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Microbiological Quality, Antibiotic Susceptibility and Stability of Human Breast Milk Microbiota Stored at Refrigerated Temperature

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ABSTRACT

Microbiota in human breast milk is important for developing and strengthening infants' immune systems. This study aimed to identify microbial isolates, determine their antibiotic susceptibilities, and assess the microbiological quality of human breast milk during refrigerated storage. Participants (n = 19) were healthy, regularly breastfeeding mothers from Universiti Putra Malaysia with infants aged 0-24 months. Samples were manually expressed, diluted, and streaked on selective media for bacterial isolation. Isolates were identified using Gram staining and biochemical tests, then profiled for antibiotic susceptibility using the disc diffusion method. Microbiological quality was evaluated using total plate count (TPC), total Coliform count (TCC), and total *Staphylococcus* count (TSC). Eight bacterial isolates were presumptively characterised and assigned to putative genera: *Staphylococcus, Escherichia coli, Bacillus, Lactobacillus, Enterobacter, Citrobacter, Streptococcus,* and *Klebsiella*. Inhibition-zone measurements were interpreted descriptively because compliance with applicable Clinical and Laboratory Standards Institute (CLSI) testing and interpretive requirements could not be verified. During refrigerated storage, 15 of 19 samples (78.9%) consistently had TPC values below 5 $\log_{10}$ CFU/mL. TSC values also remained below 5 $\log_{10}$ CFU/mL in samples reported to meet this criterion. However, 12 samples (63.2%) had TCC values above the reference limit applied in this study. Bacterial counts generally declined during storage, although several samples had elevated coliform counts. These findings describe changes in breast

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milk microbiological quality during refrigerated storage but do not establish that storage at 4 °C for seven days is microbiologically safe. They also highlight the importance of hygienic practices during milk collection, handling, and storage.

Keywords: Antibiotic susceptibility, commensal bacteria, human breast milk, refrigerated temperature, stability

INTRODUCTION

Human breast milk is considered the optimal nutrition for neonates. It is naturally sterile and responsible for the development of neonatal gut microflora, especially for the preterm infant (Xiang et al., 2023). Exclusive breastfeeding for six months for infants, and continued breastfeeding for ≧ 24 months with nutrient-dense foods for young children, has been recommended by the World Health Organization for breastfeeding mothers. Some studies have found that breast milk can protect infants against infectious diseases, such as asthma and other atopic disorders (Capra et al., 2025). Furthermore, breastfeeding has been associated with reduced childhood obesity, potentially through modulation of obesity-related genes (Danaie et al., 2024). Additionally, the synergistic effects of components in colostrum and milk such as immunocompetent cells, immunoglobulins, fatty acids, polyamines, oligosaccharides, lactoferrin, lysozyme, other glycoproteins, and antimicrobial peptides may contribute to the protective properties of breast milk (Fernández et al., 2013). Colostrum and secretory IgA in breast milk are the main providers of commensal, mutualistic, and probiotic bacteria to the infant's gut, besides conferring immunoprotection by inhibiting pathogens from adhering to or penetrating the gastrointestinal (GI) tract (Mollova et al., 2023). During breastfeeding, bacterial genera such as Lactobacillus, *Staphylococcus*, Enterococcus, and Bifidobacterium were transferred from mother to infant (Murphy et al., 2017). Furthermore, breastfeeding has been reported to benefit not only the infant's well-being but also the mother's health. Health complications such as postpartum bleeding, Type II diabetes, and breast and ovarian cancers could be prevented through breastfeeding practices (Masi & Stewart, 2024).

About 5 to 33% of lactating mothers commonly experience mastitis, or inflammation of the breast tissue. The combination of poor breast drainage, low maternal resistance, and the presence of a colonising organism makes breastfeeding mothers vulnerable to bacterial infection. Infectious mastitis is most commonly caused by bacterial strains such as *Staphylococcus aureus, Klebsiella pneumoniae, Streptococcus dysgalactiae,* and *E. coli* (Gondkar et al., 2024). Antibiotic consumption by breastfeeding mothers is one of the measures to manage mastitis. However, antibiotic resistance in bacteria is a major concern, and it is one of the food safety challenges related to public health. Bacterial species such as *Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter*

spp*., and Klebsiella pneumonia* have been frequently reported to exhibit increasing antimicrobial resistance, leading to serious infectious diseases (Mancuso et al., 2021). Moreover, current works have indicated that gut commensal bacteria can act as reservoirs for antibiotic resistance genes and may contribute to the spread of antibiotic resistance to pathogens (Despotovic et al., 2023; Lamberte & van Schaik, 2022)

According to the Australian National Health and Medical Research Council guideline (2012), the appropriate storage of human breast milk is 6-8 h at room temperature (26 °C and lower), and no more than 72 h for storage in a refrigerator (5 °C or lower). Centers for Disease Control and Prevention (2024) recommended storing freshly expressed human breast milk at room temperature (up to 26 °C) for a maximum of 4 h, in the refrigerator (at 4 °C) for up to 4 days, and in the freezer (at −18 °C or lower) for 6 to 12 months. Grazziotin et al. (2016) reported that 12 h of storage at 4-8 °C or at -20 °C for 2 weeks preserved the nutritional value and energy content of breast milk samples. Breast milk is normally stored at frozen temperature after expression. However, some mothers store the milk briefly at refrigerated temperatures to avoid freeze-thaw cycles. Hence, the microbial stability of the breast milk stored at refrigerated temperatures is mostly unknown. Therefore, this study was carried out to determine the microbiological quality, bacterial antibiotic susceptibility, and stability of human breast milk at refrigerated storage.

MATERIALS AND METHODS

Materials

MacConkey Agar (MCA), Polymyxin Mannitol Egg Yolk Phenol Red (PMYPA) Agar, Plate Count Agar (PCA), Chromocult Coliform Agar (CA), Mannitol Salt Agar (MSA), Simmon's Citrate Agar (SCA), amoxicillin, ciprofloxacin, gentamicin, penicillin, chloramphenicol, ampicillin and streptomycin were purchased from Oxoid (Hampshire, UK). Motility Agar (MA), Tryptone Broth (TB), Christensen's Urea Agar (CUA), lactose, sucrose, glucose and filter paper were purchased from Fisher (Loughborough, UK). Nutrient Agar (NA) and Nutrient Broth (NB) were purchased from Merck (Darmstadt, Germany).

Sample Collection

All participants were briefed on the study protocol (Ethic Approval No. FSTM/EXP15/P144) prior to obtaining their written consent. Healthy lactating mothers (n = 19; 24 to 36 years old) who regularly breastfed their infants aged 0 to 24 months were among staff and students of Universiti Putra Malaysia, Serdang, Selangor. Manually expressed breast milk (30- 50 mL) via hand or breast pump was collected in a sterile 120-mL polypropylene container (labelled and individually packed). Samples were immediately placed in an icebox, transported to the laboratory and stored at 4 °C. The duration of sample collection,

from breast milk expression to refrigerated storage in the laboratory, was within 4 h. Breast milk samples were coded alphabetically. Microbiological analyses were conducted within three days after sample collection.

Isolation and Identification of Bacteria

Breast milk samples were inoculated onto selective media, including MacConkey Agar (MCA), Polymyxin Mannitol Egg Yolk Phenol Red (PMYPA) Agar, Mannitol Salt Agar (MSA), and Chromocult Coliform Agar (CA), and incubated at 37 °C for 24 h for bacterial isolation using conventional culture-based methods. Briefly, 1 mL of breast milk from each coded sample was aliquoted into an Eppendorf tube and serially diluted. A volume of 0.1 mL from the appropriate dilutions was spread onto Plate Count Agar (PCA), Chromocult Coliform Agar (CA), and Mannitol Salt Agar (MSA) for the enumeration of total plate count (TPC), total coliform count (TCC), and total *Staphylococcus* count (TSC), respectively. Representative colonies were subcultured onto Nutrient Agar (NA) and incubated at 37 °C for 24 h. The isolates were then presumptively characterised using conventional biochemical tests, following the procedures described by AOAC International (2012) and Omemu et al. (2018), where applicable. The identification approach was also considered in relation to the relevant UK Standards for Microbiology Investigations (Public Health England, 2020). Because molecular or other confirmatory identification was not performed, the bacterial identities were considered presumptive. Following isolation, the bacterial isolates were presumptively characterised using colony morphology, Gram staining, and biochemical tests, including motility, indole, catalase, oxidase, urease, citrate utilisation, and sugar fermentation. The procedures were based on the laboratory methods described by Omemu et al. (2018) and, where applicable, on the relevant microbiological identification guidance. The resulting characteristics were used to assign the isolates to putative bacterial genera.

Gram Staining

Gram staining and biochemical tests, including motility, indole production, catalase, oxidase, urease, citrate utilisation, and sugar fermentation, were performed using conventional microbiological procedures. The resulting reactions were interpreted together with colony morphology and Gram-staining characteristics to support the presumptive characterisation of the bacterial isolates (AOAC International, 2012; Omemu et al., 2018). With a sterile loop, a bacterial colony from NA was smeared onto a glass slide and gently passed through the flame (heat fixation). Next, crystal violet was used to flood the smear, followed by iodine. Decolourisation was done by dropping 95% ethyl alcohol onto the smear until no dye ran. The smear was then flooded with safranin for 45 s to counterstain. Then, the smear was tissue-blotted, allowed to air-dry, and examined under the light microscope (up to 1,000× magnification). Gram-positive and Gram-negative bacteria

produced violet and pink colour, respectively. All reagents were added to the smear for 1 min and cleared with water rinsing after each step.

Biochemical Characterisation of Bacterial Isolates

Bacterial isolates obtained from 18- 24 h cultures were subjected to conventional biochemical tests, including motility, indole, catalase, oxidase, urease, citrate utilisation, and sugar fermentation tests. For these tests, bacterial growth was transferred from the culture using a sterile inoculating loop, following the respective test procedures. The inoculum was not standardised to a specified cell concentration or McFarland turbidity standard. Therefore, the amount of bacterial growth transferred was not quantitatively standardised, potentially limiting the reproducibility of the inoculation procedure. The biochemical test results were used for the presumptive characterisation of the bacterial isolates.

Motility Test

A heavy inoculum from 18 to 24 h bacterial culture was stabbed into the MA tubes. The tubes were then incubated for 24 h at 37 °C. Bacteria that grew away from the stabbed line were considered motile, and bacteria that grew along the stabbed line were considered non-motile (Aditi et al., 2017).

Indole Test

A heavy inoculum from 18 to 24 h bacterial culture was inoculated into TB and incubated at 37 °C for 18 to 24 h. Kovác's reagent (five drops) was added directly down the inner wall of the tubes to test for indole production. A positive indole test was indicated by the formation of a bright red colour at the interface of the reagent within seconds after adding the reagent. The reagent layer which remained yellow or slightly cloudy indicated negative results for the indole test (Darkoh et al., 2015).

Catalase Test

The slide method was used to perform the catalase test (Fortin et al., 2003). A heavy inoculum from an 18- to 24-h bacterial culture was smeared onto the glass slide using a sterile inoculating loop. A positive catalase test was indicated by bubble formation after addition of 3% hydrogen peroxide (H_2O_2).

Oxidase Test

The filter paper test method was used for the oxidase test (Kumar et al., 2016). Basically, 1% of freshly prepared Kovác's reagent was used to soak a small piece of filter paper and

allowed to dry. A heavy inoculum from an 18 to 24 h bacterial culture was rubbed onto the treated filter paper using a sterile cotton swab. A colour change to dark purple after 5 to 10 s indicated an oxidase-positive result.

Urease Test

The CUA slants were prepared in the Universal tubes. A heavy inoculum from an 18 to 24 h bacterial culture was streaked over the CUA slant surface and incubated with a loosened cap at 37 °C for 18 to 24 h, and the colour changes were observed. Bright pink and yellowish colour indicated the positive and negative results of the urease test, respectively (Wan et al., 2024).

Citrate Test

A heavy inoculum from 18 to 24 h bacterial culture was streaked over the SCA slant surface and incubated at 37 °C for 24 to 48 h. Citrate-positive showed visible bacterial growth on the surface and a colour change from green to intense Prussian blue (Gebremichael et al., 2024).

Sugar Fermentation Test

Three types of sugar were used for the sugar fermentation test, namely glucose, sucrose and lactose (Verma et al., 2022). A heavy inoculum from an 18 to 24 h bacterial culture was inoculated into the test tubes containing the three different sugars with a Durham tube. The test tubes were incubated at 37 °C for 24 to 48 h, and the medium was observed for colour change. Red indicated acid production, yellow or colourless indicated no fermentation and gas production was indicated by the production of a bubble in the Durham tube.

Antibiotic Inhibition Zone Assessment

The inhibition zone measurements of the bacterial isolates against seven antibiotics were assessed using the disc diffusion procedure performed in the reported study (Heller & Spence, 2019). The antibiotics evaluated were amoxicillin (25 µg), ciprofloxacin (5 µg), gentamicin (10 µg), penicillin G (6 µg), chloramphenicol, ampicillin, and streptomycin. According to Heller & Spence's (2019) experimental procedure, chloramphenicol, ampicillin, and streptomycin were prepared from antibiotic powders, and 6-mm sterile filter paper discs were loaded with 10 µL of the respective antibiotic solutions. The bacterial suspensions were prepared in 0.85% saline, adjusted to a 0.5 McFarland standard, and spread onto nutrient agar plates. The antibiotic discs were placed on the inoculated agar

surfaces, then incubated at 37 °C for 24 h. The diameters of the inhibition zones, including the disc diameter, were measured to the nearest millimetre.

The Clinical and Laboratory Standards Institute (CLSI) provides standardised guidance for antimicrobial disk diffusion testing (Clinical and Laboratory Standards Institute (CLSI, 2024). The available methodological information did not establish whether all requirements for standardised disc diffusion testing under the applicable Clinical and Laboratory Standards Institute (CLSI) guidelines were fulfilled. Therefore, the inhibition-zone measurements are presented descriptively and are not interpreted as definitive susceptible, intermediate, or resistant classifications. The findings should be considered preliminary observations of bacterial growth inhibition under the experimental conditions employed.

Microbiological Quality

The microbiological quality of the milk samples was assessed daily over seven days of storage at 4 °C. The storage temperature was monitored daily. Briefly, 1-mL of breast milk was aliquoted into an Eppendorf tube and serially diluted (10^{-1} to 10^{-5}). A volume of 0.1 mL from each dilution was spread onto Plate Count Agar (PCA), Chromocult Coliform Agar (CA), and Mannitol Salt Agar (MSA) for the enumeration of total plate count (TPC), total coliform count (TCC), and total *Staphylococcus* count (TSC), respectively. The surface-plating procedure for TPC was described with reference to the general principles of microbial colony enumeration by surface plating (International Organization for Standardization [ISO], 2013). The use of Chromocult Coliform Agar for coliform enumeration was supported by published work describing the detection and enumeration of coliforms using this medium (Stinson et al., 2022). Inoculated plates were incubated at 37 °C for 24 h, and plates containing 25- 250 colonies were used for enumeration. The resulting counts were expressed as $\log_{10}$ CFU/mL.

Statistical Analysis

Microbiological counts were expressed as $\log_{10}$ CFU/mL. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS), version 16.0. Analysis of variance (ANOVA) was used to assess differences in bacterial counts, with statistical significance set at $p<0.05$. Where applicable, significant differences between means were compared using Duncan's multiple range test. Microsoft Excel was used to organise the data and prepare graphs and charts. As the same milk samples were measured over multiple storage days, the statistical analysis should account for the repeated-measures structure of the data. Results below the detection limit were considered when determining the appropriate statistical approach.

RESULTS AND DISCUSSION

Isolation and identification of bacteria

Breast milk samples were collected and inoculated onto MCA, PMYPA, MSA and CA plates to isolate and identify the presence of bacteria using the conventional culture-based method. There was appearance and growth of *E.coli, Bacillus* spp., *Staphylococcus* spp. and *Coliforms*. The unknown colonies were subcultured onto NA and further examined through Gram staining and biochemical tests to observe the morphological and chemical characteristics of the bacterial isolates. Characterisation and presumptive bacterial genera isolated from human breast milk samples were performed in accordance with the UK Standards for Microbiology Investigations (Public Health England, 2020). As shown in Table 1, the presumptive bacterial genera identified included *Staphylococcus spp.*, *Streptococcus spp.*, *Lactobacillus spp.*, *Klebsiella spp.*, *Citrobacter spp.*, and *Enterobacter spp.* It was observed that the shapes of colonies of bacterial isolates were mostly small and round. The surface characteristics were smooth, and the margins of the colonies were entire and convex. The colonies, which were selected visually by naked eye, were observed to be whitish and cream-coloured. However, yellow-coloured colonies were also observed. All colonies were observed to be opaque. Among the six strains isolated, three were Gram-negative, with two displaying cocci morphology and one exhibiting a rod shape, whereas the remaining three Gram-positive strains were all rod-shaped. The bacterial isolates were presumptively characterised based on colony morphology, Gram staining, and biochemical reactions. The observed characteristics were used to assign the isolates to putative bacterial genera. As molecular or other confirmatory identification methods were not performed, the reported identities should be interpreted as presumptive rather than definitive species-level identifications.

Although human breast milk is naturally sterile, it can serve as a carrier of commensal, mutualistic, or potentially probiotic bacteria to the infant's gut, including *Staphylococcus, Streptococcus, Lactobacillus, Escherichia*, and *Enterobacter*, which is consistent with the majority of isolates identified in this study (Notarbartolo et al., 2022). It is suggested that the microbe isolated in this study may originate from the skin that was in contact with the milk during suckling or pumping (Lee et al., 2024). Furthermore, a mechanism involving gut monocytes during lactation may cause several bacteria from the maternal gut to reach the mammary gland through oromammary and enteromammary pathways. The mechanism would involve dendritic cells (DCs) and CD18 cells, which could take up bacteria from the gut lumen and carry them to other locations, including the lactating mammary gland (Mira & Rodriguez, 2017). In addition, during breastfeeding, a certain degree of milk may flow back into the mammary ducts; thus, the exchange of bacteria from the infant's mouth into the mammary gland could happen during such backflow (Wu et al., 2025). Moossavi et al. (2019) identified that breastfeeding mode is a key determinant of milk microbiota, and

Table 1
Morphological and biochemical characterization of bacterial isolates from human breast milk

Characteristics	Isolates							
	L-002	**O-01**	**N-01**	**N-02**	**Q-01**	**Q-02**	**R-01**	**R-02**
	Observation							
Margin	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Colour	Creamy	Creamy	Creamy	Creamy	Creamy	Yellow	Creamy	Creamy
Shape	Small, round	Small, round	Small, round	Small, round	Small, round	Small, round	Small, round	Small, round
Cell type	Cocci	Cocci	Rod	Rod	Rod	Rod	Rod	Rod
Cell arrangement	Single	Single	Single	Single	Single	Single	Single	Single
Gram Reaction	+	+	+	−	−	−	−	−
	Sugar Fermentation Test							
Glucose	+	−	+	−	−	−	+	−
Sucrose	+	+	+	+	−	−	+	−
Lactose	+	+	+	+	−	−	+	+
	Biochemical Test							
Catalase	+	−	−	+	+	+	+	+
Oxidase	−	−	−	−	−	−	−	−
Indole	−	−	−	−	−	−	−	−
Urease	+	−	−	−	−	−	+	+
Citrate	−	+	+	+	+	+	+	+
Presumptive microorganism	*Staphylococcus epidermis*	*Streptococcus* spp.	*Lactobacillus* spp.	*Klebsiella* spp.		*Citrobacter* spp.	*Enterobacter* spp.	

pumped breast milk is a consistent source of multiple microbiota, including augmentation of potential pathogens and lessening of bifidobacteria.

Antibiotic Inhibition Zone Assessment

In this study, eight bacterial isolates from human breast milk were assessed for inhibition-zone formation in response to seven antibiotics: amoxicillin (AMX), ciprofloxacin (CIP), gentamicin (GM), penicillin G (P), chloramphenicol (C), ampicillin (AM), and streptomycin (S). As shown in Figure 1, inhibition zones ranging from 9 to 35 mm, including the 6-mm disc diameter, were observed among the isolates tested. These measurements indicate bacterial growth inhibition under the experimental conditions employed. However, because compliance with the applicable Clinical and Laboratory Standards Institute (CLSI) testing requirements and interpretive criteria could not be verified, the results should not be interpreted as definitive evidence of antibiotic susceptibility or resistance.

Differences in inhibition-zone diameters were observed among the antibiotics tested and the bacterial groups examined. For the coliform group, no statistically significant differences ($p>0.05$) were observed among AMX, CIP, and C, whereas differences were observed for the other antibiotics. For *Staphylococcus, Bacillus, Enterobacter, Citrobacter*, and *Lactobacillus,* differences in inhibition-zone diameters were also reported among the antibiotics. For *Escherichia coli,* the reported comparison involved ampicillin, AMX, and chloramphenicol; however, the statistical significance and corresponding p-values should be verified against the original analysis. Any differences in inhibition-zone diameter should

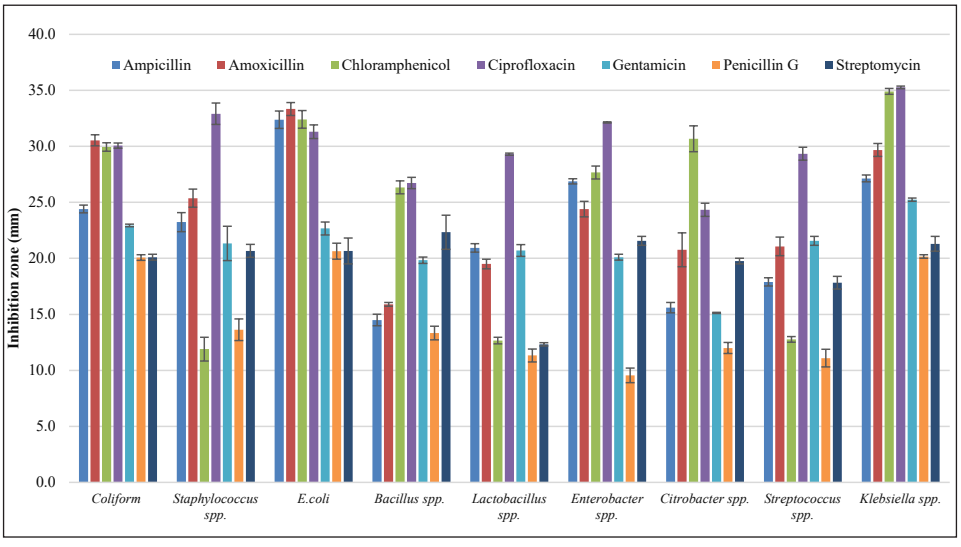

Figure 1. Inhibition-zone diameters of bacterial isolates from human breast milk in response to seven antibiotics. The measurements are presented descriptively and should not be interpreted as definitive classifications of susceptible, intermediate, or resistant. Data are expressed as mean ± SD of triplicates ($n = 3$)

be interpreted with caution, as zone sizes cannot be compared directly across different antibiotics as a measure of relative clinical effectiveness without appropriate standardised testing and organism-specific interpretive criteria.

Antibiotic resistance is an important public health concern because resistance determinants may be carried by mobile genetic elements, including plasmids and transposons, facilitating their potential dissemination among bacteria (Uddin et al., 2021). Previous studies have reported antibiotic resistance among bacteria isolated from human breast milk. For example, *Staphylococcus epidermidis* isolates from human breast milk have been reported to exhibit resistance to several antibiotics, including mupirocin, erythromycin, clindamycin, and oxacillin (Delgado et al., 2009; Begovic et al., 2013). Jiménez et al. (2013) reported virulence-associated characteristics and antibiotic resistance among *Enterococcus faecalis* and *Enterococcus faecium* isolates obtained from breast milk. Other studies have also reported resistance among breast-milk-associated *Enterococcus* spp. to selected antibiotics, including ciprofloxacin and gentamicin (Kıvanç et al., 2016). Chen et al. (2016) similarly reported resistance to antibiotics such as erythromycin, oxacillin, and clindamycin among *Enterococcus* spp. These findings highlight the importance of investigating the antimicrobial resistance characteristics of bacteria associated with human breast milk. Nevertheless, direct comparisons with the present study are limited because the bacterial species and antibiotics examined differed among studies.

Resistance patterns have also been reported among *Staphylococcus* spp*.,* although responses vary by species, antibiotic, and testing conditions. The potential clinical relevance of resistant bacteria in early life warrants attention, particularly because premature infants may receive prolonged antibiotic treatment. Such exposure may disrupt the developing gut microbiota and contribute to the selection of antimicrobial-resistant bacteria (Baker et al., 2023). However, the present study did not establish the presence or absence of antibiotic resistance in the tested isolates. Further investigation using a validated antimicrobial susceptibility testing procedure, with appropriate quality-control measures and organism-specific interpretive criteria, is therefore required to establish their susceptibility profiles and clarify the potential implications for infant health.

Stability of Human Breast Milk Microbiota at 4 °C

The microbiological quality of human breast milk during refrigerated storage was evaluated by determining the total plate count (TPC), total coliform count (TCC), and total *Staphylococcus* count (TSC). The results are presented in Tables 2, 3, and 4, respectively. Bacterial growth was detected in the TPC analysis of all samples, although no statistically significant differences ($p > 0.05$) were observed across the storage period for 10 of the 19 samples. For TCC, seven samples showed no detectable coliforms throughout the seven-day storage period, whereas the remaining 12 samples had coliform counts ranging from 2.11

Table 2
Total plate count (log_{10} CFU/mL) of human breast milk stored at refrigerated temperature (4 °C) for seven days

Sample	Aerobic Counts (log_{10} CFU/mL)							
	Day							
	0	1	2	3	4	5	6	7
1	3.60 ± 0.05^{a}	3.55 ± 0.01^{a}	3.38 ± 0.13^{ab}	3.43 ± 0.18^{a}	3.29 ± 0.21^{ab}	3.22 ± 0.35^{bc}	2.96 ± 0.05^{bc}	2.77 ± 0.00^{c}
2	5.60 ± 0.44^{a}	5.67 ± 0.42^{a}	5.09 ± 0.12^{a}	5.58 ± 0.00^{a}	4.64 ± 0.63^{a}	4.73 ± 0.00^{a}	4.81 ± 0.00^{a}	4.51 ± 0.16^{a}
3	4.21 ± 0.27^{a}	4.21 ± 0.18^{a}	4.24 ± 0.23^{a}	4.30 ± 0.17^{a}	4.19 ± 0.18^{a}	3.43 ± 0.51^{a}	3.34 ± 0.36^{a}	3.13 ± 0.32^{a}
4	5.38 ± 0.00^{de}	5.72 ± 0.10^{cde}	5.68 ± 0.34^{abc}	6.32 ± 0.03^{ab}	6.46 ± 0.23^{a}	5.38 ± 0.24^{de}	5.19 ± 0.03^{e}	5.80 ± 0.16^{bcd}
5	4.70 ± 0.03^{a}	5.14 ± 0.31^{a}	4.80 ± 0.39^{a}	4.72 ± 0.23^{a}	5.17 ± 0.23^{a}	4.69 ± 0.05^{a}	3.57 ± 0.20^{b}	3.68 ± 0.34^{b}
6	3.25 ± 0.06^{b}	3.24 ± 0.07^{b}	3.19 ± 0.38^{b}	3.22 ± 0.33^{b}	3.05 ± 0.08^{b}	5.18 ± 0.27^{a}	4.87 ± 0.05^{a}	4.51 ± 0.29^{a}
7	3.61 ± 0.25^{a}	3.39 ± 0.02^{a}	3.49 ± 0.19^{a}	3.45 ± 0.14^{a}	3.35 ± 0.15^{a}	3.33 ± 0.14^{a}	0.47 ± 0.02^{a}	3.41 ± 0.02^{a}
8	3.88 ± 0.24^{a}	3.47 ± 0.00^{ab}	3.49 ± 0.20^{ab}	3.23 ± 0.01^{bc}	2.90 ± 0.00^{bcd}	3.29 ± 0.58^{ab}	2.58 ± 0.15^{cd}	2.38 ± 0.29^{d}
9	4.39 ± 0.05^{a}	4.36 ± 0.32^{a}	4.51 ± 0.06^{a}	4.36 ± 0.24^{a}	4.23 ± 0.12^{a}	4.31 ± 0.08^{a}	0.32 ± 0.17^{a}	4.25 ± 0.12^{a}
10	3.56 ± 0.36^{a}	3.67 ± 0.16^{a}	3.51 ± 0.07^{a}	3.52 ± 0.04^{a}	3.54 ± 0.01^{a}	3.56 ± 0.38^{a}	0.34 ± 0.14^{a}	3.06 ± 0.11^{a}
11	3.77 ± 0.16^{a}	3.76 ± 0.28^{a}	3.73 ± 0.11^{a}	3.63 ± 0.09^{a}	3.69 ± 0.32^{a}	3.64 ± 0.29^{a}	3.66 ± 0.30^{a}	3.64 ± 0.33^{a}
12	5.57 ± 0.00^{a}	5.61 ± 0.00^{a}	5.24 ± 0.33^{a}	5.73 ± 0.27^{a}	5.37 ± 0.49^{a}	5.46 ± 0.44^{a}	5.25 ± 0.58^{a}	5.47 ± 0.20^{a}
13	5.02 ± 0.50^{a}	4.92 ± 0.39^{a}	4.96 ± 0.39^{a}	5.19 ± 0.47^{a}	5.85 ± 0.03^{a}	5.78 ± 0.15^{a}	5.66 ± 0.17^{a}	5.59 ± 0.15^{a}
14	4.78 ± 0.16^{ab}	4.56 ± 0.67^{b}	4.66 ± 0.13^{ab}	4.76 ± 0.16^{ab}	5.03 ± 0.49^{a}	4.82 ± 0.23^{ab}	4.75 ± 0.31^{ab}	4.63 ± 0.14^{ab}
15	3.70 ± 0.15^{a}	3.56 ± 0.17^{ab}	3.41 ± 0.06^{ab}	3.28 ± 0.14^{abc}	3.26 ± 0.13^{abc}	3.11 ± 0.00^{bc}	3.09 ± 0.08^{bc}	2.87 ± 0.12^{c}
16	3.91 ± 0.30^{a}	3.98 ± 0.22^{a}	4.00 ± 0.28^{a}	3.90 ± 0.19^{a}	3.90 ± 0.16^{a}	3.79 ± 0.03^{a}	3.77 ± 0.02^{a}	3.45 ± 0.19^{a}
17	4.16 ± 0.32^{a}	3.81 ± 0.21^{ab}	3.68 ± 0.21^{b}	3.82 ± 0.14^{ab}	3.76 ± 0.23^{ab}	3.79 ± 0.10^{ab}	3.65 ± 0.18^{b}	3.54 ± 0.12^{b}
18	4.18 ± 0.01^{a}	4.27 ± 0.00^{a}	4.37 ± 0.21^{a}	4.13 ± 0.13^{a}	4.15 ± 0.05^{a}	3.87 ± 0.00^{a}	3.73 ± 0.19^{a}	3.53 ± 0.13^{a}
19	3.60 ± 0.04^{a}	3.55 ± 0.01^{a}	3.38 ± 0.13^{ab}	3.42 ± 0.18^{abc}	3.29 ± 0.21^{abc}	3.22 ± 0.35^{ab}	2.96 ± 0.05^{abc}	2.77 ± 0.00^{c}

Note. Data are expressed as mean ± standard deviation (SD) of three experiments (n=3). Mean values with different superscripts in the same row are significantly different ($p<0.05$)

Table 3
Total coliform count (log_{10} CFU/mL) of human breast milk stored at refrigerated temperature (4°C) for seven days

Sample	log_{10} CFU/mL							
	Day							
	0	1	2	3	4	5	6	7
1	5.72 ± 0.12^{ab}	5.46 ± 0.13^{ab}	5.67 ± 0.18^{ab}	5.49 ± 0.22^{ab}	5.85 ± 0.07^{a}	5.35 ± 0.10^{b}	5.59 ± 0.12^{ab}	5.63 ± 0.11^{ab}
2	4.81 ± 0.16^{ab}	5.08 ± 0.07^{a}	4.48 ± 0.08^{abc}	4.57 ± 0.12^{abc}	4.79 ± 0.07^{ab}	3.78 ± 0.00^{c}	4.13 ± 0.24^{bc}	4.51 ± 0.15^{abc}
3	nd*	nd	nd	nd	nd	nd	nd	nd
4	5.38 ± 0.00^{ab}	5.72 ± 0.10^{a}	5.68 ± 0.34^{ab}	6.33 ± 0.04^{ab}	6.46 ± 0.23^{ab}	5.38 ± 0.24^{ab}	5.19 ± 0.03^{b}	5.80 ± 0.15^{ab}
5	3.31 ± 0.19^{a}	3.35 ± 0.29^{a}	3.06 ± 0.00^{a}	2.85 ± 0.00^{a}	2.66 ± 0.08^{ab}	3.26 ± 0.05^{a}	2.00 ± 0.43^{b}	3.28 ± 0.03^{a}
6	2.77 ± 0.00^{b}	2.58 ± 0.00^{c}	2.46 ± 0.00^{d}	2.38 ± 0.00^{f}	2.38 ± 0.00^{e}	2.28 ± 0.00^{g}	2.11 ± 0.00^{h}	3.04 ± 0.00^{a}
7	nd	nd	nd	nd	nd	nd	nd	nd
8	nd	nd	nd	nd	nd	nd	nd	nd
9	3.32 ± 0.13^{bc}	3.61 ± 0.27^{abc}	3.42 ± 0.03^{bc}	3.53 ± 0.20^{bc}	4.60 ± 0.51^{a}	4.09 ± 0.05^{ab}	3.69 ± 0.35^{bc}	2.97 ± 0.52^{c}
10	3.08 ± 0.05^{a}	3.11 ± 0.23^{a}	2.83 ± 0.18^{a}	2.80 ± 0.28^{a}	2.62 ± 0.00^{a}	2.64 ± 0.28^{a}	2.41 ± 0.16^{a}	2.46 ± 0.02^{a}
11	nd	nd	nd	nd	nd	nd	nd	nd
12	3.87 ± 0.40^{b}	3.80 ± 0.54^{b}	4.59 ± 0.56^{a}	5.59 ± 0.19^{a}	5.61 ± 0.22^{a}	5.63 ± 0.16^{a}	5.74 ± 0.29^{a}	5.52 ± 0.06^{a}
13	4.01 ± 0.24^{a}	4.25 ± 0.24^{a}	4.34 ± 0.35^{a}	3.74 ± 0.01^{a}	4.64 ± 0.09^{a}	4.85 ± 0.54^{a}	4.60 ± 0.03^{a}	4.62 ± 0.29^{a}
14	4.59 ± 0.28^{b}	3.78 ± 0.03^{c}	4.17 ± 0.08^{bc}	4.39 ± 0.11^{b}	4.81 ± 0.53^{a}	4.27 ± 0.01^{bc}	4.21 ± 0.08^{bc}	3.91 ± 0.00^{bc}
15	nd	nd	nd	nd	nd	nd	nd	nd
16	nd	nd	nd	nd	nd	nd	nd	nd
17	nd	nd	nd	nd	nd	nd	nd	nd
18	3.59 ± 0.22^{a}	3.46 ± 0.61^{ab}	3.27 ± 0.32^{ab}	2.99 ± 0.00^{ab}	2.85 ± 0.00^{ab}	2.69 ± 0.00^{b}	2.90 ± 0.00^{ab}	3.12 ± 0.00^{ab}
19	3.04 ± 0.00^{ab}	3.02 ± 0.00^{ab}	3.21 ± 0.00^{a}	2.91 ± 0.00^{b}	2.83 ± 0.00^{b}	2.30 ± 0.13^{d}	2.47 ± 0.10^{c}	2.25 ± 0.69^{cd}

Note. nd = not detected; Data were expressed as mean ± standard deviation (SD) of three experiments (n = 3). Mean values with different superscripts in the same row are significantly different ($p<0.05$)

Table 4
Total Staphylococcus *count (log_{10} CFU/mL) of human breast milk stored at refrigerated temperature (4°C) for seven days*

Sample	log_{10} CFU/mL							
	Day							
	0	1	2	3	4	5	6	7
1	nd	nd	nd	nd	nd	nd	nd	nd
2	nd	nd	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd	nd	nd	nd	nd
4	4.44 ± 0.00^{a}	4.33 ± 0.40^{a}	4.71 ± 0.00^{a}	4.59 ± 0.14^{a}	3.51 ± 0.11^{b}	3.61 ± 0.21^{b}	3.10 ± 0.28^{b}	3.41 ± 0.03^{b}
5	nd	nd	nd	nd	nd	nd	nd	nd
6	3.12 ± 0.00^{ab}	3.07 ± 0.00^{a}	2.94 ± 0.00^{ab}	2.54 ± 0.00^{ab}	2.44 ± 0.00^{ab}	2.30 ± 0.00^{ab}	2.18 ± 0.00^{ab}	1.95 ± 0.00^{b}
7	2.92 ± 0.00^{bc}	2.72 ± 0.00^{cd}	2.74 ± 0.00^{cd}	2.74 ± 0.00^{cd}	3.07 ± 0.00^{b}	3.00 ± 0.84^{a}	2.57 ± 0.05^{d}	2.47 ± 0.06^{d}
8	2.84 ± 0.00^{b}	2.83 ± 0.00^{d}	2.77 ± 0.00^{e}	2.83 ± 0.00^{c}	2.92 ± 0.00^{a}	2.84 ± 0.00^{h}	2.76 ± 0.00^{f}	2.65 ± 0.00^{g}
9	2.92 ± 0.00^{a}	2.93 ± 0.00^{a}	3.17 ± 0.50^{a}	3.15 ± 0.44^{a}	3.44 ± 0.00^{a}	3.16 ± 0.57^{a}	2.82 ± 0.26^{a}	2.91 ± 0.43^{a}
10	3.28 ± 0.00^{a}	3.32 ± 0.00^{a}	3.55 ± 0.00^{a}	3.22 ± 0.00^{a}	3.08 ± 0.00^{a}	2.96 ± 0.00^{a}	2.78 ± 0.00^{a}	2.98 ± 0.00^{a}
11	3.37 ± 0.26^{a}	3.23 ± 0.14^{a}	3.26 ± 0.37^{a}	3.11 ± 0.36^{a}	3.16 ± 0.39^{a}	3.02 ± 0.26^{a}	3.22 ± 0.36^{a}	2.95 ± 0.60^{a}
12	3.80 ± 0.26^{a}	3.67 ± 0.07^{a}	3.58 ± 0.16^{a}	3.47 ± 0.37^{a}	3.80 ± 0.39^{a}	3.60 ± 0.20^{a}	3.66 ± 0.25^{a}	3.89 ± 0.15^{a}
13	4.91 ± 0.14^{a}	4.85 ± 0.22^{a}	4.94 ± 0.30^{a}	4.96 ± 0.30^{a}	4.37 ± 0.20^{ab}	3.72 ± 0.34^{b}	3.54 ± 0.11^{b}	3.65 ± 0.28^{b}
14	3.96 ± 0.16^{a}	4.07 ± 0.16^{a}	3.47 ± 0.35^{a}	3.49 ± 0.46^{a}	3.46 ± 0.50^{a}	3.44 ± 0.44^{a}	3.43 ± 0.43^{a}	2.83 ± 0.00^{a}
15	3.54 ± 0.09^{a}	3.51 ± 0.01^{a}	3.37 ± 0.09^{ab}	3.25 ± 0.01^{b}	3.24 ± 0.01^{b}	2.76 ± 0.01^{c}	2.25 ± 0.00^{e}	2.50 ± 0.00^{d}
16	3.18 ± 0.01^{a}	3.11 ± 0.05^{ab}	2.86 ± 0.00^{bc}	3.01 ± 0.14^{ab}	2.70 ± 0.11^{cd}	2.61 ± 0.00^{cd}	2.47 ± 0.00^{d}	2.00 ± 0.00^{e}
17	3.51 ± 0.20^{a}	3.56 ± 0.12^{a}	3.43 ± 0.03^{a}	3.32 ± 0.00^{a}	3.39 ± 0.00^{a}	2.95 ± 0.00^{a}	2.86 ± 0.00^{a}	2.97 ± 0.00^{a}
18	3.48 ± 0.17^{a}	3.32 ± 0.01^{a}	3.41 ± 0.08^{a}	2.87 ± 0.04^{b}	2.94 ± 0.00^{b}	2.53 ± 0.00^{c}	2.30 ± 0.10^{c}	1.95 ± 0.00^{d}
19	3.18 ± 0.01^{a}	3.11 ± 0.05^{ab}	2.86 ± 0.00^{bc}	3.01 ± 0.14^{ab}	2.70 ± 0.11^{cd}	2.61 ± 0.00^{cd}	2.47 ± 0.00^{d}	2.00 ± 0.00^{e}

Note. nd = not detected; Data were expressed as mean ± standard deviation (SD) of three experiments (n = 3). Mean values with different superscripts in the same row are significantly different ($p<0.05$)

to 5.85 $\log_{10}$ CFU/mL. The TSC results indicated that *Staphylococcus* was not detected in some samples; however, the number of samples with detectable and undetectable TSC values should be verified against Table 4 to ensure consistency among the samples.

Microbiological reference limits are important for interpreting the quality of milk and milk products. European Commission (2005) provides microbiological criteria for several bacterial groups, while the Malaysian Food Regulations (1985) specify microbiological limits for milk and milk products. However, these criteria should be interpreted with caution when applied to human breast milk, as the applicable regulations do not specifically establish microbiological safety limits for human breast milk in the context of this study. Using the reference criteria applied in the present study, 15 of the 19 samples (78.9%) consistently exhibited TPC values below 5 $\log_{10}$ CFU/mL throughout the seven-day storage period at 4 °C. In contrast, 12 samples (63.2%) had TCC values above the reference limit, while seven samples (36.8%) showed no detectable coliforms throughout storage. These findings indicate that the microbiological quality varied among samples and that the TCC results warrant particular attention.

Elevated TCC values may be associated with contamination introduced during milk expression, collection, handling, or storage. Potential contributing factors include inadequate hand hygiene before milk expression, insufficient cleaning of collection equipment and storage containers, and contamination during sample transfer or handling (Aliyo et al., 2022). Although sterile containers were used to collect and store the milk samples, contamination could still have occurred during other stages of collection and handling. Nevertheless, hygiene practices were not directly assessed in this study, and the source of the observed coliforms was not investigated. Therefore, poor hygiene cannot be identified conclusively as the cause of the elevated TCC values.

These findings emphasise the importance of maintaining appropriate hygiene throughout the collection and handling of human breast milk. Washing hands before milk expression, ensuring that collection equipment and containers are appropriately cleaned and sanitised, minimising contact with potentially contaminated surfaces, and handling samples carefully may help reduce the risk of contamination. Maintaining appropriate refrigeration is also important; however, refrigeration alone does not establish microbiological safety when elevated coliform counts are present. Consequently, the elevated TCC values observed in several samples warrant caution when interpreting the microbiological quality of the milk throughout the seven-day storage period.

Overall, bacterial counts declined during storage, although some samples showed minor increases or fluctuations. These observations describe changes in the measured bacterial populations under the storage conditions employed. Low temperatures can slow the growth of many microorganisms, although the response depends on the microbial species and the sample conditions (Al-Sharify et al., 2025). The observed decline in bacterial counts

should not, however, be interpreted as evidence that all microorganisms were eliminated or that the samples became microbiologically safe.

The antimicrobial properties of human breast milk may also be relevant when interpreting changes in bacterial counts during storage. Sharma et al. (2018) reported changes in the bactericidal activity of human breast milk during refrigerated storage. Lactoferrin, which binds iron and may limit its availability to microorganisms, is one of the components that contribute to the antimicrobial properties of breast milk (Kell et al., 2020). Lysozyme may also contribute to the regulation of bacterial populations through its antimicrobial activity (Aquino-Domínguez et al., 2025). However, the concentrations and activities of these components were not measured in the present study; therefore, their contribution to the observed bacterial-count trends remains speculative.

The relatively high initial bacterial counts observed in some human breast milk samples, ranging from approximately 3 to 5 log CFU/mL, may reflect bacteria originating from maternal skin or contamination introduced during milk expression and handling (Reyes et al., 2025). Although sterile containers were used in this study, their use alone cannot exclude contamination during collection or subsequent sample handling. These findings support the importance of appropriate hygiene practices and careful handling throughout the collection and storage process.

Previous research has investigated approaches to reducing the microbial load of human breast milk, including high-pressure processing and high-temperature short-time (HTST) pasteurisation (Wesolowska et al., 2019). Grazziotin et al. (2016) also discussed storage conditions in relation to the preservation of the nutritional and energy content of human breast milk. These studies provide useful context for understanding breast milk preservation; however, their findings should not be used as direct evidence of the microbiological safety of the samples examined in the present study.

In conclusion, the results describe changes in TPC, TCC, and TSC during seven days of refrigerated storage at 4 °C. Although bacterial counts generally declined, the elevated TCC values in 12 of the 19 samples highlight the need to consider hygiene during milk collection and handling and to interpret the findings cautiously. Further studies incorporating direct assessment of hygiene practices, appropriate microbiological criteria for human breast milk, and validated approaches to evaluating storage safety are needed to determine the implications of refrigerated storage duration for microbiological quality.

CONCLUSION

Eight bacterial isolates from human breast milk were presumptively characterised using conventional culture-based methods and biochemical tests, with the isolates assigned to putative bacterial genera, including *Staphylococcus, Escherichia coli, Bacillus, Lactobacillus, Enterobacter, Citrobacter, Streptococcus* and *Klebsiella*. Inhibition zone

measurements were obtained for seven antibiotics; however, these measurements were interpreted descriptively because compliance with the applicable CLSI testing and interpretive requirements could not be verified. Therefore, definitive conclusions regarding the antimicrobial susceptibility or resistance of the isolates cannot be drawn from these results.

During refrigerated storage at 4 °C for seven days, most breast milk samples exhibited total plate count (TPC) and total *Staphylococcus* count (TSC) values below the reference limits applied in this study. However, 12 of the 19 samples (63.2%) showed total coliform counts (TCC) above the reference limit. Although bacterial counts generally declined during storage, elevated coliform counts in several samples highlight the importance of hygienic practices during milk collection, handling, and storage. Overall, these findings describe changes in the microbiological quality of human breast milk under the experimental storage conditions but do not establish that storage for seven days at 4°C is microbiologically safe. Further studies using validated microbiological methods and appropriate safety criteria are needed to clarify the implications of the duration of refrigerated storage for human breast milk.

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ETHICS APPROVAL

The study was approved by the Ethics Committee for Research (Ethics Approval No. FSTM/EXP15/P144)

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