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Bifidobacterium longum, a Predominant Bifidobacterium in Early-life Infant Potentially Used as Probiotic

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Abstract. In early life, Bifidobacteria are reported as dominant bacteria in the human digestive tract. Bifidobacterium is potential as a probiotic. The probiotic property of Bifidobacterium is strain-specific. This study aimed to identify the Bifidobacterium (isolated from less than one-month-old healthy infant stool that potentially used as probiotic) based on the 16S rRNA gene and determining their similarities among Bifidobacteria. The probiotic-potentially Bifidobacterium was re-characterized by performing a Gram's staining and catalase test. The DNA extraction process was followed by the 16S rRNA amplification using 27F-1492R primers. Sequence similarity was checked by using the BLAST program in the GenBank. The phylogenetic tree was constructed by using a neighbor-joining (NJ) method within the MEGA version 7.0 package. The 16S rRNA gene was presented at 1,500 bp length. Bifidobacterium strains have a 91.14-94.26 % sequence similarity to *B. longum* subsp. *longum* strain CCUG30698 which is considered as insufficient for species and genus identifications. However, those isolates could be assigned in a phylogenetic position. This present study suggested the *B. longum* as the dominant strain of Bifidobacterium in the gut of early-life infants which has potential as a probiotic and is considered as an ideal probiotic for human consumption. This study is useful as basic information for other related research, as well as its application in industrial or community service fields.

Key words: 16S rRNA; *Bifidobacterium longum*; Infant Stool; Probiotic

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INTRODUCTION

The microorganism ecosystem plays a key role in the human body (Gouba et al., 2019). The composition of microorganisms expresses the health level of the human digestive tract. The microbial colonization begins in the first few days after birth (Attri et al., 2018) and continues to be more complex during feeding. Bifidobacteria colonize the digestive tract of infants starting from the first week after birth (Oishi et al., 2013). Also, Bifidobacteria are reported as dominant bacteria in the human digestive tract (Milani et al., 2013; Pacheco et al., 2015; Roger et al., 2010; Walker et al., 2015) based on both culture-based techniques and molecular analysis (Milani et al., 2013).

The vertical transmission from mothers to their children during birth is thought to be the main source of Bifidobacterium (Sirilun et al., 2015). *B. adolescentis, B. bifidum, B. catenulatum, B. longum,* and *B. pseudocatenulatum* were found to be monophyletic between individual mother and infant pairs (Makino, 2018; Murphy et al., 2017). Bifidobacteria are commonly found in the human milk and feces. Makino (2018) reviewed that Bifidobacterium can be found in infant feces at a concentration as high as 10^{10} cells/g. It is suggested that infant gut and feces are a potential source for probiotic discovery.

Probiotic confer health benefits, such as balancing the gut microbiota, maintaining the host immunity, and keeping the homeostasis of host metabolism (Devaraj et al., 2013; Lomasney et al., 2014; Nagpal et al., 2018). Bacteria, yeast, and mold that can act as probiotics include Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, Enterococcus, Bifidobacterium, Bacillus, *Saccharomyces cerevisiae, Candida pintolopesii,* and *Aspergillus oryzae* (Sornplang & Piyadeatsoontorn, 2016). Lactic Acid Bacteria (LAB) isolates from the human digestive tract are considered safe to be added to food and beverage products (Aloisio et al., 2012). Therefore, many products have been supplemented with LAB (Russell et al., 2012).

The probiotic property of Bifidobacterium is strain-specific. It is suggested that Bifidobacterium identification both microbiologically and molecularly is considered essential. The most common genotypic identification is 16S rRNA gene-based identification. The 16S rRNA gene is commonly used to construct the phylogenetic tree because the nucleic acid among rRNA molecules can be compared correctly (Kasi et al., 2019).

This present study aimed to identify the Bifidobacterium (isolated from less than one-month-

old healthy infant stool that potentially used as probiotic) based on the 16S rRNA genes and know their similarities among Bifidobacteria. Recently, research has focused on isolating and characterizing new potential strains of probiotic from different sources, especially from the human gastrointestinal tract. The ideal probiotic for human consumption should have a human origin because the probiotic can already effectively colonize the digestive tract of the host.

Our previous study isolated LAB from the human gut aimed to obtain probiotic bacteria that are able to inhabit and perfectly grow in the human digestive tract. This present study is useful as basic information for other research in the fields of nutrition, microbiome, metabolite production, microbial phylogenetic, and others. In addition, potential probiotic isolates that obtained from this study can be used for the production of functional food and beverages in the industrial or community service fields.

METHODS

Sample sources

The stool samples were collected from 3 less than one-month-old healthy infants in Banyumas area i.e. Purbalingga, Banyumas, and Sokaraja District with no exclusions based on delivery or feeding mode. Mothers and infants were in good health (self-reported). Subjects were excluded if the infant had a gastrointestinal disorder or had taken antibiotics in the previous 14 days, as well as if the infant had been ill in the previous 7 days, or was administered oral probiotics. Fresh stool samples were collected using a sterile Falcon tube 15 mL by the participants and immediately stored in home freezers until being delivered to the experimental laboratory within 24 hours of sample collection. Samples were placed in labeled collection tubes and stored at -20˚C until the analysis.

Bacterial cultivation and re-characterization

The culture stock of Bifidobacterium isolates (Bb1B, Bb2A, and Bb2E) was cultured on the MRS Agar medium (Oxoid) at 37 °C for 3 days, anaerobically. The growing colonies were further morphologically characterized and checked by performing a Gram's staining and catalase test.

Extraction of bacterial DNA

The Bifidobacterium isolates were inoculated into Nutrient Broth medium (Merck) at 37 °C for 3 days, anaerobically. Next, 1 mL of Bifidobacterium culture was centrifuged (Thermo Scientific) at 10,000x*g* for 3 minutes. This stage was repeated until 50-100 mg pellets were obtained. The DNA extraction was carried out by following the manufacture protocol of Zymo Research Quick DNA^{TM} Fungal/Bacterial Miniprep Kit with modification. The pellet was added by 200 µL of phosphate-buffered saline pH 7.4 (0.2g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4, and 800 mL distilled water for a final volume of 1 L solution) to the ZR BashingBead Lysis Tube, then 750 µL of BashingBead Buffer was added. The solution was vortexed for 15 seconds and centrifuged for 1 minute at 10,000x*g*. The supernatant (400 µL) was transferred into Zymo-spin^{TM} III-F, then centrifuged for 1 minute at 8,000x*g*. This stage was carried out 2 times. The filtrate was collected in a 2 mL tube, then 1 mL of Genomic Lysis Buffer was added. The solution (800 µL) was transferred into the Zymo-spin^{TM} IIC column then centrifuged at 10,000x*g* for 1 minute. This stage was carried out twice. The filtrate was removed, then the Zymo-spin^{TM} IIC column was transferred to a new collecting tube. A total of 200 µL of DNA pre-Wash Buffer was added then centrifuged at 10,000x*g* for 1 minute. A total of 200 µL DNA Wash Buffer was added into the Zymo-spin^{TM} IIC column and centrifuged at 10,000x*g* for 1 minute. The Zymo-spin^{TM} IIC was transferred to a sterile 1.5 mL micro-centrifuge tube and added with 60 µL of Elution Buffer, then incubated at room temperature for 5 minutes. Next, it was centrifuged at 10,000x*g* for 30 seconds. The extracted DNA was stored at -20°C as DNA stock. The DNA was electrophoresed by using a 1 % agarose gel (100 V for 45 minutes).

DNA quantification

A total of 3 µL extracted DNA was dripped on a NanoDrop (NanoVue) machine. The data was used to determine DNA quantity and purity.

The 16S rRNA gene amplification

The gene amplification was performed by using forward (27F) primer (3'-AGAGTTTGATC**M** TGGCTCAG-5') and reverse (1492R) primer (3'-GG**Y**TTACCTTGTTACGACTT-5'). The mixture of PCR reaction was as follows: 19 µL of Nuclease-Free Water, 25 µL of NZYTaq II 2x Green Master Mix, 2 µL of 27F primer (5 µM), 2 µL of 1492R primer (5 µM), and 2 µL of DNA samples. PCR amplification conditions were 95 °C for 2 min, 35 cycles [95 °C for 30 sec, 51 °C for 30 sec, 72 °C for 90 sec], and then 72 °C for 5 min, then left at 8 °C for 3 min. The amplification product was electrophoresed by using a 1 % agarose gel (100 V for 45 minutes).

Sequencing

A total of 20 µL PCR product was sent to the 1st Base Pte. Ltd. Company, Singapore to do the Sanger sequencing.

Sequence analysis

Sequence analysis was conducted by using BioEdit. Sequence similarity was checked by using the Basic Local Alignment Search Tool (BLAST) program in the database of GenBank which is available on http://www.ncbi.nlm.nih.gov. The phylogenetic tree was constructed by using a neighbor-joining (NJ) method within the MEGA version 7.0 package. A 1,000 bootstrap replication was used to construct the evolution distance. The obtained 16S rRNA sequences were compared with some Bifidobacterium strains (*B. adolescentis* strain LCR4, *B. faecale* strain HBUAS55035, *B. stercoris* JCM15918, *B. longum* subsp. *longum* strain CCUG30698, *B. dentium* 16S ribosomal RNA, *B. catenulatum* subsp. *kashiwanohense* strain APCKJ1, *B. indicum, B. minimum* strain LMG11592, *B. pseudocatenulatum* strain IMAUFB090, *B. psychraerophilum, B. bohemicum* JCM18049, *B. hapali* strain MRM914) and an outgroup strain (*Escherichia coli* E142).

RESULTS AND DISCUSSION

A healthy gut consists of a highly diverse microbiome population (Wong & Santiago, 2017). In our previous study, we have successfully isolated 22 Bifidobacterium isolates from 3 less than one-month-old healthy infant stool. As in classifying Bifidobacteria by classical methods, *B. catenulatum, B. minimum,* and *B. indicum* were dominant isolates followed by *B. asteroides, B. galicum*, *B. coerinum,* and *B. dentium* (Figure 1). Some of those strains were grouped as human-specific Bifidobacteria i.e. *B. catenulatum* and *B. dentium* (Liu et al., 2013; Roger et al., 2010; Sun et al., 2015). At the time of writing, the genus Bifidobacterium was represented by over 30 species and subspecies from infant feces and healthy adults (Attri et al., 2018; Duranti et al., 2017; Mariat et al., 2019; Morita et al., 2011; Narayanan & Subramonian, 2015).

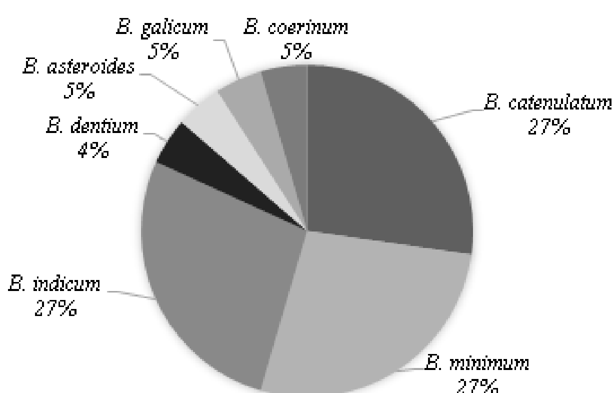

Figure 1. The diversity of Bifidobacterium isolated from less than one-month-old healthy infant stool based on the classical methods

There were three Bifidobacterium isolates (Bb1B, Bb2A, and Bb2E) potentially used as probiotic which were screened primarily for their tolerance to acidity, resistance to lysozyme, antimicrobial activity against pathogenic microorganisms (*Escherichia coli* and *Candida albicans*), and capability in producing exopolysaccharide (Kusharyati et al., 2020). Other studies suggested that probiotic isolated from human and animal gut exhibit higher adhesion activity, and more likely to be resistant to low pH levels and high concentration of bile than other probiotic sources (Sornplang & Piyadeatsoontorn, 2016).

The potential Bifidobacterium isolates were recultured on the MRS Agar medium at 37 °C for 3 days, anaerobically. The growing colonies were observed as round shaped, creamy white, shiny surface, raised elevation, and flat edge (data not shown) colonies. The single colony was examined by Gram's staining, morphological characterization, and catalase test. Morphological characterization re-confirmed that all isolates were Gram-positive and rod-shaped cells.

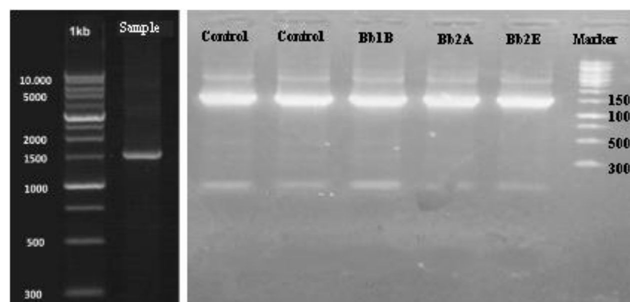

Figure 2. Visualization of the amplification product of the 16S rRNA gene (marker: 1 kb DNA ladder)

The most common method to identify unknown microorganism is a culture-based method, which observed the phenotype characteristics including morphological, physiological, and biochemical tests following Bergey's Manual of Bacteriology. The patterns of sugar fermentation provide a guideline for microbiological identification of Bifidobacteria. However, it has resulted in some taxonomic uncertainties, since it has been shown to have low sensitivity and specificity (Benga et al., 2014). The culture-based method associated with the biological molecular technique is a way for more effective identification.

The 16S rRNA gene was shown to be conserved within the genus and species (Kasi et al., 2019). Some studies have developed various primer sets of 16S rRNA genes to target Bifidobacterium strains (Kolondam et al., 2020; Ryandini et al., 2018; Sim et al.,

2012). In this study, the genome extraction of Bifidobacterium isolates yielded a 40.5-66.5 $\mu g.mL^{-1}$ of extracted DNA which presented at more than 10,000 bp length during visualization (data not shown). The amplification product of the 16S rRNA gene was presented at 1,500 bp length during visualization (Figure 2). The obtained 16S rRNA sequences length of Bb1B, Bb2A, and Bb2E isolates were 1448, 1445, and 1418 nucleotides, respectively (Table 1).

Table 1. Analysis of sequence similarity accessed from GenBank database

Isolate	Nucleotide length	Identical species	Query (%)	E-value	Identity (%)	Accession
Bb1B	1448	*Bifidobacterium longum* subsp. *longum* strain CCUG30698, complete genome	92	0.0	94.26	CP011965.1
Bb2E	1418		89	0.0	92.58	
Bb2A	1445		89	0.0	91.14	

In the present study, Bifidobacterium isolates are shown to have a high similarity to *B. longum* subsp. *longum* strain CCUG30698 (Table 1). Those similarity percentages which ranged from 91.14-94.26 % is considered as insufficient for species and genus identifications. However, those isolates could be assigned in a phylogenetic position. In general, an isolate is confirmed as identical species when it has more than 99 % sequence similarity to its comparable strains in GenBank (Da-Silva et al., 2013; Malviya et al., 2011). Since the sequence similarity was less than 97 %, a further study is needed.

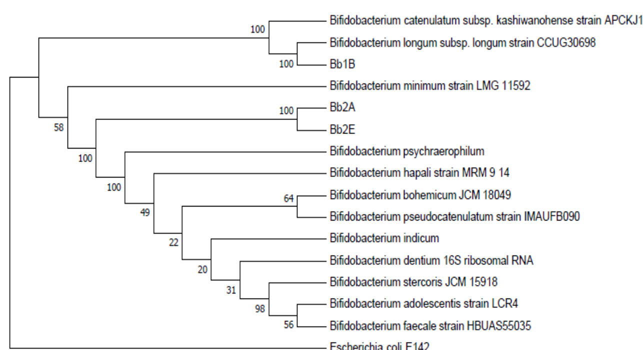

Figure 3. Similarity index of isolated *Bifidobacterium* spp. among Bifidobacteria (The phylogenetic tree was constructed by using a neighbor-joining (NJ) method and 1,000 bootstrap replication within MEGA 7.0 version)

A phylogenetic tree can assess the sequence similarity as well as estimate the evolutionary distance among strains. Bifidobacterium Bb2E and Bb2A isolates were phenotypically identic. Both similarity and distance parameters placed the Bb2E and Bb2A isolates in a different clade with Bb1B isolate (Figure 3). The dendogram also showed that Bifidobacterium Bb1B isolate was identic to *B. longum* subsp. *longum* strain CCUG30698, which were closely related and placed in the same clade with *B. catenulatum* subsp. *kashiwanohense* strain APCKJ1. These species are commonly isolated from adults, but *B. longum* is occasionally found in infant feces (Liu et al., 2013; Roger et al., 2010).

Bifidobacterium Bb1B isolate was the most dominant strain found in infant stool (Kusharyati et al., 2020), which in this present study, has a 94.26 % sequence similarity to *B. longum* subsp. *longum* strain CCUG30698 (Table 1). Another study conducted by Walker et al. (2015) found that more than 99 % of total Bifidobacterial sequences isolated from breastfed babies are matched to *B. longum*. Our finding suggested that *B. longum* as the dominant strain in the infant's gut (Table 1), since the microbial populations of the stool sample may reflect the microbial population in the gut.

B. longum dominancy might be correlated with their ability in utilizing the nutrient. In the breastfeed-infants gut, lactose and human milk oligosaccharides (HMOs) encourage the growth of Bifidobacterium (Lawson et al., 2020; Murphy et al., 2017). Typical infant species such as *B. bifidum* and *B. longum* effectively utilize HMOs, whereas adult-type Bifidobacteria such as *B. adolescentis* is less efficient in utilizing HMOs (Makino, 2018). Eshaghia et al. (2017) argued that *B. longum* localizes in the infant's gut at the few first days after birth. This finding suggesting that a considerable number of Bifidobacteria isolates could pass through the infant newly developed gastrointestinal tract and be confined.

In short, Bifidobacterium Bb1B isolate is considered as an ideal probiotic for human consumption, because they can already effectively colonize the digestive tract of the host. *B. longum* is generally the most common and prevalent species found in breast milk samples and the digestive tract of infants and adults (Chaplin et al., 2015; Kolondam et al., 2020; Martin et al., 2016; Oki et al., 2018). Some studies have also recommended the *B. longum* as a probiotic strain with many health benefits (Sugahara et al., 2015; Wong et al., 2019) which considered safe to be added to food and beverage products. In the future, *Bifidobacterium longum* Bb1B can be applied for the production of functional food and beverages in the fields of research, industrial, and community service.

CONCLUSION

This present study suggested the *Bifidobacterium longum* as the dominant strain of Bifidobacterium in the gut of less than one–month-old healthy infants

which has potential as a probiotic and is considered as ideal probiotic for human consumption because they can already effectively colonize the digestive tract of the host.

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