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SCREENING, ISOLATION, AND PROBIOTIC POTENTIAL OF LACTIC ACID BACTERIA FROM EGYPTIAN HUMAN AND COW MILKS

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ABSTRACT

Human and cow milk are recognized as rich sources of beneficial microorganisms that contribute to the development of infant gut microbiota. This study aimed to isolate, identify, and evaluate novel lactic acid bacterial (LAB) strains from human and cow milk for their probiotic and technological potential. A total of fourteen LAB isolates were identified using 16S rRNA gene sequencing. All isolates exhibited high survival rates under simulated gastrointestinal conditions, showed resistance to lysozyme, and demonstrated cholesterol assimilation capacities ranging from 21.35% to 38.50%. Antagonistic activity assays confirmed that all isolates exhibited moderate inhibitory effects against pathogenic bacteria, indicating significant antimicrobial potential. Overall, *Enterococcus faecium* C_1, *Lacticaseibacillus rhamnosus* C_8, *Enterococcus mundtii* H_2, and *Enterococcus hirae* H_{10} possesses desirable probiotic characteristics, including gastrointestinal resilience and pathogen inhibition. These findings highlight their potential application as novel starter or co-cultures in functional dairy products. This study provides a basis for future investigations into LAB strains as probiotic candidates for improving the safety, quality, and health benefits of functional dairy foods.

Keywords: Antimicrobial activity; Cow milk; Human milk; Lactic acid bacteria; Molecular identification; Probiotics properties

INTRODUCTION

The field of probiotics has garnered increasing attention over the past decade due to mounting evidence of their diverse health benefits. Further, there is a growing demand for novel Lactic acid bacterial strains that possess probiotic properties for improving the quality of food consumed **(Gul and Durante-Mangoni, 2024).** This lactic acid bacteria LAB can be obtained from a variety of natural ecological niches that remain unexploited. Additionally, LAB are a focal point of extensive universal research due to their fundamental role in the majority of fermented foods and their ability to produce a variety of antimicrobial compounds and stabilize gut microflora **(Biswasroy *et al.*, 2021).** Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" **(Hill *et al.*, 2023).** Probiotics have been shown to improve the bioavailability of nutrients, reduce the risk of diarrhea and certain cancers, immunomodulate, lowering of serum cholesterol levels and have an antagonistic effect against pathogens **(Nami *et al.*, 2022b).** To exert their beneficial effect, probiotics must be resistance to low pH and bile salts in order to survive in the upper digestive tract, and subsequently colonize via adhesion to the intestinal epithelial cells. Other crucial characteristics to consider for probiotic strain selection include antibiotic susceptibility, and consumer safety **(Ahire *et al.*, 2024).** Human breast milk is widely recognized as a complex and unique biological matrix and is considered a natural functional food. In addition to essential nutrients, it contains hormones, growth factors, immunoglobulins, cytokines, and enzymes that support infant health. Importantly, human milk also harbors a diverse microbial community with significant probiotic potential **(Liu et al., 2020; Moossavi and Azad 2020)**. Human breast milk has been demonstrated to be a reliable source of probiotics due to it's approximately 10^3-10^4 CFU/ mL commensal microbes and its continuous availability to infants. Further it would fulfill some of the main recommended criteria generally for human probiotics, such as human origin, and a history of safe. However, the origin of human's breast milk bacteria is still debated **(Anjum et al., 2020)**. Raw Cow milk can be good source of novel LAB strains with the potential desirable properties for use in the production of novel fermented dairy products (**Bettera *et al.*, 2023).** Furthermore, its high nutritional content combined with high water activity at a pH close to neutral encourages the development of a wide range of microorganisms. Despite the complexity of the microbial population in milk, various raw milk isolates can contribute to the technical qualities of dairy products and their health-promoting capacities **(Bettera *et al.*, 2023).** In Egypt, limited information is available regarding the diversity and probiotic potential of indigenous LAB associated with raw cow milk and human breast milk. Native probiotic strains may be better adapted to the local population and dietary habits, which highlights the importance of investigating local microbial resources. Therefore, further research is needed to isolate and characterize indigenous LAB strains from Egyptian sources.
In current study we aimed to isolate LAB from Egyptian Cow milk and human breast milk of native Egyptian women and estimate their potential as probiotics using a series of *in vitro* tests. The long-term goal would be to use beneficial bacteria or their products as an extra and sustainable strategy for disease control or probiotic fermented dairy food production with human health applications.

MATERIALS AND METHODS

Sample collection

Human milk samples were collected from Egyptian healthy lactating mothers visiting different public sector hospitals in Kafr El- Sheikh, Cairo, and Giza Governorates (Fig S1) for vaccination of their children. Subjects receiving any antibiotic or probiotic supplements, at the time of sampling or during the last 2 months, were not included in the study. The age of participants was between 23 and 36 years (28 ± 5 years) and a total of forty milk samples with sample volume (25 mL) were collected manually in sterile tubes after cleaning the mammary areola and nipples with sterile water and the first drops of milk were discarded. From *Palidi* (Egyptian Cows), forty Cow milk samples (50 mL) with varying lactation periods were obtained from the Dairy Technology Unit of the Faculty of Agriculture at Cairo University in Giza, Egypt. By traditional milking method in sterile containers under suitable conditions, Samples were aseptically transported to the laboratory in an anaerobic icebox.

Enrichment and, isolation of LAB

LAB was isolated according to the method described by **Nor *et al.* (2015)** with slight modifications. Milk samples were enriched by adding 1mL to 50 mL MRS and M17 (Difco Laboratories, India) broth. The enriched samples were then incubated at 37 ºC for 48h in 5% carbon dioxide incubator. Presumptive lactic acid bacteria were isolated from milk samples and spread over solidified MRS and M17medium supplemented with 1.0% $CaCO_3$ (w/v) for enumeration. The plates were incubated at 37°C for 48-72 h. Colonies that generated a surrounding clear zone with distinct morphological differences based on (color, shape, size, rough or

smooth surface) were selected and then purified using another agar plate of the same culture medium.

Prefatory identification of LAB

As previously described by **Hassan *et al*. (2023),** the presumptive LAB isolates were distinguished using Gram staining, cell morphology, catalase activity, oxidase activity, and milk fermentation. Under safety conditions, the non-LAB isolates have been discarded. Gram-positive isolates showed negative catalase and oxidase activities were further sub-cultured onto MRS and M17 plates. The viable cultures were stored in MRS broth supplemented with 15% glycerol before being stored at −20°C.

Sample preparation for scanning electron microscope

Bacterial cells suspensions of both broth (M17 & MRS) media were centrifuged at 1500x*g* for 5 min. Samples of pellets were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 hr. then washed several times in 0.1 M phosphate buffer (pH 7.2) for 15 min intervals. Samples were dehydrated in a series of aqueous ethanol solutions (25%, 50%, 75%, 95% and 100%) for 5 min each and dried to critical point using CO_2 in a Critical Point Dryer (Polaron, Waterford, England), samples mounted on aluminum SEM stubs, sputter-coated with 92 gold (Spi module sputter coater, spi supplies division of structure probe. Inc.) and examined at 10 - 25 kV through Scanning Electron Microscope as previously described by **Elzeini *et al*. (2021).** Image analysis Scanning Prop Image Processor (SPIP) program (6.0.6 BETA, Denmark) was used for micrographs analysis.

Molecular identification by 16S rRNA analysis

Polymerase chain reaction (PCR) and DNA sequencing of 16S rRNA gene was done according to the method mentioned by **Ahmed *et al*. (2021).** Briefly, all isolated cells were harvested in a microcentrifuge tube, after centrifuging for 10 min at 5000 x*g*. After washing the pellets for three times using 0.85% NaCl solution, genomic DNA was extracted using Gene JET Genomic DNA purification Kit (Thermo Fisher Scientific, Republic of Lithuania). The integrity of the extracted DNA was assessed using gel electrophoresis (Agilent technologies, Santa Clara, CA, USA) while a spectrophotometer (Nano Drop 1000;ThermoFisher Scientific) was used to determine the purity of extracted DNA. The 16S rRNA gene fragments of LAB isolates were amplified by using F-27 (5′-AGAGTT TGATCMTGGCTCAG-3′) and R-1494 (5′-CTACGG YTACCTTGTTACGAC-3′) primersin a 25 µL reaction volume containing 12.5 µL 1X Go Taq Green Master Mix (containing bacterially derived Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers) (Promega, Madison, UK), 1 µL of 100 mM forward primer (F 27), 1 µL 100 mM reverse primer (R 1429), 9.5 µL nuclease free water and 1 µL of approximately 300–500 ng/µl genomic DNA. The amplification step was performed using Thermal cycler PCR (Bio-rad T100, USA). The PCR conditions for 16S rRNA gene were initial denaturation step at 95 °C for 12 min, followed by 30 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min, and one extension step at 72 °C for 10 min at the end of PCR reaction. The PCR products were checked via agarose gel electrophoresis then purified using gel extraction kit and sequenced by Macrogen, Inc., Seoul, South Korea using automatic ABI 370 × 1 DNA Sequencer (Applied Biosystem, USA). Databases were screened for similarities by using BLAST program (NCBI, USA). A Phylogenetic tree was constructed based on 16S rRNA gene sequences using Phylogeny.fr software (http://www. Phylogeny. fr). The tree showed the relationship between the isolates and other closely related sequences of NCBI GenBank reference taxa.

Screening of strains with *in vitro* probiotic characterization

Preparation of inoculums

Isolated lactic acid bacteria were sub-cultured in MRS or M17 (Difco Laboratories, India) respectively at 37 °C for 18 h in 5% carbon dioxide incubator. Cell pellet of each culture was collected by centrifugation (3000 x*g* for 5 min at 4 °C) and the viable cells were assessed. 1 g of cell pellet of each culture having (10^8 CFU/mL) was maintained for further analysis.

Lysozyme resistance

One gram of cell pellet of each culture was resuspended in 9 ml of 0.06 mol/L phosphate buffer saline (PBS) pH 6.2 supplemented with 1% NaCl then 100 µg/mL lysozyme was added. The cell suspensions were aerobically incubated at 37 ºC for three hours as mentioned by (**Ramalho *et al*., 2019)**

Simulated gastric juice resistance

One gram of cell pellet of each culture having (10^8 CFU/mL) was resuspended in 9 mL of simulated gastric juice (3g pepsin in 1L saline, pH 3). The cell suspensions were incubated in 5% carbon dioxide incubator at 37 ºC for three hours (**Shahidi *et al*., 2008).**

Simulated intestinal juice resistance

One gram of cell pellet of each culture was resuspended in 9 ml of simulated intestinal juice (1 L intestinal juice contained 1.25 g trypsin, 7.5 g bile salts, and 8.4 g $NaHCO_3$) followed the method described by **Chávarri *et al*. (2010**). The cell suspensions were incubated in 5% carbon dioxide incubator at 37 ºC for three hours, the number of viable cells was determined.

Cholesterol assimilation

The cholesterol assimilation by different treatment was determined using MRS or M17 broth medium supplemented with water soluble cholesterol at final concentration 2 mg/mL in addition to 0.2% sodium taurocholate (Merck, Darmstadt, Germany) as previously described **El-Dieb *et al*. (2010)**.

Cold storage resistance

Sterilized skim milk was fermented by activated isolates individually and incubated at 37 °C in 5% carbon dioxide incubator till coagulation with final pH 4.6 as suggested by **Ematollahi *et al*. (2016).** The viable cell count of bacterial strains was performed by using the standard plate count method after 1 day (D1) and 14 days (D14) post–fermentation (storage period). The results of viable cell counts were expressed as log CFU per g of fermented milk (log CFU/g).

Antagonistic behavior

The culture of LAB was inoculated (10^9CFU/mL) in MRS and M17 broth (pH 6.4) and incubated for 48 h at 37 ºC. The incubated culture was centrifuged at 16,000 x*g* for 15 min. The acquired cell free supernatant (CFS) was sterilized with a 0.22 µm cellulose acetate filter and separated in a sterile tube for further use. Antibacterial activity of CFS was examined using agar well diffusion method against three clinical indicator strains *Bacillus* (*B.*) *subtilis* EMCC 1121, *Staphylococcus* (*S.*) *aureus* EMCC 3114 and *Escherichia* (*E.*) *coli* EMCC 7661(as indicator bacteria). Nutrient broth (Merck, Darmstadt, Germany) was used as propagation medium for indicator bacteria as previously mentioned by **Abdelrazik and Elshaghabee, (2021)** .After the incubation of 24 h at 37 ºC, a clear zone of inhibition around wells was measured and interpreted following **Yadav *et al*. (2017)** method.

Characterization of antibiotic resistance

Four commonly used antibiotics, including penicillin G (10 mg), streptomycin (30 mg), vancomycin (30 mg), and chloramphenicol (25 mg) were tested in this assay using the agar disc diffusion method. MRS or M17 agar plates were prepared, in which 100 µL of freshly grown culture was mixed with 10 mL media. Antibiotic discs were placed on the solidified agar surface. The plates were incubated at 37 ºC for 48 hr. The zone of inhibition was measured in millimeters (mm). Isolates were categorized as sensitive or resistant following the cut-off points given by the CLSI document (**CLSI, 2017).**

Minimum Inhibitory Concentration (MIC) assessment

The MIC of Chloramphenicol, Streptomycin, penicillin and Vancomycin antibiotics were determined applying the broth dilution method. Each antibiotic was serially diluted: Penicillin (PEN; 0.032 to 64), Streptomycin (STR; 2 to 128), Vancomycin (VAN; 0.125 to 256), Chloramphenicol (CMP; 0.125 to 256), in MRS broth. The isolated LAB was grown at 37 °C for 24 h in MRS broth supplemented with the given concentrations of antibiotic. After incubation, at 37° c for 24 h, the calculated MIC was compared with antibiotic breakpoint specified by the European Food Safety Authority **(EFSA, 2012**) or Clinical and Laboratory Standards Institute **(CLSI, 2017)** to categorize the isolates as susceptible or resistant to the tested antibiotic.

DNase and Coagulase activities

LABs were streaked on DNase agar medium to check the production of DNase enzyme. After 48 h of incubation at 37˚C the plates were examined for the zone of DNase activity. Clear and pinkish zone around colonies were considered as positive for DNase activity (**Yadav *et al*., 2017**).The Coagulase slide test was performed by mixing bacterial cell suspension into EDTA-treated rabbit plasma (1:1) on a microscope slide. Formation of clumps was examined using a light microscope (UNICO co., IP730-2102 10X Achromat, NJ 08810 USA).

Statistical analysis

Results of triplicate samples were collected, tested for normality and homogeneity using *Levene's* test, andanalysed using analysis of variance (ANOVA) by MSTAT-C software (Michigan State University, East Lansing, Michigan, USA). In all data, means are shown as means ± standard deviations of three replicates. Differences between means are deemed statistically significant at $P \leq 0.05$

according to Duncan's multiple range tests. Heatmap correlation matrix plot was carried out using R statistical program (version 4.4.2).

RESULTS

Isolation and preliminary characterization

In this study, Twenty-seven isolates were initially recovered and screened using preliminary phenotypic criteria commonly applied for presumptive LAB bacteria. Isolates exhibiting Gram-positive reaction and negative catalase activity were considered eligible for further characterization, whereas Gram-negative and catalase-positive isolates were excluded. Accordingly, fourteen isolates were obtained from Egyptian Mothers' and cow milk fulfilled these selection criteria were subjected to subsequent analyses. Seven isolates (H_1, H_2, H_5, H_7, H_9, H_{12}, and H_{14}) from human milk while, others (C_1, C_4, C_3, C_7, C_8, C_{10}, and C_{12}) from cow milk with different morphological characteristics showed a transparent zone of acid production on CaCO3 (1%) were selected for further analysis. Upon incubation of the isolates in MRS and M17 agar media for 24 h, milky white colonies, creamy, opaque, and circular colonies were observed for the isolates C_4, C_8, C_{10}, H_2, H_9, H_{12}, and H_{14}, whereas C_1 and H_5 exhibited beige, rounded, and smooth colony shape and C_3, C_7, C_{12}, H_1, and H_7 colonies appeared as grayish white in color with smooth edges (Table S1). The coagulation test was carried out by inoculating the isolates into sterile skim milk and the coagulation time was recorded. All isolates showed good coagulation properties without causing textural defects such as gas bubbles and these results confirmed the homofermentative characteristics of the isolated strains. The isolates of C_1, C_3, C_8, H_5, H_9 coagulated milk after 14 - 17 h, whereas C_4, C_7, C_{10}, C_{12}, H_1, H_2, H_{12}, H_{14} coagulated it after 18 - 24 h, While H_7 coagulated milk after 36 h. Moreover, changes in milk pH were recorded after 24 h of incubation for all isolates. The pH of the blank was 6.54, where the pH values of isolates were ranging from 4.50 to 4.7. Ten bacterial isolates were examined by SEM and subsequent SEM micrographs (Figure 1) revealed that all ten bacterial species were cocci shaped with the exception of C_8 which was rod. Interestingly, the cel1 edges were, in many cases, difficult to distinguish and therefore measurement was impossible. For those with a straight cell and clearly visible, their length varied considerably between 0.93 ± 0.08 to 1.33 ± 0.15 µm (Table S2). Average cell diameters of the ten bacterial species were ranging from 0.87± 0.07 to 1.37± 0.28 µm.

16S rRNA-based identification and phylogenetic analysis

Selected isolates were identified to species level by 16S rRNA gene sequencing and the results were further confirmed in phylogenetic analysis. The phylogenetic tree was constructed using 16S rRNA sequences that stipulated relative phylogeny between sequenced strains using the neighbor-joining method. Based on the 16S rRNA sequence isolates C_3, C_7, and H_9 were identified as *Enterococcus durans* while, C_{10} and H_5 were identified as *Enterococcus hirae*. On the other hand, C_1, H_1, and H_7 were identified as *Enterococcus faecium* and C_{12}, H_{12} were *Lactococcus lactis,* and Data is presented in Table 1. Furthermore, the 16S rRNA sequence revealed that the isolates C_4, C_8, H_2, and H_{14} showed 98.45, 96.94, 98.12, 98.76% similarity to *Pediococcus pentosaceus, Lacticaseibacillus rhamnosus, Enterococcus mundtii,* and *Enterococcus lactis*, respectively by employing the BLAST option (attainable at http://www.ncbi.nlm.nih.gov/) (Table 1, Figure S2). Strains were submitted to NCBI and had a newly accession numbers which are *Enterococcus* durans C_3 PP091953, *Enterococcus* durans C_7 PP091954, *Enterococcus* durans H_9PP091959, *Pediococcus pentosaceus* C_4 PP101248, *Enterococcus faecium* C_1 PP091952, *Lacticaseibacillus rhamnosus* C_8 PP101249, *Enterococcus faecium* H_1 PP091956, *Enterococcus faecium* H_7 PP091958,*Enterococcus hirae* C_{10} PP091955**,** *Enterococcus hirae* H_5 PP091957, *Lactococcus lactis* C_{12} PP101250 *Lactococcus lactis* H_{12} PP091960, *Enterococcus mundtii* H_2 PP101251 and *Enterococcus lactis* H_{14} PP101252. Additionally, Phylogenetic tree was completed by employing the computer Phylogeny.fr software of the neighbor joining method (http://www. Phylogeny.fr) based on the 16S rRNA gene sequence analysis (Figure 2)

Screening of strains with *in vitro* probiotic characterization

Lysozyme resistance

Lysozyme is an antibacterial enzyme found in the gastrointestinal tract (GIT) that plays a crucial part in enhancing innate immunity and has the ability to lyse gram-positive bacteria. Lysozyme resistance of fourteen LAB isolates during three hours of incubation at 37 ºC is presented in Table 2. Only the viable count (log CFU/mL) of *Lactococcus lactis* C_{12} was significantly decreased (p = 0.01) after two hours exposure of lysozyme as shown in Table 2.

Simulated gastric and intestinal juice resistance

The viable counts of *Lacticaseibacillus rhamnosus* C_8, *Enterococcus hirae* C_{10}, *Enterococcus lactis* H_{14}, *Enterococcus durans* C_3, and *Enterococcus hirae* H_5 were not significantly (p = 0.23) reduced after 180 minutes of simulated gastric juice exposure (Table 2). Additionally, all isolates were resistance to simulated colonic solution except the two lactococci isolates *Lactococcus lactis* H_{12} and *Lactococcus lactis* C_{12}. However, their viable counts were remaining above 10^6 CFU/mL. The tested *Enterococcus* isolates showed variable tolerance to simulated gastrointestinal conditions, suggesting a strain-specific response.

Cholesterol assimilation

Excess cholesterol is associated with cardiovascular diseases (CVD), Probiotic bacteria have demonstrated potential to lower cholesterol levels by different mechanisms. Data in Figure 3 show the ability of different LAB isolates from either human or cow milk to assimilate cholesterol in broth medium. The highest cholesterol assimilation activity was observed in *Lacticaseibacillus rhamnosus* C_8, *Pediococcus pentosaceus* C_4, and *Enterococcus hirae* C_{10} followed by *Enterococcus* sp. H_5 and H_{14} isolated from human milk. On the other hand, *Lactococcus lactis* C_{12} showed the lowest cholesterol assimilation activity (Fig.3).

Cold storage resistance

Figure 4 shows a significant decrease for isolated strains during storage at 5±2 °C for 14 days. This decline varied among bacterial species because of different sensitivity to environmental stresses such as low temperature. Considering Figure 4, the highest survival throughout the storage belonged to *Enterococcus faecium* C_1, *Enterococcus durans* C_3, and *Lacticaseibacillus rhamnosus* C_8, respectively. Therefore, strains of C_1, C_3, and C_8 were the most suitable probiotic strain to use in probiotic fermented dairy products stored at low temperature. While *Lactococcus lactis* H_{12} maintained the same as its initial viable population at the end of storage and the viable counts of *Enterococcus lactis* H_{14} was decreased by < 2 log cycle during cold storage. After 14 days of refrigerated storage the count of > log 7 CFU/g (minimum recommended level) in all tested LAB isolates was observed.

Antagonistic behavior

Data in Table 3 show that all isolates of LAB from either human or cow milk had antibacterial activity against tested indicator bacteria. No significant differences were observed between LAB isolates from cow milk and those isolates from human milk (p = 0.10). However, *Lactococcus lactis* C_{12} had the lowest antibacterial activity. The antibacterial activity of the tested isolates may be attributed to the production of organic acids, as evidenced by the reduction in pH of the growth media (MRS for *Lactobacilli* and M17 for *Lactococci* and *Enterococci*), which ranged from 4.20 to 3.80 after 18 h of incubation. Nevertheless, the specific organic acids produced by these novel strains have been previously identified and analyzed (**Abd Rabo *et al.*, 2025**)

Characterization of antibiotic resistance

Table 4 and 5 presents the antibiotic resistance of the LAB isolates against four antibiotics commonly used in medical treatment in humans. According to the antibiotic breakpoints reported by EFSA (2012) and CLSI (2014 & 2017), the minimum inhibitory concentration (MIC) of four antibiotics of putative probiotic LAB was specified. As shown in Table (4), all isolates were sensitive or moderate susceptible to penicillin except *Enterococcus faecium* H_1, *Enterococcus lactis* H_{14} and *Enterococcus durans* C_3, which exhibited resistance. Additionally, *Enterococcus lactis* H_{14} showed a resistance to chloramphenicol while all tested isolates were sensitive. In general, isolates from human milk were sensitive to Vancomycin with the exception of *Enterococcus durans* H_9 and *Lactococcus lactis* H_{12}. LAB isolated from cow milk showed a resistance effect to streptomycin with the exception of *Enterococcus hirae* C_{10}.

DISCUSSION

Researchers are continuously searching for effective probiotic strains with potent antibacterial and health-promoting properties for application in food and pharmaceutical industries (Yaacob ***et al.*, 2018**), However, many previously identified probiotic strains still exhibit limitations in practical applications, particularly low survival rates and poor proliferation under gastrointestinal conditions **(El-Dieb *et al.*, 2010**). Therefore, ongoing research focuses on isolating novel strains with enhanced functional properties and improved health benefits. Additionally, the direct assessment of potential probiotics *in vivo* is frequently both costly and time-consuming. Consequently, *in vitro* evaluation as major criteria for probiotic selection is to find the most effective and appropriate strain with optimal beneficial properties. In the present study, fourteen lactic acid bacterial isolates obtained from human and cow milk fulfilled the preliminary probiotic selection criteria, including Gram-positive reaction and negative catalase and oxidase activities, as described in Bergey's Manual of Determinative Bacteriology (**Holt, 1977**). Human and cow milk are considered valuable natural sources of probiotic microorganisms with potential beneficial activities. Ten isolates from distinct species were selected for morphological analysis using SEM and images produced from scans were analyzed using SPIP software. Morphological parameters measured coincide with those obtained by **Yaacob *et al.* (2018).** However, **Hardie**

and Whiley, (1997) stated that cocci group had spherical cells ranging in diameter 0.5-3.5 µm which is wider than what is obtained in the present work. These differences in cell diameters may be due to the preferential growing conditions preferred between the strains. Probiotics have to successfully transit through the gastrointestinal tract and be able to withstand low pH and high bile salt concentrations. Another essential feature in a potential probiotic candidate is resistance to acid, lysozyme, and bile. Lysozyme causes cell death by hydrolyzing the bacteria cell wall. In this study, the discrepancies in lysozyme resistance of isolates may be attributed to variances in cell wall structures and layers **Dudley, (2022).** These results are in agreement with the finding of **Zago *et al.* (2022)** who showed that lysozyme resistance varied with 24.0- 99.9%. Furthermore, counts of *Lacticaseibacillus rhamnosus* isolated from human milk did not significantly decrease after lysozyme exposure as mentioned by **Rajoka *et al.* (2019).** In another study of lysozyme resistance **Inayah *et al.* (2022)** reported that lactobacilli could well survive after 120 min of lysozyme exposure. Another major requirement to be considered to qualify potential probiotic candidates, it must be able to withstand high levels of acidity and maintain an adequate population of bacteria for two to three hours after entering the stomach. Acid tolerance of bacteria is important not only for withstanding gastric stresses, but it also enables the strain to survive for longer periods in high acid carrier foods, such as yogurt. **Vinderola *et al*. (2000)** reported that the reduction in the growth of LAB isolates is attributed to the impact of H^+ ions produced by acid on cell wall and metabolisms of isolates. In general, the mechanisms of acid tolerance are strain and species dependent which may elucidate the variation in acid tolerance among our isolates. Generally, *lactobacilli* are more adaptable for acidic condition and presence of bile salt than *lactococci* and *bifidobacteria* as reported by **Abushelaibi *et al.* (2017).** These results were confirmed by **El-Dieb *et al.* (2010)** whereas *Lactobacillus casei* -01 showed the highest survival rate under simulated GIT condition. In contrast, results obtained by **Abushelaibi *et al* .(2017)** showed that *Lactococcus lactis* KX881768, *Lactobacillus plantarum* KX881772, *Lactococcus lactis* KX881782 and *Lactobacillus plantarum* KX881779 had the survival rates among twenty-three of bacterial isolates whereas camel milk was the source of isolation. Bile salts are toxic for living cells and bile salt tolerance is considered one of the essential properties required for lactic acid bacteria to survive in the small intestine (**Singhal *et al.*, 2019**). In the same way, it is expected that the proposed probiotic will demonstrate a tolerable level of tolerance to bile salts in the human gut. The variation in bile tolerance observed in this study is consistent with previous report **Elzeini *et al*. (2021).** Increase levels of plasma cholesterol are associated with cardiovascular disease (CVD) and different symptoms of atherosclerosis **Tarride *et al*. (2009).** Different probiotic microorganisms could reduce levels of cholesterol through different mechanisms such as assimilation, lowering cholesterol absorption and bile salt hydrolase **Tarride *et al.* (2009).** Additionally, data of different literatures showed a variation between different lactic acid bacterial isolates in their ability to assimilate cholesterol from broth media indicating a strain-dependent rather than genus-wide trait. In the present study, *Lacticaseibacillus rhamnosus* C_8 and *Pediococcus pentosaceus* C_4 showed the highest cholesterol assimilation activity followed by C_{10}, and *Enterococcus hirae* H_5, *Enterococcus lactis* H_{14} isolated from human milk while, the two *lactococci* isolates were the lowest. Similar findings were also reported by **Tomaro-Duchesneau *et al*. (2014**). Who found that *Lactobacillus reuteri* NCIMB 702656 assimilated over 39% in broth media more than *Lactobacillus rhamnosus* ATCC 53103 (over 30% cholesterol assimilation). However, *Lactococcus lactis* KX881782 could assimilate over 40% of cholesterol **Tomaro-Duchesneau *et al.* (2014**). Different *Enterococcus faecium* strains assimilated more than 50% of cholesterol in media without adding bile salt **(Singhal *et al*., 2019). Nami *et al.* (2019a)** demonstrated that *Enterococcus hirae* ES 28 isolated from traditional yoghurt assimilated 33.31% of cholesterol. *Pediococcus acidilactici* LRCC5307 assimilated cholesterol by 30.5% and this percentage was increased to 74.5% when bile salt was added to the fermentation media (**Kim *et al.*, 2021**). Several mechanisms for cholesterol assimilation by probiotic microorganisms have been proposed **(Ziar *et al*., 2014**) including cholesterol incorporation in the cell wall, conversion of cholesterol to coprostanol by reductase, and disruption of cholesterol micelle in the intestine by deconjugated bile salts. The probiotic-related properties observed in the present study, including cholesterol assimilation and gastrointestinal tolerance, should be considered strain-specific rather than generalized characteristics at the genus level. Although previous studies have reported similar functional properties among members of lactic acid bacteria, considerable variability may exist among strains within the same genus or species. Therefore, the beneficial effects observed in this study are specifically associated with the tested isolates and cannot be universally attributed to all strains belonging to the same genus. The viability of probiotic bacteria during storage is an important factor influencing their functional efficacy. In the present study, viable counts of most isolates gradually decreased during storage; however, bacterial populations remained above the recommended minimum level of 6 logs CFU/g required to confer probiotic health benefits. Similar observations were previously reported by **Gueimonde *et al.* (2004).** The reduction in bacterial viability during storage may be attributed to acid accumulation and metabolic by-products generated during fermentation (**Donkor *et al*., 2007).** Antibacterial activity is a key consideration for choosing which lactic acid bacteria to use as probiotics. Several mechanisms have been proposed to explain the antibacterial action of lactic acid bacteria including organic acids, hydrogen peroxide, and bacteriocins (**Kumar and Kumar, 2015**). In the present study, antimicrobial activity differed amongst isolates which could be related to variations between species and strains. Several workers have reported that antimicrobial activity is species- and strain-dependent (**Kumar and Kumar, 2015; Sharma *et al*., 2017**). According to **Sharma *et al.* (2017)** *lactobacilli* from human milk showed substantial diversity in antibacterial activity, but no inhibition zone was observed for some lactobacilli isolates against *S. aureus* and *Listeria monocytogenes*. In contrast, lactic starter cultures that were originally isolated from dairy products had antibacterial activity against *Staph. aureus* and *Bacillus subtilis* (**Abdelrazik and Elshaghabee, 2021)**. The observed inhibition zones in the present study were relatively moderate and should be interpreted cautiously in the absence of comparative reference strains or neutralized supernatant controls. Nevertheless, the obtained results indicate detectable antibacterial potential among several isolates. Evaluating the antibiotic susceptibility is considered as the second important safety aspect regarding employing the bacteria as probiotics in food and animal feed. Regarding antibiotic resistance, vancomycin resistance is well documented in all LAB isolated from cow milk which is attributed to the synthesis of modified cell wall peptidoglycan precursors that end in a depsipeptide D-alanine-D-lactate instead of the dipeptide D-alanine-D-alanine, the target for vancomycin activity as reported by **Handwerger and Tomasz, (1985).** Furthermore, the resistance observed against some antibiotics tested implies that these strains wouldn't be affected by therapies. This may contribute to maintain the natural balance of intestinal microbiota during antibiotic treatments. These results agreed with that found by **Senan *et al.* (2015**). Additionally, the observed resistance of some *Enterococcus* isolates to clinically relevant antibiotics, particularly vancomycin, highlights the importance of comprehensive safety evaluation before probiotic application. Since antibiotic resistance traits may potentially be transferable, further molecular investigations are required to confirm the safety of these isolates and exclude the presence of transferable resistance genes or virulence determinants. All tested isolates from human and cow milk were screened for DNase and coagulase activities and none of the strains showed these activities. Because the strains show no signs of pathogenicity indicating they are safe for consumption (**Saroj *et al.*, 2016).** The interrelationships among the studied measurements and the effect of isolated LAB strains on these interrelationships were assessed and the obtained data are plotted in heat map data visualization pattern (Fig. 5). Color indicates the interrelationship among different parameters where red color reveals high interrelationship, while light color points out a low or weak correlation. From heat map data plot, there was a high correlation between simulated colonic solution and simulated gastric juice resistance, an intermediate correlation was observed among antibacterial activity and simulated colonic solution, simulated gastric juice resistance, or lysozyme resistance. On the other hand, the differences in the isolated LAB strains had a positive effect ($p = 0.02$) on the interrelationship between antibacterial activity and antibiotic susceptibility profiles and the interrelationship between simulated colonic solution and lysozyme resistance. Also, the isolated LAB strains exhibited a great effect ($p = 0.001$) on the correlation between antibacterial activity and simulated colonic solution, simulated gastric juice resistance, or lysozyme resistance.

CONCLUSION

Lactic acid bacteria were isolated from Egyptian human and Cow milk and screened for various probiotic characteristics. Twenty-seven isolates of LAB were obtained from Egyptian human and Cow milk. Of them, fourteen isolates, were identified as potential probiotic candidates and were identified by 16S rRNA sequences homology as *Enterococcus durans* C_3, C_7, and H_9, *Enterococcus hirae* C_{10} and H_5, *Enterococcus faecium* C_1, H_1, and H_7, *Lactococcus lactis* C_{12} and H_{12} and *Pediococcus pentosaceus* C_4, *Lacticaseibacillus rhamnosus* C_8, *Enterococcus mundtii* H_2, and *Enterococcus lactis* H_{14}. Results showed that *Enterococcus hirae*H_5, *Enterococcus lactis* H_{14}, *Enterococcus durans* C_3, *Lacticaseibacillus rhamnosus* C_8 and *Enterococcus hirae* C_{10} were highly resistant to simulated gastric and intestinal juice, whereas *Lactococcus lactis* C_{12} was sensitive to lysozyme. Strains were highly susceptible to chloramphenicol and penicillin with antibacterial activities against clinical pathogens. This study is the first one to isolate *Enterococcus mundtii* and *Enterococcus hirae* from human milk in Egypt and to investigate their probiotics characteristics. In conclusion, this research establishes a robust foundation for the use of *Enterococcus faecium* C_1, *Lacticaseibacillus rhamnosus* C_8, *Enterococcus hirae* H_5 and *Enterococcus mundtii* H_2 in food and health applications. In order to verify its probiotic efficacy, safety, and prospective therapeutic benefits, future research should concentrate on in vivo evaluations. Furthermore, exploring its genomic attributes and optimizing fermentation conditions can further expand its applications in the food industry.

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writing – review and editing **Amira M. Abdel-maged**: Conceptualization; formal analysis **Ashwak Abdel moneim Hassan**: Supervision ;Data curation; methodology; resources; writing –review and editing

Statements and Declarations

Ethical statement: All procedures were approved by Cairo university, Egypt with approval number: CU/I/F/29/21 and performed according to the guide of sampling and use. Human milk collection was made with the help of clinicians at lactation centers located in Egypt.
Availability of data and material: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.
Competing interests: The authors declare that they have no competing interests.
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