INHIBITION OF BIFIDOBACTERIUM sp ISOLATED FROM INFANTS FECES TOWARDS ADHESION OF SALMONELLA TYPHI ON BALB/c MICE ENTEROCYTE

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ABSTRACT

Diarrhea, up to the recent year remains a cause of high morbidity and mortality worldwide, especially in developing countries including Indonesia. Research concerning of management, prevention, and medication of the disease have been continually improved. The aim of this research is searching *Bifidobacterium* sp isolated from infants feces. This Bifidobacterium was then applied as an anti-adhesion of *Salmonella typhi* in the hope to gain a cure of diarrhea. This research employed two research designs, namely descriptive exploration and true experimental. Exploration was applied in order to obtain isolation and characterization of *Bifidobacterium* isolated from infants feces. Adherence ability of this Bifidobacterium sp towards *Salmonella typhi* adhesion on mice entherocyte was then carried out by applying *Randomized Posttest-Only Control Group Design*. In this research, average *Bifidobacterium* sp adhesion index of 1950 on entherocyte was obtained. In simple word, there are 19.5 *Bifidobacteria* adhere to any single entherocyte cell. This adhesion index value is higher compare to *Salmonella typhi* adhesion of 1504. Conclusions that can be drawn from this research are the finding of *Bifidobacterium* sp isolated from infants feces. This Bifidobacterium sp has an ability to inhibit adhesion of Salmonella typhi on BALB/c mice enterocyte. Future work that can be carried out are further researches concerning whether these bacteria have an ability to inhibit adherence of other pathogen bacteria. More over, searching of cell wall adhesin of *Bifidobacterium* sp that can be used as a replacement of life probiotic bacteria is also a great interest of research to be carried out.

Keywords: diarrhea, adhesion, *Salmonella typhi*, *Bifidobacterium* sp.

INTRODUCTION

Diarrhea is a disease characterized by a frequent increase of defecation (> 3 time/day) followed by changes of stools concistence, with/without gross blood and/or mucus. Diarrhea, up to the recent year remains a cause of high morbidity and mortality worldwide, especially in developing countries including Indonesia. Therefore, research concerning of management, prevention, and medication of the disease have been continually improved. WHO indicates that 4 billion cases occured worldwide during the years of 2000. Of these, 2.2 million were killed, whithin the most are children under 5 years.[1,2] In Indonesia, the morbidity rate of accute diarrhea are in the range of 200-400 cases per 1000 people per year. Of the most, 70-80%

are children under 5 years. This group is experiencing diarrhea more than once per year. A part of this patient (1-2%) will be ended with dehydration and if there is no sufficient aid, 50-60% of them could be died.[1]

It was reported that the main cause of diarrhea dominated by enteropathogenic bacteria, including *E. coli* diarrheagenic involving ETEC and EPEC, *Salmonella spp*., *Shigella spp*., and *Vibrio spp*.[3] Diarrhea caused by *S. typhi* were initiated by adherence of *Salmonella typhi*. This adhesion induces neutrophile transepithelial migration and villi entherocyte damage followed by membrane destruction on the site of addhesion. This damaging membrane subsequentially followed by endocytosis and internalization.

It was well established that there are much more bacteria including diarrhea bacteria are resitance towards antibacterial.[4,5] This condition has endorsed researches for establishing research to obtain an alternative cure to replace antibacterial that has already used clinically. Obtaining an agent that can affect or protect bacteria adherence is one of researhers strategy to overcome this situation. These kind of agents are known as anti-adhesion. Other strategics are obtaining vaccine for diarrhea. Anti-adhesion agents are not bactericide or antibiotic, therefore, their propagation and occurrence for therapy will not leading to resistence.[5,6]

MATERIAL AND METHOD

Research Design

Two research designs were employed, i.e explorative and true experi mental. Explorative was carried out to explore isolation and characterization of *Bifidobacterium* isolated from infants feces. Experimental study with Randomized Posttest-Only Control Group Design was employed to obtain inhibition ability of *Bifidobacterium* towards adhesion of *Salmonella typhi* on enterocyte.

Research Procedure

Isolation of *Bifidobacterium* from infants feces

Isolation of *Bifidobacterium* from infants feces was carried out following Beeren, (1990) in Hadadji.[7] About 1 g of infants feces was placed on a flask added with 9 mL NaCl (0.9%) containing 0.2 % cystein-HCl. This suspension was then homogenized for 2 minutes. Around 0.1 mL of this suspension was inoculated on MRS broth media. All plates were then anaerobically incubated using oxoid gas jar at temperature of 37 ^{0}C with 5 % CO_2 for 18-24 h. The *Bifidobacterium* obtained was then separated using solid MRS broth media. The clonal was then selected and indentified for further treatment.

Isolation of Balb/c mice enterocyte

Enterocyte isolation was carried out based on Weisler method taken from Nagayama.[8] An healthy BALB/c mice age of 3 months was collected and treated for this experiment. Mice was killed using chloroform and resected. The intestine was taken out, cleaned and cut into 5 cm. Lumen intestine was then resected and cleaned using PBS solution containing dithiotreitol 1 mM. The lumen was immersed on a solution containing of 1.5 mM KCl; 9.6 mM NaCl; 2.7 mM Na-citrate; 8 mM KH_2PO_4 and 5.6 mM Na_2PO_4, pH 7.3 and placed on water bath at temperature of 37 ^{0}C, and shaked for 30 minutes. All solution was drained and replaced with PBS with pH of 7.4 containing 1.5 mM EDTA and 0.5 mM dithiothreitol. Intestine tissues were shaked on water bath within temperature of 37 ^{0}C for 20 minutes. Solution was drained and the intestine was cleaned using PBS pH. Of 7.4 by centrifugation at 1000 rpm, at temperature of 4^{0}C for 5 minutes. The cleansing was carried out three times and suspended on PBS pH of 7.4 and shaked. Solution containing enterocyte characterized by cloud

filtrate was taken using sterilized pippetes and store on steril tube, counted using leucocyte counting chamber and made up the concentration of 10^8 enterocyte/mL.

Adhesion test of *Bifidobacterium* on enterocyte

Nagayama method was employed for testing of *Bifidobacterium* adhesion on enterocyte.[8] A number of 100 μL of the *Bifidobacterium* suspension with concen tration of 10^8 cells/ml mixed with 100 μL enterocyte cells of 10^8 cells/mL. The mixture was then incubated on shaker water bath and shaked at temperature of 37^0C for 30 minutes. Cells were collected by centrifugation at 3000 rpm for 2 minutes. The precipitate was then collected for sweft preparate and gram staining. The preparate was observed under microscope with 1000 time zoom, to obtain the type and adhesion index.

RESULTS

Isolation of *Bifidobacterium* from Infants feces

Isolation of *Bifidobacterium* from infant feces was carried out following Beeren, (1990) in Hadadji.[7] Fecal suspension on NaCl solution with cystein-HCl was inoculated on broth media. The grown Bifidobacteria were separated using solid broth media. The clonal growth were then selected for identification (Figure1).

Enterocyte Isolation

Enterocyte isolation was carried out on the basis of Weisler method in Nagayama.[8]

Adhesion of *Bifidobacterium* sp on Enterocyte

Nagayama method was employed to observed adhesion of Bifidobacterium on enterocyte (Figure 2 and Table 1).[8] Adhesion indices of *Bifidobacterium* sp on enterocyte were listed on Table 1. In this experiment, adhesion of Salmonella typhi on enterocyte was also performed. The result was drawn on Figure 3.

Inhibition test of *Bifidobacterium* towards Adhesion of *Salmonella typhi* on enterocyte

Inhibition test of *Bifidobacterium* sp towards *Salmonella typhi* on enterocyte was performed and the test result was presented on Figure 4.

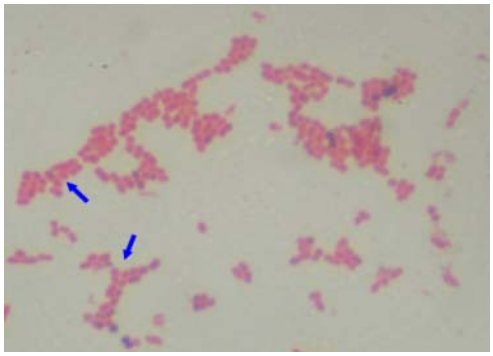

Figure 1 *Bifidobacterium* sp isolated from infants feces indicated by short rod with Y and V shape (1000 X).

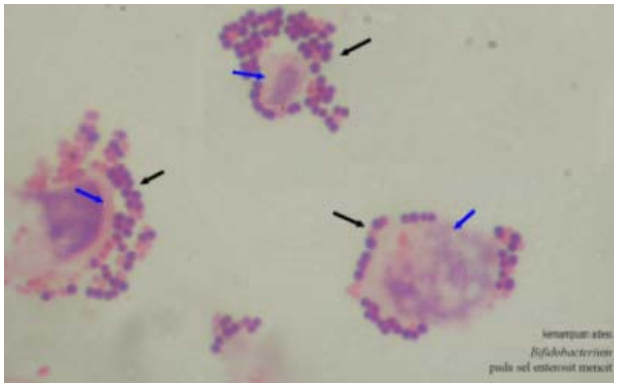

Figure 2 Adhesion of *bifidobacterium* on enterocyte,
→ *bifidobacterium* → enterocyte
(zoom of 1000 X).

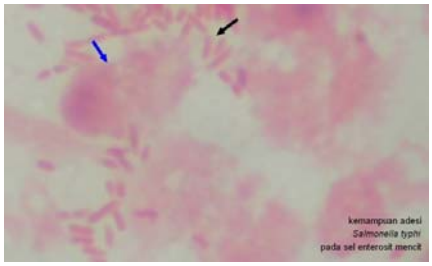

Figure 3 Adhesion of *Salmonella typhi* on enterocyte, → *S. typhi*
→ enterocyte (zoom of 1000 X).

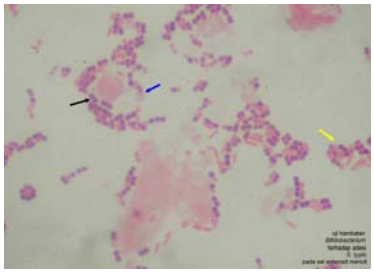

Figure 4 Inhibition of *Bifidobacterium* sp towards adhesion of
Salmonella typhi on enterocyte
→ *Bifidobacterium* → *Salmonella typhi*
→ Interaction between *Bifidobacterium* with
Salmonella typhi (zoom of 1000 X).

Table 1 Adhesion indices of *Bifidobacterium* sp on enterocyte

No	Number of *Bifidobacterium* per 100 enterocyte	Number of *Bifidobacterium* sp per enterocyte
1	2000	20
2	1900	19
3	1800	18
4	2100	21
5	2000	20
6	1900	19
Average	1950	19.50

DISCUSSION

Bifidobacterium

In this experiment, bacteria isolated from infant feces are *Bifidobacterium* sp. This can be seen clearly from the characteristic of the bacteria as a short rod with Y and V shape (Figure 1). *Bifidobacterium* was initially discovered on the year of 1889 by Tissier, a researcher from Pasteur Institute French. It was obtained that the bacteria are a gram positive, anaerobe, short rod pleomorphism of Y and V shape, without spora, and originally named *Bacillus bifidus communis* and categorize into *Lactobacillus* and named as *L. Bifidus*.[9-12] Then, in 1960 these bacteria were categorize into a special genus known as *Bifidobacterium*, characterized by their ability to produce lactate and acetic acids from glucose. Compotition of guanine and sitosine DNA of the bacteria are between 54 and 67% per molecule, and the bacteria are sacarolityc.[9-12]

Adhesion of *Bifidobacterium* sp on Mice Enterocyte

The adhesion of *Bifidobacterium* sp on enterocyte mice was carried out to evaluate the ability of the bacteria to adhere on mice enterocyte. It can be seen from Figure 4.3, that the adhesion model of *Bifidobacterium* sp on enterocyte is diffuse adhesion. This model was marked by the bacteria are homogenously spread out on cell surface. It was known that there are three types of adhesion model, namely, 1) localized adherence, 2) aggregative adherence, and 3) diffuse adherence.[13,14] Localized adhesion was characterized by grouping bacteria colonization on the receptor cell surface. Moreover, aggregative adherenece was marked by stacked-brick model of bacteria adherence both in tissue culture and on the base of object glass. In addition, for the last adhesion model was characterized by the adhesion of the bacteria are hogenously spread out on the cell surface not on the surface of object glass.[15]

Bifidobacterium on their comensal function should have an ability to interact with intestitinal epithelial cells and inducing fragment product that trigered and activated the cells. This condition was proven in this research. The interaction induces secretion of IL-6 and IL-10. TLR is playing an important role during these interaction and induction.[16] There are four important things concerning of induction of intestinal

immune response caused by *Bifidobacterium* adhesion, namely: (i) their interaction to epithelial cells, (ii) bacteria pathway of internalization on intestinal track, (iii) signal induction on intestinal immune cells to enhance cytokine production and appro priate producing cells, and (iv) improvement of IgA-producing cells on other mucosal cells, i.e. bronchus and mammary glands.[16,17]

On the other hand, concerning of Salmonella typhi adhesion on mice enterocyte as depicted on Figure 4, adhesion models observed is localized adhesion indicated by rod shape of Salmonella typhi adherence on enterocyte receptor. There should be an exposure and adherence of Salmonella typhi to enterocyte before invation of the bacteria as indicated on Figure 4. Adhesion of Salmonella typhi is a prerequisite for colonization and infection in gastrointestinal tract and becoming a major factor for occurence of invation and secretion of infection factors.[18,19]

GIT mucosal surfaces are known to be the widest area of the human body, i.e. 200-300 m^2 that can be exposed with external environment. The content of the GIT mucosal are epithelial cells, immune cells, and inhabitance of natural microorganism.[20] Adhesion ability of Salmonella typhi on enterocyte is the first step of infection, therefore, the bacteria can affect and explore the presence signal after adherence and then secreting virulence factors.[18,21]

Disturbance of host natural activities leads to entrance of Salmonella typhi into epithelial barrier.[22] Host cytosceleton is taken by enteric microbial pathogens, including Salmonella typhi as a media during cells penetration. In other words, this cytosceleton was exploited by the pathogen for entry site into the cells, move along and intra cells or rebuild the vacuola which leads to protection and survival of the bacteria.[23]

It can be seen from Figure 4, that the presence of Bifidobacterium sp inhibited adhesion of Salmonella typhi on enterocyte. Meanwhile, as can be seen on Figure 3, Salmonella typhi itself has an ability to adhere on enterocyte, proven by its index adhesion of 1504 compare to about 1950 for Bifidobacterium. There are also interaction or competition between these two bacteria on enterocyte as indicated on the same figure. *Bifidobacterium* sp adhesion ability on enterocyte is consider to become a self difence towards pathogen on GIT. The adherence of *Bifidobacterium* sp is also play an important role for competition adhesion site and nutrient with pathogen. Adhesion is also allowing *Bifidobacterium* sp to produce antimicrobial compounds and nutrient metabolism to produce volatile fatty acids and bile salt metabolits and leading to inapproriate environment for pathogen. Adhesion of *Bifidobacterium* on enterocyte can be consider as protection of intestinal mucosa and giving a steric hindrance effect, therefore, pathogen bacteria can not contact to intestinal mucosa. This phenomena is known as competition exclution effect.[18]

This research is in line with Moroni finding, they found that there was adhesion of *B. thermophilum* sp. *infantis* RBL67 and *B. thermacidophilum* sp. *suis* strain RBL68 and RBL70 on enterocyte cells.[24] Adhesion ability of *Bifidobacterium* strain RBL67 and RBL70 were found bigger compare to adhesion of *bifidum* RBL71 which is reported before having the highest adhesion[25] as well as for *B. pseudolongum* ATCC 25526.[26] On the other hands, adhesion of *B. thermacidophilum* sp *suis* strain RBL68 was found comparable to *B. bifidum* RBL71. All Bifiobacteria strain tested were not invading the ephitelial cell, therefore, *Bifidobacterium* is included as non-invasive bacteria.

CONCLUSSION AND FUTURE WORKS

Conclussion

1. Bacteria isolated from infants feces were exactly Bifidobacterium sp as indicated by their short rod like and V and Y shape.
2. Bifidobacterium sp isolated from infants feces has an ability to inhibit adhesion of Salmonella typhi on mice enterocyte.

Future Works

1. Further researchs concerning of extraction of cell wall adhesin proteins of Bifidobacterium sp need to be carried out.
2. Any further researchs should be carried out in term of commercially use of these adhesin proteins including synthesis, production, and marketing, therefore it become usefull for replacing life probiotic bacteria.

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