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Isolation and Identification of Lactic Acid Bacteria from Human Milk with Potential Probiotic Role

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Abstract In recent years, there has been an increasing interest in the field of research into the characterization of new probiotics with potential application to the health and disease prevention. Therefore, the aim of this study is to isolate and identify bacteria in human colostrum and mature milk to analyze its possible probiotic potential. We isolated and identified ten strains of bacteria in human mature milk, by molecular biology; from which, five of these strains were selected to evaluate their ability to survive *in vitro* simulated conditions of gastrointestinal stress, the antimicrobial effect, adhesion capacity and resistance to different pHs and temperatures. The results showed that three of the five selected strains, identified as *Lactobacillus fermentum* JCM 3, TW56 *Leuconostoc mesenteroides* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, were resistant to digestive enzymes, showed resistance to low pH values (2 and 3) having adhesion capacity and viability at temperatures of 40 °C. Therefore, these bacteria may could be considered as potential probiotics for the pharmaceutical and food industry.

Keywords: *probiotics, isolation, human milk, lactic acid bacteria*

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1. Introduction

Human breast milk consists of high amounts of necessary nutrients for infants, including carbohydrates, essential fatty acids, proteins, vitamins and minerals, due to this has been recognized as the gold standard of infant feeding (Sherman, [9,23,41]. It plays an important role in supporting the survival and development of infants not just because of nutrient supply but due to transfer of microflora originated in breast milk [28]. Several researchs have reported that human breast milk contains a wide spectrum of indigestible nutrients that are not utilized by the infants but exert numerous potent bioactive functions on establishment of infants' indigenous microflora [17,46].

In recent years, more than 200 different species have been described in human milk [16]. Breast milk has been shown to be a continuous source of commensal, mutualistic and⁄or probiotic bacteria to the infant gut, including staphylococci, streptococci, bifidobacteria, and, lactic acid bacteria [1,26,27,28,42]. The genera Lactobacillus, Pediococcus and Lactococcus belong to the lactic acid bacteria (LAB), and the strains of these genera are frequently used on a large scale in the production and preservation of many foods or as probiotics for human and animals [15,32]. LAB with probiotic activity are generally enteric flora and are believed to play a beneficial role in the ecosystem of the human gastrointestinal (GI) tract. There are many definitions of probiotics, but the prevailing one is that adopted according to an international scientific consensus in 2002 by the WHO and FAO. It states that probiotics are live microorganisms which, when they are administered in adequate amounts, provide a benefit to the health of consumers [13].

Certain quality requirements have been established in order to ensure efficiency, effectiveness and benefit to the host from those microorganisms, including among those features are non pathogenic or have toxic effects, contact stability and bile acid and adhesion to the intestinal mucosa [41]. Probiotics may be one of the most effective therapies for the prevention of several diseases in the new born. At birth, an infant's gastrointestinal tract is sterile and colonization of the gastrointestinal tract starts immediately after birth with the initiation of enteral feedings and is well established within the first few days of life [12]. It have been considered that in breastfed infants, bacteria of the genus Bifidobacterium and Lactobacillus predominate, with other enteric organisms being present less frequently [43,44]. Conversely, in formula-fed infants, it have been reported that coliforms, enterococci, and bacteroides predominaely colonize the intestinal tract. Moreover, preterm infants are particularly susceptible to abnormal colonization. A combination of antibiotic use, delayed initiation of enteral feedings, and exposure to the unusual microorganisms that populate the neonatal intensive care unit may lead to abnormal patterns of colonization. Gewolb et al., [11], reported that the gastrointestinal tract of extremely low birth weight infants is colonized by

fewer than 3 bacterial species by the tenth day of life and that species of Bifidobacterium and lactobacillus could be found in the stool of fewer than 5% of patients studied within the first month of life. Feeding oral probiotic bacteria may be an effective way to change this pattern of colonization. On the other hand, it has been postulated that introducing probiotics to preterm infants might be beneficial to avoid overgrowth of pathogenic organisms. Probiotic supplementation has been proposed to enhance enteral feeding and prevent diseases and nosocomial infections in preterm infants. The proposed beneficial effects of probiotic administration come from potentially competing with other organisms for binding sites and substrate in the bowel, which increases the production of antiinflammatory cytokines, decreases the production of proinflammatory cytokines, reduces intestinal permeability, and enhances enteral nutrition [3], [29] On the other hand, there is increasing interest in some lactic acid bacteria which are considered as potentially probiotic species present in breast milk such as *L. gasseri*, *L. salivarius*, *L. rhamnosus*, *L. plantarum* and *L. fermentum* [22].

It has been shown that human milk from healthy women contains approximately 10^3-10^4 CFU/mL representing a continuous source of potential commensal bacteria for the infant [13,27], and that some of the lactic acid bacteria strains isolated from this biological fluid have the ability to inhibit the growth of a wide spectrum of pathogenic bacteria by competitive exclusion and⁄or through the production of antimicrobial compounds such as bacteriocins, organic acids or hydrogen peroxide [2,13,27]. Therefore, the aim of this work was to isolate and identify bacteria in human colostrum and mature milk to analyze its possible probiotic potential.

2. Material and Methods

2.1. Collection of Milk Sample

Human milk and colostrum used for this study were donated by the Civil Hospital of Guadalajara. Samples were taken according to the selection criteria provisions of the ethics commission of the Civilian Hospital and the Health Secretariat of Guadalajara, Jalisco, Mexico. Ten voluntarily healthy mothers were selected with a period of 1 to 4 months after giving birth, between the ages of 24-38 years. The human milk samples were taken and collected in sterile flasks and transported to the Industrial Microbiology Laboratory of the University of Guadalajara in refrigerated containers under cold line (15 °C).

2.2. Isolation of Lactic Acid Bacteria

For the isolation and identification of lactic acid bacteria, 1 mL of samples of human milk were serially diluted in sterile NaCl (0.75 %) and dilution were placed in Petri dishes containing MRS (de Man, Rogosa and Sharpe, Bioxon®) agar using the pour plate technique described by Dunne [7]. The dishes were then incubated for 24-48 hours at 37 °C.

2.3. Phenotypic Identification

The isolated colonies that grew on MRS agar and presented phenotypic characteristics such as morphology of their colonies and Gram staining were subjected to biochemical tests such as with catalase and oxidase, employing methods proposed by Morrow [30].

In order to investigate carbohydrate fermentation, were cultivated the bacterias on MRS agar and the API 50 CHL test was used. Inoculum was prepared with a culture diluted to match a 2 McFarland turbidity standard (5041 API Sistem, S.A. La Balme les Grottes, Montalieu-Vercieu, France). All tests were read after 24 and 48 hours of incubation at 37°C.

2.4. Molecular identification

Genomic DNA of the isolated bacteria were extracted wit Genomic DNA Purification Kit Brand WISAR Promega® for isolation of Gram positive and Gram negative bacteria follow the manufacturer instructions. Amplification of DNA extracted from the isolated bacteria was carried out using the following universal oligonucleotides:

pA: 5´-AGAGTTTGATCCTGGCTCAG- 3´

pB: 5´-AAGGAGGTGATCCAGCCGCA- 3

Finally, phylogenetic trees were constructed employing sequences with 1500 bp aproximately obtained in a FASTA file, The sequences were edited and compared with reported sequences in the GeneBank database (http://www.ncbi.nlm.nih.gov/BLAST) available at the National Center for Biotechnology Information web-site. Sequences representing the best matches were retrieved and aligned using the clustal method.

***In vitro* tests to determine the probiotic potential of bacterial isolates.**

The selected bacteria with the profile of lactic acid bacteria according to the biochemical test described above, were grown in MRS broth (37°C) and before each test, the bacterial concentration was adjusted to the 0.5 McFarland equivalent to 10^8 CFU/mL. Assessment of the potential probiotic role of different isolates was conducted as follows:

Tolerance to acid pH.

Tolerance to acidic condition was evaluated against two pH levels in 5 mL of MRS broth, adjusting the pH with HCl (1N). These broths were inoculated with fresh cultures of each bacterium isolated until a turbidity of 0.5 McFarland was achieved. The broths were incubated at 37 °C for 4 hours and after this time 1 mL was taken and placed in a Petri dish with MRS agar using pour plate technique and incubated at 37 °C for 24 h.

Tolerance to bile salts.

For this test the growth was evaluated against two bile (SIGMA-ALDRICH®) concentrations (0.2%, 0.4%), in 5 mL of MRS broth. Fresh broth cultures of each isolated bacteria was inoculated until a turbidity of 0.5 McFarland was achieved and then incubated at 37 °C for 4 hours. After this time 1 ml was taken and placed in a Petri dish with MRS agar using the pour plate technique and incubated at 37 °C for 24 h.

Tolerance to enzymes.

Isolates were tested against different enzymes (α-amylase from *Aspergillus oryzae* 300 U/mg; proteinase of *Aspergillus melleus*, Type XXIII; trypsin from porcine pancreas, Type II-S); all enzymes were obtained from SIGMA-ALDRICH®. Isolated bacteria were incubated in MRS broth (24 hours, 37 °C); for each enzyme solution (1

mg/mL) in NaCl (0.5% w/v, pH 2). The pH was adjusted with HCl or NaOH (0.5 N). One mL of each enzyme solution was taken and added to a tube containing 0.2 mL of bacterial cell suspension (10^8 CFU/mL), vortexed and incubated at 37°C for 3 and 6 hours and a sample taken at the end of each incubation time. This was sown by pour plate technique and incubated at 37°C for 24 h to finally quantify the number of CFU/mL survivors.

Ability to auto-aggregation.

The auto-aggregation phenomenon is visually manifested by the formation of cell sedimenting aggregates. Isolated bacteria were grown in 5 mL of MRS broth and incubated at 37 °C for 24 h, brought to a concentration of $1x10^8$ CFU/mL. The pellet obtained by centrifugation was dissolved in 2 mL of PBS (20 mM) and vortex mixed for 30 seconds, then, 0.1 mL of sample was taken and diluted in 1.9 mL of PBS (20 mM) and initial OD was read at 600 nm. The microorganisms were incubated at room temperature for 4 hours and 0.1 mL of sample was extracted every hour, and OD read at 600 nm.

OD results of were used to express the extent of autoaggregation as a percentage using the following expression:

$$Autoagregation(\%) = 1 - \frac{A_t}{A_0} * 100$$

Where:

A_t represents OD at 1, 2, 3 and 4 h

$\mathbf{A_0}$ represents OD at time = 0 h

Hydrophobicity.

Test hydrophobicity has been used to assess bacterial adhesion to surfaces such as the intestinal epithelium *in vitro* through MATH (Microbial Adhesion to Hydrocarbons) partitioning technique in organic solvents simulating the adhesion thereof to nonpolar solvents. Isolated strains were grown in MRS broth and incubated at 37 °C for 24 h, and were brought to a concentration of 10^8 CFU/mL and then centrifuged at 5000 rpm, 10 min at 4 °C to obtain bacterial biomass; the supernatant was discarded. The resulting pellet was washed twice with phosphate buffer (20 mM), and re-suspended in 4 mL of PBS (0.2 M). This was mixed by vortexing and the initial OD was read at 600 nm. Toluene (0.8 mL) was added to the bacterial suspension, mixed with a vortex for 2 min and then incubated at room temperature for 20 min. Finally, the aqueous phase was removed and the OD was measured at 600 nm.

The hydrophobicity percentage (H %) was calculated according to the following equation:

$$\text{Hydrophobicity } (\%) = \left(\frac{A_0 - A}{A_0} \right) * 100$$

Where:

$\mathbf{A_0}$: represents OD before toluene extraction

A: represents OD after toluene extraction

Antimicrobial effect.

The isolated bacteria were grown in MRS broth for 24 hours at 37 °C, at the end of which the broth was separated by centrifugation (10 minutes, 5000 rpm, 4 °C), and heated at 70 °C for 30 min to inactivate proteases that it may contain. Medium pH was adjusted to 6.5 using NaOH (1 M) and filtered using a 0.45 micron filter. The well diffusion method was employed, preparing Petri dishes seeded with indicator bacteria inoculated in Mueller-Hinton agar (Bioxon ®). Wells of 6 mm approximately were cut into previously seeded agar and 100 µL of broth of each assessed bacteria obtained as mentioned above, were placed in each well. Petri dishes were incubated for 24 h at 37 °C and an inhibition test was considered positive if an inhibition halo was observed and negative if not. A broadspectrum antibiotic (Bactrim ®) was used as a positive control.

Quantification of organic acids by High Performance Liquid Chromatography (HPLC).

Broth samples belonging to each isolated strain were analyzed for organic acid production using High Performance Liquid Chromatography (HPLC), HPLC was equipped with a binary pump (Varian Prostar 210), a refractive index detector (Agilent Model G1362A) and a Meta Carb H Plus column. Mobile phase was HPLC grade water was used with H_2SO_4 (0.01N), at a 0.6 mL/min flow rate.

3. Results

Isolation and identification of lactic acid bacteria. Five strains with phenotypic characteristics that distinguish the LAB lactic acid bacteria were isolated from human milk, as shown in Table 1. According to results depicted in Table 1, API 50 CHL test were performed obtained from 98 to 99 % of probability respect to genus and specie of isolated bacteria. Later, isolated bacteria were identified molecularly and the results of this identification is shown in Table 2.

Table 1. Morphology, Gram strain and biochemical tests on isolated strains of Human Milk

Bacteria	Morphology	Gram	Catalase	Oxidase
d	Short rod	+	-	-
σ	Short rod	+	-	-
a	Short rod	+	-	-
Lacto-1	Short rod	+	-	-
8	Short rod	+	-	-
7	Short rod	+	-	-
13	Short rod	+	-	-
Lacto-2	Long rod	+	-	-

Table 2. Isolated strains from human milk, codes and real names.

Code	Bacteria
a y σ	*Lactobacillus fermentum* JCM3
Lacto-1	*Lactobacillus delbrueckii subsp. bulgaricus*
7 y 8	*Streptococcus salivarius* 8618
Lacto-2	*Bacillus subtilis subsp. subtilis* S04
d y 13	*Leuconostoc mesenteroides* TW56

Tolerance to acid pH.

The evaluated strains, *Lactobacillus fermentum* JCM3, *Leuconostoc mesenteroides* TW56 and *Lactobacillus delbrueckii* subsp. *bulgaricus* showed tolerance to the acidic conditions (pH 2 and 3). There was a growth of about $1x10^8$ CFU/mL as these strains were selected by a bile salt tolerance assay.

Tolerance to bile salts.

Those strains that showed acidity tolerance were subjected to a bile salt tolerance test. Those three strains were *Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 and *Lactobacillus delbrueckii* subsp. *bulgaricus* they also showed tolerance to bile salt concentrations of 0.2% and 0.4% (w/v).

Tolerance to enzymes.

Regarding to the tolerance to enzymes, Strains that survived to acid pH and bile salts (*Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 and *Lactobacillus delbrueckii* subsp. *bulgaricus*) were evaluated in order to assess their tolerance to enzymes. Results of enzyme tolerance tests are shown in Table 3; tolerance was expressed as positive (+) if growth after exposure to enzymes in the medium was observed and negative (-) if no growth was observed. All strains tested survived to the contact with the different enzymes evaluated.

Table 3. Enzyme tolerance of isolated strains

Strain	α-amilase	Tripsin	Proteinase
Lactobacillus fermentum JCM3	-	+	+
Lactobacillus delbrueckii subsp. *bulgaricus*	-	+	+
Leuconostoc mesenteroides TW56	-	+	+

Ability to auto-aggregation.

Sedimentation values of the strains under study, measured during a 4h incubation period, proved to be of low autoaggregation, based on data reported by Vallejo [47], with values lower than 65 %. The highest autoaggregation value obtained was of 39.6% corresponding to the strain *Leuconostoc mesenteroides* TW56, at 4 h of incubation. Observed auto-aggregation ability was related to cell surface components and this property was slightly affected after washing and resuspending the cells in phosphate buffer. The results of autoaggregation ability of LAB in the study are shown in Table 4.

Table 4. Autoaggregation ability of isolated strains.

Strain	Absorbance OD $_{600}$					
	L. fermentum JCM3		*L. mesenteroides* TW56		*L. delbrueckii* subsp. *bulgaricus*	
Time (h)	X	%	X	%	X	%
0	0.447±0.06	--	0.214±0.09	--	0.438±0.18	--
1	0.290±0.02	35.2	0.187±0.05	12.7	0.333±0.10	23.8
2	0.315±0.05	29.6	0.186±0.06	13.1	0.319±0.14	27.2
3	0.292±0.03	34.7	0.168±0.04	21.5	0.310±0.08	29.3
4	0.325±0.03	27.3	0.166±0.04	22.5	0.335±0.07	23.6

X: Average of the absorbance readings (**n**=3): replicants; ±SD. Estandar deviation. **(%):** Autoaggregation percentage = 1 - (At/A_0) X 100; $\mathbf{A_0}$: absorbance at time 0 h; **At**: absorbance after 1 hour.

Hydrophobicity.

The results obtained in the hydrophobicity test were very similar in the strains evaluated. The strains with the highest percentage of hydrophobicity were *Lactobacillus fermentum* JCM3 and *Lactobacillus bulgaricus* sp. *bulgaricus* with a hydrophobicity percentage of 75.83% and 82.85% respectively. *Leuconostoc mesenteroides* TW56 strain had the lowest hydrophobicity percentage (61.33%) of the three strains that showed tolerance to low pH and bile salts. Based on studies carried out by other authors made by Nader-Macías [31], hydrophobicity activity of isolates was classified as high (51-100%), medium (30-50%) and low (0-29%), so accordingly, these strains showed high hydrophobicity (>51-100%), on the bacterial surface. This contributes to cell interaction with gastrointestinal tract LAB. Table 5 shows the percentages of hydrophobicity of the strains mentioned above, as well as the absorbance obtained during the study for the determination of hydrophobicity.

Table 5. Adherence of isolated strains (hydrophobicity %)

Strain	$A_{0\,(X)}$	$A_{(X)}$	H% (Toluene)
***Leuconostoc mesenteroides* TW56**	0.110	0.026	77.65%
***Lactobacillus fermentum* JCM3**	0.115	0.020	82.85%
Lactobacillus delbrueckii* subsp. *bulgaricus	0.097	0.032	75.83%

$\mathbf{A_0}$: Absorbance time 0 h; **A**: absorbance after 20 minutes; **H%= ((A_0 – A)/A_0) x 100**

(X): Average of the absorbance readings (n=3): Replies.

Antimicrobial effect.

The ability to inhibit pathogenic microorganism growth is one of the mechanisms by which probiotic bacteria serve to protect the host. Probiotic bacteria produce various substances with antimicrobial effects such as organic acids, fatty acids, hydrogen peroxide, bacteriocins and related substances. Therefore to determine if the bacteria under study could inhibit pathogens, they were studied *in vitro* by measuring the inhibition zone diameter. *Leuconostoc mesenteroides* TW56 was able to inhibit *E. coli*, *Salmonella* and *S. aureus*, *L. delbrueckii* subsp. *bulgaricus* was able to inhibit only *E. coli* and *L. fermentum* JCM3 showed inhibitory activity against *E. coli* and *Salmonella*. Antagonistic activity was typified as: (+++) = 3 cm; (++) = 2 cm and (+) = 1cm. The results of the antimicrobial effect are shown in Table 6.

Table 6. Isolated strain pathogen inhibition ability.

Strain	*L. delbrueckii*	*L. mesenteroides*	*L. fermentum*
E. coli	+	+	+
Salmonella	-	+	+
S. aureus	-	+	-
Listeria	-	-	-

Inhibition diameter: (+++) =3, cm (++) = 2 cm, (+) = 1cm ;(+) Positive Inhibition (-) Negative Inhibition.

Production of organic acids.

HPLC analysis indicates that the selected strains produce acetic, lactic and butyric acids with a high production of lactic acid by the three strains evaluated. The strain *Lactobacillus fermentum* JCM3 showed the highest concentration in the production of the three acids of interest (5.59 g/L of lactic acid, 4.54 g/L of acetic acid and 4.21 g/L of butyric acid). The results for the other two bacteria were very similar but less than those of *L.*

fermentum JCM3. Lactic Acid Bacteria isolated in this study are classified as heterofermentative [18], lactic acid being not the only source of organic acids. According to data reported by Jayashree [18], bacteria in the study indicate as being heterofermentative lactic acid is not the only source of acid production by these organisms although the concentration of the acid production has been different. Concentrations of acid produced per each bacteria are shown in Table 7.

Table 7. Organic acid produced by Lactic Acid Bacteria isolated from human milk (g/L)

Organic acid (g/L)	***Lactobacillus fermentum***	***Leuconostoc mesenteroides***	***Lactobacillus delbrueckki* subsp. *bulgaricus***
Lactic	5.59	4.54	3.62
Ácetic	2.848	2.25	1.87
Butiric	3.97	2.86	2.22

4. Discussion

It has been reported that breast milk is a good source to provides all the nutritional requirements for the rapidly and healty growing of infants and it contains a variety of protective factors, such as immunoglobulin (IgA), immunocompetent cells, fatty acids, oligosaccharides, lactoferrin or lysozyme, that protect breast-fed infants against infectious diseases; and also it is a source of beneficial bacteria such as *Lactobacilli*, *Lactococci*, *Enterococci* and *Leuconostoc* spp., whichare the usual commensal bacteria present in breast milk and they play an important role in the defense system of the infant [13,27]. The LAB lactic acid bacteria isolated in this study from human milk include *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus fermentum* or *Enterococcus faecium*, all of which are considered to be all among the potential probiotic bacteria. In fact, some of the lactic acid bacteria strains with this origin have already been shown to possess probiotic properties, including the inhibition of a wide spectrum of infant pathogenic bacteria by competitive exclusion and/or through the production of antimicrobial compounds, such as bacteriocins, organic acids or hydrogen peroxide [2,24]. In the present work, the mean relative abundance of *Lactobacillus* was generally less than 50 % of isolated strains. Five strains showing phenotypic characteristics that distinguish lactic acid bacteria, were isolated from human milk of healthy mothers; however, only three of the isolated bacteria shown to have potential to be used as probiotic microorganisms. Biochemical identification was made by Gram stain, catalase and oxidase tests. The United Nations Food and Agriculture Organization and the World Health Organization [37] define the word probiotic as "living organisms that ingested in adequate amounts confer a health benefit on the host" with respect to this and certain quality requirements have been established for these microorganisms which ensure efficiency, effectiveness and benefit to the host. Among these features are they should not be pathogenic or toxic; they should show stability to contact with bile and acid and adhesion to the intestinal mucosa [37]. The microbiota of the gastrointestinal tract is regulated by several factors, including the interaction between microorganisms, gastric and pancreatic enzymes, pH, peristalsis and bile salts [47]; that is why, it is considered that in the selection process of probiotic strains of interest, it is essential to check the ability of tolerance to adverse conditions as it passes through the gastrointestinal tract. Although in the stomach a pH of 1 can be reached 1, most trials used were with a pH of 2 as in Shah, 2000 [40]; in this case, three of eight strains isolates *Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 and *Lactobacillus delbrueckii* subsp. *bulgaricus* showed tolerance to acidity conditions (pH 2 and 3). That tolerance was verified with positive growth in MRS agar seeded after strains were exposed to an acid pH. In another study, the *L. plantarum* LP9 strain isolated from buffalo milk was observed to survive at low pH, which is found in the adult human stomach. The isolate strain survived at low pH with only marginal decrease of 0.5 log cycle at pH 2.0. Lankaputhra and Shah, [20], also observed acid-dependent strain tolerance in *Lactobacilli* and *Bifidobacteria* at pH values of 1.5-3.0. Our Results of the present study suggested that our isolated strains *Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 y *Lactobacillus delbrueckii* subsp. *bulgaricus* can tolerate low pH (pH 2.0) without significant loss in cell count during the passage through the stomach. Maragkoudakisa [25] assessed probiotic potential of strains isolated from dairy products. Eight strains did not survive after 3 h of exposure to pepsin, *L. rhamnosus* ACA-DC 112 and *L. paracasei* subsp. *paracasei* ACA-DC 130 shown the best survival ability an the rest of the strains displayed loss of viability of >3 log cycles. The pH in human stomach ranges from 1 during fasting, to 4.5 after a meal, and food ingestion can take up to 3 h. Since *Lactobacillus* strains are known to survive at pH 4.6, which is the common final acidity of many fermented dairy products, lower pH values (1 and 3) were examined. Although all of the examined strains were completely resistant to pH 3 even after 3 h of exposure, most of the strains displayed loss of viability when exposed to pH 1 for 1 h. These results are in agreement with those obtained from previous similar studies where *Lactobacillus* strains were able to retain their viability when exposed to pH values of 2.5-4.0, but displayed loss of viability at lower pH values [4,7,8]. In the present study, strains *Bacillus subtilis* subsp. *subtilis* S04 and *Streptococcus salivarius* 8618 were not able to survive in acidic conditions, due to this therefore these strains were not assessed in their ability to tolerate bile salt concentrations. These result are very important, due to as before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach [14]. Moreover, the isolate ability to tolerate bile salts and the capability to grow at acidic pH were evaluated. The relevant physiological concentrations of human bile range from 0.3% to 0.5% [7,48]. It has also been reported that good bile tolerance benefits probiotic strain colonization in the host GI tract [24]. In this regard, it is important to evaluate the ability of potential probiotics to survive in the presence of bile. In the present work, 2 bile concentrations were evaluated (0.2 % and 0.4 % w/v). The same strains that showed growth at low pH (*Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 and *Lactobacillus delbrueckii* subsp. *bulgaricus*), also showed positive growth to bile salt concentrations. It has been

reported that lactic acid bacteria of the genus *Lactobacillus* and *Leuconostoc* are capable of producing the enzyme known as hydrolase bile salt (HBS) which catalyzes the hydrolysis of conjugated bile salts with glycine and taurine allowing that bacteria to survive to contact with bile in the gastrointestinal tract [21,38].

It have been reported that during the passage through the gastrointestinal tract, the bacteria come into contact with a number of enzymes (such as α-amylase which is present in salivary glands and stomach, the trypsin present in the duodenum, and proteases in the small intestinal lumen) that affect their viability of them to reach the target organ. It was decided to assess bacterial ability to survive acidic conditions and bile salt, and to grow in the presence of these enzymes. Strains *Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 and *Lactobacillus delbrueckii* subsp. *bulgaricus* were incubated in the presence of different enzymes at a concentration of 10^8 CFU/mL at the end of the experiment. No significant change was detected in the CFU/mL of assessed strains, which indicates that the bacteria isolated in this study tolerate the presence of these enzymes. One of the most accepted criteria for the selection of probiotic bacteria is the ability to adhere to gut epithelial tissue, as it is a step to intestinal colonization.

The physicochemical properties of the bacterial cell wall and the autoaggregation mechanism of characteristics involve proteins and lipoproteins located on the surface of cells are involved in aggregation, as well as forming the surface to which it adheres. The autoaggregation ability of probiotic strains seems to be a necessary requirement to ensure adherence to intestinal epithelial cells, occupying specific places to avoid potential colonization by pathogenic microorganisms [36]. In this regard, Sedimentation values of the strains under study, measured during a 4 h incubation period proved to be low according to data reported by Vallejo [47], with values lower than 65%. The highest adhesion value obtained was 39.6% corresponding to the strain *Leuconostoc mesenteroides* TW56, and the lowest value (23.6%) corresponding to the strain *Lactobacillus delbrueckii* subsp. *bulgaricus*. Moreover, physicochemical characteristics of the cell surface such as hydrophobicity may affect autoaggregation and adhesion of bacteria to different surfaces [5,35]. Additional to assessing the autoaggregation ability, the determination of surface hydrophobicity of the strains isolated by affinity for the organic solvent of cells grown in two-phase system water-organic solvent, as a predictive measure of its ability to adhere to epithelia, was also carried out. According to described by Sanchez *et al.*, [36], in their investigation, there is evidence that lipoteichoic acids provide hydrophobicity of the bacterial cell wall which is directly related to adherent strains. The highest percentage of hydrophobicity was 82.85% (*Lactobacillus fermentum* JCM3), followed of the strain by *Lactobacillus delbrueckii* subsp. *bulgaricus* with a percentage of 75.83%; and the strain with the lowest percentage of hydrophobicity was *Leuconostoc mesenteroides* TW56 with a hydrophobicity percentage of 77.65%. These hydrophobicity percentages can be considered as high, according to data published by Nader-Macias [31] which classified bacteria hydrophobicity percentage as high (51-100%), medium (30-50%) and low (0-29%). The results from this study are very similar to those found by Kaushik [19] which observed values of 47% and 57–58% respectively for that strains *L. johnsonii* LA1 and *L. acidophilus* LA7 isolated from human milk under identical conditions. *L. plantarum* isolated from goat showed surface hydrophobicity of 47–69% depending upon the solvent used Draksler [6]. Cell surface hydrophobicity of some strains of *L. johnsonii* and *L. acidophilus* has been reported as high as 23–88% and 74–95%, respectively [45]. However, some strains of lactobacilli including those from *L. acidophilus* group, which also includes *L. johnsonii*, showed surface hydrophobicity as low as 2–5% [41]. The large differences in the cell surface hydrophobicity could be due to variation in the level of expression of cell surface proteins among strains of a species as well as due to environmental conditions which could affect the expression of surface proteins [5,34]. On the other hand, ability to inhibit the growth of pathogenic microorganisms is one of the mechanisms by which lactic acid bacteria contribute to the protection of the host. Probiotic microorganisms produce antimicrobial substances such as lactic and acetic acids and acidification of the intestine helps to inhibit the proliferation of some pathogenic microorganisms; they are also sources of metabolites such as hydrogen peroxide, diacetyl and bacteriocins [45]. Antibacterial activity of strains *Leuconostoc mesenteroides* TW56, *Lactobacillus fermentum* JCM3 and *Lactobacillus delbrueckii* subsp. *bulgaricus* was assessed against common pathogens like *E. coli*, *Salmonella tiphy*, *Listeria monocytogenes* and *S. aureus*, the antibacterial activity of strains mentioned was measured as a zone of inhibition against these pathogens seeded in agar. The crude extract correspondig to the broth were bacteria growth overnight exhibited varying zones of inhibition depending upon the tested pathogen and isolated bacteria. *Leuconostoc mesentero*ides TW56 was able to inhibit *E. coli*, *Salmonella* and *S. aureus*; *L. delbrueckii* subsp. *bulgaricus* was only able to inhibit *E. coli*. and *L. fermentum* JCM3 showed inhibitory activity against *E. coli* and *Salmonella.*

Forestier [10] assessed the the antibacterial activities of cell-free Lcr35 supernatant was examined against some human pathogenic bacteria (*K. pneumoniae*, *Shigella flexneri*, *Salmonella typhimurium*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Clostridium difficile).* Strain *Lactobacillus casei rhamnosus* Lcr3 was able to inhibit growth of these pathogenic strains. Differences among strains of this study and the present one with respect to growth inhibition are in agree with to those reported byVignolo and Jacobsen [19,39], who reported that the inhibitory effect of *Lactobacillus* strains is variable even within a same species.

Because of the wide range of activity of *Lactobacillus casei rhamnosus* Lcr35, the antimicrobial mechanism involved is unlikely to be the production of classic bacteriocins, proteinaceous compounds produced by lactic acid bacteria with a bactericidal effect against taxonomically closely related bacteria [22]. In the present study, inhibitory activity was not detected in all pathogenic strains, due to and this we believe that this inhibitory effect is thought to be due a bacteriocin instead of lactic, or another, organic acid. If inhibitory activity

was due to an organic acid produced, we were inhibition was observed in all pathogenic strains.

Peréz *et al*., [33] describes in his study that lactic acid bacteria (homo- and heterofermentative produced organic acids such as lactic, acetic, butyric, and propionic acid) which reducing intestinal pH and preventing colonization by undesirable bacteria do not proliferate to such effect. Analysis by HPLC indicates that the selected strains are producing acetic, lactic and butyric acid, lactic acid being the main organic acid produced by three strains that showed to be tolerance to acidic conditions, bile salts and enzymes. *Lactobacillus fermentum* had the highest concentration of the three organic acids (5.59 g/L of lactic acid, 4.54 g/L of acetic acid and 4.21 g/L of butyric acid). The results for the other two bacteria were very similar but less than those of *L. fermentum*. According to data reported by Jayashree [18], the bacteria in the study indicate be as heterofermentative lactic acid is not the only source of acid production by these microorganisms, although the concentration of acid production has been different.

As has been stated above, the heterofermentative characteristic of the LAB isolated in this study indicates that lactic acid is not the only source of organic acids and the concentration each one attains is specific.

5. Conclusions

Five bacteria, being molecularly identified as *Lactobacillus fermentum* JCM3, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Leuconostoc mesenteroides* TW56, *Bacillus subtilis* and *Streptococcus salivarius* TM85 were isolated from human milk. From these isolated bacteria, the strains identified as *Lactobacillus fermentum* JCM3, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Leuconostoc mesenteroides* TW56 showed the ability to grow at low pH in the presence of bile salts, in addition to surviving gastric enzymes and showing ability to inhibit the pathogenic bacteria growth. Results obtained from strain *Lactobacillus fermentum* JCM3 allow us to assume that this bacteria has a potential as probiotics, however, it is important to continue the characterization in *vivo* to determine whether the above strain can be considered as probiotic microorganism to be used in gastrointestinal disorders.

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