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Characterization of Bifidobacteria from infant feces with different mode of birth at Purwokerto, Indonesia

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Abstract. *Hendrati PM, Kusharyati DF, Ryandini D, Oedjijono. 2017. Characterization of Bifidobacteria from infant feces with different mode of birth at Purwokerto, Indonesia. Biodiversitas 18: 1265-1269.* Bifidobacteria belongs to the so-called beneficial intestinal flora. Before attempting to raise their intestinal levels to improve the health status of the host, it is importance to know about physiological variations in the Bifidobacterial colonization of the human intestine. Birth process influenced the diversity of *Bifidobacteria* in infant feces**.** This research was intended to isolate and characterize *Bifidobacterium* spp. as well to evaluate their presence in the feces of infants who were born by mode of normal, caesarean and premature. The research was conducted by survey method and data were analyzed with descriptive analysis. *Bifidobacterium* character was observed include colony and cell morphology. The biochemical test included catalase, oxidase, indole, Voges-Proskauer, different pH growth, and resistance to lysozyme. *Bifidobacterium* metabolites obtained tested its bacterial activity to *Salmonella typhimurium* and *Escherichia coli*. The result of this research showed that 35 isolates are suspected *Bifidobacterium* group and after API 20 A test showed 17 isolates are rally genera of *Bifidobacterium* spp.and all isolates come from infant feces with caesar and premature delivery. These isolates inhibited the growth of *S. typhimurium* and *E. coli* with different inhibitory capabilities*.* This finding is very important for science and medical point of view and could be developed with further research.

Keywords: Bifidobacteria, Characters, Mode of birth, Infant feces

INTRODUCTION

Probiotics are living microorganisms that provide health benefits to the host when ingested in adequate amounts (Bermudez-Brito et al. 2012). In recent years, many studies have shown that probiotics can cure a variety of diseases, such as diarrhea, colitis, urinary tract infection, hypertension, allergies, and cancer. Probiotics benefits are important from an early age. The previous few years, the safe test of probiotic bacteria are on the group of Bifidobacteria, i.e. *Bifidobacterium lactis*. The probiotics use for children was focused on the prevention of diarrhea and allergies as well as increasing the number of Bifidobacteria in the gut. Bifidobacteria is the dominance microorganism in the human digestive tract of adults and infants (Holzapfel et al. 2001). The dominance of Bifidobacteria in the human digestive tract is about 10% in adults and up to 90% in infants (Harmsen et al., 2000a) that were analyzed from stool samples. Bifidobacteria colonize the intestine of infants during birth and the first week after birth (Heidarpour et al. 2008). Narayanan and Subramonian (2015) showed that there are about 29 Bifidobacteria species that have been identified, including eleven species isolated from infant feces. The most frequently isolated Bifidobacteria species in infant feces are *B.bifidum, B.longum, B. infantis* and *B. breve.* These organisms are Gram-positive, non-motile, non-spore forming, anaerobic, and pleomorphic rods. Some researchers suspect that the presence of intestinal Bifidobacteria in infants is affected by birth process (Mariat et al. 2009). During vaginal birth process (normal), the contact with vaginal and intestinal flora are important resources to begin the colonization in infants, while during a cesarean birth process, direct contact of infant's mouth with vaginal and intestinal microbiota are not happen, and the intestinal colonization is derived from the skin of his parents, workers, and medical equipment (Lif Holgerson et al. 2011).

The composition of the microbiota at the beginning of human life can affect the health for several months. Biasucci et al. (2008) stated that the composition of microbiota in infants plays an important role in the development of the post-partum immune system. The development of fecal microbiota in neonates is important because those bacteria are the first to colonize the sterile intestine of the neonates and, thus, have a significant effect on the host. Bifidobacteria are predominant in the fecal microbiota of infants, and, therefore, they are important to an understanding of how commensal Bifidobacteria is established in the intestine of infants (Mikami, Kimura, and Takahashi.2012). The important of *Bifidobacterium* is the ability to produce antimicrobicrobial compounds called bacteriocin (Aly et al. 2006).

The problem of this research is "how is the dominance of *Bifidobacterium* sp. from the feces of infants with a different birth process (normal, cesarean, and premature)?". Information of *Bifidobacterium* isolated from newborn feces is still lack in Indonesia. This reason encourages researcher to explore *Bifidobacterium* from infant feces

especially in Purwokerto. The research on Bifidobacteria as the agent of probiotics is currently being developed by isolating the potential isolate from infants feces with the different birth process. Vaginal birth process and breastfeeding will stimulate the presence of Bifidobacteria in the gastrointestinal tract of infants. The research was intended to isolate and characterize the *Bifidobacterium* spp. as well as to evaluate their presence in the feces of infants who were born by modes of normal, caesarean and premature.

MATERIALS AND METHODS

The research was conducted at Laboratory of Microbiology, Faculty of Biology, Jenderal Soedirman University using survey method. The feces were taken from 10 until 12 days old infant with the different birth process (normal, cesarean, and premature). Samples were taken randomly, i.e. feces from 2 normal, 2 cesar, and 1 premature birth process. The data was analyzed with descriptive analysis. Determination of *Bifidobacterium* spp. was based on the morphological, chemical, and sugar test. Manual characterization refers to Bergey's Determinative Bacteriology, Cowan and Steel's Manual for the Identification of Medical Bacteria, World Journal of Dairy & Food Sciences, International Journal of Food Microbiology, Applied and Environmental Microbiology, and followed by API 20A(Biomériux) test

Procedures

Isolation

Isolation of *Bifidobacterium* sp. from infants' feces used the Modification of Gronlund (Modification of Gronlund et al., 2009). Samples were taken aseptically from 5 feces of under one month-aged infant with the different birth process (normal, cesarean, and premature). A 5 g of feces was added with 45 mL of sterile distilled water, then homogenized and diluted for up to 10^{-4}. The last two dilutions were used to isolate the bacteria using de Man, Rogosa, Sharpe Agar (MRSA) Oxoid with a spread plate method in duplicate and were anaerobically incubated at 30^0C for 24 hours.

Identification

Morphological characterization. Morphology of colony. Colony characterization was performed in MRSA, such as shape, size, surface, elevation and edge formation of the colony.

Cellular characterization. Gram staining. One loop of the single colony was taken, and then was smeared on a glass object. Crystal violet dye was dropped for 1 minute, and then was washed. Mordant solution (Lugol's iodine) was dropped as the second dye for one minute, and then was washed. Ethanol 96% was dropped gently, until it was lightly crisp, and then was washed. Safranin was dropped for one minute, and then was washed. The glass object was air-dried, then was examined under the microscope. The cells of Gram-positive bacteria were purple in color. Meanwhile, Gram-negative bacteria were red. Motility test. An ose of the single colony was stab inoculated on Sulphide Indole Motility Agar (SIMA) medium, and then was incubated at room temperature for 2x24 hours. The spread of growth was observed. A positive result was interpreted with pellicle formation on the surface of the medium.

Biochemical test

Catalase test. One loop of the single colony was smeared on glass object, and then was dropped with the H_2O_2 reagent. A positive result was interpreted with gas bubbles formation.

Test indole. One loop of single colony was inoculated into indole medium (Tryptone Broth) for 2x24 hours at 35-36^0C. Then, the medium was added with Kovac's Indole reagent. Positive the result was interpreted with pink rings formation on the surface of the medium.

Voges-Proskauer test. One loop of the single colony was inoculated into Methyl Red Voges Proskauer (MR-VP medium, and then was incubated for 2x24 hours at 30^0C**.** A total of 3 drops of 40% KOH and 2 drops of alpha-naphthol reagent were added to the medium. A positive result was interpreted as color change into the pink medium.

Oxidase test. One loop of the single colony was covered with a piece of filter paper, and then was added by 1-2 drops of reagent (tetramethyl-D-phenylenediamine dihydrochloride). Positive result was interpreted with a color change to maroon blue.

Measurement of Total Lactic Acid. A total of 2 mL of 24 hours bacterial culture was taken, and then was diluted with 20 ml of distilled water and was shaken. Then, 3 drops of phenolphthalein (pp 1%) were added and titrated with 0.1 N NaOH solution until a light pink color formation.

Resistance test to Lysozyme. MRSA medium was added with the white part of the egg containing lysozyme (200 $mg.ml^{-1}$). The medium was poured into a sterile dish and allowed to solidify. A 1 mL of bacterial culture was inoculated on *Bifidobacterium* medium, then was anaerobically incubated for 24 hours at 35-36^0C. The bacterial resistance was shown by the growth of *Bifidobacterium* in the medium

The pH range Growth test. Strains isolated MRS broth was grown in media with pH 4,7,9 and was incubated for 24 hours at a temperature of 37^0C.

API 20A (Biomériux) test

The colony isolated from infants feces was re-cultured on MRSA medium and anaerobically incubated for 24 hours, then 3 loops of the colony was taken and identified with kit of API 20A. Indole test was added with mineral and anaerobically incubated for 24 hours. Indole test interpretation needs the addition of XYL and EHR reagent. The sugar test, glucose-arabinose, and glycerol-trehalose need the addition of BCP reagent. Catalase test was added by H_2O_2. The data was analyzed in the web of API 20

Inhibition test against pathogenic bacteria

Bacterial culture. *Salmonella typhimurium* and *Escherichia coli* were re-cultured in 100 mL Nutrient Broth (NB), and incubated in the shaker of incubator at 150 rotary per minute (rpm) for 24 hours.

Antibacterial activity. Suspension of *S. typhimurium* and *E. coli* were inoculated on 10 mL Nutrient Agar (NA) medium (spread plate method). 0.1 mL supernatant was dropped on Sterile paper disc with the diameter of 6 mm. Then, the paper disc was placed on the surface of NA medium (which had been inoculated with *S. typhimurium* and *E. coli*), then incubated for 24 hours at 30^0C. Antimicrobial 1% of 24 hours culture of *Bifidobacterium* spp. was inoculated into MRSB medium, and centrifuged at 13,000 rpm for 15 minutes. The supernatant was adjusted with 1 N of NaOH to pH 6.5(neutralized), and heated at 100^0C for 15 minutes. Antimicrobial activity was determined by measuring the clear zone around the paper disc after incubation.

RESULTS AND DISCUSSION

Characterization test

Based on the results of the morphological and biochemical test, there are 35 isolates belonging to the genus *Bifidobacterium.* Morphology of colonies is similar in color, shape, surface and elevation, but quite different on the size and edge formation. The milky white colonies have a flat edge/ smooth (entire), some are uneven like wool, round shape, shiny surface, convex elevation with varying sizes. These isolates have morphological characters referring to genus *Bifidobacterium* , i.e. colony with milky white color or slightly creamy, rounded form with the diameter of 0.1-0.5 mm as well as Gram-positive (Wasilewska and Bielecka 2003; Lievin 2000; Hadadji et al. 2005).

Biochemical tests

The results of biochemical tests of 35 isolates by Gram staining test were confirmed as Gram-positive bacteria as the purple cell. Gram-positive bacteria do not undergo decolorization so it stays shiny purple with color of crystal violet staining and the end is not stained by safranin and Gram-positive bacteria contains a peptidoglycan thick. Motility test showed that all isolates showed no spreading pellicle formation on the surface of the medium and without turbidity;therefore it was interpreted as nonmotile bacteria. Garrity et al. (2005) showed that the motility test positive if growth isolates form a pellicle widespreading on the surface of the medium, while it didn't show the spread of pellicle on the medium surface, named nonmotile. The positive reaction of catalase test is indicated by the formation of O_2 gas bubbles as the breakdown of H_2O_2 by catalase enzyme produced by the bacteria. The negative reaction to calatase test is indicated by no formation of gas bubbles. According to Hadadji et al.(2005), *Bifidobacterium* is catalase negative-bacteria. Indole test showed a negative result, indicated by no formation of the pink ring on the surface of the medium. Zinedine and Faid (2007) stated that *Bifidobacterium* is negative in indole test.Voges-Proskauer test showed the negative result, indicated by color change into pink or dark red after a few drops of 40% KOH and alpha-naphthol 5%. Venkatesn et al (2012) stated that *Bifidobacterium* shows a negative result in VP tests, because the bacteria do not ferment mixed acid or butanediol fermentation. Oxidase test showed the positive result, indicated by blue-black color formation after a few drops of tetramethyl-D-phenylenediamine dihydrochloride. The color change is due to the cytochrome enzyme oxidase the reagent. According to Feliatra et al. (2004), *Bifidobacterium* shows a positive result in oxidase test. Our data of lactic acid concentration from 35 isolates were ranged from 0.05-0.224%. According to Silalahi and Ikhsan (2010), longer fermentation leads to the greater production of lactic acid. This is due to the increase length of fermentation time that increases the number of cell, therefore it increases the microbial activity to produce lactic acid.

Lysozyme test results showed that all isolates were able to grow on de Man, Rogosa and Sharpe Agar (MRSA) medium supplemented with the white part of the egg (containing lysozyme). According to Gagnon et al. (2004), the *Bifidobacterium* is more resistant to white-egg lysozyme or digestive tract than other bacteria, even at the concentration of 300μg/ ml which is higher than the concentration of intestinal lysozyme. The test results showed that the pH range for the better growth of *Bifidobacterium* spp. is at pH 7 than pH 4 and pH 9. According to Biavati et al. (2000), the *Bifidobacterium* is an acid-tolerant microorganism with an optimum pH range of 6.5-7.0

API 20A test

Indole test, after the addition of XYL reagent (2-3 min) and EHR (5 min), indicated a positive result with red color and the negative result with yellow color. Urease test, after the addition of XYL reagent (2-3 min) and EHR (5 min), indicated a positive result with red color and the negative result with yellow color. The test of Glucose, mannitol, lactose, saccharose, maltose, salicine, xylose, arabinose, glycerol, cellobiose, mannose, melezitose, raffinose, sorbitol, rhamnose, and trehalose, after the addition of BCP reagent, showed that the positive result was indicated by yellow or yellow-green, and the negative result was indicated by purple color. Gelatin positive test was indicated by the occurrence of black pigment with diffusion and negative result was without diffusion. Esulin positive test was shown in black brown and fluorescent, the negative result was shown in yellow without fluorescent. The result of API test showed that 17 of 35 isolates were confirmed as genus *Bifidobacterium* namely BC4, BC5, BC6, BC7, BC8, BC10, BC13, BC14, BC15, BC16, BC17, BC18, BC19, BP1, BP6, BP7, dan BP9 (Figure 1). In normal birth process, contact between infant and nonpathogenic bacteria in mother's vagina bacteria lead to colonization of probiotic bacteria, while in caesar and premature birth processes, infant doesn't have a chance of that contact, therefore colonization of probiotic bacteria doesn't occur. In this research, the feces of infants in normal birth process did not contain any Bifidobacteria in their fecal samples. This is presumably due to fecal samples of infants having mothers who live in rural areas that their modes of feeding do not fulfill the nutrition during pregnancy (Figure 1, 2 and 3).

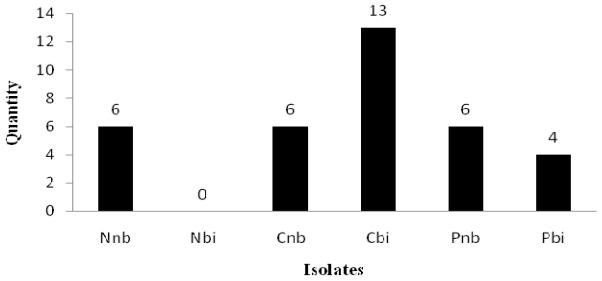

Figure 1. Distribution of 35 isolates suspected of *Bifidobacterium* after API 20A test. Note: Nnb: normal birth, non *Bifidobacterium,* Nbi: normal birth, *Bifidobacterium,* Cnb: Caesarean birth, non *Bifidobacterium,* Cbi: Caesarean birth, *Bifidobacterium,* Pnb: Premature birth, non *Bifidobacterium,* Pbi: Premature birth, *Bifidobacterium*

Inhibitory activity test

Figure 2 and Figure 3 show that the inhibitory activity test of 17 isolates belonging into *Bifidobacterium* spp. against *E. coli* and *S. typhimurium.* The diameter of the inhibitory zone of supernatant with NaOH 1 N addition against *E. coli* ranges from 6.00-13.8 mm and diameter of the inhibitory zone of supernatant without the addition of NaOH 1 N against *E. coli* ranges from 6.8-12.5 mm. The diameter of the inhibitory zone of supernatant with NaOH 1 N addition against *S. typhimurium* ranges from 6.00-11.5 mm and diameter of the inhibitory zone of supernatant without the addition of NaOH 1 N against *S. typhimurium* ranges from 7.19-12 mm.

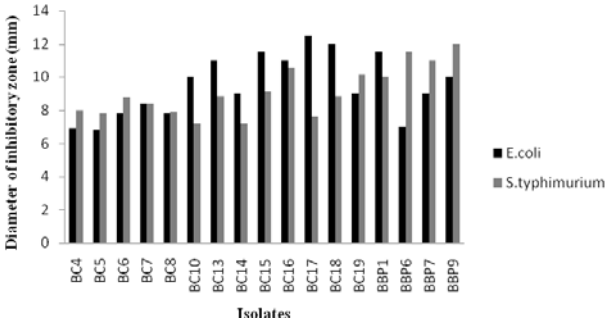

Figure 2. Diameter of inhibitory zone secondary metabolites of *Bifidobacterium* without the addition of NaOH

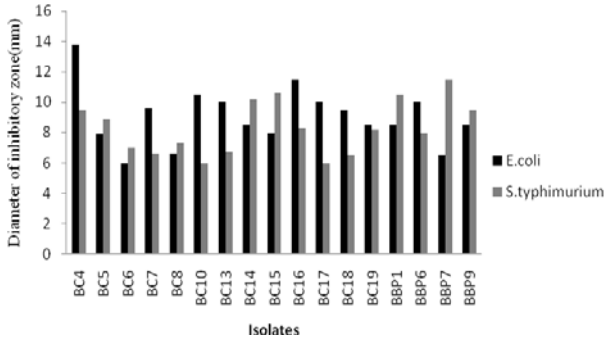

Figure 3. Diameter of inhibitory zone secondary metabolites of *Bifidobacterium* with the NaOH addition

Discussion

The results showed that the diameters of the inhibitory zone of supernatant without NaOH 1 N addition are varied. This result is due to the present of lactic acid as the antimicrobial agent. The present of Lactic acid from the fermentation of the *Bifidobacterium* spp. has several advantages, such as high solubility, low toxicity and as an effective antibacterial property (Lye et al.2009). The lactic acid can suppress the growth of pathogenic bacteria, and reduce the infection and carcinogenic effects (Venkatesan et al. 2012).

The clear zone formation indicates the ability of metabolite of *Bifidobacterium* spp. in inhibiting *E. coli* and *S. typhimurium*. The NaOH addition to pH 6.5 aims to eliminate the influence of the produced acid during metabolism, therefore the inhibition of *E. coli* and *S. typhimurium* is expected due to the activity of bacteriocins. Supernatant consisting of bacteriocins produces a clear zone with clear boundaries because bacteriocins are bactericidal which produce acid or other antimicrobial components which are bacteriostatic. According to Ray and Field (1992), bacteriocins produce a really clear zone. If there is no clear zone, it is estimated as the action of hydrogen peroxide, acid or diacetyl. Hydrogen peroxide is produced by lactic acid bacteria that can damage the structure of the microbial lipid membrane, thus will increase the membrane permeability, and then it will damage the structure of nucleic acids and proteins of the cell (De Vuyst and Leroy 2007).

Bacteriocin is secondary metabolites of lactic acid bacteria. Bacteriocins have the same way of working with antibiotic, which is able to inhibit the growth of certain bacteria. Bacteriocins in performing the antimicrobial activity will attack the cytoplasm (Naidu and Clemens, 2000). Mechanism of bactericidal activity of bacteriocins generally starts from a molecule bacteriocins that come into direct contact with the cell membrane, therefore the process of this contact causes the disturbation of membrane potential, i.e. destabilization depolarization of the cytoplasmic membrane, therefore the cells cannot survive. Membrane fluidity impacts the formation of holes or pores in the cell membrane through the disruption of Proton Motive Force (PMV) (Rodney et al. 2014).

In conclusion, there are 35 isolates expected to be *Bifidobacterium* group. After the API 20A test, 17 isolates from normal birth process, 14 isolates from cesarean birth process, and 3 isolates from premature were confirmed to be genus *Bifidobacterium* spp.. All these isolates inhibited the growth of *S. typhimurium* and *E. coli* and can also be used as potential inoculum in fermented healthy beverages.

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