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ORIGINAL ARTICLE

In vitro evaluation of probiotic properties and antioxidant activities of Bifidobacterium strains from infant feces in the Uyghur population of northwestern China

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Abstract

Purpose: *Bifidobacterium* is an important probiotic used in food and medicine production. The probiotic properties of bifidobacteria are strain specific, so it is necessary to evaluate the probiotic properties of bifidobacteria isolated from specific populations, especially when developing products suitable for specific populations. The objective of this study was to evaluate the probiotic potential and safety of bifidobacteria isolated from healthy Uyghur infants from northwestern China.

Methods: In this study, antimicrobial activity, antibiotic sensitivity, hemolytic, acid and bile tolerance, hydrophobicity, co-aggregation, auto-aggregation, and antioxidant activity were evaluated.

Results: Based on antagonistic activity spectrum against seven intestinal pathogenic bacteria, 14 excellent strains were initially selected. Among 14 strains, four bifidobacteria strains (BF17-4, BF52-1, BF87-3, and BF88-5) were superior to strain *Lactobacillus rhamnosus* GG in cell surface hydrophobicity and auto-aggregation percentages and close to strain GG in co-aggregation with *Escherichia coli* EPEC O127: K63 (CICC 10411). The antioxidant activities of each of the 14 bifidobacteria strains varied with the cell components. Most of the strains were sensitive to all the antimicrobials tested, except kanamycin and amikacin.

Conclusion: BF17-4 and BF52-1 are good candidates for further in vivo studies and further used in functional foods.

Keywords: *Bifidobacterium*, Probiotic properties, Antioxidant activities, Infant intestines

Introduction

Bifidobacteria are one of the most important probiotics and widely used in food and medicine industries (Ouwehand et al. 2011). Bifidobacteria have been recommended for infants with diarrhea (Milani et al. 2017). Later, numerous studies have indicated that bifidobacteria are key symbionts during the interaction between humans and microorganisms and play an important role in maintaining the health of the human gastrointestinal tract, such as lactose intolerance and relieving constipation. It also plays a role in serum cholesterol reduction, immune regulation and anti-cancer ability (Masco et al. 2007; Souza et al. 2013).

Bifidobacterium breve is a bifidobacteria that forms the intestinal flora of infants and is used as a probiotic in preterm infants and children with congenital surgical conditions (Underwood, 2019). An observational study of 483 VLBW infants showed that the *B. infantis* EVC001 administration was associated with a significant

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reduction in the risk of NEC and NEC-related mortality (Tobias et al. 2022). Interestingly, *Bifidobacterium* plays an important role in improving intestinal microbiome homeostasis. Studies have found that by providing *B. infantis* EVC001, breastfed infants could be stably frozen at high levels, with significant changes in overall microbiome composition lasting more than a month, regardless of whether the infants were delivered vaginally or by cesarean section (Frese et al. 2017). Particularly, most *Bifidobacterium* species, such as *B. bifidum*, *B. breve*, and *B. longum ssp. Infantis* in human intestinal tract are obligative endosymbionts (Martino et al. 2018).

The primary requirement for bifidobacteria as a potential probiotic is that it must have certain characteristics, including resistance to gastric acid, bile salts, colonization of the intestine, and safety to the host. It is confirmed that bifidobacteria have antagonistic effects on *Escherichia coli*, *Listeria monocytogenes*, and *Bacillus subtilis* (Delcaru et al. 2016; Toure et al. 2003); and some research has shown that the bactericidal effect of *Bifidobacterium longum* subsp. *infantis* to pathogenic bacteria that may be related to the broad-spectrum non-organic acid substances produced during the growth and metabolism process (Scott et al. 2014). With further studies on the probiotic performance of bifidobacteria, it has been found that the addition of bifidobacteria to infant formula has an excellent co-therapeutic effect on children with allergies, diarrhea, and other diseases (O'Sullivan et al. 2015).

One study found that human aging and some diseases are closely related to free radicals in the human body (Vina et al. 2013). Aerobic organisms can produce oxygen free radicals during oxygen metabolism, which are one of the root causes of physiological aging and pathological changes. It is reported that the imbalance between free radical production and scavenging capacity can cause aging and various diseases such as tumor, atherosclerosis, myocardial infarction, diabetes, hypoxia, or ischemic injury (Martinez-cayuela, 1995). Some studies reported that some probiotic possess strong antioxidative activity that can decrease the risk of reactive oxygen species (ROS) over production. Bifidobacteria can also help the body reduce oxidative damage stress related diseases from external sources through oral route (Amaretti et al. 2013). Thus, searching for safe and natural alternative effective antioxidants from the wild bifidobacteria that might complement or replace current synthetic antioxidants draws great interest.

Obviously, bifidobacterial strains of human origin are safer and feasible for the human than the other source, in view of the physiological needs and the colonization in the human intestine (Prasanna et al. 2014). Numerous studies focused on bifidobacteria isolated from breast milk or from infant stool samples and its application in functional foods (Zacarias et al., 2011). However, an increasing number of studies recently authenticated that probiotic effect of bifidobacteria have individual specificity (Gupta et al. 2017). In addition, *Bifidobacterium* community varied depending on the national groups, and their prevalence differed significantly among regional populations. Therefore, the development of probiotics and their preparations targeting different ethnic groups should be as personalized as possible.

Xinjiang, situated in Northwest China, is a multi-ethnic region with a large tract of land, where Uighur have developed a unique lifestyle and food culture. To date, in terms of dominant factors shaping the human microbiome, the geographical/racial variations and some of cultural/behavioral features like diet, hygiene, environmental exposure, etc., were widely believed to overshadow host heredity. Uighur villages in Xinjiang have always maintained their traditional way of life and eating habits, have their own language, and do not marry outside the ethnic group. Naturally, it can be speculated that the bifidobacteria isolated from the feces of Uygur people have more potential to be developed into probiotic products suitable for this ethnic group.

Studies on the probiotic properties of human bifidobacteria have been reported. Liu et al. (2020) isolated and identified *Bifidobacterium* and *Lactobacillus* from human colostrum and screened out two lactic acid bacteria with potential probiotics. Liu et al. (2013) also evaluated the probiotic characteristics of bifidobacteria isolated from human fecal samples from different ethnic regions of China, as well as its fermentation efficiency and storage characteristics, and finally screened out at least 4 potential dairy probiotics. However, there are few reports on the development of probiotics among ethnic minorities in China, especially for the anti-oxidation bifidobacteria. Therefore, the main purpose of this study is in vitro to evaluate the safety and effectiveness of indigenous *Bifidobacterium* strains isolated, by examining their probiotic properties with the emphasis on antioxidant activities, which provides an opportunity to develop new probiotics suitable for the intestinal physiological characteristics of Xinjiang Uygur.

Materials and methods

Isolation of bacteria from feces samples

Bifidobacteria is the most abundant species in the intestinal microbiota of infants, and its diversity decreases with the increase of age (Turroni et al., 2018). Therefore, this study selected the specific group of infants of Uygur nationality. Samples were taken from the feces of 28 breast-fed infants from the Uighur ethnic group of Xinjiang in northwest China. Volunteers

were enrolled according to the following criteria: (i) healthy infants without present or past underlying conditions; (ii) infants and they mother had not been prescribed antibiotics for at least 3 months prior to the study or had taken any probiotics; (iii) normal, full-term vaginal pregnancy; and (iv) absence of infant and/or maternal perinatal problems, including mastitis (Zuo et al., 2016). This study was conducted according to the guidelines of the Declaration of Helsinki. All procedures involving human subjects were then adopted by the Ethics Committee of the First Affiliated Hospital, Shihezi University School of Medicine (2017-117-01). In the preliminary research work, written informed consent was issued by all parents or guardians of participating infants. Explain relevant matters to the baby's guardian and obtain explicit informed oral consent before sampling. Samples were collected with a stool sampler, stored in a vehicle refrigerator at −20°C and quickly transported back to the laboratory for separation.

Stools were serially diluted in sterile saline water. Respectively put 200 μL of each dilution into modified deMan, Rogosa, and Sharpe (MRS, Sigma) medium (supplemented with 0.05 g/L L-cysteine hydrochloride, 0.0025 g/L mupirocin, 0.005 g/L norfloxacin; pH 6.0) and incubated at 37°C under anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) for 48–72 h. Colonies with Y or V shape Gram-positive, catalase-negative were picked up. Purification was repeated by thrice on modified MRS medium in order to ensure the purity of the cultures. Stock cultures were maintained at −20°C in MRS broth with 50% (v/v) glycerol. Except for the modified MRS medium used in the process of strain isolation, MRS medium was used in the following experimental process for strain cultivation.

Identification of selected bacterial

16S rRNA analysis was carried out as described by Yu et al. (2011). Genomic DNA was extracted according to the instructions of the bacterial genomic DNA extraction kit (TIANGEN). Polymerase chain reaction (PCR) was performed using universal primers 27F (5′-AGA GTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGT TACCTTGTTACGACTT-3′). Then, PCR products were purified and sequenced by Shenggong Biotechnology Co., Ltd., Shanghai, China. Based on strain grouping result obtained by 16S rRNA gene analysis, all isolates were subjected to accurate genetic identification throughout multiplex-PCR, using species-specific primers targeting a conserved region of the *tuf* gene (Ventura et al., 2002) and rep-PCR (Sheu et al., 2013). The nucleotide sequences were compared with sequences available at GenBank database using the BLAST program.

Antimicrobial activity

The antagonism towards various bacterial pathogens and common bacteria present in the GI tract was evaluated according to the Oxford cup method (Argyri et al., 2013). Respectively, the cell-free supernatant (CFS) produced from seventy-five isolates were tested in duplicate against the indicator bacteria (*Escherichia coli* EPEC O127: K63 (CICC 10411), *Escherichia coli* (ETEC) O78: K80 (CICC10421), *Escherichia coli* EHEC O157: H7 (CICC 21530), *Salmonella typhimurium* CICC 10420, *Listeria monocytogenes* CGMCC 1.9136, *Salmonella enterica* subsp. *enterica* CGMCC 1.1754, and *Staphylococcus aureus* CICC 21600). The three different *Escherichia coli* species were selected because they have different pathogenic effects in humans and are representative of the diarrheagenic *Escherichia coli* species. Those indicator bacteria were obtained from the China Industrial Microbial Culture Management Center.

Acid and bile salts resistance assay

Acid and bile salts tolerance test were determined as described by Arboleya et al. (2011) with some modifications. Briefly, strains were grown 28 h on MRS broth at 37°C under anaerobic conditions, and the cultured cell fluid was centrifuged at 4000g for 5 min at 4°C and washed twice with 0.85% (w/v) sterile saline solution. The cell pellet was re-suspended in PBS solution with different pH, i.e., 2.5, 3.5, and 4.5 incubated for 0h and 4 h. Then, the absorbed 100 μL of these dilutions were applied to MRS agar plates at 37°C for 28 h under anaerobic conditions, and the number of colonies on the plate was used to determine the survival rate (the viable cell count was recorded and compared with initial viable cell count, i.e., the number of viable cells treated for 0 h). For bile salt tolerance, survival rates for these strains were estimated at different pig bile salt concentrations (0.2, 0.4, and 0.6%). The experiment was carried out using the same experimental method as the acid resistance test and Compared with the treatment group cultured for 0 h under the same conditions (same bile salt concentration). In this case, the experiment was conducted in triplicates and take the average.

Tolerance of strains to a simulated gastric and intestinal fluid

The survival rate of strains after the exposure of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were assessed according to Liao et al. (2019) with some modifications. MRS broth, which incorporated 3

g/l pepsin (Sigma-aldrich), was adjusted to pH 2.5 with 12 M HCl for fresh SGF. SIF was prepared with 3 g/L bile salt and 1 g/L pancreatin (Sigma-aldrich) dissolved into MRS broth (pH 7.4) adjusted by 0.1 M NaOH. The strain fermentation liquid (1 mL) washed with 0.85% (w/v) sterile saline solution was added to simulated gastric juice (9 mL) and cultivated at 37°C for 0, 60, and 120 min under anaerobic conditions. Then, the samples that were exposed to simulated gastric juice (120 min) were harvested and washed with 0.85% (w/v) sterile saline solution and then they were added into 9 ml of simulated intestinal juice (pH7.4). The SIF containing bacteria was cultivated at 37°C for 60 and 120 min under anaerobic conditions. Samples were taken at each of the above time points for counting viable bacteria (CFU/mL) and evaluating the survival rate (the viable cell count was recorded and compared with initial viable cell count). The commercial probiotic *Lactobacillus rhamnosus* GG was treated in the same way as above for comparison.

Hydrophobicity, co-aggregation, and auto-aggregation assay

Hydrophobicity (H%) of the fourteen selected strains was assayed as described by Mohanty et al. (2019) with some modifications. Chloroform, xylene, and ethyl acetate were used to assess the electron donor (basic), hydrophobic, and electron acceptor (acidic) characteristics of bacterial surface, respectively (Kos et al., 2003). The hydrophobicity is calculated according to the following equation:

$$H\% = (1 - A_1/A_0) \times 100 \quad (1)$$

where A_1 represents the absorption value at 580 nm of the aqueous phase after mixing and phase separations and A_0 represents the absorption value at 580 nm of original suspension.

Auto-aggregation (A %) ability of the fourteen selected strains was assayed according to Abdulla et al. (2014) and was expressed as percent decrease in the absorbance (A_{600}) after 5 h (A_t) relative to that of original suspension (A_0). It was expressed using the following:

$$A\% = (1 - A_t/A_0) \times 100 \quad (2)$$

Co-aggregation of bifidobacteria with pathogenic bacteria *Escherichia coli* EPEC O127: K63 (CICC 10411) was evaluated as described by Solieri et al. (2014).

Antioxidant activities

Preparation of test samples

Extracellular fermentation supernatant (EFS): The strains were grown in MRS broth at 37°C for 28 h to logarithmic growth phase under anaerobic conditions, and the culture solution were centrifuged at 4000g, 4°C for 10 min. The supernatant was collected for antioxidant activity test.

Intact cells (IC): The bacterial cells obtained by centrifugation were washed thrice with PBS solution (pH 7.4) and then resuspended in an equal volume of PBS solution. The obtained solution was the bacterial solution required for antioxidant activity test.

Intracellular cell extracts (ICE): After the bacterial cells washed thrice with PBS solution, collected and sent to low temperature ultrasonic disruption (400 W, working time 5 s, interval 5 s, 80 times), then centrifuged at 8000g for 20 min, the supernatant were collected as intracellular cell extract (ICE) for antioxidant activity test.

DPPH-free radical scavenging assay

DPPH radical scavenging ability was measured with some modification (Fan et al., 2017). The 1-mL sample (EFS/IC/ICE) was mixed with 1 mL ethanol DPPH solution (0.4 mmol/L) and incubated for 30 min at 37°C in the dark to determine the absorbance value at 517 nm. The DPPH-free radical scavenging activity was counted as follows:

$$scavenging\ activity\% = 1 - (A_1 - A_2)/A0 \times 100 \quad (3)$$

where A_0 represents the Abs of control group replaced the sample solution with distilled water, A_1 is the Abs of test sample reacting with DPPH, and A_2 is the Abs of blank group replaced the DPPH solution with an equal volume of absolute ethanol.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging test was conducted with modified method (Ahire et al., 2013). The mixture is used to prepare hydroxyl radical which are composed of 1 mL of 0.75 mM 1,10-phenanthroline, 1 mL of 0.75 mM $FeSO_4$, 1 mL of H_2O_2 (0.01%, vol/vol), and 1.5 mL of 0.15 M sodium phosphate buffer (pH 7.4). The 1-mL sample (EFS/IC/ICE) was mixed with the solution containing hydroxyl radical and incubated at 37°C for 30 min. The absorbance of the mixture was measured at 536 nm. The hydroxyl scavenging activity was calculated as follows:

$$scavenging\ activity\% = (A_1 - A_0)/(A_2 - A_0) \times 100 \quad (4)$$

where A_0 represents the Abs of control group replaced the sample solution with distilled water, A_1 is the Abs of sample solution reacting with hydroxyl radical, and A_2 is the Abs of blank group replaced the H_2O_2 solution with distilled water.

Superoxide anion scavenging assay

The activity of scavenging superoxide anion was determined by a modified method (Kuda et al., 2014). The

superoxide radical was generated in 3 mL of 0.1 M sodium phosphate buffer (pH 7.4) which contains 156 μM NADH, 52 μM NBT, and 20 μM PMS. The 1-mL sample (EFS/IC/ICE) was mixed with the solution containing superoxide anion and incubated at 25°C for 5 min. The absorbance of the mixture was measured at 560 nm. Scavenging activity was counted as follows:

$$scavenging\ activity\% = 1 - (A_1 - A_2)/A_0 \times 100 \quad (5)$$

where A_0 is the Abs of the Tris-HCl buffer reacting with pyrogallicacid, A_1 is the Abs of the sample solution reacting with superoxide anion, and A_2 is the Abs of Tris-HCl buffer and sample solution without pyrogallicacid.

Determination of total reducing capacity

The total reducing power of samples performed was according to with minor modifications (Xing et al., 2018). The 0.2-mL sample (EFS/IC/ICE) was mixed with equal volume 1% potassium ferricyanide and 20 mmol/L sodium phosphate buffer (pH 7.0) and incubated at 50°C for 20 min. The 0.2 ml of 10% (wt/vol) trichloroacetic acid was added to the mixture to terminate the reaction and centrifuged at 780g at 4°C for 10 min. The top of the supernatant was mixed with 0.1 mL 0.1% (wt/vol) ferric chloride and 0.4 mL distilled water. The absorbance value was measured at 700 nm and l-cysteine was used as the standard.

Antibiotic susceptibility

Antibiotic susceptibility of the selected strains was assayed by using the drug sensitive paper agar diffusion method (K-B method) (Bauer et al., 1966). 200 μL (10^7 CFU/mL) of cultured 28 h strain suspension was spread to the MRS plates. In this study, 18 antibiotics were selected, among which rifampicin, clindamycin, penicillin, and vancomycin were selected because of their wide clinical application, while the other 14 antibiotics were all broad-spectrum antibiotics. The 18 commercial antibiotic pills (OXOID) of tetracycline (30 μg), minocycline (30 μg), rifampin (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), kanamycin (30 μg), chloramphenicol (30 μg), ergomycin (15 μg), clindamycin (2 μg), ampicillin (10 μg), penicillin (10 μg), streptomycin (300 μg), vancomycin (30 μg), amikacin (30 μg), oxacillin (1 μg), cockalin (30 μg), ampicillin (10 μg), and gentamicin (120 μg) were placed on the above MRS agar medium. After incubation under anaerobic condition at 37°C for 48 h, the plates were observed for the diameter (mm) of clear zone around the pills. The resistance and sensitivity were expressed according to the guidelines of the American Clinical and Laboratory Standards Institute (CLSI) (2014).

Hemolytic activity

Blood hemolysis was examined on MRS agar plates supplemented with 5% sheep blood, which were incubated at 37°C under anaerobic conditions for 28 h. The blood plates were confirmed for β-hemolysis (clear zones around colonies), α-hemolysis (greenish zones around colonies), or γ-hemolysis (no zones around the colonies). *Lactobacillus rhamnosus* GG was used as negative control.

Statistical analysis

The values were expressed as the mean ± the standard deviation (SD) based on three independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) using SPSS 19.0. Differences were considered to be statistically significant if $P<0.05$ by applying Duncan's multiple range tests.

Results

Strains identification

In this study, a total of 28 stool samples from healthy Uyghur infants from northwestern China were collected. Among the 122 isolates, 75 *Bifidobacterium* species strains were characterized by using a combination of rep-PCR, species, and/or subspecies-specific PCR based on the *tuf* gene and 16S rRNA gene sequencing as *B. bifidum* (15 isolates), *B. pseudolongum* (12), *B. pseudocatenulatum* (13), *B. kashiwanohense* (11), *B. longum* subsp. *infantis* (18), and *B. breve* (6).

Antimicrobial activity

In the present study, antimicrobial activity was chosen as criteria screening the strains for the further experiments. Among the 75 *Bifidobacterium* strains investigated (Table S1), fourteen strains had significant antibacterial activity against seven common pathogenic bacteria (Table 1). All of the strains showed forceful bacteriostatic ability against *Escherichia coli* EPEC O127: K63 (CICC 10411), *Escherichia coli* ETEC O78: K80 (CICC 10421), and *S. typhimurium* CICC 10420 (with an inhibition zone higher than 8.0 mm in diameter) as *L. rhamnosus* GG. Interestingly, *B. longum* subsp. *infantis* (BF48-2, BF17-4, BF67-13) and *B. bifidum* (BF87-11, BF52-1) could inhibit all pathogenic bacteria in this experiment which had a broad spectrum of inhibition. While five strains (BF8-3, BF66-2, BF13-15, BF88-5, BF96-5) had no inhibitory effect on *Salmonella enterica* subsp. *enterica* (CGMCC 1.10754), *Listeria monocytogenes* (CGMCC 1.9136), and *Staphylococcus aureus* CICC 21600.

Resistance to acid and bile salts

To determine whether the isolated bifidobacteria can survive the acidic environment of the stomach, the acid and

Table 1 Antimicrobial activity of the *Bifidobacterium* strains against the enteropathogenic bacteria

Strain	*E. coli* (EPEC) O127: K63 CICC 10411	*E. coli* (ETEC) O78: K80 CICC 10421	*E. coli* (EHEC) O157: H7 CICC 21530	S. *typhimurium* CICC 10420	*S. enterica* CGMCC 1.1754	L. *monocytogenes* CGMCC 1.9136	*S. aureus* CICC 21600
B. pseudocatenulatum							
BF5-10	+	++	+	+	+	-	+
BF8-3	++	+	+	++	-	-	-
B. longum subsp. *infantis*							
BF48-2	+	+	+	++	-	+	-
BF17-4	+	++	+	+	+	-	+
BF66-2	++	++	++	+	-	-	-
BF67-13	+	+	+	++	-	+	-
B. bifidum							
BF87-11	++	+	-	+	-	-	+
BF87-3	++	+	-	+	-	-	+
BF87-12	++	+	++	++	+	-	+
BF52-1	+	++	++	++	++	+	+
B. kashiwanohense							
BF13-15	++	+	+	+	-	-	-
BF88-5	++	+	+	++	-	-	-
BF98-12	+	+	-	+	-	-	+
BF96-5	+	+	-	+	-	-	-
L. rhamnosus GG	++	+	+	+	+	+	+

Diameter of the zone of inhibition (including that of the oxford cup of 8 mm): (−) less than 8 mm, negative; (+), 9-16 mm, mild; (++), 17–24 mm, strong

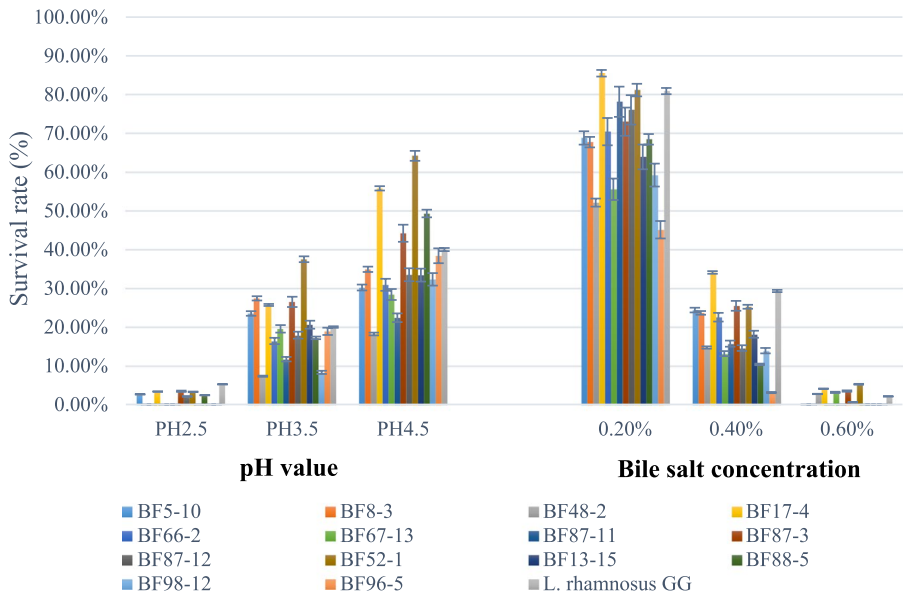

Fig. 1 Survival rate (%) of the *Bifidobacterium* strains in low pH and bile salts. (Error bars indicate standard deviation from three independent experiments)

bile salts tolerance of the isolates was tested. As shown in the Fig. 1, the test strains were grown in MRS medium with different pH values and bile salt concentrations, the number of colonies on the plate was used to determine the survival rate. Two of the fourteen strains had a survival rate of more than 50% at pH 4.5 and five strains

showed above 30% of the survival rate at pH 3.5. Only six of the strains (BF5-10, BF17-4, BF87-3, BF87-12, BF52-1, BF88-5) had a survival rate of 1 to 5% at pH 2.5. Meanwhile, the results indicated that the survival rate decreases with a rise in bile salt concentrations. It can be seen from the growth condition that fourteen strains were able to survive at a bile salt concentration of 0.2%, with the gradual increase of bile salt concentrations, the growth of most bifidobacteria were inhibited, only five of the strains (BF48-2, BF17-4, BF67-13, BF87-3, BF52-1) had very low survival rate at a bile salt concentration of 0.6%.

Resistance to simulated gastric and intestinal fluid

The fourteen bifidobacteria strains were tested for the tolerance in vitro simulated experiments in the GI tract, which presented a course of 120 min cultivation in SGF and followed by growth for 120 min in SIJ. *L. rhamnosus* GG was used as positive control (Fig. 2). A successive decrease survival rate of strains was observed. All strains had a survival rate of exceed 68% in simulated gastric fluid for 60 min and eight strains showed above 50% of the survival rate after expose to SFG for 120 min. However, it was observed that the survival rate of strains was declined rapidly after transferring into the intestinal simulation. Only three of the strains (BF17-4, BF87-3, BF52-1) survived sequential cultivation in simulated gastric and intestinal fluid for 240 min.

Hydrophobicity, auto-aggregation and co-aggregation assay

Cell surface hydrophobicity was evaluated by the partitioning ratio of cells between aqueous and aromatic (chloroform, xylene, and ethyl acetate) layers and was measured in a two-phase system. Results are illustrated in Fig. 3. When compared to the hydrophobicity, the affinities with chloroform were significantly increased in the 14 *Bifidobacterium* strains, while those with ethyl acetate were decreased ($P< 0.05$). The best affinities with chloroform were observed in *B. bifidum* BF52-1 (65.44%), *B. bifidum* BF87-3 (60.28%), and *L. rhamnosus* GG (57.42%), while the least affinities were observed in *B. longum* subsp. *infantis* BF67-13 (20.11%) and *B. kashiwanohense* BF88-5 (24.68%). Unlike chloroform, the bacterial adhesion to ethyl acetate was low, ranging from 3.42 to 9.64%.

The auto-aggregation rate of 14 strains ranged from 20.22 to 67.56% after incubation at room temperature for 5 h (Fig. 3). Four strains (BF17-4, BF52-1, BF87-3, and BF88-5) exhibited excellent auto-aggregation properties, with an aggregation percentage higher than 50%. In addition, *B. longum* BF48-2, *B. longum* subsp. *infantis* BF66-2, and *B. bifidum* BF87-12 showed auto-aggregation rate ranging from 36.11 to 46.40%.

Results of co-aggregation ability of the thirteen selected strains with the pathogen *E. coli* O127: K63 (CICC 10411) are illustrated in Fig. 3. Except for 4 strains (BF48-2, BF66-16, BF88-5), all tested strains

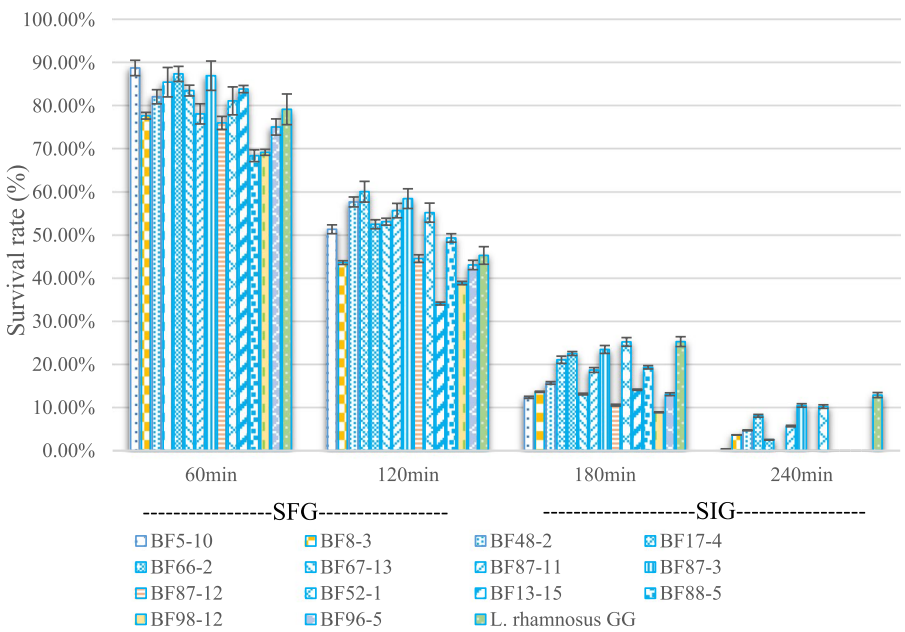

Fig. 2 Survival rate(%)of the *Bifidobacterium* strains after sequential cultivation in simulated gastric and intestinal fluid. (Error bars indicate standard deviation from three independent experiments)

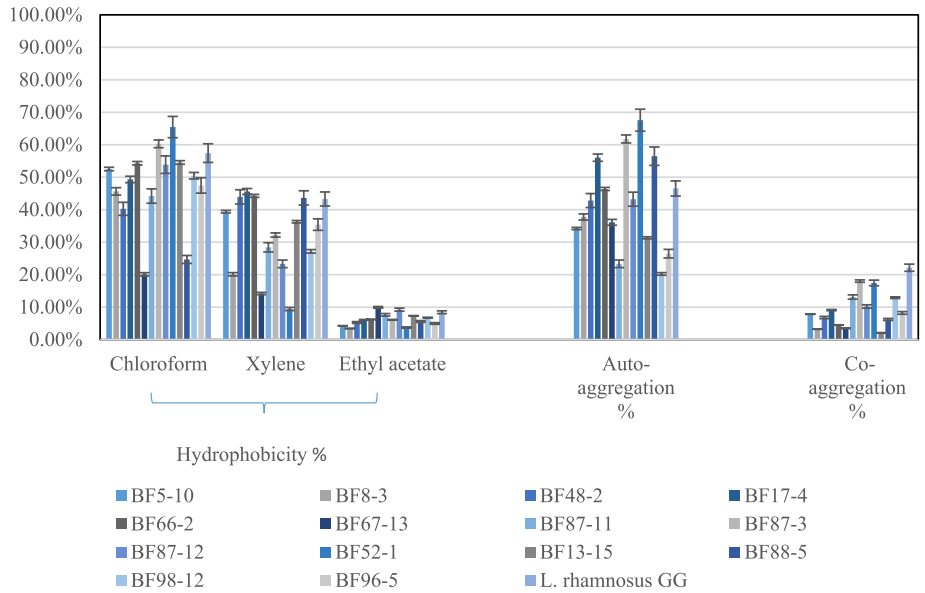

Fig. 3 Hydrophobicity (chloroform, xylene, ethyl acetate), auto-aggregation and co-aggregation with *Escherichia coli* EPEC O127: K63 (CICC 10411) percentage of the *Bifidobacterium* strains. (Error bars indicate standard deviation from three independent experiments)

showed above 30% of percentage of co-aggregation. The highest percentages were displayed by the *B. bifidum* BF87-3(18.11%), BF52-1(17.45%), and *L. rhamnosus* GG (22.14%).

Antioxidant activity

Scavenging of DPPH radicals

The DPPH-radical scavenging activity of the isolated strains is shown in Fig. 4. Results indicated that the ICE of bifidobacteria had the highest average for radical scavenging activity, which reached 42.11%, *B. longum* subsp. *infantis* BF17-4 had the highest scavenging rate, with 56.25%. Besides, BF87-11, BF13-15, and GG strain scavenging rates were higher than 50%. Meanwhile, the DPPH average scavenging rate for EFS of the tested strains was 16.54%. Strain BF66-2 had the highest scavenging rate of 25.68%, followed by *L. rhamnosus* GG and BF87-3. The bifidobacteria IC had the lowest level of

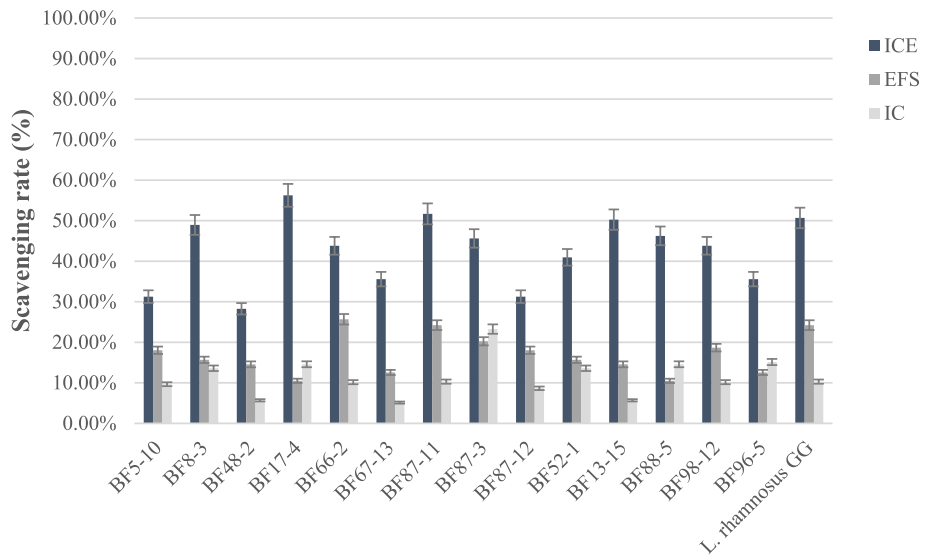

Fig. 4 The ability of the *Bifidobacterium* strains' intracellular cell extract (ICE), extracellular fermentation supernatant (EFS), and intact cells (IC) to scavenge DPPH free radicals. (Error bars indicate standard deviation from three independent experiments)

DPPH scavenging rate with an average scavenging rate of 11.44%.

Scavenging of hydroxyl radicals

As shown in Fig. 5, the hydroxyl radicals scavenging ability of IC (average scavenging rate of 44.23%) was significantly different from the ICE and EFS scavenging rates and these differences were statistically significant ($p < 0.05$) between strains. The strain with the highest radical scavenging activity in the bacterial IC was BF17-4 (57.05%), and the lowest activity was found for BF67-13 (21.62%). Therefore, we concluded that the number of substances that scavenge hydroxyl radicals in bifidobacteria cells was strikingly higher than in other parts.

Scavenging of superoxide anion radicals

The scavenging ability of superoxide anion radicals is confirmed as shown in Fig. 6. Remarkably, the bacterial

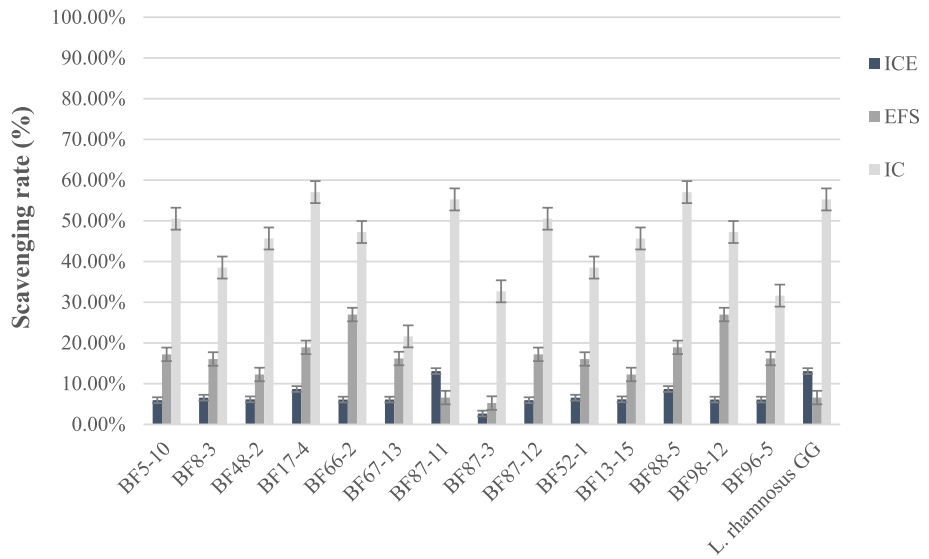

Fig. 5 The ability of the *Bifidobacterium* strains' intracellular cell extract (ICE), extracellular fermentation supernatant (EFS), and intact cells (IC) to scavenge hydroxyl free radicals. (Error bars indicate standard deviation from three independent experiments)

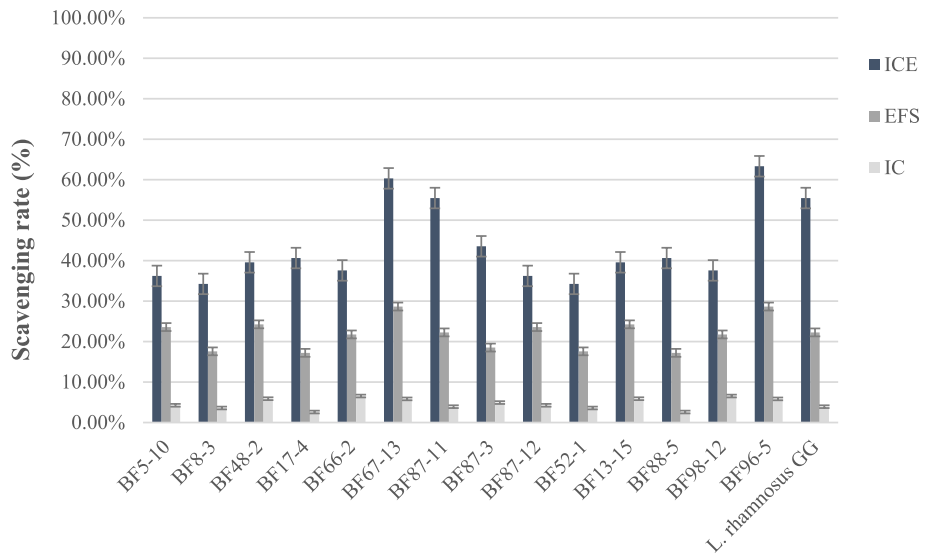

Fig. 6 The ability of the *Bifidobacterium* strains' intracellular cell extract (ICE), extracellular fermentation supernatant (EFS), and intact cells (IC) to scavenge superoxide anion free radicals. (Error bars indicate standard deviation from three independent experiments)

IC had little ability to scavenge superoxide anion (scavenging rate average scavenging rate 4.74%), and the ICE (scavenging rate average scavenging rate 42.80%) was better than the EFS (average scavenging rate 21.92%). Among all the strains, BF67-13 had the highest scavenging ability of superoxide anion with the scavenging rate of ICE and EFS reaching 60.32% and 28.65%, respectively. Thus, we hypothesized that the substance of scavenging superoxide anion in the bifidobacteria were mainly distributed in the bacterial cells, which may be associated with the presence of cell-surface proteins or polysaccharides can improve the antioxidant activity of intact cells.

Assay of total reducing power

In the total reducing power test, antioxidants have the capacity to reduce Fe^{3+} /ferricyanide complex to its Fe^{2+} form. And [Fe (CN) 6]$^{-4}$ would react with Fe^{3+} to create Prussian blue, which has the strongest absorbance at 700 nm. The reducing ability of distinct parts of the 14 isolates tested is shown in Fig. 7, average absorbance (0.78) of the EFS at 700 nm was strikingly higher than the ICE (0.32) and IC (0.14). The absorbance of BF52-1 in the EFS was significantly ($p<0.05$) higher than that of other strains (reaching 1.29). And the reducing power of the BF98-12 and BF87-11 were higher than the standard probiotic strain *L. rhamnosus* GG (0.94±0.01).

Properties related to safety

The results for the antibiotic susceptibility analysis to 18 antibiotics for the *Bifidobacterium* strains are shown in Table 2. All tested strains exhibited resistant to kanamycin and amikacin. On the contrary, fourteen strains were sensitive to tetracycline, minocycline, rifampin, chloramphenicol, ergomycin (except BF17-4), clindamycin (except BF17-4), ampicillin, penicillin, streptomycin, vancomycin (except BF88-5), oxacillin (except BF48-2), cockalin (except BF88-5), ampicillin, and gentamicin. Regarding the antibiotics ciprofloxacin and norfloxacin, 5 strains were semi-tolerant to ciprofloxacin and 6 strains were semi-tolerant to norfloxacin, as *L. rhamnosus* GG. Results of hemolysis activity of *Bifidobacterium* strains are reported in Table 2, none of the strains was able to hydrolyze sheep blood, proving non-hemolytic activity.

Discussion

There are increasing experimental data that support long-term health benefits elicited by the infant gut microbiota and that also implicate the early human gut microbiota in modulating risk factors related to particular adult health conditions (Relman, 2012). This realization has in turn fueled the development of nutraceutical products (e.g., probiotics and/or prebiotics). In this study, 75 strains of bifidobacteria isolated from feces of Uygur infants were evaluated for their probiotic potential, including *B. bifidum*, *B. pseudolongum*, *B. pseudocatenulatum*, *B. kashiwanohense*, *B. longum* subsp. *infantis*, and *B. breve*. Bifidobacteria is regarded as an important probiotic group in the human gut, which have biological antiseptic properties, and may prevent and inhibit the invasion and colonization of pathogenic microorganisms by biological antagonism or by reducing pH (Ben Braiek et al., 2018).

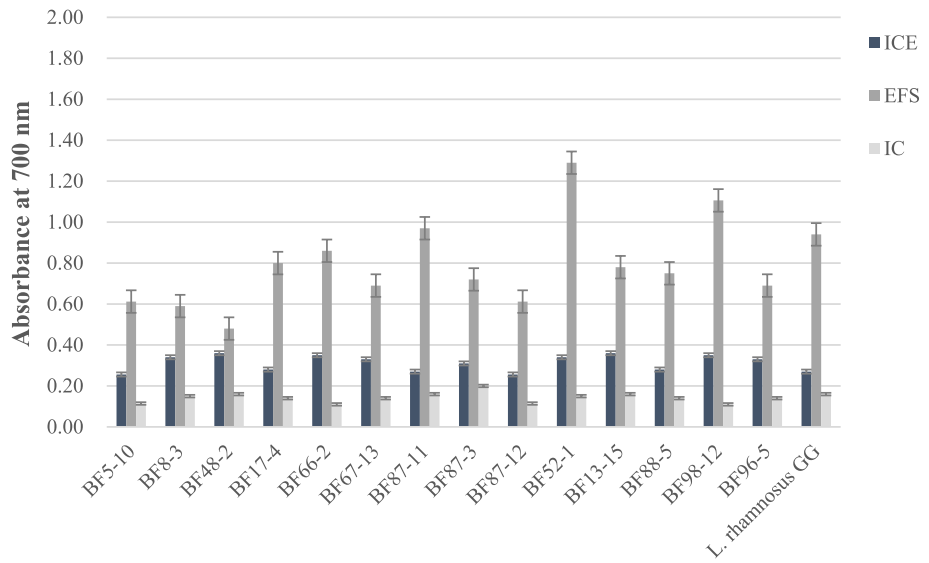

Fig. 7 The total reducing power of the *Bifidobacterium* strains' intracellular cell extract (ICE), extracellular fermentation supernatant (EFS), and intact cells (IC) of the strains. (Error bars indicate standard deviation from three independent experiments)

Table 2 Antibiotic-resistant and hemolytic activity of the *Bifidobacterium* strains

Strain	Antibiotics[a]																	Hemolytic activity[b]
	TE	MH	RD	CIP	NOR	K	C	E	DA	AML	P	S	VA	AK	OX	TEC	AMP	CN	
BF5-10	S	S	S	I	I	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF8-3	S	S	S	R	I	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF48-2	S	S	S	I	I	R	S	S	S	S	S	S	S	R	I	S	S	S	-
BF17-4	S	S	S	R	R	R	S	R	R	S	S	S	S	R	S	S	S	S	-
BF66-2	S	S	S	R	I	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF67-13	S	S	S	I	R	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF87-11	S	S	S	R	I	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF87-3	S	S	S	R	R	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF87-12	S	S	S	R	R	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF52-1	S	S	S	R	R	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF13-15	S	S	S	I	R	R	S	S	R	S	S	S	S	R	S	S	S	S	-
BF88-5	S	S	S	I	R	R	S	S	S	S	S	S	R	R	S	R	S	S	-
BF98-12	S	S	S	R	R	R	S	S	S	S	S	S	S	R	S	S	S	S	-
BF96-5	S	S	S	R	I	R	S	S	S	S	S	S	S	R	S	S	S	S	-
GG	S	S	S	S	I	R	S	S	S	S	S	S	S	R	S	S	S	S	-

[a] *TE* tetracycline; *MH* minocycline; *RD* rifampin; *CIP* ciprofloxacin; *NOR* norfloxacin; *K* kanamycin; *C* chloramphenicol; *E* ergomycin; *DA* clindamycin; *AML* ampicillin; *P* penicillin; *S* streptomycin; *VA* vancomycin; *AK* amikacin; *OX* oxacillin; *TEC* cockalin; *AMP* ampicillin; *CN* gentamicin. *S* susceptible; *I* intermedio; *R* resistente; [b] -: No hemolysis

Fourteen representative *Bifidobacterium* strains showed forceful activity against *E. coli*, *S. typhimurium*, and *S. enterica*. This may be caused by acid or ablastins produced during the metabolic process of bifidobacteria. The exact inhibitory mechanism will be further confirmed in the subsequent studies. Similarly, Eshaghi et al. (2017) demonstrated that *Bifidobacterium* strains isolated from Iran mother's milk and infant stool could inhibit the food-borne pathogens growth of *Shigella dysenteriae*, *S. typhimurium*, *E. coli*, and *L. monocytogenese*, as well Delcaru et al. (2016) revealed bifidobacteria isolated from infant feces inhibited the growth of *E. coli*. Clinical trials have indicated that the selected *Bifidobacterium* strains used in these clinical studies are a safe and effective treatment for children with acute infectious diarrhea (Isolauri et al., 2002; Rosenfeldt et al., 2002). *S. typhimurium*, *E. coli*, and *S. enterica* are the most common diarrhea-causing pathogens in children and young people (Peltier et al., 2009), so we speculate that the bifidobacteria strains resistant to these pathogens in this study have the potential to treat diarrhea caused by pathogenic bacteria.

Probiotic bacterial strains have to overcome the harsh digestive environment (stomach acid, bile, and digestive enzymes) in order to exert their probiotic properties in the gastrointestinal tract (Larsen et al., 2018). Among the 14 strains of *Bifidobacterium* species with bacteriostatic properties in the present study, three of strains (BF17-4, BF87-3, BF52-1) were resistant to pH 2.5 and 0.6% bile salts, the latter being slightly higher than that reported by Zuo et al. (2016), where bifidobacteria survived in 0.5% bile salts. Similar to previous studies, we observed differences in the sensitivity among the different bifidobacteria species to a simulated gastrointestinal environment (de los Reyes-Gavilan et al., 2011). In this contest, three strains (BF17-4, BF87-3, BF52-1) can survive after sequential cultivation in simulated gastric and intestinal fluid for 240 min. Our results revealed a wide range of heterogeneity among tested strains in relation to simulated gastrointestinal fluid tolerance. Moreover, those isolated *Bifidobacterium* strains tolerance to the simulated gastrointestinal fluid may be related to the unique lifestyle of Uighurs who often eat hand-made dairy products and exclusively breast feed their infants.

The ability of bacteria to adhere to the intestinal epithelial cells plays an important role in the gastrointestinal colonization, preventing the bacterial numbers from decreasing due to peristalsis (Crociani et al., 1995). Criteria such as bacterial aggregation and the ability to adhere to host intestinal epithelial cells are prerequisites for the colonization of probiotic strains in the gut. Cell adhesion is also usually related to cell surface properties, such as hydrophobicity, which may affect the aggregation and adhesion of bacteria on different surfaces.

We measured the hydrophobicity of 14 isolates using different organic solvents: chloroform, xylene, and ethyl acetate. Chloroform, xylene, and ethyl acetate were

used to assess the electron donor (basic), hydrophobic, and electron acceptor (acidic) characteristics of bacterial surface, respectively (Kos et al., 2003). The hydrophobicity of all tested strains against chloroform was significantly higher than that for ethyl acetate, indicating all the strains were electron donors, which further proved that these bifidobacteria have hydrophobicity.

The ability to auto-aggregation is required for probiotics to adhere to the intestinal epithelial wall. In this work, the auto-aggregation rate of 14 strains ranged from 20.22 to 67.56% after incubation at room temperature for 5 h (Fig. 3). Co-aggregation of bacteria with harmful microorganisms has been considered as a protective mechanism against infection and has been described in *Lactobacillus* (Garcia-Cayuela et al., 2014) and in *Bifidobacterium* (Crociani et al., 1995) from human origin. This work revealed that *Bifidobacterium* strains with high co-aggregation capacity with the pathogen *Escherichia coli* EPEC O127: K63 (CICC 10411), in accordance with previous data (Pithva et al., 2014).

One of the detrimental effects of aging is caused by an increased formation of free radicals, and is accompanied by oxidative damage of the cellular structure and function. In the present study, the intracellular cell extracts of bifidobacteria had the highest average for DPPH and superoxide anion radical scavenging activity, in agreement with Lin and Chang (2000) who also found the ICE of *B. longum* ATCC 15708 had high scavenging activity toward DPPH radicals. The intact cell had the highest activity to scavenge hydroxyl radicals, and the extracellular fermentation supernatant had higher reducing ability than other extract mixture, in accordance with other previous studies (Han et al., 2017). The results of Ahire et al. (2013) showed that both intact cells and intracellular cell extracts of *Lactobacillus helveticus* displayed significant antioxidant effects on DPPH and hydroxyl radical scavenging in vitro, and Kaizu et al. (1993) showed the intracellular cell extracts also has antioxidative activity in vivo. Possible reasons for the antioxidant effect of these strains are (1) that the species of antibacterial substances in bifidobacteria are different, (2) the distribution sites are nonuniform, and (3) the action mechanism of each antioxidant substance were different, thus resulting in a species- and strain-specific activity (Wang et al., 2017). From antioxidant experiment results, it can be concluded that 14 *Bifidobacterium* strains possess an antioxidative potential and can be used as a commercial probiotic supplement to boost the antioxidative status of the host.

Intestinal microorganisms can acquire resistance genes through spontaneous mutation or horizontal transfer from another species in the gut or colon, resulting in antibiotic resistance (Davies, 1994). Although none of the identified bifidobacteria have been associated with human clinical diseases, the safety of selected strains should be assessed before use (Sharma et al., 2014). The present study evaluated the resistance of 18 antibiotics to bifidobacteria isolated from infant stool. Fourteen strains showed sensitivity to or semi-tolerant to sensitive to tetracycline, minocycline, rifampin, chloramphenicol, ergomycin, clindamycin, ampicillin, penicillin, streptomycin, vancomycin, oxacillin, cockalin, ampicillin, gentamicin, ciprofloxacin, and norfloxacin. All tested strains were known to be resistant to kanamycin and amikacin, in accordance with previous data (Masco et al., 2006). These results may be related to the geological location of the stool sample donors. The stool sample were collected from Uygur infants in Xinjiang, northwestern China, where people are away from the urban area and rarely use antibiotics. Moreover, the safety properties of the promising probiotic strains were also confirmed none of the *Bifidobacterium* strains showed hemolysis, in accordance with Awasti et al. (2016)

Conclusions

Human intestines represent an excellent source of potential probiotics. In this study, 75 strains of bifidobacteria were isolated from the feces of 28 healthy Uygur infants in northwest China. According to the antagonistic activity spectrum of 7 intestinal pathogens, 14 excellent strains were screened. Two *Bifidobacterium* strains (BF17-4 and BF52-1) with probiotic potential were screened out of 14 strains by further analysis of cell surface hydrophobicity, agglutination capacity, antioxidant capacity, and survival rate in simulated gastric and intestinal fluid. Moreover, these strains were confirmed to be safe for the absence of resistance to most clinical antibiotics and no hemolysis. Further in vivo investigation will aim to elucidate both their potential health benefits to human and their technological properties before allowing their utilization as probiotics in functional foods and drugs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-022-01670-y.

Additional file 1: Table S1. Antimicrobial activity of 75 *Bifidobacterium* strains against the enteropathogenic bacteria.

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Authors' contributions
JC: writing—final draft, data analysis. QN, WT, and LW: conceptualization, review, and editing. JC and JB: methodology. JB: writing—review. The authors read and approved the final manuscript.

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Availability of data and materials
The data were obtained by the authors.

Declarations

Ethics approval and consent to participate
All procedures involving human subjects were then adopted by the Ethics Committee of the First Affiliated Hospital, Shihezi University School of Medicine (2017-117-01). Explicit informed oral consent was obtained from the parents of the infants who provided the samples used in this study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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