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Otros

In vivo assessment and characterization of lactic acid bacteria with probiotic profile isolated from human milk powder

Evaluación y caracterización in vivo *de bacterias acidolácticas con perfil probiótico aisladas a partir de leche materna en polvo*

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Abstract

Introduction: breast milk (MH) contains nutrients and bioactive compounds for child development, including probiotic bacteria, which contribute to intestinal maturation. This benefit accompanies the individual until adulthood. There are new methods such as spray drying that give this compound a good conservation without loss of microbiota.

Objective: the aim of this study was to analyze the viability of lactic acid bacteria isolated from human milk with probiotic potential after the spray drying process, as well as to evaluate the possible adhesion in the colon of mice of the Balb/C strain after feeding them powdered human milk and a commercial formula milk.

Method: we isolated and identified the presence of lactic acid bacteria with possible probiotic potential in powdered human milk using the MALDI-TOF MS technique. Powdered human milk and a commercial formula milk were fed to mice of the Bald/C strain for 14 weeks. Glucose level and weight were measured in the mice. The feces were collected to verify the presence of lactic bacteria. The mice were sacrificed and their intestines were weighed, isolating the lactic acid bacteria both from the intestines and from the feces. The strains isolated from mice fed human milk were evaluated for their probiotic potential, analyzing their ability to inhibit pathogens, resistance to pH, temperature, adhesion, and hydrophobicity.

Results: the presence of *Lactobacillus fermentum* LH01, *Lactobacillus rhamnosus* LH02, *Lactobacullis reuteri* LH03, and *Lactobacillus plantarum* LH05 in powdered human milk was identified. All strains showed a possible probiotic profile due to the ability of bacteria to resist low pH, bile salts, and exposure to gastric enzymes, as well as their hydrophobicity and self-aggregation capacity, and their failure to show hemagglutination or hemolysis activity in a culture medium rich in erythrocytes. We observed that the consumption of powdered human milk prevented weight gain and constipation in mice.

Conclusions: after spray drying, strains with possible probiotic potential may be preserved in human milk. The consumption of powdered human milk with probiotic bacteria prevents constipation and weight gain in mice, when compared to those fed a commercial formula milk.

Keywords:

Human milk. Probiotic. Spray drying. Lactic acid bacteria. Probiotic activity.

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Resumen

Introducción: la leche materna (HM) contiene los nutrientes y compuestos bioactivos necesarios para el desarrollo infantil, incluidas bacterias probióticas, que contribuyen a la maduración intestinal.

Objetivo: el objetivo de este estudio fue analizar la viabilidad de las bacterias acidolácticas aisladas de la leche humana con potencial probiótico, después del proceso de secado, así como evaluar su posible adhesión en el colón de ratones (BAlb/C) alimentados con leche humana en polvo y leche de una fórmula comercial.

Método: se aislaron e identificaron mediante la técnica de Maldi-Tof-MS las bacterias acidolácticas con posible potencial probiótico en la leche humana en polvo. Se alimentó con leche humana en polvo y leche de una fórmula comercial a ratones de la cepa Bald/C durante 14 semanas. Se midieron el nivel de glucosa y el peso. Las heces se recolectaron para verificar la presencia de bacterias lácticas. Los ratones se sacrificaron y se pesaron los intestinos, aislando las bacterias lácticas tanto de los intestinos como de las heces. En las cepas aisladas de la leche humana se evaluó el potencial probiótico analizando su capacidad para inhibir patógenos, resistir distintos pH y temperaturas, adherirse y mostrar hidrofobicidad.

Resultados: se identificó la presencia de *Lactobacillus fermentum* LH01, *Lactobacillus rhamnosus* LH02, *Lactobacullis reuteri* LH03 y *L. plantarum* LH05 en la leche humana en polvo. Todas las cepas mostraron resistencia a los pH bajos, a las sales biliares y a la exposición a enzimas gástricas, así como una buena hidrofobicidad y capacidad de autoagregación. Además, no presentaron actividad de hemaglutinación o hemólisis en un medio de cultivo rico en eritrocitos. Observamos que el consumo de leche humana en polvo evita en los ratones el aumento de peso y el estreñimiento.

Conclusiones: después del secado por aspersión, las cepas con posible potencial probiótico pueden conservarse en la leche materna. El consumo de leche humana en polvo con bacterias probióticas evita el estreñimiento y el aumento de peso en los ratones, en comparación con los alimentados con leche de una formula comercial.

Palabras clave:

Leche humana. Probiótico. Secado por pulverización. Bacterias acidolácticas. Actividad probiótica.

INTRODUCTION

Breast milk represents an important source of microorganisms that use oligosaccharide substrates for their growth, favoring the development of the intestinal microbiota in children (1,2). Probiotic bacteria play a very important role in the early colonization of a baby's intestine since they protect it against diseases and at the same time confer immunomodulatory effects in its future life (3,4).

An example of this statement is the study by Maldonado et al. (5), which showed that the administration for 6 months of a strain of *Lactobacillus fermentum* CECT5716 from breast milk to infants led to a reduction in the incidence rate of gastrointestinal infections to 46 %, of infections of the upper respiratory tract to 27 %, and of the total number of infections to 30 %. Likewise, it has been studied that the administration of probiotic bacteria to the infant can exert beneficial effects against diarrheal and respiratory diseases, and can reduce the risk of developing some diseases such as diabetes or obesity (6).

Necrotizing enterocolitis is another of the serious diseases present in newborns that affect the colon. Bacteria such as *Lactobacillus reuteri* sp. and *Lactobacillus plantarum* sp. have been studied for their ability to combat this type of disease through various mechanisms. The former produces a potent compound called reuterin, which inhibits a broad spectrum of Gram-positive and Gram-negative bacteria, fungi, and protozoan microorganisms. In *Lactobacillus plantarum* sp. an ability to survive gastric transit and therefore colonize the intestinal tract of humans and other mammals has been observed, with a protective effect against pathogenic bacteria (1).

For those infants whose mothers cannot feed with their milk, there are human milk banks where milk is generally pasteurized to prevent potential transmission of pathogenic microorganisms. However, some authors have described negative effects with the application of this treatment (7,8,9). There is another treatment that could avoid the loss in this case of microorganisms considered beneficial to the health of newborns, namely spray drying, which is an economical method used in food industry. Milk dehydration permits long term preservation, provides more stability in microbiological terms as compared to fresh milk, and requires no refrigeration as well as a smaller storage volume (10). The relatively short drying time, compared to other preservation processes, makes dehydration suitable for heat-sensitive materials such as enzymes, blood plasma, and milk proteins (11).

Due to the advantages that spray drying offers to products such as milk, and the relevance of probiotic bacteria in human milk, in this study spray drying of human milk was used with the aim of preserving human milk in powder form while keeping the viability of bacteria with probiotic potential after the spray-drying process. Also, to evaluate the effect of feeding mice powdered human milk versus a commercial formula in order to observe its influence on the gut microbiota.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

The human milk (HM) samples used in this study were obtained from the Human Milk Bank at the Fray Antonio Alcalde Civil Hospital in Guadalajara, Mexico, where they were collected with the informed consent of healthy donor mothers. This study was approved by the Ethics and Research Committee at Fray Antonio Alcalde Civil Hospital in Guadalajara in May 2018.

For the *in vivo* study, a commercial formula was used and, according to the information on the label, every 100 g of powder contains 58.7 g of carbohydrates, 27.7 g of lipids, and 9.5 % g of protein.

SPRAY-DRIED HUMAN MILK

Human fresh milk was homogenized by stirring at 150 rpm at 25 °C, and subsequently dried with a LabPlant SD-Basic Spray Dryer (USA). Operation adjustments included: 2 mL/min feed rate, at 170 °C air inlet temperature and 80 °C air outlet temperature (12). A powdered product was obtained and, according to the nutritional analysis carried out, every 100 g of powdered human milk contained 6.25 ± 0.69 g of carbohydrates, 2.46 ± 0.46 g of lipids, and 1.53 ± 0.45 g of protein.

ANALYSIS IN BALB/C MICE

BALB-C/cAnNHsd substrain male mice, obtained from the UNAM-Envigo RMS Laboratory in Mexico City, were housed with light-dark cycles of 12/12 h at 22 ± 2 ºC and 50 ± 4 % humidity, with free access to food (Rodent Laboratory Chow, Purina Cat. 5001, Mexico) and water. The animals were handled with proper care, and used in accordance with the Rules for Research in Health Matters (Mexican Official Norms NOM-062-ZOO-1999, NOM-033-ZOO-1995) (13).

Mice weighing 20-25 g were used for this test, divided into three groups with 6 mice each: the control group (CG) was supplied with commercial feed plus water; the GPHM group was supplied with 50 µL of human milk powder, reconstituted with water every 12 hours; the GMF group was supplied with 50 µL of a powdered commercial milk formula, reconstituted with water every 12 hours. The dose of 50 µL of powdered human or commercial milk corresponds to 5 mg of milk/mice/day, which was calculated followed the manufacturer's instructions in the commercial powdered milk product.

Each mouse was weighed monthly (Precision Digital Analytical Scale 200 x 0.001 g Profession®, USA.) and blood glucose was measured monthly by pricking the tip of the mouse tail (Glucometer Kit On Call Plus Total Medic On Call®, Mexico). Mice feces were collected every week to perform total microbiological counts and LAB isolation. After three months, the mice were sacrificed using the decapitation euthanasia method, with prior intraperitoneal sedation using sodium pentobarbital at 40 mg/kg of live weight, according to the specifications of the American Veterinary Medical Association (14). The organs were removed, and an intestinal scraping was subsequently performed to isolate and identify mainly lactic bacteria in MRS agar.

MICROBIOLOGICAL ANALYSIS

The microbiological analysis of human milk was performed on 1 mL samples of diluted milk; 1 mL of each dilution was taken and added to a Petri dish containing potato dextrose agar (PDA; Difco TM®, BD 232100, USA), Standard count agar (SCA; SIGMA-ALDRICH®, Canada), MacConkey agar (Mc; BD Bioxon®, LOT 5839203, Mexico), and MRS agar (MRS; BD Bioxon®, LOT 6530221, Mexico). The plates were incubated (Hinotek DHP-9052 Heating Incubator, China) for 24 to 48 hours at 37 °C (under anaerobic conditions in the case of MRS agar), and PDA at 30 °C. After this time microbial growth was observed.

ISOLATION OF STRAINS

LABs were isolated from human milk before drying, powdered human milk, and stools and intestinal scrapings of mice, carried out by means of dilutions and the pour-plate method, until colonies of a pure strain were obtained. The isolated strains were subjected to tests such as Gram staining, catalase, and oxidase; subsequently, an inoculum matching a 2 McFarland Turbidity Standard equivalent was prepared, and a miniature system API® 50 CHL test was used. The diluted cultures were incubated for 48 hours at 37 °C (14).

IDENTIFICATION OF STRAINS

Strain identification ensued using MALDI-TOF MS, which was performed at the Centre for Microbiological and Immunomolecular Diagnostics in Guadalajara, Jalisco, Mexico. For molecular biology identification, the 16S rDNA ribosomal gene was used as molecular marker. DNA extraction was carried out following the instructions for Gram-positive bacteria of a Genomic DNA Purification Kit (Wizard Promega®, A1120, Canada) on a 1 mL overnight culture. The 16S gene was amplified using 28F and 1492R primer base pairs. The polymerase chain reaction (PCR) products were purified using a GeneJET PCR purification kit (Thermo Scientific, K0702, USA). Amplicons were sequenced at the Institute of Biotechnology, Mexican Autonomous National University (UNAM), México. The sequences were analyzed with the NCBI BLASTN web site (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastn), and the MEGA 6 software (Molecular Evolutionary Genetics Analysis X Version 10.1, available at: https://www.megasoftware.net/) was used for constructing phylogenetic trees (15).

DETERMINATION OF POTENTIAL PROBIOTIC PROPERTIES

Strains isolated and identified as lactic acid bacteria (LAB) were grown in MRS broth incubated at 37 °C for 24 h. Then, the culture was adjusted to a 0.5 McFarland Turbidity Standard equivalent of 10^8 CFUs/mL using sterile MRS culture medium for dilution and adjustment of cell concentration, and subsequently used to carry out the following studies.

pH resistance

The pH resistance test was carried out according to Aguilar-Uscanga et al. (16), with some modifications. The pure strains were seeded in 5 mL MRS broth (pH 6.5) for 24 hours at 37 °C to obtain biomass. The cultures were centrifuged for 10 min at 5000 rpm to

obtain the bacterial pellet, which was resuspended in MRS broth at pH 2 and 3. The cultures at different pH values were incubated at 37 °C for 4 h, and subsequently a 1-mL sample (10^8 CFUs/mL) was plated on MRS agar using the pour-plate method, incubating the plates at 37 °C for 48 hours to observe LAB growth.

Bile salt tolerance

The study of LAB tolerance to bile salts was carried out using the method described by Pieniz et al. (17), with modifications. The cultures in MRS broth were centrifuged (Hermle, Z300K, Labortechnik, Germany) at 5000 rpm for 10 min at 4 °C, and the cell pellet was dissolved in new MRS media supplemented with 0.4 % and 0.5 % bile salt, and further incubated at 37 °C for 4 h. After incubation, the viable cell count was determined on MRS agar by the pour-plate method, expressed as CFU/mL.

Enzyme resistance

For evaluation of enzyme resistance, the method of Botes et al. (18) was used with some modifications. Strains were cultured in MRS broth for 24 h at 37 °C. The cultures were centrifuged at 5000 rpm for 10 min at 4 °C, the pellet washed with 0.2 M PBS buffer, pH 7.0, and resuspended in 2 mL of the same buffer. The reaction mixture contained 100 mL of cell suspension at 10^8 CFUs/mL. Strain cultures were evaluated with different enzymes: α-amylase (300 U/mg *Aspergillus oryzae*); proteinase (*Aspergillus melleus*, Type XXIII), and trypsin (pancreas portion, Type II-S). All enzymes were obtained from SIGMA-ALDRICH®, Canada. The different cultures with these enzymes were incubated at 37 °C for 4 h, and subsequently a 1 mL sample was plated on MRS agar using the pour-plate method, incubating the plates at 37 °C for 48 hours to observe LAB growth (18).

Antimicrobial effects

The evaluation of antimicrobial effects by the bacteria isolated from HM against indicator strains, including *E. coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* ATCC 49217, *Listeria monocytogenes* HPB 2739, and *S. aureus* ATCC 29213, was carried out by means of the pour-plate technique (16), with Mueller-Hinton agar inoculated with 10^6 CFUs/mL of each indicator strain. A series of wells were made in the agar, which contained the indicator strain, and 80 µL of supernatant from a previously MRS culture were added for each BAL isolated from HM. Afterwards, the plates were incubated at 37 ºC for 24 h for the observation of inhibition halos. The inhibitory activity of the crude extract against pathogenic bacteria was taken as positive in case of growth-inhibition clear zones appeared around the pathogenic bacteria. Amoxicillin 250 mg and clavulanic acid 62.5 mg (concentration of the antibiotic in 5 mL) were used as positive control (Augmentin, GlaxoSmithKline, England No. 3888792).

Hydrophobicity test

The hydrophobicity test was carried out according to Del Re et al. (19). LABs isolated from HM were grown in MRS broth at 37 °C for 24 h, were then centrifuged at 5000 rpm for 10 min at 4 °C to separate the biomass. The cells were washed with 0.2 M PBS buffer, pH 7.2, and subsequently resuspended in 4 mL of the same buffer. Xylene (0.8 mL) was added to the bacterial suspension, mixed for 2 min and incubated at room temperature for 20 min; the aqueous phase was removed, and the optical density at 600 nm was measured with a UV spectrophotometer (Scinco, S-3100, Lab Pro Plus Software, Korea). The decrease in absorbance of the aqueous phase was taken as a measure of cell surface hydrophobicity. The hydrophobicity percentage (%H) was calculated according to the equation:

$$(\%)\ \text{Hydrophobicity} = (A0\text{-}A\ /\ A0) * 100$$

where A0 = optical density before xylene extraction, and A = optical density after xylene extraction.

Autoaggregation

Autoaggregation capacity was determined according to Kos et al. (20). The BALs isolated from HM were grown in MRS broth (5 mL), incubated (24 h, 37 °C), taken to a concentration of 1 x 10^8 CFUs/mL, washed with phosphate buffer (0.2 M), and centrifuged at 5000 rpm for 15 min at 4 °C; the supernatant was discarded, 0.2 M PBS (2 mL) was added, mixed, and a 0.1 mL sample was extracted, which was mixed with 1.9 mL of PBS; the initial OD was read at 600 nm with a spectrophotometer (Scinco, S-3100, Lab Pro Plus Software, Korea), microorganisms were incubated at room temperature for 4 hours, with 0.1 mL samples being collected at each hour, and OD was read under the conditions already mentioned. The percentage of autoaggregation was calculated using the equation:

$$\%\ \text{Autoaggregation} = 1 - \frac{At}{A0} * 100$$

Where At = optical density at 1, 2, 3, 4 h, and A0 = optical density at 0 h.

Hemagglutination and hemolysin activity

The determination of both hemagglutination and hemolysin activities was started following the method described by Devaki & Kandi (21), using an overnight culture of 5 mL of brain-heart infusion broth BD® for both tests. The following day this culture was centrifuged at 10,000 rpm for 5 minutes, collecting only the sediment, which was suspended in PBS (5 x 10^{10} cells/mL concentration). A bacterial suspension (25 mL) and 25 mL of chilled red blood cells at a concentration of 3 % were added to microtiter polystyrene plates, and incubated at 4 °C for 1 h; hemagglutina-

Table I. Microbiological analysis

Treatment	Liquid human milk				Spray-dried human milk			
Culture	MacConkey	PDA	SCA	MRS	MacConkey	PDA	SCA	MRS
CFU/mL	95 ± 45	47 ± 18	393 ± 52	348 ± 33	0	0	12 ± 3	15 ± 1

PDA: potato dextrose agar; SCA: standard count agar; MRS: Man, Rogosa, & Sharpe agar; CFU: colony forming unit. The experiments were performed in triplicate.

Table II. Probiotic activity of lactic acid bacteria strains isolated from human milk powder, formula milk, and mice feces and intestine

Strains	pH		Bile salts %		Enzyme (+/-)			Antimicrobial effect				%H
	2	3	0.2	0.4	α-amylase	Trypsin	Proteinase	*E. coli*	*Listeria*	*Salmonella*	*S. aureus*	
L. fermentum LH01[1]	+	+	+	+	+	+	+	+	+	+	+	91.6
L. rhamnosus LH02[1]	+	+	+	+	+	+	+	+	+	+	+	66.4
L. plantarum LH03[2]	+	+	+	+	+	+	+	+	+	+	-	65.1
L. reuteri LH05[1]	+	+	+	+	+	+	+	+	+	+	+	34.9
B. subtilis sp[3]	+	+	+	+	+	+	+	+	+	+	-	34.4

%H: hydrophobicity; [1]Isolated from human milk powder (HMP); [2]Isolated from liquid human milk (LHM); [3]Isolated from mice stool and intestine of control group (CG). The experiments were performed in triplicate.

tion was subsequently observed. For the hemolytic activity, 200 µL of culture were introduced into each well in the microtiter plate, and 200 µL (1 % W/V) of washed human red blood cells from different blood groups were added. The plate was covered with aluminum, incubated at 37 °C overnight, and hemolysis activity was observed. Only when there was no hemolysis after 24 hours, the plate was cooled down to 6 °C for an additional 24 hours, and hemolysis was observed. Controls were 200 µL of sterile brain-heart infusion broth and 200 µL of washed human red blood cells from different blood groups.

STATISTICAL ANALYSIS

Data were analyzed in triplicate and for each replicate 3 samples were analyzed. The data were reported as mean ± standard deviation (SD) or standard error of the mean (SE). The data obtained from the experiments were subjected to a one-way analysis of variance (ANOVA) using IBM SPSS software Statistics, version 24 (IBM Inc., Chicago, IL, USA) and GraphPad Prism v6.01 (2014 Inc. 2236 Beach Avenue Jolla, CA 92037, USA). Duncan's test was used to determine any significant differences between mean values at $p \leq 0.05$.

RESULTS

Table I shows the growth of microorganisms found in liquid human milk and dry human milk in different media (MacConkey, PDA, SCA, and MRS agar); it shows there was a significant reduction of CFU/mL in human milk after spray drying ($p < 0.05$) by approximately 98 % of total bacterial content; however, LABs manage to survive after spray drying.

The probiotic activity of microorganisms isolated from human milk is shown in table II. All strains were shown to be potentially probiotic, although neither *L. plantarum* LH03 nor *B. subtilis* sp. could inhibit the growth of *S. aureus*; also *B. subtilis* sp. showed the lowest percentages of hydrophobicity in comparison with the rest of strains, and something similar happened with *L. reuteri* LH05. The results of autoaggregation as part of the tests performed to measure probiotic potential are shown in figure 1, which shows that the highest values were obtained by *L. reuteri* LH03 (57.2 %)

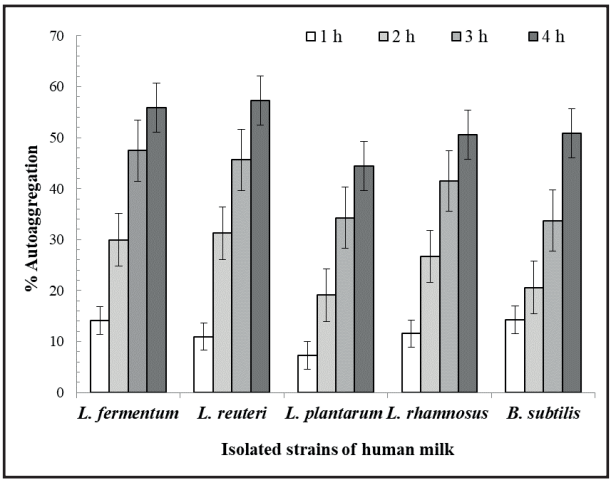

Figure 1.
Autoaggregation capacity.

followed by *L. fermentum* LH01 (36.6 %) and *L. plantarum* LH05 (53.6 %). On the other hand, the bacteria isolated did not present hemagglutination or hemolysis in a culture medium rich in erythrocytes. This result is considered a safety criterion, allowing to identify bacteria whose activity does not trigger any blood conditions that may compromise the health of the consumer.

Table IV shows the names of the strains that were identified using MALDI-TOF MS in liquid human milk, powdered human milk, formula milk, mice stools and mice intestine. In the case of formula milk it was only possible to isolate a strain, while in powdered human milk at least half of the strains isolated from liquid human milk could be preserved; these microorganisms were also found to have survived in the stools and intestine of the study mice. This result is important because, according to table II, they possibly have probiotic potential. Thanks to the molecular identification of microorganisms, the strains isolated from human milk could be verified by phylogenetic analysis, and a phylogenetic tree was made to locate each bacterial strain found with a 99 % similarity (Fig. 2).

The *in vivo* study was carried out with a group of 6 mice that were fed powdered human milk (HMP), a group of 6 mice fed an infant formula milk (FM), and a group of 6 mice fed a staple food for mice (CG) or control group. Weight and blood glucose levels were measured in these mice for 3 months (Table III). Glucose levels showed no significant differences ($p > 0.062$) between mice groups; however, regarding weight, the mice fed the formula

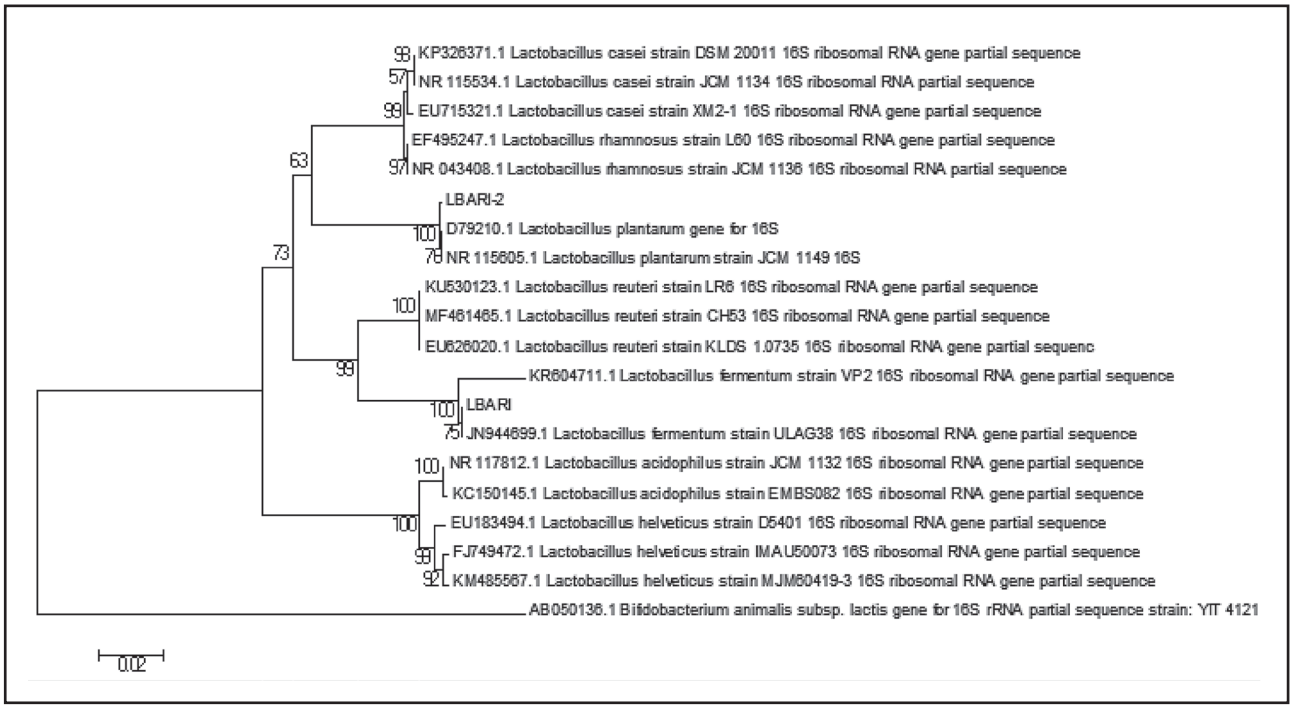

Figure 2.

Phylogenetic tree of strains identified by MALDI-TOF MS.

Table III. Measurement of weight and glucose content in mice fed spray-dried human milk and a commercial formula milk

	Weight (g)			Glucose (mg/dL)		
Month	**HMP**	**FM**	**CG**	**HMP**	**FM**	**CG**
0	26.42 ± 1.83	26.59 ± 1.33	25.75 ± 1.32	83.2 ± 13.44	83.1 ± 12.55	83.4 ± 12.43
1	26.26 ± 1 1.89	26.89 ± 1.43	28.54 ± 1.72	82.6 ± 16.16	98.4 ± 7.63	103.8 ± 11.88
2	26.59 ± 2.33	27.88 ± 1.31	29.57 ± 1.26	85.8 ± 22.55	97.6 ± 13.11	108.2 ± 6.91
3	26.44 ± 2.06	29.36 ± 1.61	30.22 ± 1.40	85.6 ± 14.81	98 ± 15.68	105 ± 8.52

HMP: human milk powder; FM: formula milk; CG: control group. The number of mice in each group was 6.

Table IV. Identification of strains in liquid human milk, powdered human milk, formula milk, and mice intestine and stools

Liquid human milk	Powdered human milk	Formula milk	Mice stools			Mice intestine		
LHM	HMP	FM	HMP	FM	CG	HMP	FM	CG
L. fermentum LH01	*L. fermentum* LH01	*L. reuteri* sp.	*L. fermentum* LH01	*L. reuteri* sp.	*B. subtilis*	*L. fermentum* LH01	*L. reuteri* sp.	*B. subtilis* sp.
L. rhamnosus LH02	*L. rhamnosus* LH02		*L. rhamnosus* LH02	*B. subtilis* sp.	*B. cereus* sp.	*L. rhamnosus* LH02	*B. subtilis* sp.	*S.haemolyticus* sp.
L. plantarum LH03	*L. reuteri* LH05		*L. reuteri* LH05			*L. reuteri* LH05		*B. cereus* sp.
L. paracasei LH04			*B. subtilis* sp.			*B. subtilis* sp.		
L. reuteri LH05								
L. brevis LH06								

LHM: liquid human milk; HMP: group of mice fed with human milk powder; FM: group of mice fed with a commercial formula milk; CG: control group.

milk and the control group increased their weight (by 11 %) when compared to mice fed powdered human milk.

As regards the weight of both intestines (small and large) in order to evaluate the different treatments, in small intestine weight no significant differences were observed between groups; however, the large intestine of the mice fed a formula milk (FM) showed a significant weight gain by 35 %, when compared to the control group (CG) and to the group fed human milk powder (HMP).

DISCUSSION

According to table I, the result clearly shows how spray drying reduces total bacterial content by approximately 98 %, and that bacteria with probiotic potential manage to survive spray drying ($p \leq 0.05$). It is important to mention that the colonies observed in PCA were the same LAB colonies observed in MRS agar. All the lactic acid bacteria isolated were Gram-positive, catalase- and oxidase-negative organisms, and tested positive for *Lactobacillus* with API tests.

Something similar was reported by Gardiner et al. (22), who evaluated the survival of *Lactobacillus paracasei* NFBC 338 and *Lactobacillus salivarius* UCC118d in 20 % (w/vol) skimmed cow milk dried by spraying. The results showed that *L. paracasei* NFBC338 is considerably more resistant (3.23×10^9 CFUs/g) to heat than *L. salivarius* UCC18 (5.23×10^7 CFUs/g), demonstrating that spray drying can be cost-effective to produce large quantities of LABs at low cost for the food industry, which could be used also for the preservation of probiotic cultures in milk.

In general, all strains showed resistance to pH 2 and 3, as well as to enzymes and bile salts; therefore, they could be considered bacteria with probiotic potential since tolerance to adverse conditions along the gastrointestinal tract is key to ascertain the probiotic capacity of any given microorganism. In the quest to acquire better probiotics with bioactive properties to improve health, diverse lactic acid bacteria (*Lactobacillus fermentum*, *Lactobacillus pentosus*, and mainly *Lactobacillus plantarum*) have been isolated from unconventional sources (plants, fermented foods, beverages, and human feces), that are often attractive targets for the industry (23). However, this study demonstrates that bacteria with such probiotic characteristics may be obtained from human milk.

Regarding antimicrobial activity tests, all the bacteria isolated in this study showed antimicrobial activity against pathogen strains (Table II), except *L. plantarum* LH03 (isolated from human milk) and *B. subtilis* sp., (isolated from mice stools and the intestine of mice in the control group), which failed to show antimicrobial activity against *S. aureus* sp. This may happen because Gram-positive bacteria such as *S. aureus* are covered by a mucoid layer that provides enhanced adherence to the intestinal mucosa, and also serves as a kind of camouflage to avoid being recognized and phagocytized (24). Therefore, these organisms are more difficult to eliminate. In the case of *B. subtilis* sp., the organism has no probiotic characteristics but has been documented as a potential antagonist due to its production of lytic enzymes, antibiotics, and substances with biocidal activity (25). The ability to inhibit the growth of pathogenic microorganisms is one of the mechanisms by which lactic acid bacteria contribute to host protection, which they provide by producing various substances such as acids with antimicrobial effects, including organic acids, hydrogen peroxide, and bacteriocins (26).

The measurement of autoaggregation is important to ascertain the ability of bacteria to adhere to the intestinal epithelial tissue, a step in the process of intestinal colonization by the microorganism, and to block potential colonization by pathogens (27). Kos et al. (28) investigated the aggregation and adhesiveness properties of *Lactobacillus acidophilus* M92. Their results showed that these bacteria had a strong autoaggregating (40 % to 70 %) phenotype, and a high degree of hydrophobicity (56 % to 73 %). In our study, strains isolated from the intestines of mice fed with HM demonstrate that there is a relationship between autoaggregation and adhesiveness, maybe mediated by protein components on the cell surface.

With these results it is possible to suggest that spray drying could be considered a good treatment for milk preservation, as it has been recently reported that this process permits to preserve the viability of probiotic cultures (29), and even significantly improves the resistance of these bacteria to simulated gastrointestinal digestion, as compared with fresh cultures. The authors conclude that spray drying is a promising technological tool for the development of probiotic cultures in milk, as long as the bacteria present in the milk are able to overcome the thermal treatment involved in dehydration (30).

Concerning the weight of the intestines, it shows that formula milk possibly agglomerated in the large intestine or was probably digested more slowly than human milk, causing increased weight in the colon of mice. The same problem has been seen in children who consume this kind of products, causing constipation and poor nutrient absorption. In general, it is known that babies fed with breast milk are less susceptible to constipation, while those fed a formula milk have a greater tendency to produce hard stools when compared to breast-fed infants. At the age of six months, some infants experience symptoms of constipation due to their being fed with formula milk in the first few months after birth (31). Constipation is a common problem in commercial formula-fed infants. Unfortunately, the marketing of infant formulas is promoted worldwide as a harmless, similar alternative to breast milk. Studies carried out by Baker et al. (32) show worrying increases in the use of infant formulas to replace human milk, and report that from 2003 through 2018 total infant formula sales grew by 40.8 % worldwide; the authors also observed that using an infant formula represents a dramatic dietary change that results in problems for the proper development and health of infants.

Nowadays, infant formulas supplemented with probiotics, prebiotics, and other additives are manufactured to improve intestinal tolerance to enteral feeding in preterm infants, despite uncertainties regarding their efficacy (33). The ESPGHAN Committee on Nutrition considers that formula milk supplementation with probiotics and/or prebiotics is an important field of research; nevertheless, it is of the utmost importance that well-designed, carefully controlled trials are performed, with relevant inclusion and exclusion criteria and adequate sample sizes, to achieve a good result (34).

CONCLUSIONS

L. fermentum LH01*, L. rhamnosus* LH02*, L. reuteri* LH03, *and L. plantarum* LH05, isolated from human milk before and after spray-drying treatment, show a potential probiotic profile because of the ability of these bacteria to resist low pH, bile salts, and exposure to gastric enzymes, as well as as due to their hydrophobicity and self-aggregation capacities, and absence of hemagglutination or hemolysis in a culture medium rich in erythrocytes. The *in vivo* results of this work showed that the consumption of powdered human milk with probiotic bacteria prevents constipation and weight gain in mice when compared to those fed a commercial formula milk. However, these results could be different in humans, and there is a need to reproduce this trial in humans. After spray drying, bacteria with probiotic characteristics were preserved in human milk; therefore, this process should be considered a new method to be implemented in human milk banks.

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