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Original article

Potential probiotics from human breast milk with promising cholesterol-reduction and anti-tumour effects

Amany Abd El Mawla[1], Nayra Shaker Mehanna[2], Marwa Kamal Sallam*[3], Zeinat Kamel [1], Mahmoud S.M. Mohamed [1]

1- Department of Botany and Microbiology, Faculty of Science, Cairo University, 12613, Giza, Egypt.
2- Probiotics Lab, Central Laboratories Network, Institute of Food Science and Nutrition, National Research Center, Dokki, Egypt
3- Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University, Egypt

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ABSTRACT

Background and rationale: The objective of this study is to isolate probiotic bacteria from human breast milk and test their health benefits as cholesterol reduction and human DNA protection. **Methods**: Potential probiotic bacteria were isolated from human milk. The probiotic properties were tested namely their ability to survive acidic pH, tolerate bile salts and antimicrobial activities. Serum cholesterol reduction ability and cytoprotective effect on Hep-2 cells were studied. **Results**: A total of 48 different isolates were purified from 12 breast milk samples. They all tolerated bile salts while 81% were proved to be resistant to acidic pH and had antimicrobial effects against different pathogens. Three isolates showed high ability to reduce cholesterol from 19.4-22.6%. This percentage of cholesterol reduction was improved in presence of bile salt to be 48.7-54.7%. Four isolates were proved to have a protective effect against the H_2O_2 cytotoxicity by decreasing tail moment up to 2.8 and 15.6% DNA concentration in the tail of the examined cells. Phenotypic characteristics and 16S rDNA sequence analyses were used to identify the promising isolates as *Pediococcus pentosaceus*, *Enterococcus durans*, and *Enterococcus faecium* strains. **Conclusion**: Probiotic strains isolated from human milk in the study had a dual beneficial role; human health benefit as well as cytoprotective activities.

Introduction

Human milk is an exceptionally distinctive combination of nutrients and immune modulatory proteins that stimulate the proper development of the newborn's gut and his entire immune system [1]. Infant intestinal normal flora establishment is a progressive step which provides the main source of microbial stimuli for the developing baby.

Breast milk was long supposed to be sterile [2], where the existence of bacteria was expected to be a contamination. Then researchers started to isolate living bacterial strains from human milk [3–9]. Some of those bacterial genera discovered include lactobacilli, lactococci, streptococci staphylococci, micrococci, enterococci and bifidobacteria, are modulating the different functional properties and metabolic activities among the intestinal biome members in humans [10]. Breast milk is now considered one of the main initial sources of microbes transferred to the infant gut; after birth canal; where studies reported that average consumption of bacterial cells by breast fed infants is around (8×10^5) on daily basis [11].

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* *Corresponding author:* Marwa Kamal Sallam
E-mail address: *m_kamal@kasralainy.edu.eg*

Many studies recommended that microbial bi-products and metabolism of bile acids took place by gut microbiota can alter blood lipids levels (mainly cholesterol) [12]. Moreover, Probiotics were suggested to have ability towards modulating cancerous cell's apoptosis and proliferation. Therefore, the possible submission of the demonstrated properties of probiotic bacteria in innovative treatments may perhaps be used as alternative therapy to radiotherapy or chemotherapy [13].

Properties of a strain to be a potential probiotic is to confer health benefits like reducing effect of cholesterol levels, anti-cancerous activity and anti-microbial activity [14,15].

Probiotics properties are strain-specific. Some strains isolated from breast milk were recommended to be good candidates for use as probiotics [6]. Moreover, different authorities like the EFSA (European Food Safety Authority) and the FDA (Food and Drug Administration) have granted the isolated bacteria from human milk as a safe for human consumption [1,16].

The main requirements settled by the FAO (Food and Agriculture Organization) and WHO, includes stress resistance and antimicrobial activity inside the body of the host [17], Therefore, for a strain to be considered as a probiotic it should reach its final destination in the intestine alive, i.e. it should pass the stomach acidity, tolerate the bile in intestine to colonize it, have antimicrobial activity, and to be sensitive to antibiotics, so it can be controlled if it overgrows [18]. Therefore, this work aimed to find prospective probiotic bacteria after isolation from human milk samples that are capable of resisting the low pH, tolerate bile salts, and possessing antimicrobial activities. Moreover, studying the effect of the selected probiotic bacteria on cholesterol reduction as well as their cytotoxicity prevention was evaluated.

Materials and Methods

Study design

Isolation of bacteria from human milk using suitable media and identification by morphological and biochemical characterization of selected bacteria. Then study of the probiotic properties of bacterial isolates for their antimicrobial effect, effect on cholesterol levels and effect on cancer cell line. Further identification of the most promising isolates using molecular biology techniques.

Microorganisms' isolation from milk samples:

Twelve human milk samples were collected in sterile airtight screw-capped vials; each were containing 0.5 ml of thioglycolate transport medium to guarantee the anaerobic condition, then were cultured immediately after being delivered to the laboratory of the Microbiology and Immunology Department, Faculty of Medicine, Cairo University according to the work of **Sallam et al**. [19].

The isolation and purification of bacteria were performed by inoculating one ml of the milk samples into 9 ml of Man, Rogosa and Sharpe (MRS) broth media tubes (Conda, Madrid, Spain), and then they were serially ten folds diluted. One ml aliquots from each sample and its dilutions were plated using the pour plate method on different medias; MRS agar media for isolation of lactobacilli, MRS agar media supplemented with 0.05% (wt/vol) L-cysteine hydrochloride and 50 µg Mupirocin (Delchimica, Italy) per liter to prepare MRS-Cys agar plates for isolation of bifidobacteria and M17 agar media plates (Oxoid, UK) for isolation of lactic acid producing streptococci. All Inoculated MRS and MRS-Cys plates were incubated anaerobically using Oxoid AnaeroGen compact gas packs (Oxoid, UK) at 37°C for 48-72 h in an anaerobic jar, whereas inoculated M17 plates, were incubated aerobically at 37°C for 24 h [19].

Microorganisms' initial identification

Sixty pure isolates were initially collected. Following phenotypic and biochemical methods in Bergey's manual of determinative bacteriology [20], 48 bacterial isolates were selected according to their colony characteristics, being Gram positive and having characteristic cell morphology under the microscope. Further biochemical analysis of isolates was evaluated regarding acid production, and being oxidase and catalase negative [21]. All isolates were sealed and conserved at -20 °C in Brain–Heart Infusion (BHI) (Merck, Germany) broth containing 50% (v/v) glycerol till next use.

Biochemical identification

API kit (bioMérieux, Marcy l'Etoile, France) was used for the biochemical identification. The used kit includes identification of streptococci and enterococci by API® 20 Strep, lactobacilli by API® 50 CH in combination with 50 CHL liquid media and anaerobic bacteria by using API® 50 CH, API® 20A and API® ZYM. Manufacturer protocol

was followed beside their analytical profile index and the identification software (Apiweb) [19].

Probiotic properties testing the of the isolates. Bacterial isolates were grown overnight in Luria-Bertani (LB) broth, then bacterial cultures were adjusted to 0.5 McFarland (1.5 × 108 CFU/ml) standard to test their ability for different probiotic properties.

Resistance to low pH

Bacterial resistance to low pH was tested by inoculating all purified bacterial isolates to MRS broth, then 30 ml of overnight cultures growth were centrifuged at 8000 rpm for 20 min, washed twice in sterile phosphate-buffered saline (PBS; NaCl: 9 g/L, Na2HPO4.2H2O: 9 g/L, KH2PO4: 1.5 g/L, pH 6.2) then resuspended in 3 ml PBS. Aliquot of washed cell suspension (1ml) was centrifuged at 10000 rpm for 5 min and the cells were resuspended in 10 ml of MRS and M17 broth adjusted to different pH values (2.0, 3.0 and 4.0) by using 1 N HCl and then incubated for 3h. The viability of each bacterial isolate was confirmed by incubation at 37 °C for 24 h on standard MRS and M17 agar plates. Cultures grown on MRS and M17 broth without pH modification were used as controls [22].

Tolerance to bile salt

The ability of bacteria to tolerate bile salts was tested, 1 ml aliquot of washed cell suspension from each isolate was inoculated onto MRS and M17 broth containing different bile salt concentrations (0.5, 1 and 2%) and incubated for 24 h at 37 °C [22]. The viability of tested isolates was confirmed by surface plating on MRS and M17 agar plates and incubation at 37 °C for 24 h. Cultures grown on MRS and M17 broth without bile salts were used as control.

Antibiotic susceptibility

Bacterial isolates susceptibility to antibiotics was evaluated on Müller Hinton agar (MH; Laboratories Conda SA, Madrid, Spain) according to the Clinical and Laboratory Standards Institute (CLSI, 2021) recommendations by antibiotic disc diffusion method from (Oxoid, Basingstoke, UK). Antibiotic discs were Cephalexin (CL 30 µg), Gentamicin (CN 10 µg), Doxycycline (DO 30), Norfloxacin (NOR 10), Ciprofloxacin (CIP 5) and Cefoxitin (FOX 30). Ampicillin (50 mg/ml) was used as standard. After incubation at 37 °C for 24 h, zones of inhibition were measured, and isolates were classified as sensitive (S) or resistant (R) according to the criteria of the CLSI guidelines [23].

Antimicrobial activity

All isolates were evaluated for having antimicrobial activity by testing the ability of the cell-free supernatant (CFS) of 24 h bacterial culture extract to inhibit pathogenic microbial strains growth. The CFS of isolates were prepared from 0.5 McFarland (1.5×10^8 CFU/ml) culture centrifugated at 10,000 rpm then filtered by 0.45 µm pore diameter filter. The pH of CFS was adjusted to be 6.5 to eliminate the possible inhibition effects due to organic acids then examined to record any inhibition zones by using agar-well-diffusion assay [24]. Indicator pathogens of *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 9027, Salmonella typhi ATCC 6539, *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* ATCC 7644 and *Candida albicans* ATCC 10231, purchased from Faculty of Agriculture, Ain Shams University, after being incubated at 37 °C for 24 h, was adjusted to 0.5 McFarland standard and 100 µl were spread on Müller-Hinton (MH) agar plates. A volume of 100 µl of each isolates CFS were filled into a 5 mm diameter punched wells. As a positive control, streptomycin (10 µg/ml) was used as standard antibacterial agent while fluconazole (10 µg/ml) was used as standard antifungal agent and sterile water was used as negative control. All the inoculated plates were incubated at 37 ºC for 48 h and zones of inhibition were recorded (mm) [24].

The effect of selected probiotic isolates on cholesterol reduction

The cholesterol reduction assay was carried out in the Probiotic laboratory, Dairy Science Department, National Research Center (NRC) Egypt, as per description of previous studies [25,26] with minor modifications. MRS broth was supplemented with filter-sterilized cholesterol solution (poly-oxyethanyl-cholesterol sebacate; Sigma-Aldrich, St. Louis, USA) adjusted to (100 µg/ml) concentration. After that, it was inoculated with 1% overnight grown culture suspension and incubated anaerobically at 37 °C at time intervals (0, 24, 36 and 48 h). Incubation was followed by centrifugation of the mixture for 20 min at 4000 rpm at 4 °C, then the supernatant was collected. Uninoculated MRS broth was used as a control. A method described by [27] was used for the determination of supernatant's initial and remaining amount of cholesterol. Briefly, one ml of

supernatant aliquot was mixed with 1 ml of KOH (33% w/v) and 2 ml of absolute ethanol, vortexed for 1 min and then heated at 37 °C for 15 min. After cooling at room temperature, 2 mL of distilled water and 3 ml of hexane were added and mixed for 1 min. The upper hexane layer was separated and evaporated under nitrogen gas and the residue immediately dissolved in 2 ml of o-phthalaldehyde solution (Sigma-Aldrich St. Louis, USA). After that 0.5 ml of concentrated sulfuric acid (98%) was added, mixed for 1 min and left for 10 min before reading the absorbance at 550 nm.

Cholesterol reduction ability of the tested isolates was calculated as the cholesterol percentage removed at different incubation intervals using the equation:

Cholesterol reduction (%) =(cholesterol (µg/mL)0h - cholesterol (µg/mL)time intervals h)/(cholesterol (µg/mL)0h)×100

Isolates with best results from the previous mentioned assay were re-evaluated using the same method using MRS broth supplemented with 0.3% bile salt. The cholesterol reduction percentage was evaluated by the same method described in the previous step.

In vitro cytotoxicity of selected probiotic isolates

Propagation of the Hep-2 cell line

For cytotoxicity test, the human cell line (Hep-2) was purchased from cell culture department in VACSERA, Cairo, Egypt, and cultured in complete medium that contained Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and 10% fetal bovine serum (FBS) in 5% CO2 and 95% air at 37 °C. Cell culture medium was renewed twice a week. After 7 days, cells became confluent and ready to use, the propagated cell line was subdivided into 6 separate groups to test the effect of selected probiotic bacterial isolates in comparison to positive and negative control.

Comet assay

In order to assess the ability of the selected probiotic bacterial isolates for the protection of DNA in the cell, the comet assay (single-cell gel electrophoresis) was performed in alkaline conditions as early described [28]. Glass slides were cover up with a first layer of 0.5% normal agarose (Sigma-Aldrich St. Louis, USA). The H_2O_2 treatment was performed after embedding the cell-line cells in agarose to avoid loss of cells during the treatment. CFS of the selected bacterial isolates were prepared as described before and combined with Hep-2 cell line then washed with PBS. The slides were immersed in 75 µM H_2O_2 in a Coplin jar for 1h at low temperature (4 °C), then slides were washed three times, 5 min each, by cold PBS. For the negative control, Hep-2 cell line was used, while a Hep-2 cell line treated with hydrogen peroxide served as a positive control [29]. After that, the suspensions plus agarose were covered immediately with clean coverslips. Afterward the covers were removed, and the slides treated for 1h at 4 °C with freshly prepared lysis solution (100 mM EDTA; 10 mM Tris; 1 % Triton-X -100; 2.5 M NaCl; 10 % DMSO; 8 g NaOH; 1% Na lauroyl sarcosinate; NaOH to pH 10). Finally, the treated slides were washed once with PBS then placed in an electrophoresis tank filled with alkaline buffer (300 mM NaOH,1 mM EDTA, pH > 13) freshly prepared. Electrophoresis was operated for 20 min at 25 volts at room temperature. Then, neutralizing buffer (0.4 M Tris, pH 7.5) was used to neutralize the pH by washing the slides three times for 5 min. Afterwards, all samples were fixed by ethanol. Finally, 60 µl ethidium bromide (20 µl/ml) was utilized to stain DNA. Three slides were prepared for each treatment, and 50 cells were randomly chosen for the measurements using Leica fluorescence microscope supplied with Comet Assay automatic image analysis system. The obtained results were assessed by nine image-analysis parameters. The DNA damage was defined when the investigated cells had "comet" shape, whereas cells with circular shape were considered as not damaged [17]. The percentage of DNA in tail, tail moment as well as tail length calculated automatically by the Comet score software system.

Molecular identification of selected probiotic isolates

This was done in the Microbiology Laboratory, Botany and Microbiology department in Faculty of Science, Cairo University. The pure colonies of the four selected probiotic isolates with the best probiotic properties were cultivated first in three ml MRS broth medium then DNA was extracted from each isolate using Genomic DNA extraction Kit pursued from QIAGEN (Hilden, Germany) according to the kit's manual instructions. The pure genomic DNA was used as a template for the PCR reaction to amplify 16S rDNA gene using the universal primers (forward primer

27F and reverse primer 1492R) as described before [30]. The PCR products obtained from each probiotic isolate were purified then sequenced by the company Macrogen (Seoul, Korea) using the same universal primers. The obtained forward and reverse sequence reads for each probiotic isolate were assembled to single contig utilizing the software DNAStar Lasergene (Version 7). These contigs sequence were compared to references 16S rDNA sequences of other bacteria published in GenBank utilizing the BLAST server of National Center for Biotechnology Information. The phylogenetic tree was constructed for the selected probiotic isolates after the sequences were aligned using MUSCLE utilizing the MEGAX program set to the maximum likelihood method, the Kimura 2-paramater model and 1000 bootstrap replications [31]. The identified isolates were deposited in the culture collection bank at Faculty of Pharmacy, Ain Shams University.

Statistical analysis

In order to examine the significant effect of differences in probiotic bacterial isolates on quantitative parameters, one-way ANOVA was performed on the cholesterol reduction and assimilation activities data. Then Tukey's test was used to test differences between means ($p < 0.05$). The means and standard deviations in all tests were calculated from at least three independent replicates (n=3).

Results

Isolation of bacteria from human breast milk with potential probiotic characteristics

A total of 12 breast milk samples were used for isolation of probiotic bacteria, 60 isolates were successfully purified. The initial phenotypic and biochemical tests revealed 48 isolates which were catalase and oxidase negative, non-spore-forming, acid producing and Gram positive which can have the potential to be probiotics as reviewed in literature [21]. High percentage of isolates (54%) were rod-shaped in pairs or in short chains and the rest of isolates were cocci (46%). All samples were identified using API technique, results revealed that 19 isolates have been identified as Lactobacillus (40%), 13 isolates were Streptococcus (27%), 9 isolates were Enterococcus and other related genera (19%) and 7 isolates were Bifidobacterium (14%).

Testing the probiotic characteristics of selected isolates

Resistance to low pH and bile salts

To start with selection of potentially probiotic strains, survival at low pH was assessed. The 48 isolates were evaluated for their survival ability at acidic pH values (pH 2.0, 3.0 and 4.0). The results revealed that there was a diminishment in the number of resistant isolates with reduction in the pH values **(Figure 1)**. At pH 4, the total isolates surviving percentage (81%; n: 39) was greater than at pH 3.0 (69%, n: 33) and 2.0 (35%, n: 17).

Then, evaluation of the bile tolerance of bacterial isolates was assessed, different concentrations (0.5, 1.0 and 2.0 %) of bile salts were tested. The results indicated that all the 48 isolates were able to survive even after being subjected to the used bile salt concentrations. Most of the isolates proved to be resistant to the simulated conditions of the intestine.

Antibiotic susceptibility

The results of antibiotic resistance are shown in **table (1)**. All probiotic isolates recorded a sensitivity to the antibiotic doxycycline, 41 isolates to gentamicin, 26 isolates to ciprofloxacin, and 15 isolates to norfloxacin. All isolates demonstrated resistance against cephalexin and cefoxitin, 7 isolates to gentamicin, 22 isolates to ciprofloxacin and 33 isolates to norfloxacin according to CLSI M-100 (2021).

Anti-microbial activity

The results of antimicrobial activity of isolates against indicator bacteria tested are shown in **table (2)**. According to the results, 14 isolates (29%) had shown an inhibitory activity against E. coli, 12 isolates (8%) against Bacillus cereus, 3 isolates (6%) were active against Staphylococcus aureus, and only one bacterial isolate was active against Listeria monocytogenes.

Table 2, antimicrobial activities of prospective probiotic strains from human breast milk against pathogenic bacteria by agar-well-diffusion assay

Testing the effect of the isolates with promising probiotic properties to

Cholesterol reduction

Twelve selected isolates were tested for cholesterol reduction in comparison to the negative control. Three isolates had the best reduction percent of cholesterol ($p < 0.05$), AM8 reduced cholesterol by 22.6%, followed by AM1029 reduced cholesterol by 22% then AM7 by 19.4%, after 48 h, as shown in **figures (2,3)**. In presence of bile salt, the cholesterol

lowering activity was even more, where isolate AM8 reduced cholesterol by 54.7%, followed by isolate AM1029 by 50.7% then isolate AM7 by 48.7% after only 24 h, as shown in **figure (4)**.

***In vitro* cytotoxicity**

Six isolates protective effect on Hep-2 cell line were tested by treatment with H_2O_2 and were evaluated by using comet assay in comparison to positive and negative controls. In Hep-2 cell line cells treated only with H_2O_2 (positive control), DNA was distinctly damaged compared to the DNA of cells in negative control. However, the application of microbial probiotic isolates has a positive effect, where the percentage of damaged DNA was reduced when compared to the positive control result as per **figure (5)**. Four isolates showed a protective effect to cellular DNA against damage by H_2O_2, Isolate AM7 had the least percent of damaged cells showing 2.85 tail moment with 15.65 % DNA in the tail followed by isolate AM24 with 3.65 tail moment with 25.25% of DNA, AM25 with 4.05 tail moment and 37.6% of DNA and AM26 with 4.6 tail moment with 43.6% of DNA.

Molecular identification of selected probiotic isolates

Four bacterial isolates with the most promising probiotic properties were analyzed to confirm the identity using the sequence analysis of 16S rDNA genes by the universal primers (27F and 1492R). The results revealed that two probiotic isolates belonged to the species *Enterococcus durans*, one belonged to *Enterococcus faecium* and one to *Pediococcus pentosaceus*, which matched the primary results obtained from morphological and biochemical characterizations. A high sequence similarities degree was shown between the probiotic isolate's amplicons and 16S rDNA from other references of *Enterococcus durans*, *Enterococcus faecium*, *Pediococcus pentosaceus* strains in the GenBank ranged from 99–100 percentage, as per **figure (6)**. The identified isolates' (*Enterococcus durans* strain AM7, *Enterococcus durans* strain AM11202, *Enterococcus faecium* strain AM1029, and *Pediococcus pentosaceus* strain AM26) sequences were deposited into the GenBank database under the accession numbers ON739177, ON739192, ON 739184 and ON739193 respectively, then were deposited in the culture collection bank at Faculty of Pharmacy, Ain Shams University under the accession numbers CCASU-2023-56, CCASU-2023-53, CCASU-2023-54, CCASU-2023-55 respectively. The phylogenetic tree was generated with the related species using the maximum likelihood method by bootstrap analyses based on 1000 analysis **(Figure 6)**.

The probiotic isolates were separated in the phylogenetic tree into two different main groups, Pediococcus group and Enterococcus group. *Pediococcus pentosaceus* strain AM26 in one subgroup of Pediococcus group, while the *Enterococcus durans* strain AM112028 and *Enterococcus durans* strain AM7 in one subgroup of Enterococcus group where *Enterococcus faecium* strain AM1029 in parallel phylogenetic subgroup within the same Enterococcus group as per **figure (6)**.

Table 1. Antibiotic susceptibility of isolates against 6 different antibiotics showing number and percent of sensitive and resistant isolates.

	Drug class or Subclass	**Tetracycline**	**Aminoglycoside**	**Fluoroquinolone**		**Cephem**	
Antibiotic discs	**Name**	**Doxycycline**	**Gentamicin**	**Ciprofloxacin**	**Norfloxacin**	**Cephalexin**	**Cefoxitin**
	Abbreviation	**DO**	**CN**	**CIP**	**NOR**	**CL**	**FOX**
	Conc. (µg)	**30**	**10**	**5**	**10**	**30**	**30**
	Code	**19059**	**19026**	**19056**	**19035**	**19011**	**19018**
Isolates sensitivity	**Sensitive**	48 (100%)	41 (85%)	26 (54%)	15 (31%)	0 (0%)	0 (0%)
	Resistant	0 (0%)	7 (15%)	22 (46%)	33 (69%)	48 (100%)	48 (100%)

Table 2. Antimicrobial activities of prospective probiotic strains from human breast milk against pathogenic bacteria by agar-well-diffusion assay.

	Growth inhibition zone (mm) ± SD*				
Isolates	**Bacterial strains**				
	E. coli	***B. cereus***	***S. aureus***	***L. monocytogenes***	***C. albicans***
AM4	23.33±0.58	-	-	-	-
AM7	18.00±1.00	13.89±1.53	-	-	-
AM8	21.33±1.53	22.67±2.08	-	-	-
AM3	16.67±1.53	31.67±1.53	-	-	-
AM13	-	15.33±1.53	-	-	-
AM27	20.33±1.53	21.33±2.31	-	-	-
AM19	-	22.33±2.08	15.00±1.00	-	-
AM22	-	18.67±1.53	-	-	-
AM24	14.33±1.53	21.33±1.53	-	-	-
AM25	13.33±0.58	17.33±1.53	-	-	-
AM261	14±1.00	-	-	-	-
AM26	14.33±1.53	13.67±1.53	13.67±2.08	22.00±1.73	-
AM262	13.33±0.58	-	-	-	-
AM28	15.33±0.58	-	-	-	-
AM112028	17.33±1.53	21.67±1.53	-	-	-
AM1029	16.33±0.58	-	14.67±0.58	-	-
AM30	21.00±1.00	17.67±0.58	-	-	-
Streptomycin	14.33±0.58	18.67±0.58	17.33±0.58	18.33±1.15	-
Fluconazole	-	-	-	-	28.67±0.58
Water (Negative control)	-	-	-	-	-

*Measured as inhibition zone in mm diameter, -; no zone of inhibition detected

Figure 1: The percentage of isolates resistance to low pH values.

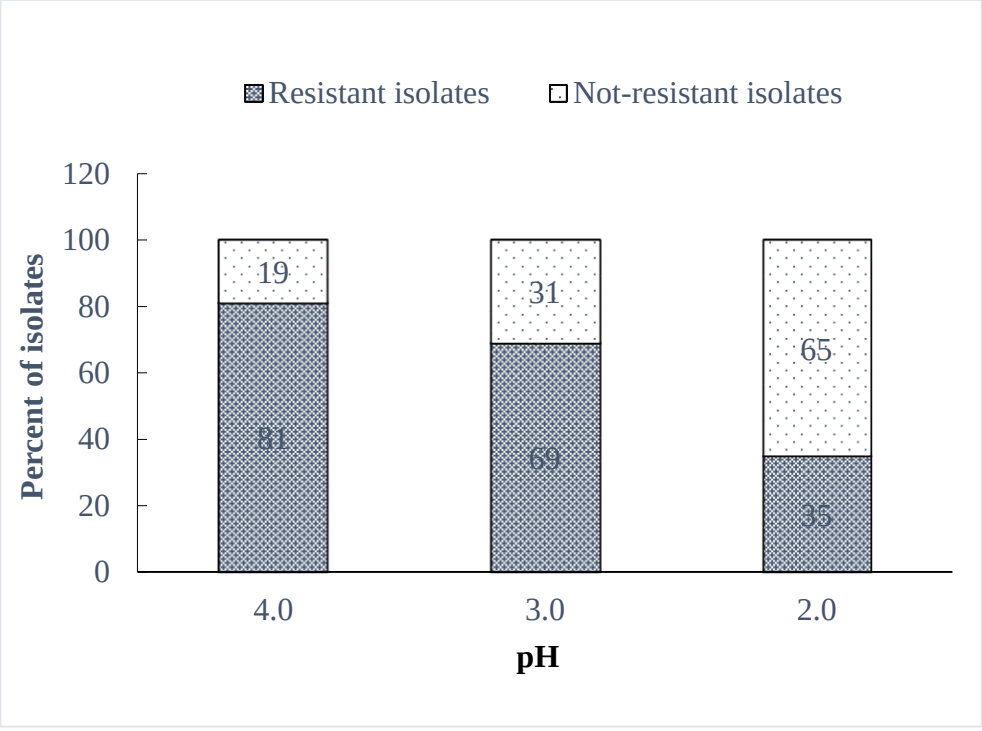


Figure 2: Cholesterol reduction ability of 12 bacterial isolates compared to initial concentration of control sample without bacteria at time intervals 0, 24, 36 and 48h. of incubation. The represented bars on the figure were mean of triplicate independent experiments (n = 3) ± standard deviation. The different letters mean significant differences ($p < 0.05$), while values that sharing at least one similar letter are not significantly different.

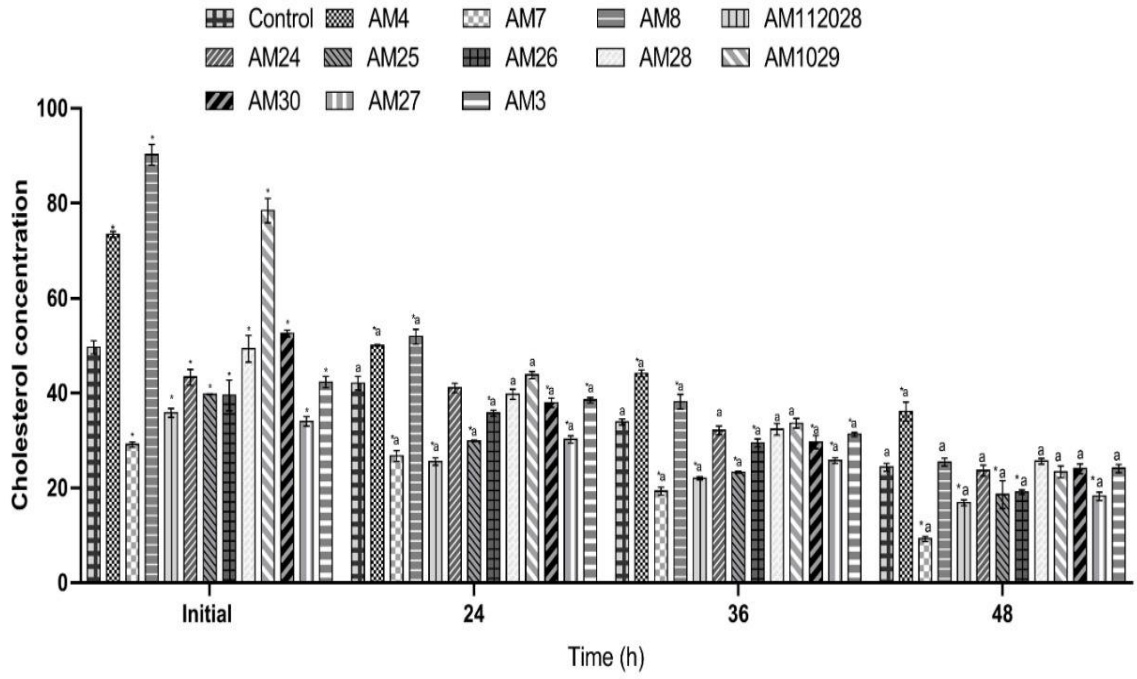


Figure 3: The percentage of cholesterol reduction activities for the bacterial isolates AM7, AM8 and AM1029 in the absence of bile salts compared to control sample without bacteria after 48h of incubation. The represented bars on the figure were mean of triplicate independent experiments (n = 3) ± standard deviation. The different letters mean significant differences ($p < 0.05$), while values that sharing at least one similar letter are not significantly different.

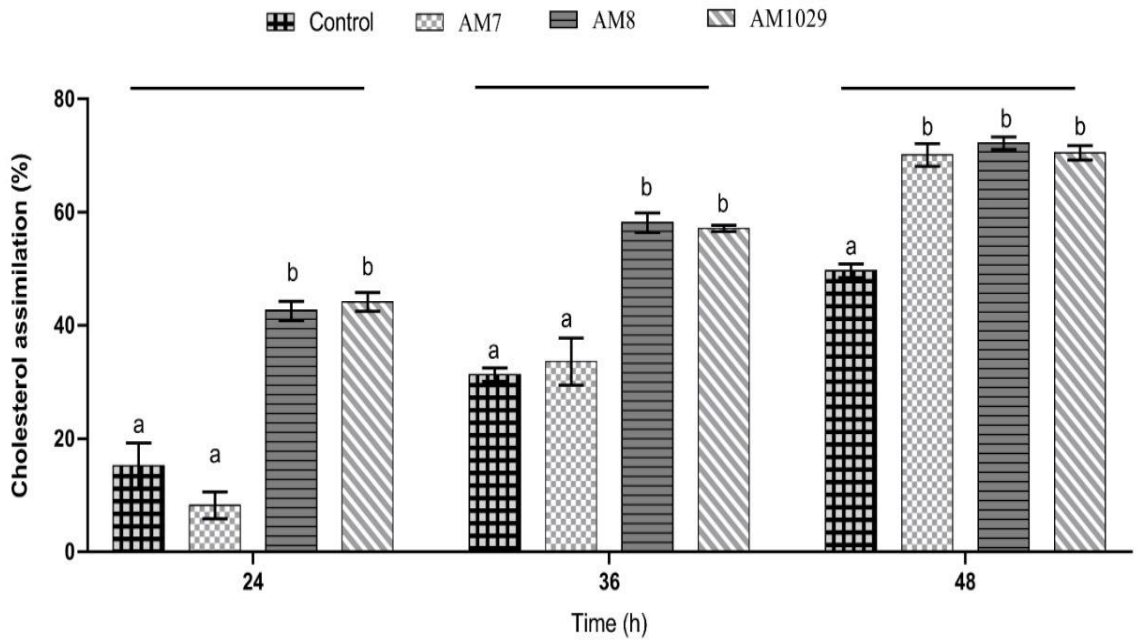


Figure 4: The probiotic isolates AM7, AM8 and AM1029 percentage of cholesterol reduction activities in the presence of bile salts in comparison to control sample without bacteria after 24h of incubation. The represented bars on the figure were mean of triplicate independent experiments (n = 3) ± standard deviation. The different letters mean significant differences ($p < 0.05$), while values that sharing at least one similar letter are not significantly different.

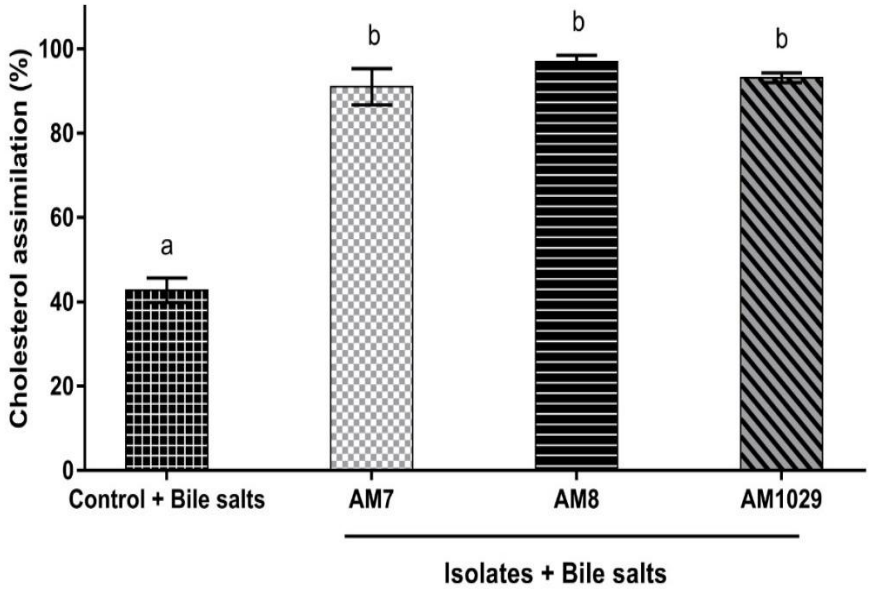


Figure 5: The anti-cytotoxic effect of six bacterial isolates was tested using Hep-2 cell line. Isolates AM7, AM24, AM25 and AM26 showed a protective effect to cellular DNA against damage by H_2O_2 compared to positive control. The represented bars on the figure were mean of triplicate independent experiments (n = 3) ± standard deviation.

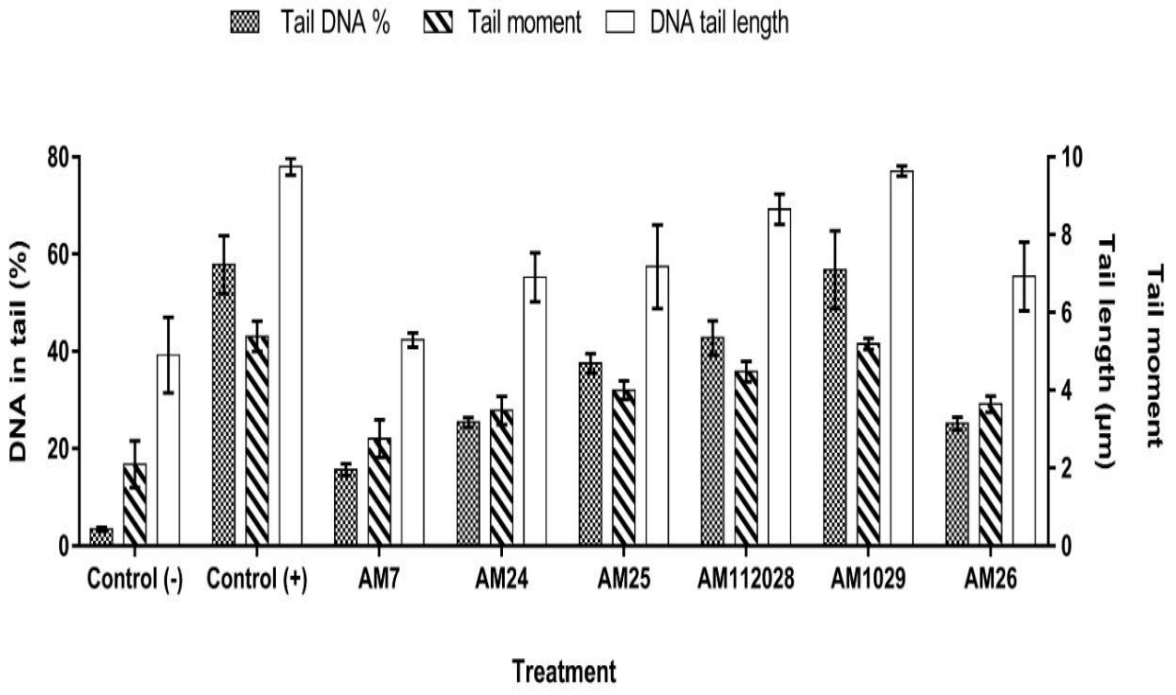
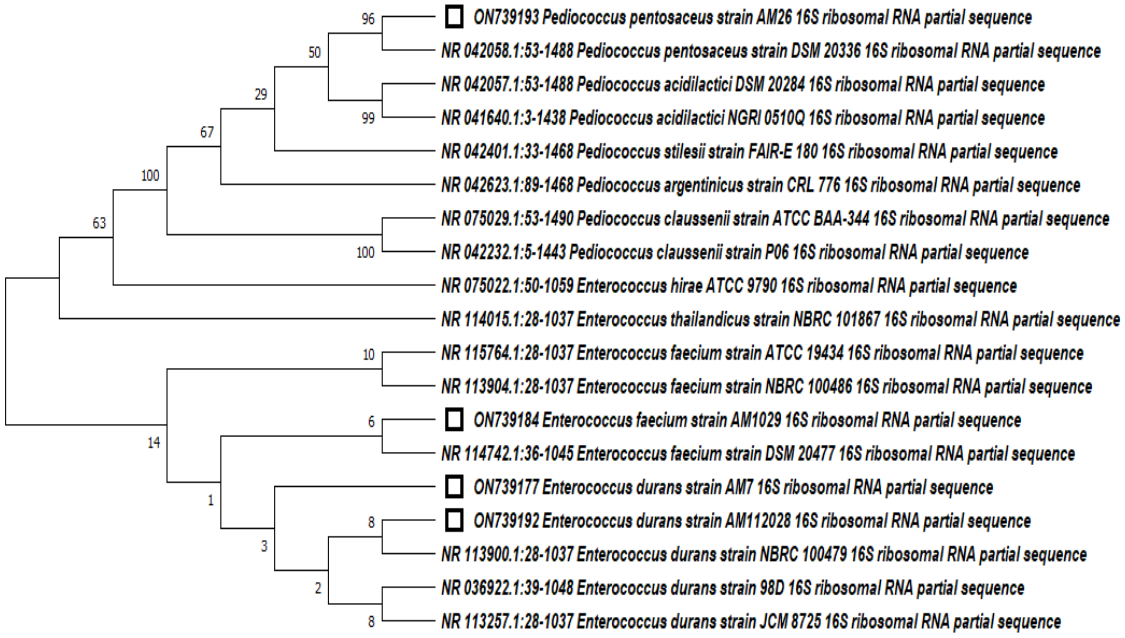


Figure 6: Evolutionary analysis of the selected probiotic isolates demonstrated by phylogenetic tree using the maximum likelihood method for the 16S rDNA sequences of *Enterococcus faecium* strain AM1029, *Enterococcus durans* strain AM7, *Enterococcus durans* strain AM112028 and *Pediococcus pentosaceus* strain AM26. The percentage values given by 1000 bootstrap analysis represented by the numbers at nodes.



Discussion

Human breast milk is an extremely unique vital fluid that was long thought to be sterile until the studies proved the isolation of living bacterial strains from it, some were probiotics [3–9,11,32]. In this study, 60 different bacterial isolates were isolated from 12 human milk samples then were used for application of different tests to identify their probiotic properties and to search for their other health potential benefits. 48 isolates were selected being conforming to the probiotic criteria Gram positive group, catalase negative type and lactic acid producing bacteria [3,21]. This was comparable to the study using the same samples number to obtain 25 bacterial isolates [33].

A potential probiotic strain should have the ability to survive acidic pH of the stomach passage and the intestinal environment. Stomach pH ranges from 1.5 to 4.5, and this range depends on the feeding intervals, food types and the duration digestion process, which can last for 3–4 h [34]. The good probiotic candidates should survive low pH conditions with a rate more than 50% and to be resistant to bile salts [35]. Accordingly, initial tolerance to low pH was tested and results revealed that most of isolates were resistant to pH 3, 4 while only 21% survived pH2. This is comparable to the results of bacterial isolates from different samples like milk, curd and bovine colostrum that exhibited the best low pH resistance at pH 4 and 5, sparse growth was observed at pH 3 however no growth was observed at pH 2 [22]. The buffering mechanism of probiotic strains could be explained by presence of exopolysaccharides structures on its external cell wall beside its acid regulatory mechanisms which contribute to the protection of the bacterial cells from the low acidity of the stomach, and so, increase the concentration of viable active cells in the gastrointestinal tract [36,37]. Bile salts destroys bacterial membranes and for a bacterium to survive gastrointestinal tract (GIT) conditions, it should have a method of resistance like producing bile salt hydrolase (BSH) enzyme

which conjugates to free bile acids. It have been agreed in many earlier studies that the threshold for bile salt tolerance of a potential probiotic is tolerating the high concentration of intestinal bile which is believed to reach 0.3% w/v and its contact time could reach 4 h [35]. Therefore, tolerance to intestinal bile salts is vital for the probiotic existence and progression in the GIT. All examined isolates were able to tolerate bile concentrations up to 2%. Where, previous reports on LAB isolated from different dairy products reported its tolerance to high bile concentrations ranged from 0.05 to 2.5% [34,35].

Being from human milk sources add to the proposed safety of the tested probiotics. However, antibiotic susceptibility profile for commonly used antibiotics should be tested for safety assessment of a probiotic strain [38]. On the other hand, antibiotic resistance property should be examined as it's essential for enabling probiotic strain to survive in the GIT during antibiotic medications [39]. All the tested isolates were susceptible to doxycycline and some of them showed sensitivity to gentamicin, ciprofloxacin and norfloxacin. However, all the tested isolates were resistant to cephalexin and cefoxitin. Though, no isolate showed resistance to all tested antibiotics. If it is resistant to one antibiotic, it was susceptible to another. Accordingly, poultry probiotics screened for antibiotic susceptibility profile to commonly used antibiotics showed different susceptibility profile against different tested antibiotics [38]. These findings indicated that the antibiotic susceptibility profile of isolated bacteria depends on antibiotics types.

Antimicrobial activity against pathogenic strains is a crucial criterion of prospective probiotics. Potential probiotics were characterized for producing of bacteriocins, hydrogen peroxide and organic acid that work as inhibitory substances responsible for preventing the growth of pathogenic bacteria in the GIT. Some of the tested isolates showed a good inhibitory activity against *E. coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes*. This was comparable to other studies that reported antagonistic activity against six tested pathogens including *E. coli* and *Listeria monocytogenes* [38], different lactobacilli inhibitory effects against different Candida species [40] and other probiotic strains with antimicrobial activity against *Bacillus cereus* species [41].

Atherosclerosis and cardiovascular diseases are associated with the elevated blood cholesterol and considered a main cause for the coronary cardiovascular diseases. The relation between bacterial metabolites and hypercholesterolemia of some probiotic strains was studied, and as a result, a long-term hypocholesterolemia properties could be achieved by the daily dietary consumption of products containing probiotic bacteria [42,43]. Three possible mechanisms have been suggested to elucidate cholesterol reduction ability by probiotic cells from the culture media: binding to bacterial cell walls, cholesterol assimilation during bacterial growth and cholesterol precipitation. However, the exact mechanisms of cholesterol reduction remains unclear [44]. Herein, the 12 isolates with the most promising probiotic properties (pH resistant, bile tolerant, antibiotic sensitive and having antimicrobial activity) were tested for cholesterol reduction in a broth medium free from bile salts, three promising isolates were selected for further analysis. This can recommend the proposed mechanism of cholesterol reduction by combination to the bacterial cell wall rather than being metabolically degraded [12]. Cholesterol-reduction ability could be associated with BSH activity, therefore, media supplemented with bile salt was used to assess cholesterol reduction activity which was much more improved by isolates AM7, AM8 and AM1029. The reduction could be attributed to one of the mechanisms suggesting cholesterol co-precipitation with free bile salts or bile salts deconjugation by BSH activity results in enzymatic assimilation of cholesterol by the cells of the probiotic strain. Moreover, isolates identifications revealed they were *Enterococcus durans* strain AM7 and *Enterococcus faecium* strain AM1029. These results are comparable to the study evaluated Enterococcus strains for their cholesterol-reduction abilities where all of them were able to assimilate cholesterol and their abilities increased tremendously with the addition of bile salts [45]. Another search detected cholesterol assimilation activity by *Enterococcus durans* species [46]. Besides, other studies reported isolates with high cholesterol reduction abilities [47,48].

Since 1970s, **Goldin and Górbach** were amongst the first to reveal the relationship between a diet supplemented with Lactobacillus and a reduced frequency of colon cancer infections. Recently, the potential application of probiotics in

combination with conventional therapies in the treatment of cancer is promising [13]. Therefore, protection of human DNA during microbial treatment was studied in the current research by comet assay using Hep-2 cell line. In the positive control, the DNA of cells treated only with H2O2, was found to be severely damaged when compared to the negative control. Where, cell line cells previously treated with microbial strains revealed a reduced damage in the cellular DNA. Four probiotics treatments; included *Enterococcus durans* strain AM7 and *Pediococcus pentosaceus* strain AM26; showed promising results that could be attributed to the possibility of extracellular antioxidants production. Remarkably. These results may pave the way for preventing the oxidative DNA damage and cellular oxidation. This is in accordance with results of probiotic microbial strains isolated from honey kefir beverage that proved protection of human DNA against hydroxyl radical damage and showed high antioxidant activity [17].

Identification and characterization of all 48 strains using API technique showed 40% Lactobacillus, 27% Streptococcus, 19% Enterococcus and other related genera and 14% Bifidobacterium. Previous studies reported the presence of similar genera from raw goat milk [39]. Four isolates with the most promising probiotic properties were further analyzed and confirmed the identity of two *Enterococcus durans*, *Enterococcus faecium*, *Pediococcus pentosaceus* strains. This is in accordance with previous studies reported the isolation of Bifidobacterium sp.; Corynebacterium sp., Enterococcus sp., Lactobacillus sp., Proprionibacterium sp., Rothia sp., Staphylococcus sp. and Streptococcus sp., also genera from the families of Micrococcaceae, Neisseriaceae and Actinomycetes from the milk of lactating mothers [8,9,11,32,49–51].

Probiotics are now recognized as appropriate candidates in the control of pathogenic infections and well known to confer different benefits. Many studies have concentrated on the isolation of probiotic strains from human breast milk for the subsequent use in human health, pharmaceutical industries and nutrition markets because of its added factor of safety and well tolerance to acidic pH and high concentrations of bile. From all the conducted experiments in this work, four probiotic strains were isolated from human breast milk and were accurately identified by molecular techniques to be two *Enterococcus durans*, *Enterococcus faecium* and *Pediococcus pentosaceus*.

They could be promising candidates for usage to confer health benefits to the host with added not only anti-microbial capabilities against pathogens but also cholesterol-reduction activity which can ultimately be anti-atherosclerosis together with DNA-protective abilities against oxidants damage which can be furtherly evaluated as anti-cancerous.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Amany Abd Al Mawla, Mahmoud S.M. Mohamed, Nayra Sh. Mehanna and Marwa K. Sallam. The first draft of the manuscript was written by Amany Abd Al Mawla and Mahmoud S.M. Mohamed and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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