathogens were cultured by inoculating a loopful of cells into nutrient broth and then incubating for 24 hours at 37 ºC. Subsequently, the 24-hour-old cultures were transferred into sterile nutrient broth to attain a turbidity matching a 0.5 McFarland standard, corresponding to approximately 1 x 10^6 CFU. Bacterial cultures were plated on solid Mueller Hinton agar for testing. Following this, wells of 6 mm diameter were uniformly created on the agar surface using a sterile cork borer. Subsequently, cell-free supernatant from the bacteria (100 µg in 20 µL) and the positive control (ciprofloxacin, 5 µg in 20 µL) were added to these wells. The plates were then placed in an incubator at 37 °C for 24 hours. After incubation, the plates were examined for visible signs of growth inhibition around the wells. The diameter of the inhibition zone was measured to quantify the extent of growth inhibition. Measurements were taken from three different angles around each well, and the average value was calculated for each inhibition zone. The entire procedure was conducted in triplicate for accuracy and consistency.

2.6 Antibiotic Susceptibility Test

The disk diffusion assay method was employed for the antibiotic susceptibility test. The culture media and all antibiogram discs were procured from Himedia (India). Fresh cultures of bacterial isolates, grown overnight, were spread onto MRS agar plates. Subsequently, 12 antibiogram disk included gentamycin (10 µg), cefixime (5 µg), penicillin (10 µg), chloramphenicol (30 µg), streptomycin (10 µg), erythromycin (15 µg), ampicillin (10 µg), ciprofloxacin (5 µg), kanamycin (30 µg), vancomycin (30 µg), Tobramycin (10 µg), and clindamycin (2 µg) were meticulously placed on the agar plates, then the plates were then incubated at 37◦C for a duration of 24 hours. The results were interpreted and reported in accordance with the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) [22].

3. RESULTS

3.1 Isolation of Bacteria

The samples of mother's milk were diluted and then introduced to MRS agar plates, where visible colonies were observed. From the collected milk samples, a total of 118 bacterial colonies, each with distinct morphology, were successfully isolated. These 118 bacterial

strains, each exhibiting varying degrees of Hemolytic Activity, were selected for further investigation (Table 1).

3.2 Hemolytic Activity

This characteristic is often considered a safety feature for probiotic selection. Most probiotic strains are expected to be non-hemolytic. In our study, six isolates exhibited no hemolytic activity (γ-hemolysis) among the tested isolates. Strains demonstrating hemolytic activity were excluded, while strains showing non-hemolysis were selected for subsequent experiments (Fig. 1).

3.3 Morphological and Biochemical Characterization of Isolated Non-Hemolytic Strains

Five bacterial isolates from mother's milk were characterized through gram staining, motility testing, and biochemical analysis, with the results detailed in Table 2. The gram staining results revealed that the bacterial isolates MM-18 and MM-103 are rod-shaped, Gram-positive, and non-motile. These isolates tested negative for Indole, MR, Gelatin, Citrate, Catalase, VP, Oxidase, and H_2S production. Based on these microscopic and biochemical characteristics, the isolates are predicted to be of the *Lactobacillus* species (Fig. 2).

Bacterial isolates MM-28, MM-38, and MM-39, which formed round-shaped colonies on a solid agar medium, are identified as rod-shaped, Gram-positive, and motile. These isolates tested positive for VP, Gelatin hydrolysis, and Catalase tests. However, they tested negative for Indole, MR, Citrate, Oxidase, and H_2S production. Given these microscopic and biochemical characteristics, the isolates are predicted to belong to the *Bacillus* species (Fig. 3).

3.4 Antibacterial Activity of Cell-Free Supernatants of Isolate Bacterial

The antibacterial activity of the cell-free supernatants from strains MM18, MM28, MM38, MM39, and MM103 was tested against selected bacterial pathogens on solid MHA plates. The cell-free supernatant from strain MM18 exhibited the highest growth inhibitory activity against *Staphylococcus aureus* (14.26±0.13 mm). This was followed by the cell-free supernatant from strain MM38, which showed growth inhibitory activity against *Streptococcus pneumoniae* (12.62±0.12 mm) and *Haemophilus influenzae* (12.03±0.04 mm). The standard antibiotic Ciprofloxacin demonstrated a zone of inhibition ranging from 18 to 20 mm (Table 3).

3.5 Evaluation of Antibiotic Susceptibility

Table 4 reveals that all strains to be resistant to penicillin. However, isolate MM39 showed resistance to penicillin and intermediate resistance to cefixime, while being sensitive to all other antibiotics. Isolate MM28 demonstrated resistance to all antibiotics, except for gentamycin, and showed moderate intermediate resistance to clindamycin. Isolate MM38 was resistant to Penicillin and showed intermediate resistance to cefixime and chloramphenicol, but was sensitive to all other antibiotics. According to the antibiogram, the strain MM-28 showed maximum resistance to the 10 antibiotics among the 12 tested.

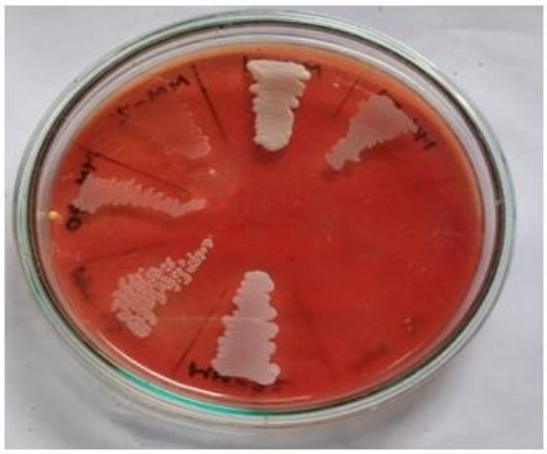
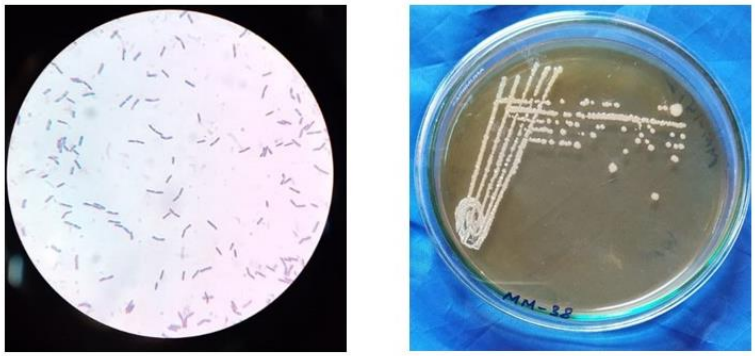


Fig. 1. Non-Hemolytic activity of MM18, MM28, MM38, MM39, and MM103 on blood agar plates



Lactobacillus sp.,

Fig. 2. Morphology of Lactobacillus sp isolates on MRS

Bacillus sp.,

Fig. 3. Morphology of *Bacillus* sp on MRS

Table 1. Isolation of probiotic bacteria from mother milk

S. No	Samples	Total colony forming units (CFU/mL x 10^6)	Total number of isolates
1	MM 1	28. 18	13
2	MM 2	27. 62	13
3	MM 3	19.04	09
4	MM 4	23.08	11
5	MM 5	27.12	12
6	MM 6	30. 36	15
7	MM 7	22.85	11
8	MM 8	30.91	16
9	MM 9	19.33	07
10	MM 10	20.09	12
Total			118

4. DISCUSSION

"Breast milk provides all the nutritional needs for infants' rapid and healthy growth. It contains a variety of protective factors, including immunoglobulin (IgA), immunocompetent cells, fatty acids, oligosaccharides, lactoferrin, and lysozyme. These factors protect breast-fed

infants against infectious diseases. Additionally, breast milk is a source of beneficial bacteria such as Lactobacilli, Lactococci, Enterococci, and *Leuconostoc spp.,* which are common commensal bacteria present in breast milk and play a crucial role in the infant's defence system" [14,15]. "In this study, the average relative abundance of Lactobacillus was generally less than 50% of isolated strains. Five strains with phenotypic characteristics that distinguish lactic acid bacteria were isolated from the milk of healthy mothers. However, only three of these isolated bacteria showed potential for use as probiotic microorganisms. Biochemical identification was conducted using Gram stain, catalase, and oxidase tests" [23]. All five isolates, namely MM18, MM28, MM38, MM39, and MM103, were screened for Gram's reaction and then observed microscopically. MM39 and MM103 were Gram-positive, rod-shaped, non-motile bacteria, while the other strains were observed as Gram-positive, rod-shaped, motile bacteria, indicating that they have characteristics of lactic acid bacteria [24].

Identifying lactic acid bacteria often proves unreliable when based solely on physicochemical and biochemical analysis, as many different bacterial species exhibit similar morphological and nutritional requirements [25]. Therefore, in this study, all three isolates were identified through 16S rRNA gene sequencing after microscopic observation. The 16S rDNA sequences were aligned using the BLAST algorithm [26]. "The lactic acid bacteria isolated in this study from human milk include *Lactobacillus sp* and *Bacillus sp*. All of these are considered potential probiotic bacteria. Some lactic acid bacteria strains of this origin have already been shown to possess probiotic properties, including the inhibition of a wide spectrum of infant pathogenic bacteria by competitive exclusion and/or through the production of antimicrobial compounds, such as bacteriocins, organic acids, or hydrogen peroxide" [16,27].

According to Jeronymo-Ceneviva *et al.* [28] "Lactobacillus strains isolated from dairy products demonstrated survival in NaCl (1–7%), indicating a high tolerance to sodium chloride. Hemolysis, a significant virulence factor for pathogenic microorganisms, was investigated in 118 strains in this study. The results showed ɣ-hemolysis (non-hemolytic), which is considered a safe characteristic for selected probiotic strains as per Araya [29]". Tanaka et al. [30] reported similar findings, where strains MM18, MM28, MM38, MM39, and MM103 isolated from mother's milk were non-hemolytic. One of the ways lactic acid bacteria contribute to host protection is by inhibiting the growth of pathogenic microorganisms. Probiotic microorganisms produce antimicrobial substances such as lactic and acetic acids, and the intestine's acidification helps inhibit certain pathogenic microorganisms' proliferation. They also produce metabolites like hydrogen peroxide, diacetyl, and bacteriocins. The antibacterial activity of strains MM18, MM28, MM38, MM39,

Table 2. Morphological and biochemical characterization of isolated non-hemolytic bacterial strains

Characters	Probiotic isolates				
	MM-18	**MM-28**	**MM-38**	**MM-39**	**MM-103**
Colony Morphology	Round	Round	Round	Round	Round
Colony Colour	white	white	Pale white	Pale white	white
Gram Staining	Positive	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod
Motility	Non-Motile	Motile	Motile	Motile	Non-Motile
Indole	-	-	-	-	-
MR	-	-	-	-	-
VP	-	+	+	+	-
Citrate	-	-	-	-	-
Gelatine	-	+	+	+	-
Catalase	-	+	+	+	-
Oxidase	-	-	-	-	-
H_2S	-	-	-	-	-
Probable organism	*Lactobacillus* sp	*Bacillus* sp	*Bacillus* sp	*Bacillus* sp	*Lactobacillus* sp

+: Positive; -: Negative

Table 3. Antibacterial activity of cell-free supernatants of isolate bacterial

Bacterial pathogens	MM-18	MM-28	MM-38	MM-39	MM-103	positive control (Ciprofloxacin 5 µg)
Haemophilus influenzae	08.76±0.52	10.43±0.35	-	-	12.03±0.04	20.25±0.44
Staphylococcus aureus	14.26±0.13	10.34±0.24	11.34±0.53	-	08.12±0.31	19.31±0.41
Escherichia coli	-	08.85±0.35	09.46±0.33	12.34±0.35	-	19.51±0.51
Streptococcus pneumoniae	07.93±0.73	-	12.62±0.12	08.23±0.47	10.22±0.55	18.56±0.76
Klebsiella pneumoniae	11.08±0.35	-	09.65±0.20	09.29±0.05	11.40±0.18	19.20±0.32

-: No zone of inhibition. The values are expressed in the mean ± standard deviation of triplicates.

Table 4. Antibiotic susceptibility of isolated probiotic strains

Antibiotic	MM-18	MM-28	MM-38	MM-39	MM-103
Gentamycin	S	I	S	S	S
Cefixime	R	R	I	I	S
Penicillin	R	R	R	R	R
Chloramphenicol	S	R	I	S	I
Streptomycin	S	R	S	S	S
Erythromycin	S	R	S	S	R
Ampicillin	R	R	S	S	R
Ciprofloxacin	S	R	S	S	S
Kanamycin	R	R	S	S	S
Vancomycin	S	R	S	S	R
Tobramycin	S	R	S	S	S
Clindamycin	S	I	S	S	R

R, Resistant; S, Sensitive; and I, Intermediate.

and MM103 was assessed against common pathogens like *Haemophilus influenzae, Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae,* and *Klebsiella pneumoniae*. The antibacterial activity was measured as a zone of inhibition against these pathogens seeded in agar. The crude extract corresponding to the overnight bacterial growth broth exhibited varying zones of inhibition depending on the tested pathogen and isolated bacteria. Strains MM18 and MM103 inhibited *Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae, and Klebsiella pneumoniae;* MM38 inhibited *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae*, and *Klebsiella pneumoniae*. MM28 inhibited *Escherichia coli, Streptococcus pneumoniae,* and *Klebsiella pneumoniae,* and MM39 showed inhibitory activity against *Escherichia coli, Streptococcus pneumoniae,* and *Klebsiella pneumoniae.*

Forestier *et al.* [31] examined "the antibacterial activities of cell-free supernatant of lactic acid bacteria against several human pathogenic bacteria (*K. pneumoniae, Shigella flexneri, Salmonella typhimurium, Enterobacter cloacae, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Clostridium difficile*). The strain *Lactobacillus casei rhamnosus* Lcr3 was found to inhibit the growth of these pathogenic strains". The differences in growth inhibition among strains in this study and the current one align with those reported by Kaushik et al. [32], Schneitz et al. [33], who noted that "the inhibitory effect of Lactobacillus strains can vary even within the same species". "Because of the wide range of activity of *Lactobacillus casei rhamnosus* L cr35, the antimicrobial mechanism involved is unlikely to be the production of classic bacteriocins, proteinaceous compounds produced by lactic acid bacteria with a bactericidal effect against taxonomically closely related bacteria" [34]. In the present study, inhibitory activity was not detected in all pathogenic strains, we believe that this inhibitory effect is thought to be due a production of lactic acid production.

Antibiotic sensitivity is deemed a crucial aspect of probiotics' safety evaluation. The disc antibiotic susceptibility method is typically favored. A modified approach, which substituted agar plates with MRS broth, was employed in this study [35]. The Minimum Inhibitory Concentration (MIC) of the three tested strains was gauged against nine antibiotics from different groups: cell wall inhibitors (azithromycin, cephalexin, penicillin G, and ampicillin) and protein synthesis inhibitors (kanamycin, streptomycin, tetracycline, chloramphenicol, and roxithromycin). Strains were deemed resistant if their MIC values exceeded the MIC breakpoints set by the European Food Safety Authority [36].

As indicated in Table 4, antibiotic susceptibility tests using the disk diffusion method revealed that all the strains were resistant to Penicillin. However, isolate MM39 was resistant to penicillin and showed intermediate resistance to cefixime, while being sensitive to all other antibiotics. Isolate MM28 was resistant to all antibiotics except gentamycin and showed moderate intermediate resistance to clindamycin. Isolate MM38 was resistant to penicillin and showed intermediate resistance to cefixime and chloramphenicol, but was sensitive to all other antibiotics.

5. CONCLUSIONS

This study investigated the potential of bacteria isolated from human milk as probiotic candidates. Among isolated strains, five exhibited non-hemolytic activity: Lactobacillus sp. MM18 and MM103, and Bacillus sp. MM28, MM38, and MM39. These non-hemolytic isolates displayed antibacterial activity against tested pathogens and exhibited resistance to most tested antibiotics. These findings suggest that the isolated probiotic strains from human milk warrant further exploration for treating gastrointestinal disorders. However, *in vitro* and *in vivo* studies are necessary to confirm their probiotic efficacy before clinical application. If further research confirms their benefits, these isolates hold promise as next-generation probiotics with potential value for the pharmaceutical industry.

ETHICAL APPROVAL

As per international standards or university standards written ethical approval has been collected and preserved by the author(s).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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https://prh.mbimph.com/review-history/3448