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Isolation and identification of *Bifidobacterium* species from human breast milk and infant feces in Indonesia

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Abstract. *Dosan R, Mudana SO, Julyanto CMP, Purnama ET, Sugata M, Jo J, Tan TJ. 2024. Isolation and identification of* Bifidobacterium *species from human breast milk and infant feces in Indonesia. Biodiversitas 25: 337-343.* There has been a growing interest in identifying emerging probiotic strains because of their benefits for human health. Many bifidobacteria originated from humans have been reported to possess probiotics properties. They are commonly found in the intestine of breast-fed infants. Hence, this study aimed to isolate and identify bifidobacteria from human breast milk and infant fecal samples in Indonesia and evaluate their probiotic properties. Twenty colonies were isolated from two independent fecal samples and two independent breast milk samples. Ten isolates (BR1-M1, BR1-B1, BR2-5, BR2-6, BR2-12, BS2-PB3, BS2-PB5, BS2-PS1, BS2-PS2, BS2-MB1) showed a compatible phenotypic character with Bifidobacteria based on the Bergey's Manual, including Gram-positive, irregular rods, no catalase activity, non-spore-forming, and non-motile. Subsequently, four isolates with similar carbohydrate fermentation patterns as *Bifidobacterium* spp. were selected for further molecular identification based on 16S rRNA gene sequencing analysis. The results showed that BR2-5 and BR2-6 were found to be closely related to *Bifidobacterium animalis* subspecies *lactis* with 100 and 98.39% similarity, respectively. Meanwhile, BS2-PS1 and BS2-PB3 were found to be closely related to *Bifidobacterium breve* with 100 and 98.26% similarity, respectively. Further investigation revealed that BR2-5 and BS2-PB3 were resistant to low pH (≥4) and could tolerate the exposure of bile salts (1%). Both isolates survived under different oxidative stress conditions (aerobic and microaerophilic). In conclusion, BR2-5 and BS2-PB3 exhibited promising characteristics as probiotic candidates, though further investigations are required to substantiate these current findings.

Keywords: Bifidobacterial, bile salt, low pH, oxidative stress, probiotic

INTRODUCTION

Probiotics are living microorganisms that, when consumed in sufficient amounts, will benefit the host's health (Markowiak and Śliżewska 2017). Many Lactic Acid Bacteria (LAB) are categorized as probiotics, a group of bacteria found in nature, fermented food, and in the oral cavities and gastrointestinal tracts (GIT) of humans and animals. The health benefits offered by probiotics lead to a great interest in identifying emerging probiotic strains. These strains must exhibit several criteria, one of which is human origin (Zommiti et al. 2020). Bifidobacteria are among the most common groups of actinomycetes in the human gastrointestinal tracts and are among the first bacteria to colonize the GIT, where they are likely to exert health-promoting properties (O'Callaghan and van Sinderen 2016; Markowiak and Śliżewska 2017).

Many *Bifidobacterium* strains have been classified as probiotics because of their valuable contributions to human health, such as giving protection against pathogens through the production of antimicrobial agents and the modulation of host immune response, helping the production of vitamin K and short-chain fatty acids, eliminating pro-carcinogenic cells, and reducing digestive problems, i.e., lactose intolerance, constipation, and diarrhea. *Bifidobacterium* spp. in the human digestive system can also produce nutrients such as riboflavin and folic acid, as well as lower the pH of the large intestine hence that pathogenic bacteria cannot survive to cause any infection (Markowiak and Śliżewska 2017; Vitellio et al. 2019). Bifidobacteria are Gram-positive pleomorphic anaerobic, non-spore-forming, non-catalase, and non-motile bacteria (Mattarelli et al. 2017). They were first discovered in the stool of breast-fed infants and named *Bacillus bifidus communis* because of their Y-shaped appearance. A comparison of the cell morphology of many *Bifidobacterium* species cultured anaerobically in a Trypticase-Phytone-Yeast extract (TPY) medium revealed that some species have different cell shapes or arrangements. The shapes of Bifidobacteria cells vary among amphora-like cells, V-shaped cells, palisade cells, the linear group of globular elements, and middle-enlarged cells (Mattarelli et al. 2017). One of the factors that can influence the morphological form of *Bifidobacterium* spp. is the concentration of amino acid and N-acetylglucosamine in the medium used during cultivation. The low concentration of amino acids and N-glucosamine cause Bifidobacteria cells to have more branches (Shah 2002).

Bifidobacteria are dominant among intestinal bacteria in breast-fed infants due to the growth-stimulating effect of

human milk oligosaccharides found in breast milk (Lawson et al. 2020). Although bifidobacteria accounts for more than 90% of total intestinal bacteria in breast-fed infants, the flora of formula-fed infants is more heterogeneous (Kumar et al. 2020; Lorenzo 2008). Supplementary diet, antibiotic exposure, and hygiene conditions affect microbial colonization (Azad et al. 2013; Boudry et al. 2021). Therefore, fecal specimens could be used to investigate microbial communities in the colon without an invasive procedure (Azad et al. 2013; Shigwedha and Jia 2013). Bifidobacteria regularly found in infant feces include *B. breve*, *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. adolescentis*, *B. bifidum*, and *B. dentium*, while bifidobacteria that are regularly found in human breastmilk include *B. breve*, *B. adolescentis*, *B. bifidum*, *B. longum*, *B. animalis*, and *B. catenulatum* (Modesto 2018; Boudry et al. 2021).

Only a few groups had reported the isolation and characterization of bifidobacteria from biological samples in Indonesia. Kusharyati et al. (2020) tried to isolate *Bifidobacterium* spp. from infant's feces in Banyumas, Indonesia and evaluated their antimicrobial activity. Although seventeen *Bifidobacterium*-like bacteria were successfully isolated, molecular identification to confirm the isolates' identities had not been done. In addition, Hanidah et al. (2019) isolated lactic acid bacteria from breast milk in Jatinangor, Indonesia, and suggested that the isolate was *Lactobacillus plantarum* based on the biochemical tests. This study aimed to isolate Bifidobacteria from human breast milk and infant feces. Subsequently, selected isolates were identified through molecular analysis, and their probiotic properties were evaluated.

MATERIALS AND METHODS

Sample collection

A total of four independent samples (two breastmilk samples and two infant fecal samples) were collected in Jakarta, Indonesia. The sample collections were done from February to March 2022. Breastmilk samples were collected from two lactating mothers (age 35 and 37 years old, respectively) using protocol from Eglash and Simon (2017). The electric breast pump and the required containers for the breast milk collection were sterilized in hot water at a temperature of 80°C. The sterilized equipment was dried using a clean paper towel. The nipple and areola area of the mother were first cleaned with sterilized water and subsequently 2% chlorhexidine-soaked cotton. Breast milk samples were collected using an electric breast pump and transferred into a sterilized Breast Milk Storage Bag (BKA, Jakarta, Indonesia).

Fecal samples were collected from infants (age 1 and 2.5 months old, respectively) according to protocol from Modesto (2018). The parents were given a fecal tube containing 5 ml of Preservation Medium with a sterile spoon and an instruction paper. Preservation medium was prepared by mixing 1 g bacteriological peptone, 2.5 g yeast extract, 0.8 g NaCl, 0.02 g KCl, 0.014 g Na_2HPO_4, and 0.024 g KH_2PO_4 in 1 L distilled water. Fecal samples were collected by putting the feces (1.0 to 3.0 g) from the diaper into the feces tube. Fresh infant fecal samples were collected immediately after defecation. The collected samples were placed in a cool box equipped with ice packs (4°C) for temporary storage during shipment to the laboratory. Once the samples arrived in the laboratory, they were processed immediately.

Isolation and characterization of the *Bifidobacterium* spp.

A modified Trypticase-Phytone-Yeast (mTPY) medium was used for the isolation. mTPY agar consists of Tryptone Soya Broth/TSB (17.6 g/L), peptone (5 g/L), glucose (5 g/L), yeast extract (2.5 g/L), tween 80 (1 mL/L), L-cysteine (0.5 g/L), K_2HPO_4, (2 g/L) and bacteriological agar (15 g/L). According to Modesto (2018), L-cysteine could promote the growth of *Bifidobacterium* species. The fecal and breastmilk samples were diluted ten to one hundred times using sterile bacteriological peptone (0.1%). The diluted samples were spread-plated on mTPY agar and incubated anaerobically at 37°C. After four-day incubation, single colonies were selected and sub-cultured using a four-way streak method several times until purified isolates were obtained. Subsequently, the isolates were characterized based on their morphology and catalase activity. Mattarelli et al. (2017) stated that *Bifidobacterium* spp. are Gram-positive bacteria without catalase activity.

Carbohydrate fermentation

Twelve sugars were tested for the carbohydrate fermentation assay: glucose, fructose, galactose, lactose, maltose, sucrose, mannose, arabinose, mannitol, sorbitol, xylose, and starch. Individual sugar (1%, %w/v) was prepared in aqueous solutions and sterile-filtered by a 0.45 µm syringe filter unit (Millipore, Germany). For the fermentation assay, nine mL of MRS without sugar (Merck, Germany) was adjusted to pH 7 and mixed with 1 mL of sugar solution in the test tube containing an inverted fermentation tube (Durham tube). Bromocresol purple as the pH indicator was also added to the medium. Each test tube was subsequently inoculated with 100 µL of liquid culture of each isolate (in sterile Phosphate Buffer Saline [PBS]). The cultures were prepared by incubating the isolate at 37°C under anaerobic conditions for 4 days. After the incubation, the culture was centrifuged (5,000 rpm; 3 minutes) and resuspended in sterile PBS. The ability of each isolate to ferment individual sugar was observed based on the changes in the medium color, the presence of gas in the Durham tube, and the bacterial growth (OD_{600}). The medium color became purple at a higher pH (≥6.8) and turned yellow at pH 5.2. At pH levels in the transition range, intermediate colors would appear. Based on the medium color, the sugar fermentation ability of the isolates to produce acid was categorized into three: no fermentation ability (pH ≥6.8), low fermentation ability (5.2<pH<6.8), and strong fermentation ability (pH ≤5.2) (Hedberg et al. 2008). Based on the turbidity of the isolate's liquid cultures, the bacterial growth was divided into two categories: good growth (OD_{600} ≥0.5) and minimal growth or no growth

(OD_{600} ≤0.5) (Watson et al. 2013). The test tube containing medium was added with PBS for the negative control.

Molecular identification

Four isolates were selected and grown overnight in mTPY broth under anaerobic conditions at 37°C. Genomic DNA was extracted using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, US). The extracted DNA was quantified with a BioDrop DUO UV/Vis spectrophotometer (BioDrop, Cambridge, UK) and used as a template for PCR, which was carried out to amplify the 16S rRNA gene of the selected isolates. All reactions were performed using universal primers (27F and 1492R). Each PCR mixture (50 µL) was composed of 10 µL of 5x KAPA HiFi PCR buffer, deoxynucleotide triphosphate (dNTP) at a concentration of 0.3 mM, a pair of universal primers at a concentration of 0.3 mM of each primer, 1 µL of KAPA HiFi DNA Polymerase, and 20 µL of template DNA. The amplification products (5 µL) were separated in 0.8% (w/v) agarose gel electrophoresis, followed by ethidium bromide staining (1 µg/mL). Subsequently, the PCR products were sent to Apical Scientific (Selangor, Malaysia) for Sanger sequencing. Sequence similarity was analyzed using the Basic Local Alignment Search Tool (BLAST) program, available at http://www.ncbi.nlm.nih.gov. The phylogenetic tree was constructed using a neighbor-joining (NJ) method within the MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura et al. 2021).

Stress tolerance

The stress tolerance assay is a critical component of characterizing probiotic bacteria, as it provides valuable insights into the ability of these microorganisms to survive and function under conditions that may be encountered during production, storage, and transit through the gastrointestinal tract. The tolerance level of the selected isolates against different environmental stress conditions, including low pH and bile salt, was evaluated using a modified method from Parlindungan et al. (2021). Meanwhile, oxidative stress tolerance was determined based on a method adapted from Watanabe et al. (2012). In brief, cell pellets from the overnight cultures were washed and resuspended in 1 mL of PBS. For acid tolerance assessment, the resuspended cells (1% v/v) were inoculated into 5 mL of de Man, Rogosa, and Sharpe (MRS) broth (Liofilchem, Italy) in which the pH was adjusted to 2.0 and 4.0. The resuspended cells (1% v/v) were inoculated into 5 mL of MRS broth containing various bile salt concentrations (0.5%, 1%, 2%) to evaluate bile salt tolerance. Each mixture was vortexed for 15 seconds and incubated anaerobically at 37°C for 4 hours. For oxidative stress tolerance, the resuspended cells (1% v/v) were inoculated into 5 mL of MRS broth and incubated under 3 conditions (aerobic, microaerophilic, and anaerobic) at 37°C for 4 hours. After exposure to various stress conditions, total viable cell count (CFU/mL) was measured by collecting 30 µL of each mixture, spreading the mixture on MRS agar, and incubating the plates at 37°C for 72 hours.

Statistical analysis

Data were presented as mean ± SD (n=3) and analyzed using SPSS (version 26.0, IBM, New York, US). Statistical significance was evaluated using Kruskal-Wallis and Mann-Whitney U tests. In addition, p-value ≤0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A total of two fecal samples and two breastmilk samples from four different donors were examined; the samples from feces and breastmilk were independent. Twenty isolates (10 isolates from fecal samples and 10 isolates from breastmilk samples) were selected based on the similarity of colony morphology with *Bifidobacterium* spp., i.e., *B. longum* subsp. *longum* colonies are smooth cream to white, convex with entire edges, and soft polished. Among those isolates, the characteristics of ten isolates (BR1-M1, BR1-B1, BR2-5, BR2-6, BR2-12 from fecal samples and BS2-PB3, BS2-PB5, BS2-PS1, BS2-PS2, BS2-MB1 from breastmilk samples) matched with cellular characteristics of *Bifidobacterium* spp., i.e., Gram-positive, irregular rods, no catalase activity and non-motile (Table 1). Figure 1 shows the representative Gram staining result of two isolates from fecal samples.

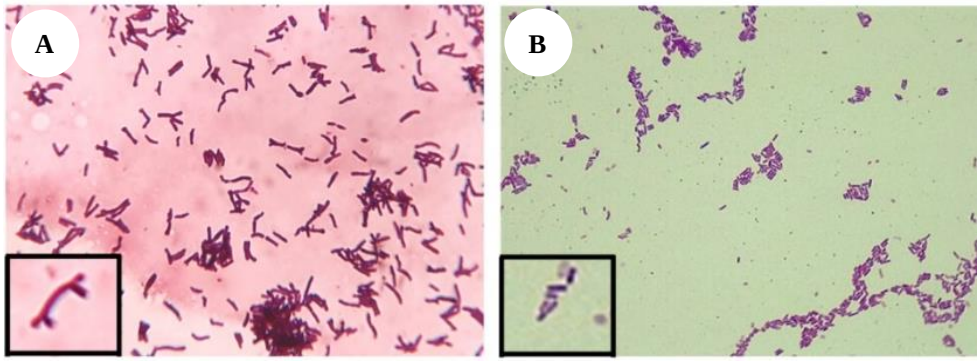

Figure 1. Representative Gram staining results of two isolates, BR2-5 (*left*) and BS2-PB3 (*right*) observed under a light microscope with a total magnification of 1000x. A: Bifid and curved globular rod; B: Small rod and middle-enlarged cell

Table 1. Ten isolates with similar profiles with *Bifidobacterium* species

Origin	Isolates code	Colony morphology				Gram	Catalase	Motility
		Shape	Margin	Texture	Elevation			
Feces	BR1-M1	Circular	Entire	Smooth	Flat	+	-	-
Feces	BR1-B1	Circular	Entire	Smooth	Flat	+	-	-
Feces	BR2-5	Circular	Entire	Smooth	Flat	+	-	-
Feces	BR2-6	Circular	Entire	Smooth	Flat	+	-	-
Feces	BR2-12	Circular	Entire	Smooth	Flat	+	-	-
Breastmilk	BS2-PB3	Circular	Entire	Smooth	Flat	+	-	-
Breastmilk	BS2-PB5	Circular	Entire	Smooth	Flat	+	-	-
Breastmilk	BS2-PS1	Circular	Entire	Smooth	Flat	+	-	-
Breastmilk	BS2-PS2	Circular	Entire	Smooth	Flat	+	-	-
Breastmilk	BS2-MB1	Circular	Entire	Smooth	Flat	+	-	-

Table 2. Sugar fermentation patterns of ten *Bifidobacterium*-like isolates

Isolate code	Tested sugars									
	Sucrose	Galactose	Lactose	Maltose	Xylose	Arabinose	Starch	Sorbitol	Mannitol	Mannose
BR1-M1	+	+	+	+	-	-	-	+	-	-
BR1-B1	+	+	+	+	-	-	-	+	-	+
BR2-5	+	+	+	+	+	+	-	-	-	+
BR2-6	+	+	+	+	-	-	-	-	-	+
BR2-12	+	+	+	+	-	-	-	+	-	+
BS2-PB3	+	+	+	+	-	-	-	+	-	+
BS2-PB5	+	+	+	+	+	-	-	+	-	-
BS2-PS1	+	+	+	+	-	-	-	+	-	+
BS2-PS2	+	+	+	+	-	-	-	+	-	+
BS2-MB1	+	+	+	+	-	-	-	+	-	-

Notes: (+) indicates a pH decrease in liquid culture (pH < 5) and a growth based on the turbidity of liquid culture (OD_{600} > 0.5)

The ability of ten *Bifidobacterium*-like isolates to ferment sugars was tested. Some sugars used in this study are considered to have prebiotic properties; thus, they are expected to support the growth of beneficial gut commensals. The MRS medium without glucose and supplemented with L-cysteine (mMRS) were used for further testing. All isolates demonstrated appreciable growth (final OD_{600} >0.5 and pH <5) on mMRS added with two different positive controls (glucose and lactose, respectively). Distinctive growth profiles were observed when the individual carbohydrate was used as the sole energy source in mMRS (Table 2). Although various sugars used for fermentation experiments were not quite conclusive; however, they still could provide some hints of fermentation patterns.

The ability of several isolates to ferment various sugars was matched with the profiles of *Bifidobacterium* species in Bergey's Manual of Bacteriology. Five isolates (BR1-B1, BR2-12, BS2-PB3, BS2-PS1, and BS2-PS2) with comparable carbohydrate fermentation ability were suspected to be *Bifidobacterium adolescentis*. Two isolates (BR2-5 and BR2-6) showed similar carbohydrate fermentation patterns to *Bifidobacterium longum* subsp. *longum*. Three isolates (BR1-M1, BS2-PB5, and BS2-MB1) displayed identical carbohydrate fermentation patterns to *Bifidobacterium breve*. Although sugar fermentation patterns could be used as a guideline for microbiological identification of *Bifidobacterium* species, it has limited sensitivity and specificity, resulting in some taxonomic uncertainties. Selective substrate selection and different carbon catabolite control mechanisms between various strains of *Bifidobacterium* spp. indicate niche-specific adaptations. Hence, the culture-based method, in connection with the biological molecular technique, such as 16srRNA sequencing, would enable better identification (Lugli et al. 2018; Mattarelli et al. 2017; O'Callaghan and van Sinderen 2016).

Four isolates (BR2-5, BR2-6, BS2-PB3, BS2-PS1) were selected for molecular identification, which was carried out based on the 16S rRNA sequencing. The 16S rRNA gene has been widely used as a valuable tool for bacterial identification, including *Bifidobacterium* spp., because it was shown to be conserved within the genus and species. According to BLAST results, BR2-5 and BR2-6 were very similar to *Bifidobacterium animalis* subsp. *lactis* with 100 and 98.39% similarity, respectively. BS2-PS1 and BS2-PB3 were very similar to *Bifidobacterium breve* with 100 and 98.26% similarity, respectively. Chun et al. (2018) state that similarity percentages above 98.7% are adequate for species identification, while lower percentages could be perceived as a new species.

A phylogenetic tree can evaluate sequence similarity and determine the evolutionary distance between strains. The phylogram shows several different and diverse *Bifidobacterium* species, possibly with distinctive genomic characteristics and capabilities (Figure 2). BS2-PS1 was closely related to BS2-PB3 and placed in the same clade as *B. breve* strain HDB7046. BR2-5 and BR2-6 were closely related to t *B. animalis* subsp. *lactis* HBM6. *Bifidobacterium breve* is the dominant species in the

breast-fed infant's gut, and *B. animalis* subsp. *lactis*, although not considered the dominant strain, may be highly expressed in some infants. The colonization of both species might be connected to their ability to utilize the nutrients in the infant's gut. Even though *B. animalis* subsp. *lactis* and *B. breve* are typically isolated from adults, they are occasionally found in infant feces. Lactose and Human Milk Oligosaccharides (HMOs) could promote the growth of *Bifidobacterium* spp. in breast-fed infants' gut. Still, adult-type bifidobacteria, such as *B. adolescentis*, are less effective in using HMOs (Chichlowski et al. 2020).

To be categorized as probiotics, the isolates must be able to endure gastric juice (stomach phase) and bile salt (intestinal phase) to thrive in the human gastrointestinal tract (City et al. 2021). Even though both gastric juice (pH 2) and bile salts are severely bactericidal, several *Bifidobacterium* strains can endure the stomach's low pH for a certain period and tolerate the effect of bile salts in the human small intestine. Shigwedha and Jia (2013) stated that pH 2.0 and 3.0 are lethal and sublethal pH values for most Lactic Acid Bacteria (LAB), including bifidobacteria. Probiotics must survive after exposure to acidic environments after ingestion. Although a drop in viability may occur, only sufficient bifidobacteria survive in the gut needed to provide health benefits; the pH of 4.0 represents fermented products (Fuochi et al. 2015; Parlindungan et al. 2021).

B. animalis subsp. *lactis* BR2-5 and *B. breve* BS2-PB3 were selected as representative strains for stress tolerance tests. Table 3 shows that BR2-5 and BS2-PB3 did not survive after exposure to pH 2 for 4 hours. Both isolates also lost a significant number of viable cells after exposure to pH 4 and 9 for 4 hours. Bifidobacteria could tolerate slight pH fluctuations, especially in the pH range of 6.0 to 9.0. The Acid Tolerance Response (ATR) is a typical bacterial response to acidic conditions in which a range of inducible molecular pathways accomplishes acid adaptation. Ruiz et al. (2013) reported that the two major cytoplasmic subunits of the F0F1-ATPase (atpA and atpD) are found to be overrepresented on 2DE patterns following acid exposure, indicating that this enzyme plays an important function in the *Bifidobacterium* ATR. This enzyme's activity was additionally higher in membranes of *B. animalis* subsp. *lactis*, compared with *B. breve*, after cultivation under acidic conditions (Shigwedha and Jia 2013; Parlindungan et al. 2021). This finding might explain the higher viability of BR2-5, as compared to BS2-PB3, after exposure to acidic conditions.

Bile salts are known to be harmful to many cells due to their disruption of the lipid bilayer structure of cellular membranes. Previous studies showed that autochthonous gut bacteria must develop mechanisms to defend themselves from bile salts. The physiological bile salt content within the human intestine is estimated to be 0.3-0.4% (%v/v). Probiotics, generally Gram-positive bacteria, are more reactive to bile salts than Gram-negative bacteria (City et al. 2021; Parlindungan et al. 2021). As shown in Table 3, both species did express satisfactorily resistant characteristics to the bile salts. The BR2-5 and BS2-PB3 survived bile salt 0.50% (w/v) with more than 1.07 x 10^5 and 8.2 x 10^4 CFU/mL, respectively, of viable cells after treatment. Probiotics, including Bifidobacteria, can perceive bile salts directly via a two-component signaling system consisting of a membrane-associated histidine kinase sensory protein and a response regulator protein (HPK-RR). When HPK detects bile salt, a signal is delivered to RR, which regulates the cellular response to the stimulus. Bacterial Bile Salt Hydrolase (BSH), which regulates the deconjugation reaction of bile salts, is believed to have an active role in reducing bile salt toxicity. The amino acids produced during deconjugation can be used as carbon and nitrogen sources for bacterial survival. BSH stimulates the integration of cholesterol into bacterial membranes, causing alteration in membrane properties, influencing bile salt tolerance, and increasing probiotic intestinal survivability (Hernández-Gómez et al. 2021).

This study revealed the impact of oxidative stress on the growth performance of selected isolates was evaluated by culturing the isolates in MRS broth under the following conditions: aerobic (shaking), microaerophilic (static), and anaerobic (static). Table 3 shows the survival of BR2-5 and BS2-PB3 under different oxygen levels. Both isolates experienced a significant cell loss in the presence of oxygen, even though no viable cells were found in aerobic conditions (data not shown). However, BS2-PB3 showed higher cell counts in all oxygen-level conditions than BR2-5; it is suggested that exposure to oxygen is one of the factors for loss in viability of probiotics. Generally, probiotics, such as lactobacilli and bifidobacteria, are microaerophilic and obligate anaerobes, respectively. Thus, they are considered highly sensitive to oxygen and have low survival rates in aerobic environments (Guo et al. 2017).

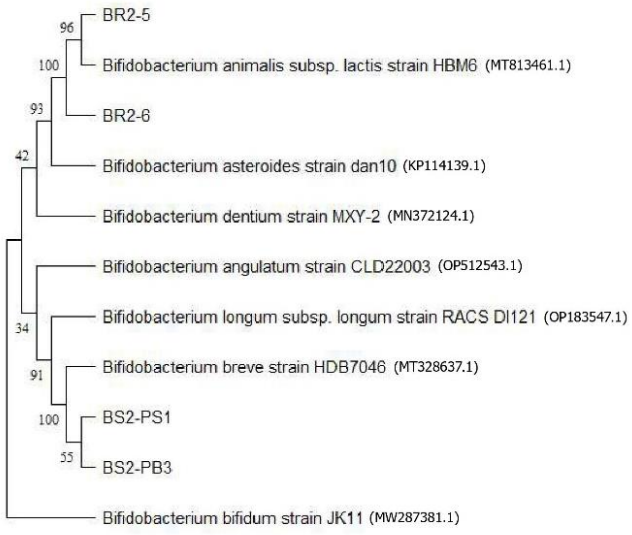

Figure 2. The phylogenetic tree was constructed using the Maximum Likelihood (ML) method based on 16S rRNA gene sequences showing the position of selected isolates among related Bifidobacterium species. Each GenBank accessions for the sequences used in this study are provided. Bootstrap values at the branches are based on 1,000 replicates. Evolutionary analyses were conducted in the MEGA 11 version

Table 3. Survival of two selected isolates under different pH, bile salt concentrations, and oxygen level conditions at 37°C for 4 hours

Isolates	Cell number (x10^5 CFU/mL)								
	pH				Bile salt			Oxygen level	
	pH 2	pH 4	pH 7	pH 9	0%	0.50%	1%	Micro-aerophilic	Anaerobic
BR2-5	0*	0.72 ± 0.08*	2.10 ± 0.07	0.94 ± 0.04*	2.06 ± 0.10	1.07 ± 0.09*	0.79 ± 0.06*	0.21 ± 0.04*	0.79 ± 0.49
BS2-PB3	0*	0.22 ± 0.03*	2.50 ± 0.18	0.37 ± 0.06*	2.56 ± 0.19	0.82 ± 0.08*	0.58 ± 0.09*	0.28 ± 0.06*	1.45 ± 0.49

Note: Data were presented as mean ± SD (n=3). Asterisk (*) indicates statistically significant differences ($p < 0.05$) of three independent experiments compared to pH 7, 0% bile salt, and anaerobic conditions, respectively.

For probiotics, growing under high oxygen levels can create Reactive Oxygen Species (ROS) such as superoxide or hydroxyl radicals due to electron leakage from Electron Transport Chain (ETC) intermediates. These ROS can cause severe cell damage by reacting with DNA, proteins, and lipids (Watanabe et al. 2012). Unlike aerobes, most obligate anaerobic microorganisms, such as bifidobacteria, cannot produce enzymes (i.e., catalase, peroxidase, and superoxide dismutase) to neutralize the harmful ROS (Zuo et al. 2014; Shibata and Toraya 2015). However, bifidobacteria have a variety of stress response systems to protect cells from harmful environmental conditions. Watanabe et al. (2012) reported that anaerobic microorganisms may adapt to ROS by generating oxidative stress pathways. Watanabe et al. (2012) and Guo et al. (2017) suggested that bifidobacteria possess key enzymes, i.e., alkyl hydroperoxide reductase a thioredoxin reductase, as well as a rapid and sensitive oxidation response system, such as NADH oxidase and NADH peroxidase system, to remove ROS.

Stimulation of oxidative stress mechanisms during growth may result in more robust probiotic cells that are more resistant to the adverse conditions encountered in the gastrointestinal tract or during cell preparation or storage for starter cultures. Furthermore, the growth ability in different oxygen level conditions, especially high oxygen levels, may favorably influence the isolate's robustness, i.e., stress tolerance, which may be useful for its usage as a probiotic and starter culture in food fermentations.

In summary, ten of the twenty isolates were Bifidobacteria-like bacteria. The molecular 16S rRNA analysis revealed that BR2-5 and BR2-6 were closely related to *Bifidobacterium animalis* subsp. *lactis* with 100 and 98,39% similarity, respectively. Meanwhile, BS2-PS1 and BS2-PB3 were closely related to *Bifidobacterium breve* with 100 and 98.26% similarity, respectively. For more precise investigation of microorganism's genetic diversity, analysis of the entire bacterial genome contents from next-generation sequencing needs to be performed. BR2-5 and BS2-PB3 were distinguished by their tolerance to pH, bile salts, and oxidative stress. In particular, BR2-5 and BS2-PB3 were able to withstand pH 4 and 1% bile salts. Under the two oxidative stress conditions, both isolates survived with minimal viability. In sum, BR2-5 and BS2-PB3 might be considered as potential probiotic strains. Future studies should focus on the further assessment of the isolate's probiotic properties and benefits.

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