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Original Research

Characterization of Candidate probionts isolated from human breast milk

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Abstract: This study was designed to isolate and identify the potential probionts present in 32 healthy mothers' breast milk. Microbial culture media and 16SrRNA sequencing were used to isolate and identify the bacteria and all isolates were analyzed for their antagonistic potential, resistance to acidic pH, bile salts and survival under simulated gastric and intestinal conditions. The colonization potential was further assessed based on adherence to human enterocyte-like Caco-2 cell lines. The breast milk samples harbored significant numbers of Gram positive and catalase negative (85%) bacteria. Based on 16SrRNA sequencing, these isolates were identified as *Lactobacillus casei*, *L.gasseri*, *L.fermentum*, *L.plantarum, Pediococcus acidilactici*, and *Enterococcus facieum*. Among the isolates, *P. acidilactici* was the most frequent species (71%) present in these samples. Few Gram and catalase positive isolates, *Staphylococcus aureus* and *S.hominiis* were also observed. The isolates were viable and unviable in pH 3 and 1.5, respectively, while all isolates survived in 1.0% bile salt. As putative probionts, *P.acidilactici* 1C showed a significantly higher percentage of adhesion to Caco-2 cells ($p< 0.05$)than the other two isolates *L.plantarum 7A* and *E.facieum* 2C. Bacterial strains isolated from human breast milk were shown to have probiotic properties including anti-infective protection and may be considered as future therapeutics for infants.

Key words: Probiotic; 16SrRNA sequencing; Caco-2 cell adhesion; Breast milk.

Introduction

Probiotics are categorized as a large group of bacteria or yeast which have several beneficial effects on host health (1, 2). Probiotic bacteria are the potential candidates to treat or prevent some human and animal disorders (3, 4). Therefore, it seems that probiotics can be considered as suitable candidates for functional foods.

Previously, intramammary human milk was thought to be free of bacterial species, however, recent studies have shown that the infant gut receives a continuous supply of commensal, mutualistic and potentially probiotic bacteria from colostrum and breast milk (5). Breast milk bacteria are among the first to colonize the intestine of the newborn, and approximately 1×10^5-1×10^7 commensal bacteria are ingested while suckling by infants consuming approximately 800 ml breast milk per day (6).

Bacterial genera naturally present in the human mammary gland are mainly *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Propionibacteria* and *Bifidobacterium* (7, 8). A number of these bacteria have been known to prevent the colonization and growth of pathogens and can thereby help to lower the risk of infection in weaning infants. Moreover, the antiviral effects of these bacteria have also been reported. According to reports, lactic acid bacteria (LAB) from human breast milk could inhibit HIV-1 virus and might therefore help protect against HIV in exposed infants receiving breast milk (9, 10). Because of their beneficial health properties, isolating these bacteria from breast milk seems a potential prospect as they meet the major requirements for use as a probiotic (7, 8). Despite the fact that breast feeding is a common practice in our country and people are highly aware of the potential influence of this biological fluid on infant health, little information is available regarding the composition of bacterial flora in the breast milk and the presence of potential probiotics.

Thus, this study was conducted with the aim of identifying and evaluating the potential probiotic bacterial species present in breast milk samples of healthy mothers. Additionally, another aim of this study was to determine the probiotic properties of isolated bacteria from human breast milk including antibacterial activity, resistance to acid and bile, resistance to simulated gastric and intestinal content and finally their ability to adhere to Caco-2 Cell lines as a model of epithelial cells.

Materials and Methods

Breast milk sample collection and bacterial isolation

After filling out the informed written consent form and approving the study protocol by the Ethics Committee of the Razi Vaccine and Serum Research Institute, thirty-two breast milk samples were collected from healthy mothers (25-30 years) referring to a healthcare centre in Tehran, Iran. The volunteers reported not being on medications, especially antibiotics, for the

last 2 weeks. Approximately 5 ml of milk samples were collected in sterile Falcon tubes after washing (soap and water) and disinfecting (chlorhexidene) the area around the nipples and mammary areola. The collected samples were transported to the laboratory on ice within 4 hrs and processed accordingly.

Pour plate technique was used to isolate the bacteria from collected milk samples. Appropriate dilutions of the milk samples were cultured on different media including Brain Heart Infusion, a general-purpose medium (BHI, Oxoid, UK), de Man, Rogosa and Sharpe (MRS) (for isolation of lactic acid bacteria), MRSs (MRS media supplemented with 0.05% filter sterilized L-cysteine and 50 mg L^{-1} mupirocin, Sigma, UK) (for selective isolation of bifidobacteria), KF agar supplemented with TTC (2, 3, 5-triphenyl-tetrazolium chloride solution, Sigma, UK) 1% final concentrations and Kanamycin Aesculin-Azide Agar (KAA, Oxoid, UK) (for selective isolation of enterococci) and Baird-Parker (BP, Biomerieux, France) (for the isolation of staphylococci). All plates in duplicate were incubated under aerobic and anaerobic conditions for 48 to 72 h at 37 ºC, respectively (11).

Phenotypic and genotypic identification

The obtained pure colonies were tested for their morphology, Gram reaction, catalase reaction and oxygen requirements. The isolates were identified to species level based on their ability to ferment carbohydrate substrates, using the API 50 CHL system (Biomerieux, Lyon, France), according to the manufacturer's recommendations.

For genotypic identification, all isolates were subjected to PCR reactions using a pair of universal primers described by Satokari *et al*. (2001) (12). The amplified products were sequenced and aligned to 16S rRNA gene sequences in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/).

Antibacterial activity

The antibacterial spectrum of the isolates against a number of Gram positive and negative pathogens listed in Table 1, were by agar well diffusion method (13, 14). The cultures of the isolates and the indicator pathogens were adjusted to McFarland Index 0.5 prior to use. The antimicrobial activity was recorded as the appearance of a clear zone around the wells and the diameter of the zones recorded in millimeters (13, 14).

Table 1 illustrates that all the isolated probiotic bacteria are able to inhibit growth of pathogenic bacteria and the widest spectrum of activities was related to *P.acidilactici* 1C and *S.hominiis* 6D as they suppress the growth of all tested pathogens.

Resistance to acid and bile

The isolates were screened for their viability under acidic conditions by the method described by Sahadeva and colleagues (15). The isolates were subjected to pH values of 1.5, 3.0, 4.0 and 6.5 (control) and their growth pattern followed by plating serial dilutions of the samples on MRS agar plates and recording cfu/ml after 0, 2.0 and 3.0 hrs.

Bile tolerance of the isolates was determined by incubating the test organisms in the presence of different bile concentrations (0.5, 0.7 and 1.0%) after 8 hrs of incubation, by the method of Walker and Gilliland (16). Coefficient of inhibition (C_{inh}) was calculated using the following formula:

$C_{inh} = \Delta_{T8\text{-}T0}$ Control $- \Delta_{T8\text{-}T0}$ Treatment / $\Delta_{T8\text{-}T0}$ Control

in which $\Delta_{T8\text{-}T0}$ represents the difference in absorbance at time zero (T0) and after 8 hrs (T8) (8). Isolates were considered as suitable candidate probionts when C_{inh} was < 0.4 (17).

Resistance to simulated gastric and intestinal content

The resistance of the selected *Lactobacillus* isolates to simulated gastric content was tested by the method described by Beumer and colleagues (18). Simulated gastric juice medium (pH 3.0) was prepared by adding pepsin (13.3 mg L^{-1}), lysozyme (0.1 mg L^{-1}), porcine bile (0.05 mg L^{-1}), and 0.5 % sodium chloride. All enzymes used in the study were purchased from Sigma, UK. The bacterial survival was calculated as follows:

R= Average of cells at 10 min interval/ Average of cells at 0 min

When no effect on the growth and survival of bacteria is seen, R=1, whereas a loss of 50% of the viability is indicated by R=0.5. Ratios > 1 indicate bacterial growth.

The viability of the selected isolates in simulated upper intestine content (UIJ) containing pancreatic enzymes was evaluated as described by Charteries *et al*., (1998) (19). The survival rate (R value) of the tested bacteria at 0, 4, 8, 12 and 24 h was determined using the aforementioned formula (19).

Adhesion to Caco-2 cell lines

The Caco-2 cell lines (ATCC) were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibc, Invitrogen, UK), with 1% non essential amino

Table 1. Antibacterial activity of isolated strains against tested pathogens.

Indicator Strains	Producer strains (zone diameter mm)						
	1A	5A	7A	1C	2C	3D	6D
E.coli ATCC 8739	27	21	32	25	32	28	25
E. faecalis PTCC 1237	22	17	20	25	0	23	21
Kl.pneumoniae RTCC1254	28	21	27	25	21	15	25
L.monocytogenes RTCC1290	30	28	30	22	24	0	35
Ps.aerouginosa ATCC 9027	32	20	29	26	19	15	25
S.typhi (local isolate)	28	0	0	27	27	0	29
Staph. aureus RTCC 1263	0	42	26	23	22	15	38
Sh. dysenteriaea (local isolate)	28	22	29	27	20	17	27

acids, 10% of fetal bovine serum (Gibco, UK) inactivated (30 min, 56°C), 100 µg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (Sigma,UK). The cells were maintained at 37 ºC with 5% CO_2 and 95% air. Cells were passaged when 90% confluence was achieved. Cell culture media were replaced on alternate days for a period of 20 days.

Twenty-one day old cells were cultured in 12 well tissue culture plates and used for the cell adhesion assays. A day prior to the experiment, the monolayers were washed twice with phosphate buffered saline (PBS) in order to remove traces of the medium and then 500λ of DMEM without antibiotics was added to each well. Later, 150λ of individual bacterial suspension with a concentration of $1x10^8$ cfu/ml was transferred to each well and incubated at 37 °C for 2 hours with 5% CO_2 and 95% air atmosphere. The supernatant was removed by micropipette and monolayer cells were washed 4 times with 1 ml sterile PBS. Two ml of methanol was added to each well and incubated for 15 min at room temperature. Methanol was completely removed and fixed cells were stained with Gram staining. The plates were air dried and examined with oil immersion microscopy. The number of bacteria in 20 random microscopic fields was counted and the bacteria were categorized as non adhesive (≤ 40 bacteria/field), adhesive (41-100 bacteria/field), or strongly adhesive (> 100 bacteria/field).

For determining the adhesion percentage, the washed monolayers were detached by trypsinization by adding 0.1% trypsin to individual wells and incubation at room temperature for 10 min. Serial dilutions of the cell suspension were plated on MRS agar plates and incubated overnight at 37 ºC. Percentage of adhesion was determined based on the given formula: (Cfu/ml of adhered bacteria/cfu/ml of initially added bacteria) x 100 (20).

Statistical analysis

All experiments in the present study were carried out in triplicate and the results indicate their mean values. For statistical analysis, SPSS software version 18 has been used and the standard errors of the means were calculated and the means were tested according to One-way ANOVA test. Value of $P<0.05$ was considered significant.

Results

Phenotypic and genotypic identification

Among 73 isolated colonies, 62 appeared Gram positive and catalase negative (71%), while seven were Gram positive and catalase positive, and the remaining four were Gram negative bacteria, respectively.

All Gram positive bacteria isolated were identified to genus level based on their carbohydrate fermentation profiles and 16S rRNA gene sequencing. An amplified PCR product of approximately 1500bp was sequenced and analyzed based on BLAST search. Based on the results, the isolates showed >97% similarity to *Lactobacillus casei* (n=3), *L.gasseri* (n=1), *L.fermentum* (n=3), *L.plantarum* (n=8), *Pediococcus acidilactici* (n=45), *Enterococcus facieum* (n=2), *Staphylococcus aureus* (n=2) and *S.hominiis* (n=5).

Antibacterial activity

The antagonistic activities of the isolates against a number of Gram positive and negative pathogens were evaluated. Table 1 depicts maximum inhibitory action of *E.faceium* 2C, *L.fermentum* 3D, *L.gasseri* 5A, *L.plantarum* 1A, *L.plantarum* 7A, *P.acidilactici* 1C and *S.hominiis* 6D against a number of tested pathogens. *P.acidilactici* 1C and *S.hominiis* 6D exhibited the broadest spectrum of activity as they were able to inhibit the growth of all tested pathogens. *L.fermentum* 3D was able to inhibit the growth of all pathogens except *L.monocytogenes,* while *L.gasseri* 5A and *L.plantarum* 7A could inhibit the growth of all pathogens except *S.typhi*. The two *L.plantarum* isolates 1A and 7A differed in their antibacterial spectrum as *L.plantarum* 7A showed slightly broader spectrum of activity compared with *L.plantarum* 1A and was able to inhibit the growth of *S.aureus* as well.

The selected seven isolates were subjected to detailed studies. Table 2 depicts the survival percentage of the selected isolates at acidic pH values at different time intervals. None of the isolates in study appeared resistant to pH 1.5, however, they showed variable degrees of resistance at pH 3.0. Only *S.hominiis* 6D was incapable of tolerating pH values of 3.0. Among all the tested isolates, *E.faceium* 2C, *P.acidilactici* 1C and *L.plantarum* 7A showed highest survival at this pH value after 3 hrs

Table 2. Tolerance of selected isolates at acidic pH values at different time intervals.

pH Value	Isolates	Total plate counts (log10 CFU/ mL)		
		0 hour	2.0 hour	3. 0 hour
1.5	1A	1.74E+06	–	–
	5A	1.98E+06	–	–
	7A	1.71E+06	–	–
	1C	1.96E+06	–	–
	2C	2.10E+06	–	–
	3D	1.95E+06	–	–
	6D	1.83E+06	–	–
3.0	1A	1.94E+06	1.46E+06	1.69E+06
	5A	2.11E+06	1.50E+06	1.70E+06
	7A	1.44E+06	4.36E+06	4.29E+06
	1C	2.11E+06	2.59E+06	4.40E+06
	2C	1.98E+06	3.43E+06	5.70E+06
	3D	1.91E+06	1.50E+06	2.70E+06
	6D	1.87E+05	–	–
4.0	1A	1.89E+06	2.46E+06	3.91E+06
	5A	1.26E+06	2.71E+06	2.97E+06
	7A	1.56E+06	3.39E+06	4.95E+06
	1C	1.26E+06	2.71E+06	4.97E+06
	2C	1.7E+06	3.64E+06	5.33E+06
	3D	1.87E+05	2.59E+06	2.89E+06
	6D	1.56E+06	2.77E+06	3.98E+06
6.5	1A	1.94E+06	2.44E+06	3.93E+06
	5A	2.11E+06	2.01E+06	3.22E+06
	7A	1.44E+06	2.78E+06	4.31E+06
	1C	2.11E+06	2.01E+06	4.71E+06
	2C	1.96E+06	4.49E+06	5.61E+06
	3D	1.96E+06	2.48E+06	3.01E+06
	6D	1.44E+06	3.56E+06	4.11E+06

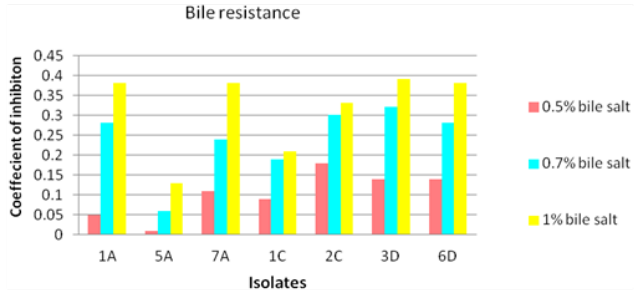

Figure 1. Bile tolerance of the selected isolates in variable concentrations of bile during 8 hrs. The figure illustrates that all isolates were considered bile tolerant as they well tolerated bile concentrations of up to 1%.

of incubation with their survival rate overcoming death rate. All isolates grew well at pH 4.0 and above with 100% survival rate.

The table illustrates that none of the isolates in the study appeared resistant to pH 1.5, while variable degree of resistance was observed at pH 3.0. All isolates grew well at pH 4.0 and above with 100% survival rate.

Resistance to acid and bile

Figure 1 shows the survival of the tested strains in the presence of variable concentrations of bile salt after 8 hrs of incubation at 37 ºC. All the isolates in this study were considered bile tolerant as they tolerated bile concentrations of up to 1%. The coefficient of inhibition (C_{inh}) for all isolates as calculated by the given formula was below 0.4. *L.gasseri* 5A and *P.acidilactici* 1C were the most bile tolerant strain as they were able to survive and grow at the highest used concentrations of bile salts with their C_{inh} recorded 0.13 and 0.29, respectively.

Resistance to simulated gastric and intestinal content

The survival rate of the selected isolates in simulated gastric and intestinal content is as shown in Table 3. According to the results, *L.gasseri* 5A was the least resistant isolate under simulated gastric conditions with survival rate below 50 %. R values above 1 indicated both survival and growth. In contrast, the survival rate of this isolate in simulated intestinal conditions was highly significant (R value 1.35). The growth rate of all the tested isolates except *L.plantarum* 7A and *S.hominiis* 6D reduced significantly during the initial 4 h in simulated intestinal conditions. However, after 8 hrs of incubation, an observable increase in the growth and reciprocally an increase in R value of these isolates were recorded. *E.faceium* 2C was the most resistant isolate in both the simulated gastric and intestinal conditions.

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Adhesion to Caco-2 cell lines

The selected seven isolates were scrutinized to select the most suitable probionts based on their overall performance under the above mentioned test conditions. Based on the results three isolates, namely *E.facieum* 2C, *L.planatrum* 7A and *P.acidilactici* 1C were selected as putative probionts and their adherence to Caco-2 cell lines was evaluated. Figure 2 shows the adherence of the selected probionts to the tested epithelial cell line as

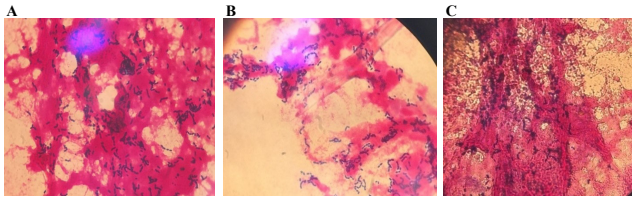

Figure 2. Adherence of *E.facieum* 2C (A), *L.plantarum* 7A (B) and *P.acidilactici* 1C (C) to Caco-2 cell lines as observed under microscope.

Table 3. Viability of tested isolates in simulated gastric and intestinal conditions.

	Time	1A	5A	7A	1C	2C	3D	6D
Intestine content	0 min	0.92	0.89	0.82	0.87	0.94	0.91	0.93
	10 min	0.72	0.60	0.62	0.51	0.91	0.92	0.94
	20 min	0.59	0.32	0.45	0.28	0.84	0.86	0.91
	30 min	0.68	0.31	0.51	0.24	1.14	0.83	0.87
Gastric content	0 h	0.84	0.91	0.92	0.91	0.92	0.88	0.90
	4 h	0.81	0.95	0.94	0.87	0.91	0.81	0.93
	8 h	0.97	0.97	0.99	0.89	0.97	0.85	0.96
	12 h	1.26	1.11	1.21	1.42	1.38	0.92	1.11

Table 4. Adherence of selected isolates to Caco-2 cell monolayers.

Bacterial species	Average number of bacteria adhesive to one Caco-2 cell	Adhesion#	Adhesive percentage
E.facieum 2C	6.42±4.2	133±23	17.41±5.2*
L.plantarum 7A	15.21±3.8	185±25	35.80±8.1*
P.acidilactici 1C	4.66±1.51	65±12	11.91±2.4*
P value	<0.001	<0.001	<0.001

observed under microscope after staining. The number, percentage, and average number of bacteria adhered to Caco-2 cells is shown in Table 4.

The isolates exhibited varying degrees of adhesion as indicated by their adhesion scores. *L.plantarum 7A* and *E.facieum* 2C appeared strongly adhesive (>185±25 bacteria/20 microscopic fields), while *P.acidilactici* 1C was moderately adhesive (65±12 bacteria/20 microscopic fields) to the Caco-2 cell lines. The adherence percentage recorded was 17.41, 35.80 and 11.90% for *E.facieum* 2C, *L.plantarum* 7A and *P.acidilactici* 1C, respectively.

Discussion

Mother's milk is a rich source of nutrients and live microorganism, playing a direct role in the development of the immune system in infants. The dominant flora present in breast milk of healthy mothers includes *Lactobacilli*, *Lactococci*, *Enterococci*, and *Leuconostoc spp.* (21). Reportedly, *Lactobacillus* species isolated from breast milk have probiotic potential comparable to or exceeding that of certain strains used commercially at large scale. Previous studies have demonstrated the probiotic potential of some of these breast milk isolates including *L.gasseri. L.rhamnosus, L.plantarum*, *L.fermentum*, *L.reuteri*, and *E.facieum* (21, 22). In this study, we were able to isolate a number of *Lactobacillus* species including: *L.fermentum, L.plantarum*, and *L.gasseri*. In contrast to a number of previous reports which indicate *P.pentosaceous* as the most frequent isolate in breast milk (5, 10), the most dominant species isolated in our studies from this biological fluid was *P.acidilactici.*

Apart from *Lactobacillus* species, the presence of *Staphylococci*, *Streptococci*, *Propionibacteria* and *Bifidobacteria* in the breast milk samples has also been reported (10, 23). Although we were unable to identify *Bifidobacteria* in the breast milk samples, a number of *S.aureus* and *S.hominis* strains were identified. Gonzalez and colleagues (2013) reported *S.epidermidis, S.homini* and *S.aureus* to be the most frequent *Staphylococcus* species isolated from breast milk (23). However, knowing the fact that *S.aureus* is a common skin contaminant present on the maternal skin and the main cause of mastitis in nursing mothers (24, 25), no further studies on these isolates were conducted.

A number of LAB are capable of antagonistic actions on other microorganisms, a property which renders them more advantageous in the competition with pathogenic bacteria. The inhibitory activities of these bacteria towards the growth of pathogenic bacteria such as *Listeria monocytogenes*, *E. coli,* and *Salmonella spp*. etc (26-28), are a well known phenomenon. In our studies, seven species showed significant antibacterial effect against important human and animal pathogens. The dominant species in the tests were *P.acidilactici* 1C and *S.hominis* 6D which were able to inhibit all the tested pathogens, thereby emphasizing their probiotic characteristics. However, *L.gasseri* 5, well known for its anti-obesity effects (29, 30) and *S.hominis* 6D showed maximum inhibitory activity towards *S.aureus*. In a previous report, *S.hominis* MBBL 2-9 isolated from vaginal microbiota of healthy women was shown to possess a strong inhibitory activity towards *S.aureus* (25).

An important criterion for selective isolation of candidate probiotic strains is their tolerance to low pH values which mimic stomach conditions. A pH value of 1.5 has been the lowest recorded pH in the stomach, mainly during fasting (31). In our studies, none of the isolates could resist the extremely acidic pH value of 1.5 and complete inhibition of their growth was seen immediately after exposure to this pH value. Similar results have been reported by Mandal *et al* (2006), who reported a decline in bacterial viability within 3 hours of incubation at low pH values of 1.5 (32). However, the most suitable probiotic species are expected to tolerate at least pH 3.0 for 3 hours (33, 34) which has been set as a threshold point to determine acid tolerance of probiotic bacteria. In accordance with these reports, all of our selected candidate probionts were able to survive at pH values of 3.0 and above. These data further support the fact that the isolates could survive in the stomach during digestion, which takes approximately 3 to 4 hours.

Besides resistance to acidic conditions, another prerequisite for probiotic strains is their ability to demonstrate bile resistance. In a report, Marteau and colleagues (2001) demonstrated that it is difficult

to study the effects of bile on probiotic strains using *in vitro* methods because the bile concentration in the human system varies over time (35). Therefore we used various concentrations of bile in this study in an effort to mimic the physiological conditions more closely. Although all tested isolates were significantly bile resistant with their coefficient of inhibition (C_{inh}) below 0.4 in 1% of bile salt concentrations, *L.gasseri* (5A) proved to be the most bile resistant isolate as it showed the highest growth rate in the used bile concentrations. Therefore, it appears that the *L.gasseri* may exhibit more probiotic properties and can be considered for further investigations into its use in food enrichment.

Generally, bacteria claimed as a probiotics should be able to survive under the conditions of the stomach and upper intestine where a combination of acid and bile exists (36). According to reports, *in vitro* and *in vivo* observations do not always correspond due to the complex nature of the human system. This was negated by other researchers who reported the difference to be insignificant and suggested *in vitro* methods for assessing the transit tolerance of the probionts (37, 38). We adopted *in vitro* methodology for assessing the effect of simulated gastric and intestinal conditions on the survival of candidate breast milk probionts. In this study, the higher survival rate of the isolates at alkaline pH values of upper intestine compared to acidic environment of gastric conditions was evident, which is similar to our previous reports (14). The most resistant strain appeared to be *E.facieum* 2C as it tolerated both gastric and intestinal conditions.

Apart from acid and bile tolerance, another important feature of suitable probionts is their ability to adhere to and colonize the intestinal epithelium, and to survive in the gastro-intestinal tract (39). We studied the adhesion of the selected three probionts using a human enterocyte-like Caco-2 cell line as a model for intestinal epithelium, thereby bypassing the obstacles related to *in vivo* testing. Caco-2 cells are considered an appropriate model for enterocytes and are often used to study

the adhesion and bacterial invasion into mammalian cells (40, 41). All three putative probionts were able to adhere to the used cell lines, but *L.plantarum* 7A showed the strongest adhesive properties. The significant adhesion of the isolates to Caco-2 cells satisfies an important prerequisite for probiotic selection.

Accordingly, it appears that each bacterial strain isolated from human breast milk has some properties which support it's use as an appropriate candidate for further investigation into its use in food enrichment. Additionally, based on the fact that the bacteria are separated from human breast milk, and newborn cases may suffer from some infectious diseases by pathogens, hence, it may be hypothesized that the probiotics may be used for probiotic therapy.

In conclusion, the results suggest that the bacterial strains isolated from human breast milk might be suitable strains to develop probiotic products for infants. Thus, it appears that using bacterial strains isolated from human breast milk may be strongly recommended to be used for enriching human foods. Additionally, it is recommended that the probiotics be isolated from other ethnics to compare the effects of ethnics on the probiotics properties.

The project has some limitations including low sample size and no *in vivo* investigations were performed to evaluate the safety of the probiotics.

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