Characterization of potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum

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ABSTRACT

Breast milk is the main source of nutrition for infants; it contains considerable microflora that can be transmitted to the infant endogenously or by breastfeeding, and it plays an important role in the maturation and development of the immune system. In this study, we isolated and identified lactic acid bacteria (LAB) from human colostrum, and screened 2 strains with probiotic potential. The LAB isolated from 40 human colostrum samples belonged to 5 genera: *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, and *Staphylococcus*. We also isolated *Propionibacterium* and *Actinomyces*. We identified a total of 197 strains of LAB derived from human colostrum based on their morphology and 16S rRNA sequence, among them 8 strains of *Bifidobacterium* and 10 strains of *Lactobacillus*, including 3 *Bifidobacterium* species and 4 *Lactobacillus* species. The physiological and biochemical characteristics of strains with good probiotic characteristics were evaluated. The tolerances of some of the *Bifidobacterium* and *Lactobacillus* strains to gastrointestinal fluid and bile salts were evaluated in vitro, using the probiotic strains *Bifidobacterium lactis* BB12 and *Lactobacillus rhamnosus* GG as controls. Among them, *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 showed survival rates of 97.25 and 78.33% after digestion for 11 h in artificial gastrointestinal juice, and they exhibited growth delays of 0.95 and 1.87 h, respectively, in 0.3% bile salts. These two strains have the potential for application as probiotics and will facilitate functional studies of probiotics in breast milk and the development of human milk-derived probiotics.

Key words: human colostrum, lactic acid bacteria, isolation/identification, artificial gastric juice, bile salt

INTRODUCTION

Human breast milk contains many essential nutrients, including immune factors, lipids, oligosaccharides, microRNAs, hormones, and many other biologically active components (Hennet and Borsig, 2016); it is considered the best source of nutrition for infants (Verhasselt, 2010). Human breast milk was long thought to be aseptic, and the presence of bacteria was presumed to be a result of contamination (Murphy et al., 2017). The microorganisms were thought to originate from pregnant women with mastitis or from the skin, vagina, intestine, or oral cavity (Kvist, 2010). However, at least some of the bacteria present in the maternal gut could reach the mammary gland via an endogenous route (Rescigno et al., 2001; Martín et al., 2004; Fernández et al., 2013). The first descriptions of the bacterial diversity of human milk from healthy women in 2003 were based on in vitro culturing methods (Heikkilä and Saris, 2003; Martín et al., 2003). Since then, other studies of bacterial communities of human milk using culture-dependent and culture-independent methods have been published (Kozak et al., 2015; Ruiz et al., 2019).

Human milk from healthy women contains bacterial flora of approximately 10^3 to 10^4 cfu/mL and is a persistent source of commensal bacteria for infants (Arici et al., 2004). More than 200 bacterial taxa have been isolated from breast milk, principally facultative anaerobes such as *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Lactobacillus*, and *Bifidobacterium* (Heikkilä and Saris, 2003; Sakwinska et al., 2016). Viable commensally mutualistic or potentially probiotic bacteria are present in healthy human milk (Fernández et al., 2013). They contribute to the maturation of the digestive and immune systems in the developing infant and play an irreplaceable role in promoting growth and

Received September 17, 2019.

Accepted December 26, 2019.

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development. Bifidobacteria in particular participate in defense against pathogens by preventing adhesion to the gut epithelium during a vulnerable period in life, when the infant's own defenses are immature (Bode, 2012; Chiu et al., 2014). In addition, breast milk bacteria can maintain a healthy gut microbiota and have the potential to promote maternal and infant health, including the prevention or treatment of mastitis (Arroyo et al., 2010; Fernández et al., 2016), the promotion of normal gut bacterial colonization in preterm neonates (Escribano et al., 2014), and the prevention of diarrhea (Shin et al., 2018).

Several species of *Bifidobacterium* (e.g., *Bifidobacterium breve* and *Bifidobacterium longum*) and *Lactobacillus* (e.g., *Lactobacillus gasseri*, *Lactobacillus salivarius*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus fermentum*) have been isolated and identified (Amezcuca López et al., 2019; Ruiz et al., 2019). The European Food Safety Authority granted them Qualified Presumption of Safety status, and the United States Food and Drug Administration granted them Generally Recognized as Safe status.

Thus far, numerous probiotics have been isolated from the human gastrointestinal tract and from dairy products. However, lactic acid bacteria (LAB) with excellent probiotic properties from human milk have rarely been identified. If bacteria with the ability to confer health benefits were isolated from breast milk, they would be attractive probiotic organisms. These bacteria would fulfill some of the main criteria generally recommended for human probiotics, including human origin, a history of safe, prolonged intake by infants, and adaptation to dairy substrates (Martín et al., 2003). Such strains could be used in the development of probiotic products and health food. Therefore, we isolated and identified LAB in human milk and screened them for potential probiotic strains.

In the present study, we isolated LAB and bifidobacteria from human colostrum using a traditional pure culture method, and we conducted gastric acid and bile salts tolerance tests in a simulated gastrointestinal environment in vitro. We evaluated the physiological and biochemical characteristics of 2 potential probiotics, such as carbohydrate utilization and growth curve. The findings point to microbial species or strains that may be suitable for development as probiotics for infants who cannot breastfeed.

MATERIALS AND METHODS

Sample Collection

Samples of human colostrum were collected from 40 healthy lactating volunteers of Han and Mongolian eth-

nicity in Inner Mongolia. Samples were collected using 50 mL sterile sampling tubes or storage bags, stored at 4°C using an ice box, and transported to the laboratory within 2 h for bacterial isolation.

Isolation and Purification of LAB

Breast milk samples of 0.5 mL were diluted to 10^{-3} and 10^{-4} in 10-fold steps in 4.5 mL of PBS (8.0 g of NaCl/L, 0.2 g of KH_2PO_4 /L, and 1.15 g of Na_2HPO_4 /L; pH 7.2), and 200 μL of each sample was coated on triplicate sets of modified de Man, Rogosa, and Sharpe agar (MRS, Oxoid, Thermo Fisher Scientific Inc., Basingstoke, UK) and M17 agar (Oxoid), and incubated anaerobically (80% N_2 , 10% H_2 , and 10% CO_2) at 37°C for 72 h. The MRS agar (Oxoid) was modified by adding 0.05% L-cysteine and 0.05 mg/mL mupirocin (Qingdao Hope Bio-Technology Co. Ltd., Qingdao, China; Ferraris et al., 2010). The colony morphology, size, color, transparency, and surface roughness were recorded (Holzapfel et al., 2001). Colonies were purified on the corresponding separation agar medium and incubated at 37°C for 72 h. Single colonies were subjected to Gram stain and cell morphology was observed under a microscope. To assay catalase activity, a single colony was mixed with a droplet of 3% H_2O_2 (Beijing Chembase Technology Co. Ltd., Beijing, China) using an inoculating needle; a lack of bubbles indicated a negative result (Angmo et al., 2016). Gram-positive and catalase-negative strains were inoculated into liquid separation medium and incubated anaerobically at 37°C for 24 h. To prepare frozen stocks, pure cultures were centrifuged at $4,000 \times g$ (M2 rotor, model T centrifuge; Regenstein Centrifuge Co., New York, NY) at room temperature (22–25°C) for 10 min and washed twice with PBS; the bacterial slurry was mixed with a cryoprotective agent (10% nonfat milk, 0.1% sodium glutamate, and 0.05% L-cysteine) and stored at -80°C for long-term preservation.

Identification of LAB by 16S rRNA Gene Sequencing

An aliquot of liquid culture of the LAB (2 mL) was centrifuged at 12,000 rpm for 1 min, the supernatant was discarded, and the microbial biomass was collected. The DNA was extracted using a DNA Extraction Kit [Tiangen Biotech (Beijing) Co. Ltd., Beijing, China], and the concentration and purity of the genomic DNA were measured using an ND-1000 micro UV spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Then, 50 μL of purified DNA was diluted to 100 ng/ μL for 16S rRNA amplification and sequencing using an automatic thermal cycler (MJ Research PTC-200, MJ Research Inc., Watertown, MA) with universal primers for the 16S rRNA gene: 27 forward

(5'-AGAGTTTGATCCTGGCTCAG-3') and 1495 reverse (5'-CTACGGCTACCTTGTACGA-3'; Delbès et al., 2007). Each PCR reaction (50 µL) consisted of 1.5 µL (10 µmol/L) of upstream and downstream primers, 2.0 µL of genomic DNA, 5.0 µL of 10× buffer, 4.0 µL of dNTP (2.5 mmol/L), 0.5 µL of *Taq* DNA polymerase, and 35.5 µL of distilled water. Then, PCR amplification was performed as follows: predenaturation at 94°C for 5 min, followed by 30 cycles (1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 2 min of extension at 72°C), with a final extension at 72°C for 10 min. The products were held at 4°C to prevent degradation. The PCR products were resolved by 1% agarose gel electrophoresis and visualized on a gel imager (Beijing Sage Creation Science Co. Ltd., Beijing, China), and a DNA band of approximately 1,500 bp was obtained from all isolated strains. Sequencing was performed by Majorbio Bio-Pharm Technology Corp. (Shanghai, China). The sequences were aligned using DNA-STAR software (DNA-STAR Inc., Madison, WI). A homology alignment analysis with the nucleic acid sequences of bacteria in GenBank was performed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A phylogenetic tree was constructed with a demarcation threshold of >99% homology by the neighbor-joining method using MEGA version 6.0 (Tamura et al., 2007).

Physiological and Biochemical Tests

Evaluation of Growth. The strains were inoculated (2%, vol/vol) in MRS broth medium (Oxoid) and incubated anaerobically at 37°C for 30 h. Viable bacteria were enumerated at 3-h intervals, and a growth curve was plotted (Feng and Chang, 2012). Three independent experiments were carried out per strain.

Analysis of Physicochemical Characteristics. Bacterial colony size and morphology were evaluated by Gram stain and catalase assay. The strains were inoculated (2%, vol/vol) into MRS broth medium (Oxoid), and their growth in air, acid (pH 4.5), alkaline (pH 9.6), high salts (6.5% NaCl), and at a range of temperatures (10, 15, 42, and 60°C for 30 min) was evaluated (Angmo et al., 2016). Next, 49 types of sugars that could be fermented by the strains were identified using a carbohydrate fermentation kit (France BioMérieux Sa Company, Marcy l'Etoile, France). Three independent experiments were performed per strain (Španová et al., 2015).

Preparation of Artificial Gastrointestinal Fluid

To prepare the artificial gastric juice, 3.0 g/L pepsin (Beijing Chembase Technology Co. Ltd.) in sterile

PBS was adjusted to pH 2.5 using 0.1 mol/L HCl that had been sterilized by passage through a 0.22-µm filter (Biosharp Biotechnology Co. Ltd., Hefei, China).

To prepare the artificial intestinal fluid, 1.0 g/L trypsin and 1.8% bile salts (both from Beijing Chembase Technology Co. Ltd.) in sterile PBS were adjusted to pH 8.0 with 0.1 mol/L NaOH and sterilized by filtration through a 0.22-µm membrane (Biosharp Biotechnology Co. Ltd.).

Evaluation of Acid and Bile Salt Tolerance

Acid Tolerance. Eight strains were incubated anaerobically in MRS broth medium (Oxoid) at 37°C for 24 h and continuously cultured for 3 generations. After centrifugation at $4,000 \times g$ for 10 min, the strains were collected, washed with sterile PBS, and resuspended in PBS to prepare bacterial suspensions. The bacterial suspensions were diluted, and the cell density (0 h) was determined by viable count. The bacterial suspensions were mixed with artificial gastric juice and incubated at 37°C for 3 h. The percentage of viable bacteria was determined by plate counting. The artificial gastric juice containing bacteria, incubated for 3 h at pH 2.5, was then transferred to artificial intestinal fluid and incubated anaerobically for 8 h at 37°C. Finally, the survival rate (%) was calculated by plate counting (Bao et al., 2010). The mean of 3 independent experiments was calculated:

$$\text{Survival rate (\%)} = N_1/N_0 \times 100,$$

where N_0 is the number of viable bacteria at 0 h (cfu/mL) and N_1 is the number of viable bacteria in artificial gastrointestinal fluid at 3 and 11 h (cfu/mL).

Bile Salt Tolerance. Fifty microliters of the strain preserved in the skim milk protectant was collected for activation. Strains with better tolerance to artificial gastrointestinal fluid were selected for further testing. Following incubation, the test strains were inoculated (1.0%, vol/vol) into MRS broth medium (Oxoid) containing bovine bile salts (0.2% sodium thioglycolate and 0.3% oxgall; Beijing Chembase Technology Co. Ltd.); MRS broth medium (Oxoid) without bovine bile salts (0.2% sodium thioglycolate) was used as the control. The strains were incubated anaerobically at 37°C, and the optical density (OD) at 620 nm was measured hourly using a UV-visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan) until it had increased by ≥ 0.3 units. The lag period was used to evaluate the ability of the strains to tolerate bile salt. Each strain was tested independently in triplicate. The lag period is defined as the difference in the time required for the

absorbance to increase by ≥ 0.3 units between the test and control strains (Walker and Gilliland, 1993).

Statistical Analysis

Results are expressed as mean $\pm$ standard deviation. The statistical significance of the differences was evaluated by 1-way ANOVA using Data Processing System software version 7.05 (Zhejiang University, Hangzhou City, China). Differences were considered significant at $P < 0.05$. Graphical presentations were generated by Origin software version 8.5 (OriginLab Corp., Hampton, MA).

RESULTS

Isolation and Identification of LAB

In total, 197 strains were isolated from 40 human colostrum samples. Sequence alignment was performed using BLAST, and species identification was performed by phylogenetic tree construction with MEGA 6.0 (Figure 1). The isolated bacteria were of the genera *Staphylococcus* (26.90%), *Streptococcus* (37.06%), *Enterococcus* (2.54%), *Lactobacillus* (5.08%), *Bifidobacterium* (4.06%), *Propionibacterium* (1.02%), and *Actinomyces* (1.52%; Figure 2). Among them, *Lactobacillus* and *Bifidobacterium* were abundant. The *Lactobacillus* species were *Lactobacillus oralis* (4 strains), *L. plantarum* (4 strains), *L. gasseri* (1 strain), and *L. rhamnosus* (1 strain). The *Bifidobacterium* species were *Bifidobacterium dentium* (2 strains), *Bifidobacterium animalis* (5 strains), and *B. animalis* ssp. *lactis* (1 strain). Sixteen identifiable *Streptococcus* species were isolated from human colostrum, including *Streptococcus gordonii*, *Streptococcus salivarius*, and *Streptococcus sobrinus*; the latter was the most abundant. Of the *Staphylococcus* species isolated from human colostrum, most were *Staphylococcus epidermidis*.

Based on the probiotic characteristics of *Lactobacillus* and *Bifidobacterium* strains, we screened 2 acid- and bile salt-tolerant strains. Based on their physiological and biochemical characteristics and 16S rRNA sequence analysis results, the 2 strains were identified as *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9, respectively.

Characteristics of the Strains

Colony Morphology. On modified MRS agar, *B. lactis* Probio-M8 produced convex, circular, milky-white colonies of 1 to 2 mm in diameter, with complete edges and a smooth surface (Figure 3A). Gram stain showed dumbbell bacilli, regular at both ends, with

many obvious nodes (Figure 3B). On MRS agar, *L. rhamnosus* Probio-M9 produced round milky-white or light-yellow colonies of 1 to 2 mm in diameter, with regular edges, a slightly raised center, and an opaque, smooth surface (Figure 4A). The Gram reaction was positive, and the strain was a short rod with regular ends that formed chains (Figure 4B). The morphology of the two strains was assessed using scanning electron microscopy (Figures 3C and 4C).

Physiological and Biochemical Variables. The physiological and biochemical properties of the 2 strains are shown in Tables 1 and 2. According to preliminary identification, Probio-M8 was a *Bifidobacterium* spp., which could grow in air and in 6.5% NaCl at pH 4.5 but not pH 9.6. As well, *B. lactis* Probio-M8 could grow at 10 to 42°C and use glucose, lactose, sucrose, and maltose efficiently and use ribose and D-xylose less efficiently. Probio-M9 was a *Lactobacillus* spp. that could grow in air and at pH 4.5, but not at pH 9.6 or in 6.5% NaCl. As well, *L. rhamnosus* Probio-M9 could grow at 42°C but not at low temperatures; it could use glucose, fructose, maltose, lactose, cottonseed sugar, xylose, melissaccharide, and other carbohydrates.

Acid Tolerance. Probiotics such as *Lactobacillus* and *Bifidobacterium* enter the mouth, pass through the acidic environment of the stomach, and colonize the gut (Tuo et al., 2012). We evaluated the acid tolerance of 18 *Lactobacillus* and *Bifidobacterium* strains at pH 2.5 for 3 h, among which 8 strains showed good tolerance. Eight strains were subjected to transport tolerance testing in artificial gastric juice at pH 2.5 and in artificial intestinal juice at pH 8.0. As shown in Table 3, *B. lactis* BB12, *L. rhamnosus* GG, and *L. plantarum* P-8 were used as controls. In the simulated gastrointestinal environment, only *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 showed high tolerance (survival rate $>80\%$). The strain *B. lactis* Probio-M8 was exposed to artificial gastric juice at pH 2.5 for 3 h (survival rate 85.38%) and artificial intestinal juice at pH 8.0 for 8 h (survival rate 97.25%); its survival rate was superior to that of the control strain *B. lactis* BB12. The strain *L. rhamnosus* Probio-M9 was exposed to artificial gastric juice at pH 2.5 for 3 h (survival rate 83.72%) and artificial intestinal juice at pH 8.0 for 8 h (survival rate 78.33%); its survival rate was superior to that of the control strain *L. rhamnosus* GG. The survival rates of the other 6 strains ranged from 6.52 to 38.06% in artificial gastric juice at pH 2.5 for 3 h, and 1 to 31.59% in artificial intestinal juice at pH 8.0 for 8 h. *Bifidobacterium animalis* GSL-1 and *L. plantarum* LN-3-1 showed little tolerance to artificial intestinal juice. As well, *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 were tolerant to gastrointestinal juices in continu-

Figure 1. The phylogenetic tree based on 16S rRNA genes of isolates and type strains.

Figure 2. Bacterial genus distribution of 197 strains from 40 human colostrum samples.

ous culture and so could survive passage through the stomach to reach the intestine.

Bile Salt Tolerance. The tolerance of the strains to bile salts was evaluated by lag period. When the OD (600 nm) value of each strain reached 0.3 units greater than the value at 0 h, the measurement was stopped immediately; the time was then recorded. The time difference for the same strain between the test and control groups was compared and recorded as the lag period. The growth of the control and test strains was inhibited to different degrees in MRS broth medium containing 0.3% bile salts, and all showed good tolerance (lag times: 0.95, 1.98, 1.87, and 2.10 h, respectively). In the presence of 0.3% bile salts, *B. lactis* Probio-M8 showed greater tolerance than the 2 control strains ($P < 0.05$); *L. rhamnosus* Probio-M9 and *B. lactis* BB12 showed similar levels of bile salt tolerance, which were greater than that of *L. rhamnosus* GG (P

< 0.05) (Table 4). Therefore, *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 could survive in the intestinal tract and reduce oxidative stress.

Growth. *Bifidobacterium lactis* Probio-M8 (1.725×10^7 cfu/mL) was inoculated into modified MRS broth medium, continuously cultured at 37°C for 30 h, and entered logarithmic growth after 6 h. The viable count peaked at 2.075×10^9 cfu/mL at 18 h, and then slightly decreased to a plateau (Figure 5A). *Lactobacillus rhamnosus* Probio-M9 (1.555×10^7 cfu/mL) was inoculated into modified MRS broth medium and underwent logarithmic growth at 3 to 18 h. The viable count peaked at 2.967×10^9 cfu/mL at 21 h and then slightly decreased to a plateau (Figure 5B).

DISCUSSION

Bacteria are present in the breast milk of healthy lactating women. Among them, the genera *Staphylococcus*, *Streptococcus*, and *Propionibacterium* are core members of the human milk microbiota; these genera appear to be common in healthy breast milk, irrespective of maternal and infant characteristics (Hunt et al., 2011; Jiménez et al., 2015; Sakwinska et al., 2016). *Staphylococcus* and *Propionibacterium* can also be isolated from the skin and may prevent the host from being colonized by more dangerous pathogens, such as *Staphylococcus aureus* (Iwase et al., 2010). The presence of potentially beneficial bacteria in human milk (e.g., *Lactobacillus* and *Bifidobacterium* species) is frequently reported (Martín et al., 2003, 2009; Abrahamsson et al., 2009; Solís et al., 2010; Arboleya et al., 2011; Murphy et al., 2017), although these species are not detected in all samples and populations studied; moreover, they usually represent a minority, albeit an important one, of the bacterial populations detected (Chen et al., 2018). For example, *Bifidobacterium* and *Lactobacillus* represented 1.70% and 0.40% of all isolated bacteria. The

Figure 3. Morphologic observation of *Bifidobacterium lactis* Probio-M8. (A) Colony morphology; (B) Gram stain; (C) scanning electron microscopy. Morphology of the bacteria was observed under an optical microscope $\times 1,000$ and under a scanning electron microscope $\times 10,000$.

Figure 4. Morphologic observation of *Lactobacillus rhamnosus* Probio-M9. (A) Colony morphology; (B) Gram stain; (C) scanning electron microscopy. Morphology of the bacteria was observed under an optical microscope $\times 1,000$ and under a scanning electron microscope $\times 10,000$.

197 strains of bacteria isolated in this study included *Staphylococcus*, *Streptococcus*, *Enterococcus*, and other species; the proportions of *Bifidobacterium* (4.06%) and *Lactobacillus* (5.08%) were small. Therefore, we confirmed that *Streptococcus* and *Staphylococcus* predominate in human breast milk, and that *Bifidobacterium* and *Lactobacillus* are present at lower levels. The infant intestinal tract progresses from sterility at birth to extremely dense colonization at the end of the first year of life. By 1 yr of age, the infant microflora retains its uniqueness but has converged toward a profile characteristic of the adult gastrointestinal tract. Breastfeeding has a profound influence on the gut microbiota; in particular, it is associated with the presence of *Bifidobacterium*. This has prompted international organizations to recommend exclusive breastfeeding during the first 6 mo of life (World Health Organization, 2003). *Bifidobacterium* was initially isolated from the feces of a breastfed infant by Dr. Tidder of the Pasteur Institute in France (Zhang, 2012). It resides mainly in the human gastrointestinal tract, along which its abundance varies

markedly and becomes dominant at d 6 to 8 after birth (Cheikhoussef et al., 2009). *Bifidobacterium* spp. are found equally frequently in breastfed infants (Benno et al., 1984; Martín et al., 2009; Chen et al., 2016) and predominate in the intestinal microflora of children; *Lactobacillus* spp. are similarly highly represented, including *L. rhamnosus* (Adlerberth and Wold, 2009), which was first isolated from the intestinal flora of a healthy human by Gorbach and Goldin in the United States, and so is also known as *L. rhamnosus* GG. It has been isolated from human feces (Gu et al., 2008; Muñoz-Quezada et al., 2013) and human intestine (Chen et al., 2014), as well as from cheese (Morales et al., 2011). In 2017, a thorough screening of *L. rhamnosus* strains from breast milk was performed to evaluate their potential use in probiotic products, and the authors found that cell-free culture supernatants of *L. rhamnosus* strains had a significant anticancer effect on HeLa cervical cancer cells via induction of cytotoxicity and apoptosis (Riaz Rajoka et al., 2017). *Lactobacillus* has a high intestinal adhesion rate and readily colonizes the intestinal tract; it can modulate the intestinal flora, prevent and treat diarrhea, prevent allergy, improve immunity, and exhibit anticancer activity (Björkstén, 2004; Österlund et al., 2007; Andrade and Borges, 2009; Hojsak et al., 2010; Riaz Rajoka et al., 2018). In recent years, scientists in China have begun to study probiotics in breast milk, with the aim of evaluating the effect of microbes in breast milk on the intestinal health of infants. Therefore, we conducted a preliminary evaluation of the potential probiotic characteristics of *Bifidobacterium* and *Lactobacillus* isolated from breast milk, in terms of survival in the host gastrointestinal tract and resistance to bile salts, to address the scarcity of probiotics originating from breast milk.

The ability of probiotics to survive in high bile salt concentrations and at a low pH are important features for successful passage through the gastrointestinal tract (Patel et al., 2014; Mandal et al., 2016). The pH of

Table 1. Physiological and biochemical variables of *Bifidobacterium lactis* Probio-M8 and *Lactobacillus rhamnosus* Probio-M9¹

Experimental assay	<i>B. lactis</i> Probio-M8	<i>L. rhamnosus</i> Probio-M9
Cell morphology	Rod	Rod
Gram stain	+	+
Motility	—	—
Spore formation	—	—
Contact enzyme	—	—
Aerobic growth	+	+
6.5% NaCl growth	+	—
pH 4.5 growth	+	+
pH 9.6 growth	—	—
10°C growth	+	—
15°C growth	+	—
42°C growth	+	+
60°C, 30 min growth	—	—

¹+, positive; —, negative.

Table 2. Carbohydrate fermentation by *Bifidobacterium lactis* Probio-M8 and *Lactobacillus rhamnosus* Probio-M9¹

Serial number	Sugar alcohol	Probio-M8	Probio-M9
0	Contrast	—	—
1	Glycerol	—	—
2	Erythritol	—	—
3	D-Arabinose	—	+
4	L-Arabinose	—	+
5	D-Ribose	+	+
6	D-Xylose	+	—
7	L-Xylose	—	—
8	D-Adonitol	—	—
9	Methyl-βD-xylopyranoside	—	—
10	D-Galactose	—	+
11	D-Glucose	+	+
12	D-Fructose	—	+
13	D-Mannose	—	+
14	L-Sorbose	+	+
15	L-Rhamnose	—	+
16	Dulcitol	—	—
17	Inositol	—	—
18	D-Mannitol	—	+
19	D-Sorbitol	—	+
20	Methyl-αD-mannopyranoside	—	—
21	Methyl-αD-glucopyranoside	—	+
22	N-Acetylglucosamine	—	+
23	Amygdaline	—	+
24	Arbutine	—	+
25	Esculine ferric citrate	+	+
26	Salicine	—	+
27	D-Cellobiose	—	+
28	D-Maltose	+	+
29	D-Lactose (bovine origin)	+	+
30	D-Melibiose	+	—
31	D-Saccharose	+	+
32	D-Trehalose	—	+
33	Inuline	—	+
34	D-Melezitose	—	+
35	D-Raffinose	+	—
36	Starch	—	—
37	Glycogen	—	—
38	Xylitol	—	—
39	Gentiobiose	—	+
40	D-Turanose	—	+
41	D-Lyxose	—	—
42	D-Tagatose	—	+
43	D-Fucose	—	—
44	L-Fucose	—	—
45	D-Arabitol	—	—
46	L-Arabitol	+	—
47	Potassium gluconate	—	+
48	Potassium 2-cetogluconate	+	+
49	Potassium 2-cetogluconate	—	—

¹+, positive; —, negative. Serial numbers were provided in the carbohydrate fermentation kit (bioMérieux SA, Marcy l'Étoile, France).

human gastric juice is typically around 3.0, and the digestion time is 1 to 3 h. The survival rate of LAB in artificial gastric juice for 3 h is evaluated to screen for tolerant strains (Chen et al., 2015). In the pres-

ent study, 10 strains of *Lactobacillus* and 8 strains of *Bifidobacterium* were screened in terms of their ability to tolerate gastric acid. Eight strains were resistant to artificial gastric juice at pH 2.5, including 6 of *Bifidobacterium*, 1 of *L. rhamnosus*, and 1 of *L. plantarum*. At least an 80% survival rate in gastric juice at pH 2.5 for 3 h is the standard for identifying potentially probiotic organisms (Liu et al., 2007; Nawaz et al., 2011). Compared with *B. lactis* BB12, *L. rhamnosus* GG, and *L. plantarum* P-8 control strains, *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 showed similar survival rates and greater tolerance. The survival rates of *L. plantarum* LN-3-1 and *B. animalis* GRS-1 exposed to gastric juice for 3 h were <40%, half the rates of *B. lactis* M8 and *L. rhamnosus* Probio-M9; the other test strains showed survival rates of <10%. The test strains also tolerated artificial intestinal juice at pH 8.0 for 8 h. The survival rates of *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 were >75% after exposure to artificial intestinal juice at pH 8.0 for 4 h; in contrast, the survival rates of the other strains decreased slightly. Surprisingly, the survival rate of *B. lactis* Probio-M8 was 97.25% after exposure to extreme alkalinity. The survival rate of *B. lactis* Probio-M8 in the gastrointestinal environment was higher than the survival rates of previously reported strains, such as *Bifidobacterium* spp., *L. rhamnosus*, and *Lactobacillus casei* (Fang, 2012; Tulumoglu et al., 2013; Tuo et al., 2013; Manini et al., 2016). Another study found that 7 strains of *Lactobacillus* isolated from breast milk showed strong acid resistance, with survival rates of >80% at pH 2.0 to 3.0 followed by transfer to bile at a concentration of 1.0% (wt/vol); indeed, the survival rate of *L. rhamnosus* was 80% and that of *L. casei* was 57% (Riaz Rajoka et al., 2017, 2018). These rates were significantly higher than those observed in a previous study of *L. rhamnosus* IMC501 and *Lactobacillus paracasei* IMC502 using a bile concentration of 0.3% (mass/vol) (Verdenelli et al., 2009). However, compared with the artificial intestinal fluid bile salt concentration of 1.8% in the present study, the concentration in the previous study was insufficient. The bile salt concentration in the intestinal tract ranges from 1.5 to 2.0% (wt/vol) during the first hour of digestion, and then decreases to 0.3% (wt/vol; Wang et al., 2014). We selected artificial intestinal fluid with a higher bile concentration to better determine the tolerance of the strains, and *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 showed a high level of tolerance to bile acids. Normally, ingested probiotics are buffered by food or some other medium in the stomach, and so are not likely to be exposed to extreme pH, except under rare circumstances, such as calcinosis and acid indigestion. Our results indicated that *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 from human breast

Table 3. Survival of *Lactobacillus* and *Bifidobacterium* strains in artificial gastric and intestinal juices

Strain	Artificial gastric juice at pH 2.5			Artificial intestinal juice at pH 8.0			
	Viable count (cfu/mL)		Survival rate (%)	Viable count (cfu/mL)		Survival rate (%)	
	0 h	3 h		4 h	8 h	7 h/3 h	11 h/3 h
<i>Bifidobacterium</i>							
BB12 (control)	4.10×10^9	3.53×10^9	86.10	2.67×10^9	2.97×10^9	75.64	84.14
LN-1	5.32×10^9	3.64×10^8	6.84	1.01×10^8	1.15×10^8	27.75	31.59
GSL-1	3.78×10^9	3.49×10^8	9.23	$<10^6$	$<10^6$	<1	<1
BJH-4	9.20×10^9	6.00×10^8	6.52	2.34×10^8	1.03×10^8	39.00	17.17
MXV-1	2.78×10^9	2.45×10^8	8.81	2.03×10^7	1.1×10^7	8.29	4.49
GRS-1	4.15×10^9	6.95×10^8	16.75	1.96×10^8	1.20×10^8	28.20	17.27
Probio-M8	3.83×10^9	3.27×10^9	85.38	3.27×10^9	3.18×10^9	100.00	97.25
<i>Lactobacillus</i>							
GG (control)	8.73×10^8	7.55×10^8	86.48	4.90×10^8	5.20×10^8	64.90	68.87
Probio-M9	4.30×10^8	3.60×10^8	83.72	1.96×10^8	2.82×10^8	76.67	78.33
P-8 (control)	3.45×10^9	3.04×10^9	88.12	2.33×10^9	2.20×10^9	76.64	72.37
LN-3-1	1.55×10^9	5.90×10^8	38.06	2.00×10^7	1.00×10^7	3.39	1.69

milk could transit through the stomach at pH 2.5 and reach the intestinal tract to exert a beneficial effect.

Bile salts are sodium or potassium salts formed by the combination of glycine or taurine and bile acid (Tanaka et al., 1999); these salts play important roles in the digestion and absorption of fat. The high osmotic pressure and surfactant activity of bile salts disrupt phospholipids and proteins in the bacterial cell membrane, altering the membrane permeability and causing damage or death (Begley et al., 2005). Resistance to cholic acid is an essential component of screening for probiotics. Generally, the concentration of bile salts fluctuates within the range of 0.03 to 0.3%; we used the highest concentration in the present study. In the presence of 0.3% bile salts, the OD of *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 increased by 0.3 units at 0.95 and 1.87 h, respectively. In the absence of bile salts, the OD of *L. rhamnosus* GG increased by 0.3 units at 4.07 h, followed by *B. lactis* BB12 and *L. rhamnosus* Probio-M9. The OD of *B. lactis* Probio-M8 increased by 0.3 units at 6.50 ± 0.02 h, more than 2 h later than the other strains. However, in the presence of bile salts,

the OD of *B. lactis* M8 increased by 0.3 units at 1 h, so this strain showed the highest tolerance to bile salts ($P < 0.05$). Therefore, *B. lactis* Probio-M8 and *L. rhamnosus* Probio-M9 could survive in the intestinal tract. A previous study isolated *B. animalis* ssp. *lactis* BZ11 from Guizhou Xiang pig feces and reported that the strain had high tolerance to oxygen, acid, and cholate. In particular, the survival rate was 92.65% compared with bacteria cultured in a medium that did not include bile salts (Zhu et al., 2017). This finding may have been due to the isolation of the selected strains from the animal intestinal tract. Another study reported that *L. rhamnosus* Z5 exhibited a survival rate of 76.14% after incubation for 6 h in 0.15% bile salts; however, its growth was severely inhibited at a higher concentration of bile salts (Gao et al., 2017). A further study reported that the growth of *L. rhamnosus* GG was completely inhibited by bile salts at 0.15% (Zhao et al., 2016). This was consistent with our finding that *L. rhamnosus* GG showed the longest delay period of the strains tested. The mechanisms of tolerance to low concentrations of bile salts by LAB have been reported. For example,

Table 4. Tolerance of *Lactobacillus* and *Bifidobacterium* strains to 0.3% bile salts in broth medium (lag time)¹

Strain	Time (h) to increase absorbance by 0.3 units at 620 nm ²		
	MRS without bile salts	MRS with 0.3% bile salts	Lag time (h)
<i>Bifidobacterium</i>			
Probio-M8	6.50 ± 0.02^a	7.45 ± 0.05^a	0.95 ± 0.03^c
BB12	4.30 ± 0.04^{bc}	6.28 ± 0.01^b	1.98 ± 0.03^{ab}
<i>Lactobacillus</i>			
Probio-M9	4.37 ± 0.08^b	6.24 ± 0.12^b	1.87 ± 0.04^b
GG	4.07 ± 0.16^c	6.17 ± 0.09^b	2.10 ± 0.07^a

^{a-c}Values in a column with different superscript letters are significantly different ($P < 0.05$).

¹Data are mean $\pm$ SD of triplicate experiments.

²MRS = de Man, Rogosa, and Sharpe medium.

Figure 5. Growth curves of strains (A) *Bifidobacterium lactis* Probio-M8; (B) *Lactobacillus rhamnosus* Probio-M9. Results are expressed as mean $\pm$ SD.

biliary salt hydrolase converts toxic primary bile salts to less toxic secondary bile salts, promoting the survival of LAB in the gastrointestinal tract. The bile saltwater lyase activity of *Bifidobacterium* is greater than that of *Lactobacillus*, and strains isolated from the intestinal tract generally have higher bile saltwater lyase activity than those isolated from natural habitats (Bustos et al., 2012).

Advances in the assessment of early hosts and breastfeeding microbes suggest that early colonization of the infant intestine by milk bacteria may promote disease prevention in children and have a positive effect on health in later life. It has been suggested that the microorganisms in breast milk aid in the maintenance of human health. However, studies in currently available strains originating from human milk are scarce. In particular, in China few reports are available concerning the screening of probiotic strains on *L. rhamnosus* and *B. lactis* from human milk. Moreover, resistance to human gastric transit is an important selection criterion for probiotic bacteria. We tested LAB isolated from human milk samples and evaluated their health-promoting potential in an in vitro model. As well, most probiotics strains used currently in China are imported from Europe and the United States, and not enough evidence is available to determine whether these strains are suitable for Chinese intestines and body types. In China, research on LAB and probiotics from human milk started late and lacked sufficient knowledge and strains. Here we provide 2 active probiotic strains of Probio-M8 and Probio-M9 isolated from human milk. These strains, with independent intellectual property rights in China, may be more suitable for Chinese people and other Asians. Our research team has also carried out a series of studies of Probio-M8/-M9, including a safety evaluation, animal experiments, and whole-genome sequencing. We found that *L. rhamnosus* Probio-M9 significantly improved the curative effect of tumor immunotherapy (PD-1) and inhibited the development of breast cancer in mice. More research will follow.

CONCLUSIONS

Lactic acid bacteria from human milk are important probiotics used frequently in related studies. By 16S rRNA gene sequencing and phylogenetic analysis, 197 strains were isolated and identified from 40 samples of human colostrum. These strains comprised 5 LAB genera and 2 other genera, as well as 10 *Lactobacillus* strains and 8 *Bifidobacterium* strains. We evaluated the ability of these strains to tolerate artificial gastric and intestinal juice in vitro to assess their ability to survive transit through the digestive tract. Eight of the 18 strains (44.44%) grew well at pH 2.5 for 3 h. Two strains showed tolerance to gastric acid and bile salts. Of these 2 strains, *B. lactis* Probio-M8 exhibited the greatest tolerance to bile salts and artificial gastric and intestinal fluid. In summary, we isolated 2 novel strains with potential probiotic characteristics,

and our work will facilitate the identification of further probiotic strains for use in the food and pharmaceutical industries.

ACKNOWLEDGMENTS

This work was supported by the Food Science and Technology Fund of the Chinese Institute of Food Science and the Technology-Youth Fund of Ketuo Hengtong Probiotics Research (No. 2018 ktjj01). All authors have declared that they have no conflicts of interest in publishing this research.

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