Preliminary selection for potential probiotic *Bifidobacterium* isolated from subjects of different Chinese ethnic groups and evaluation of their fermentation and storage characteristics in bovine milk

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ABSTRACT

A total of 29 strains of *Bifidobacterium* were isolated from 18 samples of human feces in different ethnic minority regions of China. All isolates were identified as *Bifidobacterium longum* (9 strains) and *Bifidobacterium pseudocatenulatum* (20 strains) based on 16S rRNA gene sequencing and phylogenetic analysis. These strains were preliminarily tested for their suitability to become probiotics by assessing their ability to survive adequately at low pH conditions and their tolerance of different concentrations of bile salts and simulated gastrointestinal juices. In vitro tests were sequentially used to predict the survival of these strains in the simulated conditions in the human gastrointestinal tract. These strains were first exposed to pH 2.5 for 3 h, and 7 out of the 29 strains were discriminated from the others by their high survival rates. Out of these 7 strains, 4 were found to grow and survive well at an even lower pH of 2.0 and in high bile salt concentration. Apart from the gastrointestinal survival capacity, both fermentation efficiency and storage characteristics are important criteria for selecting for suitable potential probiotic strains. Therefore, the fermentation efficiency in bovine milk and the bacterial viability during the storage in the resultant fermented milk were also evaluated for these 4 selected strains. In this study, we isolated and identified 29 novel *Bifidobacterium* strains. Based on our initial evaluation, at least 4 of them may serve as valuable resources for further dairy probiotic strain selection.

Key words: *Bifidobacterium*, human origin, potential probiotic, fermentation and storage characteristics

INTRODUCTION

China is a large country noted for its dense population and vast territory, and it has altogether 56 ethnic

groups. The majority of the population belongs to the Han ethnic group (91.95%; total of 1,159.4 million), and the other 55 ethnic groups are customarily referred to as the national minorities (together making up 8.41%; total of 106.43 million). The national minorities, although fewer in number, are scattered over a vast area, mainly distributed in the border regions from northeast China to north, northwest, and southwest China. The largest is the 12 million-strong Zhuang ethnic group in southwestern China. Yunnan Province, home to more than 20 ethnic groups, has the greatest diversity of minority people in China. Individual ethnic minority groups are characterized by having distinct physical and cultural traits, including their lifestyles, customs, eating habits, and diets. These differences may contribute to the creation of a rich and distinctive spectrum of intestinal microflora in the different ethnic groups. Therefore, it would be both important and of great scientific interest to identify and to screen for potential probiotic *Bifidobacterium* strains from these ethnic populations. The successful identification of probiotic strains not only enhances our understanding on the distinctive gut microbiota composition in the different ethnic groups, but also opens the opportunities for exploitation of novel probiotic strains that might be suitable for Chinese human intestinal tract physiological characteristics.

Probiotic bacteria are defined as “live microorganisms that when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2001). *Bifidobacteria* are gram-positive, saccharolytic, intestinal anaerobes, representing up to 25 and 80% of the cultivable fecal bacteria in adults and in infants, respectively (Picard et al., 2005). *Bifidobacterium*, as a probiotic agent in human food, has been widely used because of its generally recognized as safe (GRAS) status. Some strains have been used in fermented dairy products and exert a wide range of beneficial health effects, including the prevention or alleviation of infectious diarrhea, the inhibition of growth of pathogens and harmful bacte-

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ria, the modulation of the local and systemic immune responses, and so on. However, the viability of some of these probiotic strains for food use sharply decreases during the product shelf life. Moreover, many of these bacteria may be killed during their transit through the acidic conditions of the stomach and be degraded or inactivated by hydrolytic enzymes and bile salts in the small intestine (Playne, 1994). Therefore, the gastrointestinal survival capacity is considered as an important criterion for the initial selection for potential probiotic strains.

Fermented foods are major carriers of probiotic bacteria with positive health effect. In particular, fermented dairy products have been increasingly attracting the attention of consumers. In Japan, bifidobacteria-containing products are very popular and one-third of the total yogurt sales involve these products. In France, 11% of all yogurts sold contain added bifidobacteria (Shah, 2000). Thus, screening bifidobacterial strains with potential probiotic functions is regarded as a major basic but important task for industrial application. It is known that the resistance to acid and bile salts varies widely among bifidobacterial species, even at the level of the strain (Liu et al., 2007). Additionally, a rapid decline of viable counts in dairy food and other fermented probiotic products has been observed during the shelf life and during transit through the intestinal tract (Conway et al., 1987; Shah and Jelen, 1990; Dave and Shah, 1996). Therefore, it is necessary to select the right vehicle for the delivery of probiotics to ensure that these bacteria remain viable throughout the product shelf life and overcome the physical and chemical barriers in the gastrointestinal tract (Samona and Robinson, 1991; Vinderola et al., 2000; Del Piano et al., 2006). Adding proper probiotic strains into the fermented dairy products not only provides health-promoting effects, but also affects the physicochemical and microbial properties of the products, such as pH value, viscosity, syneresis, organic acid contents, and viable counts of the probiotics (Wang et al., 2009). In this work, 29 bifidobacteria were isolated from fecal samples of subjects from different Chinese minority ethnic groups; fermentation and storage characteristics of these selected strains in bovine milk were also evaluated.

MATERIALS AND METHODS

Sample Collection

Eighteen fecal samples were collected from 18 counties in 6 provinces of China, including Henan, Sichuan, Tibet, Yunnan, Xinjiang, and Inner Mongolia. The volunteers recruited for this study were from different

ethnic groups such as Han (distributed throughout the country and is the major population of China), Tujia (an ethnic group population, distributed widely in Hunan, Hubei, Guizhou, and Sichuan Provinces; believe in the great influence of gods and ancestors, and live on agriculture and the fishing industry), Bai (an ethnic group population; inhabit the province of Yunnan; are masters of artistic creativity and favor white clothes and decorations), Tibetan (mostly live in the Tibetan Autonomous Region, and the people believe in Tibetan Buddhism, which is also called Lamaism; butter tea, mutton, and beef are their staple foods), Kazak (mainly dwell in Xinjiang, Gansu, and Qinghai; the staple foods are meat and milk), Uighur (mainly live in Xinjiang Uygur Autonomous Regions; the people of this ethnic group believe in Islam), and Mongolian (primarily live in Inner Mongolia; often being referred to as "the minority on horseback"; the staple foods are meat and milk). The age range of the volunteers was from 18 to 35 yr old. The volunteers had no previous medical history of gastrointestinal diseases and had not taken any antibiotics 2 wk before sampling. About 50 g of fecal sample was transferred with a sterile spoon into a sterile sampling tube marked with sampling site, date, and information on the volunteer. All the samples were frozen in liquid nitrogen after collection and rapidly transported to the laboratory by aircraft.

Isolation and Identification of *Bifidobacterium*

Isolation and Preliminary Identification of *Bifidobacterium*. About 1.0 g of human feces sample was well mixed in 9 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). Decimal serial anaerobic dilutions were prepared, and 1 mL of the appropriate diluents (10^{-4} and 10^{-5}) was spread on triplicate sets of modified de Man, Rogosa, and Sharpe (MRS) and glucose-blood-liver (BL) selective media. The MRS medium (Difco lactobacilli MRS agar; Becton Dickinson, Pont-de-Claix, France) was modified by adding 100 μmol of X-gal/L (Ferraris et al., 2010), 0.5% L-cystine, 10 μg of cycloheximide/mL, and 2 g of LiCl/L. The selective BL medium (CM0785; Oxoid Ltd., Basingstoke, UK) was modified by adding neomycin sulfate (100 μg /mL), paromomycin sulfate (200 μg /mL), nalidixic acid (15 μg /mL), and cycloheximide (10 μg /mL). The inoculated agar plates were incubated under anaerobic conditions (80% N_2 , 10% H_2 , and 10% CO_2) at 37°C for 48 h (Scardovi, 1986; Jousimies-Somer et al., 2002).

The colony characteristics of bifidobacteria were more typical in modified selective MRS or BL medium, so it was easy to distinguish them from other nonrelated bacteria (Kok et al., 1996). Bifidobacterial colonies were

generally smooth, circular convex, marginal integrity, and were dark blue color on the modified MRS agar. On modified BL agar plates, they appeared as milky brown or milky white colonies of diameter between 0.6 and 1.8 mm (Scardovi, 1986). Single colonies with the described morphological characteristics were suspected to be *Bifidobacterium* and were randomly picked and inoculated into Trypticase peptone yeast extract (TPY) liquid enrichment medium using a sterile inoculating needle or loop. The pure cultures were incubated at 37°C for 24 h anaerobically. Gram-positive pure cultures were subject to a catalase test, fructose-6-phosphate phosphoketolase (F6PPK) activity detection, and further morphological observation. Detection of F6PPK activity in the cellular extracts was performed as described by Scardovi (1986). Cultures, which were catalase-negative, gram-positive, non-spore forming, and F6PPK positive, were identified as bifidobacteria as described by Hadadji et al. (2005). To prepare frozen stocks, pure cultures of bifidobacteria were centrifuged at $3,000 \times g$ for 10 min and washed 2 times with PBS, before being suspended in cryoprotective agent (10% skim milk, 0.1% sodium glutamate, and 0.5 % L-cysteine). These frozen stocks were stored at -80°C . Isolates were lyophilized for long-term storage.

Identification of *Bifidobacterium* by 16S rRNA Gene Sequencing and Phylogenetic Analysis. Cells of *Bifidobacterium* grown overnight in modified MRS broth at 30°C were used for DNA extraction and purification as described by Liu et al. (2012). Two hundred microliters of purified DNA was diluted to 100 ng/ μL for 16S rRNA amplification and sequencing. Amplification of the 16S rRNA sequences was carried out with an automatic thermal cycler (MJ Research PTC-200; MJ Research Inc., Watertown, MA) using the universal primers of the 16S rRNA gene: 27f and 1495r (Lane, 1991; Delbès et al., 2007). The primer sequences 27f and 1495r were 5'-AGAGTTTGATCCTGGCT-CAG-3' and 5'-CTACGGCTACCTTGTACGA-3', respectively. The PCR reaction mix was prepared similarly as described by Liu et al. (2012). The PCR amplifying conditions were as follows: 94°C for 5 min, 31 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, and then 72°C for 8 min and left at 4°C. The PCR products were electrophoresed on 1% agarose gel, and a DNA band of approximately 1,500 bp was obtained from all isolated strains. The DNA bands were excised, purified, and sequenced with an ABI Prism 3730XL DNA Analyzer (Applied Biosystems Inc., Foster City, CA) by Shanghai Sangni Biosciences Corporation of China (Shanghai, China). The DNA sequencing was performed twice to ensure accuracy. All sequences were then aligned using DNASTAR software package (DNASTAR Inc., Madison, WI).

Sequence similarity was checked across the GenBank database using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Phylogenetic tree construction and molecular evolutionary analyses were conducted using the neighbor-joining method within the MEGA version 4.0 package (Saitou and Nei, 1987; Kumar et al., 2004). All the obtained 16S rRNA sequences of isolates were compared with that of type strains of *Bifidobacterium* (*B. pseudocatenulatum* JCM1200^T, *B. longum* ATCC15707^T, *B. breve* ATCC15698^T, *B. catenulatum* ATCC27539^T, *B. simiae* CCUG30289^T, *B. dentium* ATCC27534^T, *B. ruminantium* JCM8222^T, *B. adolescentis* ATCC15703^T, *B. bifidum* DSM20456^T, *B. thermacidophilum* DSM20083^T, *B. asteroides* ATCC25910^T, *B. indicum* JCM1302^T, *B. coryneforme* ATCC25911^T, *B. pseudolongum* JCM1205^T, *B. magnum* JCM1218^T, and *B. minimum* ATCC27538^T) and an out-group strain (*Escherichia coli* ATCC11775^T) to construct the phylogenetic tree.

Preliminary Selection of *Bifidobacterium*

Low pH Tolerance. Fresh cultures (1.0 mL) of *Bifidobacterium* cells were collected by centrifugation at $3,000 \times g$ for 10 min, washed twice with PBS, and resuspended in saline solution to a final volume of 5 mL. Then, 100 μL of suspension was inoculated into 10 mL of MRS broth (pH 2.0, pH 2.5, and 3.0). Optical density (OD) values were recorded at 620 nm after incubation in an anaerobic environment at 37°C for 48 h. The resistant strains with survival rate over 98% were selected for further in vitro evaluation. *Bifidobacterium animalis* BB12 (Christian Hansen AS, Beijing, China), a probiotic strain, which could survive well in the presence of bile salt and had good acid tolerance, was used as a common reference strain (Dianawati and Shah, 2011). This strain was included in all the experiments of this study for comparison.

Transit Tolerance in Gastrointestinal Juices. The survival of *Bifidobacterium* in simulated gastrointestinal juices was determined according to the method described by Bao et al. (2010). Simulated gastric juice (pH 2.0 and pH 2.5) was inoculated with 1% fresh culture of the isolate to be tested and was incubated anaerobically at 37°C. Total viable counts were determined at 0, 1, 2, and 3 h of incubation. After 3 h of incubation in the gastric juice, 10% of the culture was inoculated into the simulated intestinal juice at pH 8.0 and incubated anaerobically. Total viable counts were determined at 0, 4, and 8 h of incubation to evaluate the percentage survival in the intestinal juice. Total viable counts were determined by plating the serial dilutions on MRS agar and incubating anaerobically at 37°C for 48 h.

Bile Salt Tolerance of *Bifidobacterium*. The strains with the best tolerance in gastrointestinal juices were selected to test for their bile salt tolerance. The ability of the strains to grow in the presence of bile was determined according to the method of Vinderola et al. (2008) with some modifications: MRS broth cultures supplemented with 0.5 to 2.0% (wt/vol) oxgall (Oxoid Ltd.) were freshly prepared and inoculated with 1% (vol/vol) overnight suspensions of the selected strains. Cultures were incubated anaerobically at 37°C. The absorbance at 620 nm was measured and compared with the respective control culture without bile salts.

The tests were performed in round-bottom microwell plates (Nunc). A 200- μ L volume each of MRS (pH 2.5), MRS containing 0.3% oxgall, or normal MRS, was inoculated with the strain to be tested at a level of 10^6 cfu/mL. The experiments were performed in quadruplicate. The culture broth without inoculation was used as the negative control. Changes in optical density at 620 nm (OD_{620}) were measured (Multiscan MCC/340 photometer; Labsystem, Helsinki, Finland) following 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 24 h of incubation at 37°C. Bacterial survival under the different conditions was determined after 4 h of incubation at 37°C by plate count of 100 μ L of the culture on MRS agar.

Bifidobacterium selected with high tolerance of artificial digestive juice were assessed in the next experiment. These selected strains were inoculated into modified MRS liquid medium with 2% (vol/vol) overnight suspensions of tested strains in MRS liquid medium containing bile salts (medium supplemented with 0.2 g of mercaptoacetate sodium/100 g and 0.3 g of bile salt/100 g) and non-bile-salt-modified MRS medium as a control; the cultures were incubated at 37°C. The culture turbidity was recorded every hour by monitoring OD values at 620 nm. When the OD value increased by 0.3 U, the measurement of sample was stopped. The lag times for the selected isolates and the reference BB12 strain were determined. The lag time was defined as the difference between the times needed for the bacterial culture to increase by 0.3 OD units in the presence or absence of bile salts (Walker and Gilliland, 1993).

The highest bile salt concentration that could be tolerated by each tested strain was determined. Two-percent (vol/vol) inocula of tested strains were inoculated into modified MRS liquid medium supplemented with different bile salt concentrations (0.3, 0.6, 0.8, 1.0, 1.5, 1.8, and 2.0%), and then incubated anaerobically at 37°C for 24 h. Absorbance values (620 nm) were measured at 0 and 12 h. Meanwhile, the viable counts of each strain in the different concentration of bile salts were determined on the aforementioned MRS medium after 48 h of anaerobic growth at 37°C. Each sample was prepared in triplicate.

Fermentation and Storage Characteristics of *Bifidobacterium* in Bovine Milk

Fermentation Characteristics of Selected Strains. The fermentation characteristics of selected strains were studied. Skimmed reconstituted milk was prepared by adding 1.5% of glucose into 10% skim milk. The milk was sterilized at 95°C for 10 min and cooled to 37°C; 1×10^7 cfu/mL of the tested and the BB12 strains as the starter culture were separately inoculated into the prepared skim milk for fermentation at 37°C. During the fermentation, samples were taken every 3 h to monitor the pH, titratable acidity, and the total viable count of the tested strains. The fermentation was ended if the pH value was lower than 4.6 or the titratable acidity reached 80 °T. Parallel experiments were repeated 3 times.

Storage Characteristics of Fermented Milk by the Selected Strains. The fermented milk samples were stored in the refrigerator at 4°C for 21 d. Samples were taken at 7, 14, and 21 d. The pH value, titratable acidity, and total viable count of the tested strain were measured. Parallel experiments were repeated 3 times.

Statistical Analysis

Results were expressed as mean $\pm$ standard deviation. Statistical significance was calculated by ANOVA supported by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and Identification of *Bifidobacterium*

Twenty-nine isolates obtained from 18 samples were collected from different regions of ethnic groups of Henan (6 strains), Sichuan (11 strains), Tibet (7 strains), Xinjiang (1 strain), Yunnan (3 strains), and Inner Mongolia (1 strain), as shown in Table 1. The isolates were identified by 16S rRNA sequencing and phylogenetic analysis (Figure 1). The species identification of isolates was initially determined by the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), with more than 99% similarity compared with the 16S rRNA sequences of type strains. Then, phylogenetic analysis including the representative strains and reference strains of each species was performed to reveal the species and distribution of the various isolates. Twenty-nine isolates and 17 type strains were clustered in 3 big groups presented in Figure 1, in which group I was regarded as the *B. pseudocatenulatum* group, including 19 isolates and type strains. Thus, these isolates were identified as *B. pseudocatenulatum*. The remaining isolates

Table 1. Information of sample collection and identity of isolated *Bifidobacterium*

Sampling location (province)	Sample number	No. of volunteers from city/country	Ethnic group	Isolated <i>Bifidobacterium</i> strain ¹ (no.)	
				<i>B. longum</i>	<i>B. pseudocatenulatum</i>
Henan	5	3/2	Han	6	—
Sichuan	6	3/3	Tujia	2	9
Tibet	5	3/2	Tibetan	—	7
Xinjiang	1	0/1	Uyghur	1	—
Yunnan	1	0/1	Bai	—	3
Inner Mongolia	1	0/1	Mongolian	—	1

¹— indicates that no *Bifidobacterium* was isolated.

with high similarity to type strains of *B. longum* were identified as *B. longum*, indicated by group II. Group III contained only 1 strain (*B. minimum* ATCC27538^T). *Escherichia coli* ATCC11775^T was included as an out-group reference strain. Thus, in the present study, the isolated and identified bifidobacteria included 9 strains of *B. longum* and 20 strains of *B. pseudocatenulatum*. It is worth noting that 6 out of the 9 *B. longum* strains were isolated in samples of Henan alone, from which no *B. pseudocatenulatum* was found. In contrast, samples from some other provinces, including Sichuan, Tibet, and Yunnan, were dominated by *B. pseudocatenulatum*, and *B. longum* was hardly isolated. The uneven species distribution may suggest that environmental factors such as the geographic location and regional diet were related to the microflora of the human gut, especially the composition of lactic acid bacteria and bifidobacteria.

Bifidobacterium are the crucial bacterial flora in maintaining a healthy human intestinal tract. In addition, the populations of *Lactobacillus*, *Clostridium perfringens*, and *Enterococcus* were also analyzed in the work by our research group (Sun, 2012). The results showed that the amounts of *Lactobacillus* and *Bifidobacterium* strains were significantly higher than that of *C. perfringens* and *Enterococcus* in the intestinal tract of healthy populations of Kazaks, Mongolians, and Tibetans in the provinces of Xinjiang and Tibet. It was also shown that the amounts of *C. perfringens* and *Enterococcus* in the intestinal tract of city residents were higher than those of the people living in the pastoral areas ($P < 0.05$). Correlation analysis showed that the quantity of *Enterococcus* in the intestinal gut of healthy populations was negatively correlated with *Lactobacillus* and *C. perfringens* ($r = -0.34$, $P = 0.038$; $r = -0.40$, $P = 0.011$, respectively), but was positively correlated ($r = 0.68$, $P < 0.001$) with *Bifidobacterium*. In contrast, the amount of *Bifidobacterium* was negatively correlated with *C. perfringens* ($r = 0.77$, $P < 0.001$; Sun, 2012). Thus, it can be deduced that an increase in the amount of *Bifidobacterium* in the intestinal tract can inhibit the growth of harmful bacteria such as *C. perfringens*.

It was previously reported that *B. pseudocatenulatum* and *B. catenulatum* were the most common bifidobacteria in the human intestine. They would be detected in approximately 92% of adults' fecal samples, followed by *B. longum* and *B. adolescentis* (Matsuki et al., 1999). Mullié et al. (2003) showed that *B. longum* and *B. adolescentis* were the major components of microflora in the intestinal tract of adults. Matsuki et al. (2004) studied the composition of the human intestinal bifidobacteria using quantitative PCR and fluorescence in situ hybridization methods and confirmed that *B. pseudocatenulatum* and *B. catenulatum* were the most common bifidobacteria in the intestinal tract of adults. Pang et al. (2005) analyzed the composition of bifidobacteria in healthy human guts of Chinese subjects by using the temperature gradient gel electrophoresis method. In their report, 9 out of 10 people were found to carry *B. pseudocatenulatum*; therefore, they presumed that *B. pseudocatenulatum* was a common strain in the healthy human gut in the Chinese population and proposed that it was the predominant strain. In the current study, mainly 2 species of *Bifidobacterium* were isolated and identified by pure culture method. Our results further confirmed that *B. pseudocatenulatum* and *B. catenulatum* were the most common and major species of *Bifidobacterium* in the intestinal tract of Chinese adults of different regions and ethnic groups.

Low pH Tolerance

The characteristic of tolerance of acid is a fundamental criterion for the selection for potential probiotic strains because of the low-pH environment of the stomach. It is the first challenge for the potential probiotic strains before they could successfully reach and colonize in the host gut. In most previous reports, pH 3.0 was often used to assay the tolerance of probiotic strains in vitro (Renner, 1991; Liu et al., 2007). Over 80% survival rate in gastric juice at pH 2.5 for 3 h was regarded as a standard to select for potential probiotic organisms (Liu et al., 2007; Nawaz et al., 2011). In the stomach, the pH

Figure 1. A 16S rRNA-based phylogenetic tree of 30 *Bifidobacterium* isolates and 17 type strains.

value can decrease to 1.0. However, the presence of food raises the pH value to 3.0, and the food transition time through the human stomach is about 90 min (Renner, 1991; Erkkilä and Petäjä, 2000). Therefore, probiotic strains are not likely to be exposed to the extremes of pH in the stomach because of the buffering effect of the ingested foods or other carrier matrix molecules following consumption. In the current study, we evaluated the ability of the isolated strains to tolerate low pH (pH 3.0, 2.5, and 2.0). Twenty-five out of 29 strains (86%) were able to grow well in pH 3.0 for 3 h. The survival rates of these strains were more than 96%. The selection was further carried out based on their growth at pH 2.5 and pH 2.0, respectively. Fifteen strains showed high survival rates (over 90%) after being treated at pH 2.5 for 3 h (data not shown). Among them, only 4 strains

(namely IMAUFB084, IMAUFB064, IMAUFB073, and IMAUFB077) showed relatively high survival rates of over 50% after being treated at pH 2.0 for 3 h. Our results also showed that these 4 strains were even more acid resistant than the reference strain used in this study (*Bifidobacterium animalis* ssp. *lactis* BB12). The strain BB12 was used previously in our laboratory as the reference strain for probiotic strain selection and characteristic comparison, and it showed a high level of tolerance to gastric acid and bile acids (Wang et al., 2009). This probiotic strain has been used in the industrial production of dairy starter cultures by many large dairy corporations in China and other countries. Further tests on the abilities of transit gastrointestinal tract and bile tolerance were performed on these 4 isolates from the current study.

Table 2. Viable counts and survival rates of 8 strains of *Bifidobacterium* in artificial gastric juice at pH 2.0 for different periods of time¹

Strain	Time							
	0 h		1 h		2 h		3 h	
	Viable count (log cfu/mL)	Viable count (log cfu/mL)	Survival rate (%)	Viable count (log cfu/mL)	Survival rate (%)	Viable count (log cfu/mL)	Survival rate (%)	
BB12	8.16 ± 0.09	6.10 ± 0.05	75.40	5.64 ± 0.01	69.70	4.28 ± 0.08	52.90	
IMAUFB084	8.17 ± 0.05	6.21 ± 0.05	76.00	6.17 ± 0.05	75.50	5.80 ± 0.01	70.99	
IMAUFB064	8.06 ± 0.07	6.25 ± 0.01	77.54	6.08 ± 0.07	75.40	5.69 ± 0.09	70.60	
IMAUFB073	8.08 ± 0.01	6.26 ± 0.09	77.48	6.05 ± 0.01	74.91	5.20 ± 0.04	64.36	
IMAUFB077	8.09 ± 0.08	5.62 ± 0.04	67.55	5.64 ± 0.11	69.7	4.28 ± 0.08	ND	
IMAUFB090	8.32 ± 0.03	6.05 ± 0.08	74.14	<4	0	ND ²	64.22	
IMAUFB081	8.27 ± 0.04	5.08 ± 0.16	61.43	<4	0	<4	ND	
IMAUFB066	8.31 ± 0.08	4.61 ± 0.02	55.48	<4	0	ND	ND	

¹Data are presented as mean ± SD from triplicate experiments.²ND = not detected.

Survival in Gastrointestinal Juices

The strains with the ability to tolerate low pH were further tested for their transit tolerance in artificial gastric and intestinal juices at pH 2.0 and pH 8.0, respectively. Results from the transit tolerance test are listed in Table 2. The viable counts of these 4 selected strains were more than 6.60 ± 0.03 log cfu/mL when they were cultured in artificial gastric juice at pH 2.0 for 3 h. The survival rates of all 4 strains were over 80%, and IMAUFB084 was the most acid tolerant, with a 85.98% survival rate at pH 2.0 for 3 h. Our results showed that these 4 strains could tolerate the simulated digestive juice at pH 8.0 for 8 h (Table 3); the survival rates of all strains were more than 96% after being cultured in artificial intestinal juice at pH 8.0 for 4 h. After culturing for 8 h in the same simulative digestive juice, the survival rate of these strains generally decreased slightly but remained over 89%. The strain IMAUFB090 had the highest tolerance to the artificial intestinal juice, with 97.86% survival rate at pH 8.0 for 8 h. It was shown that these 4 selected strains could survive well after a sequential passage and prolonged exposure to the simulated gastric and intestinal juices. Therefore, it is likely that these human origin isolates

could successfully transit through the stomach and reach the intestinal environment to function there effectively.

Bile Salt Tolerance

It is generally considered to be necessary to evaluate the ability of lactobacilli and bifidobacteria to resist the effects of bile acids when screening for potentially effective probiotics (Lee and Salminen, 1995; Dunne et al., 2001). The bile tolerance of the 4 gastrointestinal juice resistant strains in the presence of different concentrations of bile salts is listed in Table 4, and the lag time of bile tolerance of these strains is shown in Table 5. The bacteria were considered to be bile salt resistant if the survival rate remained over 50% after being cultured for 12 h at 37°C at the specific bile salt concentration. Among the 4 selected strains, all of them could survive well at 0.8% bile salts, 3 of them could tolerate up to 1.8% bile salts, and only 1 strain (IMIUFB084) could tolerate a high concentration of 2.0% bile salts. Four strains could grow in 1.0% bile salt. However, the delay of growth (lag time) of these 4 strains were 0.59, 0.76, 0.77, and 0.96 h, respectively (shown in Table 5). According to Chateau et al. (1994), bile salt resistance

Table 3. Survival of 5 strains of *Bifidobacterium* in artificial gastric and intestinal juices¹

Strain	Artificial gastric juice at pH 2.0			Artificial intestinal juice at pH 8.0				
	Viable count (log cfu/mL)		Survival rate (%)	Viable count (log cfu/mL)			Survival rate (%)	
	0 h	3 h		0 h	4 h	8 h	4 h	8 h
BB12	8.25 ± 0.05	7.05 ± 0.04	85.45	6.01 ± 0.02	5.94 ± 0.05	5.88 ± 0.05	98.84	97.84
IMAUFB084	8.20 ± 0.04	7.05 ± 0.02	85.98	6.05 ± 0.08	5.97 ± 0.11	5.71 ± 0.03	98.68	94.38
IMAUFB073	8.23 ± 0.06	7.05 ± 0.04	85.66	6.05 ± 0.05	5.89 ± 0.08	5.88 ± 0.02	97.36	97.19
IMAUFB064	8.17 ± 0.01	6.86 ± 0.08	83.97	5.86 ± 0.06	5.65 ± 0.06	5.27 ± 0.09	96.42	89.93
IMAUFB090	8.18 ± 0.07	6.60 ± 0.03	80.68	5.60 ± 0.01	5.54 ± 0.01	5.48 ± 0.07	98.93	97.86

¹Data are presented as mean ± SD from triplicate experiments.

Table 4. Bile salt tolerance of 5 strains of *Bifidobacterium* at different bile salt concentrations¹

Strain	Variable	Bile salt concentration (%)						
		0.3	0.6	0.8	1.0	1.5	1.8	2.0
BB12	$\Delta A_{620\text{nm}}$	1.901 $\pm$ 0.003	1.161 $\pm$ 0.031	1.089 $\pm$ 0.052	1.093 $\pm$ 0.044	0.897 $\pm$ 0.003	0.877 $\pm$ 0.022	0.977 $\pm$ 0.056
	Survival on plate	+	+	+	+	–	–	–
IMAUFB064	$\Delta A_{620\text{nm}}$	1.826 $\pm$ 0.003	1.166 $\pm$ 0.017	1.090 $\pm$ 0.045	1.131 $\pm$ 0.022	1.034 $\pm$ 0.031	0.941 $\pm$ 0.044	0.726 $\pm$ 0.027
	Survival on plate	+	+	+	+	+	+	–
IMAUFB073	$\Delta A_{620\text{nm}}$	1.542 $\pm$ 0.021	1.59 $\pm$ 0.033	1.282 $\pm$ 0.013	1.15 $\pm$ 0.052	1.002 $\pm$ 0.023	1.126 $\pm$ 0.064	0.744 $\pm$ 0.042
	Survival on plate	+	+	+	+	+	+	–
IMAUFB090	$\Delta A_{620\text{nm}}$	1.761 $\pm$ 0.015	1.337 $\pm$ 0.042	1.373 $\pm$ 0.045	0.849 $\pm$ 0.014	0.713 $\pm$ 0.022	0.577 $\pm$ 0.023	0.543 $\pm$ 0.018
	Survival on plate	+	+	+	–	–	–	–
IMAUFB084	$\Delta A_{620\text{nm}}$	2.423 $\pm$ 0.013	2.207 $\pm$ 0.015	1.821 $\pm$ 0.035	1.739 $\pm$ 0.028	1.547 $\pm$ 0.044	1.398 $\pm$ 0.051	1.265 $\pm$ 0.052
	Survival on plate	+	+	+	+	+	+	+

¹+ and – indicate that the survival rate of the strain was, respectively, >50% or <50% after culturing for 12 h at 37°C on de Man, Rogosa, and Sharpe (MRS) agar plates containing the specific concentration of bile salts; $\Delta A_{620\text{nm}}$ is the change in absorbance at 620 nm ($n = 3$; mean $\pm$ SD).

could be classified by the delay of growth or lag time in the presence of oxgall compared with its normal growth. Resistant, tolerant, weakly tolerant, and sensitive strains have delay of growth (d) or lag time of <15 min, $15 < d \leq 40$ min, $40 < d \leq 60$ min and $d > 60$ min, respectively. The delay of growth or the lag time for IMAUFB064, IMAUFB084, IMAUFB090, and IMAUFB073 were determined to be 35.4, 45.6, 46.2, and 57.6 min, respectively. From these results, only the reference strain BB12 but not any of the other *Bifidobacterium* isolates could be classified as a resistant strain. The isolate IMAUFB064 fell into the group of bile salt tolerance, whereas IMAUFB084, IMAUFB090, and IMAUFB073 were only weakly tolerant.

Fermentation and Storage Characteristics of *Bifidobacterium* in Bovine Milk

Two other important characteristics of potential fermentation starter culture strains are their efficiency in fermentation as indicated by the decrease in pH and increase in titratable acidity, as well as a high viability and a long survival time during the shelf life of the

fermented products. Therefore, these are also vital selection criteria tested in this study. In the fermentation experiments, all strains presented good acid-producing ability, as the average pH declined from about 6.45 to below 4.5 and the titratable acidity increased from 15 °T to over 80 °T during fermentation at 37°C for 21 h in bovine milk (Figure 2). Strain IMAUFB073 showed a high efficiency of acid production, as only 18 h were needed to end the fermentation at the same temperature.

To test the survival of these bacteria during product shelf life, the viability of these strains in the fermented bovine milk was followed for 21 d. It was found that, generally, the pH of the fermented bovine milks slightly decreased from about pH 4.5 to pH 4.36 (Figure 3). The pH of the fermented milk made by strain BB12 declined more than that of the others and was up to a pH change of 0.22, whereas the pH decline of fermented milks made with the 4 other strains (IMAUFB064, IMAUFB073, IMAUFB090, and IMAUFB084) were 0.12, 0.12, 0.12, and 0.13, respectively. During the first 7 d of storage, the pH change was similar and was mild in fermented milks produced by all the strains. The

Table 5. Bile tolerance (lag time) of 5 *Bifidobacterium* strains to 0.3% bile salts¹

Strain	Absorbance at 620 nm measured per hour ²		
	Modified MRS-mercaptoacetate sodium	Modified MRS-mercaptoacetate sodium + oxgall	Lag time (h)
BB12	1.86 $\pm$ 0.16	2.11 $\pm$ 0.09	0.25 $\pm$ 0.07 ^d
IMAUFB064	1.82 $\pm$ 0.08 ^b	2.41 $\pm$ 0.11 ^c	0.59 $\pm$ 0.03 ^c
IMAUFB084	2.07 $\pm$ 0.36 ^a	2.83 $\pm$ 0.25 ^a	0.76 $\pm$ 0.09 ^b
IMAUFB090	1.81 $\pm$ 0.25 ^b	2.58 $\pm$ 0.34 ^b	0.77 $\pm$ 0.09 ^b
IMAUFB073	1.82 $\pm$ 0.35 ^b	2.78 $\pm$ 0.27 ^a	0.96 $\pm$ 0.08 ^a

^{a–d}Values within a column followed by different superscript letters are significantly different from each other ($P < 0.05$).

¹Data are presented as mean $\pm$ SD from triplicate experiments.

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

Figure 2. Change of pH and titratable acidity during fermentation of bovine milk by various selected *Bifidobacterium* isolates and *Bifidobacterium animalis* BB12.

titratable acidity of the isolates from the current study increased by about 10 °T, which was lower than that of the BB12 reference strain (increased by 20.1 °T).

The changes in viable counts of bifidobacteria in the fermented bovine milks during storage for 21 d at 4°C are shown in Figure 4. Compared with the initial inoculation, the number of viable cells in all the fermented milks significantly increased when the fermentation reached the end. At the end of the fermentation, the viable counts of the fermented milks made with the reference BB12 strain and the *Bifidobacterium* isolates IMAUFB064, IMAUFB090, IMAUFB073, and IMAUFB084 were 5.13×10^8 , 3.33×10^8 , 5.44×10^8 , 6.02×10^8 , and 3.91×10^8 cfu/mL, respectively. During the 21 d of storage, the viable counts of all strains were not significantly decreased. During the first 7

d, the viable counts of bacteria BB12, IMAUFB084, and IMAUFB064 did not decline, but increased. Only the number of viable cells of the strain IMAUFB073 had a small decrease, whereas viable counts of the strain IMAUFB090 remained unchanged. After 14 d of storage, the viable counts of all the strains began to decrease, except for the reference strain BB12, which remained stable. But even strain IMAUFB084 exhibited a small decrease in viable count.

CONCLUSIONS

Twenty-nine human-originated *Bifidobacterium* strains were isolated and identified as *B. longum* and *B. pseudocatenulatum* by 16S rRNA gene sequencing and phylogenetic analysis. Preliminary in vitro tolerance tests, including acid, simulated gastrointestinal juice, and bile salt tolerance, were performed to evaluate the ability of these strains to survive in the diverse environment within the digestive tract. Twenty-five out of 29 strains (86%) were able to grow well at pH 3.0 for 3 h; however, only 7 of them could tolerate even lower acidity at pH 2.0 for 3 h. When further tests were carried out, only 4 out of these 7 strains exhibited

Figure 3. Changes of pH and titratable acidity (TA) during storage of fermented bovine milks produced by various selected *Bifidobacterium* isolates and *Bifidobacterium animalis* BB12.

Figure 4. Change in viable counts of *Bifidobacterium* strains during storage of fermented milks.

good resistance to gastrointestinal transit and bile salt tolerance. Among these 4 strains, *B. pseudocatenulatum* IMAUFB084 showed the best tolerance to acidity, bile salts, and simulated gastrointestinal juices. Moreover, all 4 strains showed good fermentation and storage characteristics in bovine milk. The fermentation time needed for these strains was less than 21 h, and was as short as only 18 h for strain IMAUFB073. The viable counts of these strains in fermented bovine milk were more than 3×10^8 cfu/mL at the end of the fermentation, and their viability did not significantly decline during longer term storage at 4°C for 21 d. Taken together, our study isolated novel strains that may serve as important resources for further dairy probiotic strain selection.

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