

Inhibition of *Bifidobacterium* Cell Wall 51.74 kDa Adhesin Isolated from Infants Feces Towards Adhesion of Enteric Pathogen *E. Coli* on Enterocyte Balb/C Mice

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Objectives: To determine 51.74 kDa adhesin of *Bifidobacterium* sp cell wall isolated from infants feces as an anti adhesion of *E. coli* on enterocyte mice.

Methods: Randomized Posttest-Only Control Group Design was employed to investigate adherence ability of this adhesin towards *E.coli* adhesion on mice enterocyte.

Results: In this research, it was obtained, that the 51.74 kDa adhesin cell wall of *Bifidobacterium* sp has an ability to inhibit adhesion of *E. coli* on mice enterocyte. The ability was increased as an increase of adhesin concentration.

Conclusions: that can be drawn from this research is the finding of 51.74 kDa adhesin cell wall of *Bifidobacterium* sp isolated from infants feces that can inhibit adhesion of *E. coli* on mice enterocyte. Future work that can be carried out are further researches concerning whether these protein can be applied to inhibit adherence of other pathogen bacteria.

Keywords: diarrhea, adhesion, *E. coli*, *Bifidobacterium* sp.

INTRODUCTION

Diarrhea is a disease characterized by a frequent increase of defecation (> 3 time/day) followed by changes of stools consistency, 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 occurred worldwide during the years of 2000. Of these, 2.2 million were killed, within the most are children under 5 years.^{1,2} In Indonesia, the morbidity rate of acute 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 neutrophil trans-epithelial migration and villi enterocyte damage followed by membrane destruction on the site of adhesion. This damaging membrane subsequently followed by endocytosis and internalization. It was well established that there are much more bacteria including diarrhea bacteria are resistance towards antibacterial.^{4,5} This condition has endorsed researches for establishing research to obtain an alter-

native cure to replace antibacterial that has already used clinically. Obtaining an agent that can affect or protect bacteria adherence is one of researchers strategy to overcome this situation. These kind of agents are known as anti-adhesion. Other strategies are obtaining vaccine for diarrhea. Anti-adhesion agents are not bactericide or antibiotic, therefore, their propagation and occurrence for therapy will not leading to resistance.^{5,6}

METHODS

Research Design

Experimental study with Randomized Posttest-Only Control Group Design was employed to obtain inhibition ability of 51.74 kDa adhesin cell wall of *Bifidobacterium* towards adhesion of *E. coli* on mice enterocyte.

Research Procedure

Isolation of *Bifidobacterium* from infant 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 °C with 5 % CO₂ for 18-24 h. The *Bifidobacterium* obtained was then separated using solid MRS broth media. The clonal was then selected and identified for further treatment.

Isolation of Balb/c mice enterocyte

Enterocyte isolation was carried out based on Weisler method taken from Nagayama.⁸ A healthy BALB/c mice age of 3 months was collected and

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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 dithiothreitol 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°C , and shaken 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 shaken on water bath within temperature of 37°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°C for 5 minutes. The cleansing was carried out three times and suspended on PBS pH of 7.4 and shaken. 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 mice

Nagayama method was employed for testing of *Bifidobacterium* adhesion on enterocyte.⁸ A number of 100 μL of the *Bifidobacterium* suspension with concentration 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 shaken at temperature of 37°C for 30 minutes. Cells were collected by centrifugation at 3000 rpm for 2 minutes. The precipitate was then collected for sweet preparate and gram staining. The preparate was observed under microscope with 1000 time zoom, to obtain the type and adhesion index.

Inhibition test of 51.74 kDa *Bifidobacterium* cell wall-adhesin towards Adhesion of *E.coli* on mice enterocyte

Inhibition test of *Bifidobacterium* sp cell wall adhesin towards *E.coli* on enterocyte was performed in the same way as inhibition test of *Bifidobacterium* sp by replacing the bacteria with the adhesin.

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 *Bifidobacterium* were separated using solid broth media. The clonal growth were then selected for identification (Figure 1).

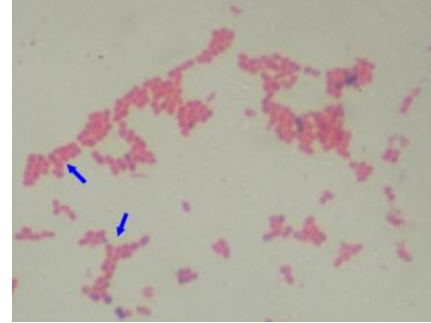


Figure 1

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

Enterocyte Isolation

Enterocyte isolation was carried out on the basis of Weisler method in Nagayama.⁸ The enterocyte obtained was pictured on Figure 2.

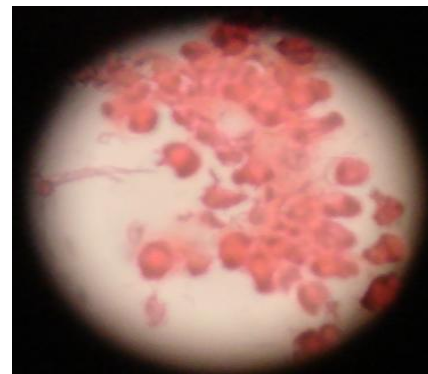


Figure 2

Enterocyte of BALB/c mice (1000x)

Adhesion of *Bifidobacterium* sp on Enterocyte

Nagayama method was employed to observed adhesion of *Bifidobacterium* on enterocyte.⁸

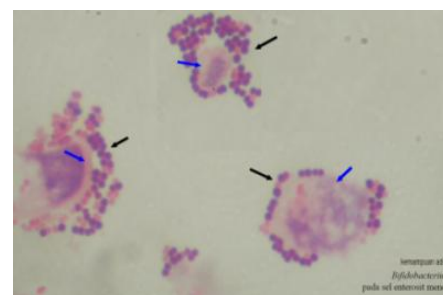


Figure 3

Adhesion of *bifidobacterium* on enterocyte, \blackrightarrow *bifidobacterium* \rightarrow enterocyte (zoom of 1000 X).

Adhesion indices of *Bifidobacterium* sp on enterocyte were listed on Table 1.

Table 1
Adhesion indices of *Bifidobacterium* sp on enterocyte

| No | Number of <i>Bifidobacterium</i> per 100 enterocyte | Number of <i>Bifidobacterium</i> sp per enterocyte |
|---------|---|--|
| 1 | 2000 | 20 |
| 2 | 1900 | 19 |
| 3 | 1800 | 18 |
| 4 | 2100 | 21 |
| 5 | 2000 | 20 |
| 6 | 1900 | 19 |
| 7 | 2000 | 20 |
| 8 | 1900 | 19 |
| Average | 1950 | 19.50 |

In this study, adhesion of *E.coli* on enterocyte was also performed. The result was drawn on Figure 4.

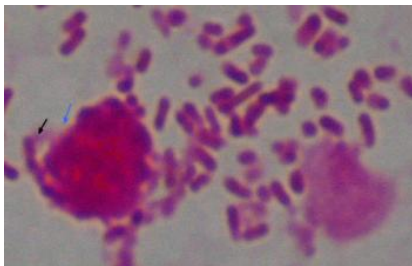


Figure 4
Adhesion of *E.coli* on enterocyte, —→ *E.coli*
—→ enterocyte (zoom of 1000 X).

Inhibition test of *Bifidobacterium* towards Adhesion of *E.coli* on mice enterocyte

Inhibition test of *Bifidobacterium* sp towards *E. coli* on enterocyte was performed and the test result was presented on Figure 5.

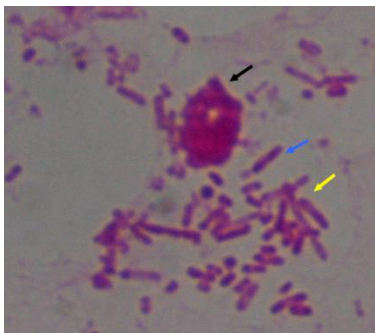


Figure 5
Inhibition of *Bifidobacterium* sp towards adhesion of *E. coli* on enterocyte —→ *Bifidobacterium*
—→ *E. coli* —→ Interaction between *Bifidobacterium* with *E. coli* (zoom of 1000 X).

Inhibition test of *Bifidobacterium* sp cell wall adhesin towards *E. coli* on enterocyte was performed in the same way as inhibition test of *Bifidobacterium* sp (Figure 6).

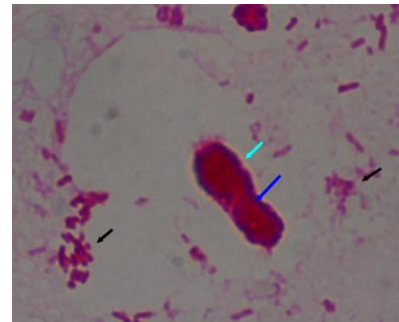


Figure 6
Inhibition of *E. coli* adhesion by *Bifidobacterium* sp cell wall adhesin. —→ *E. coli*, —→ enterocyte, —→ adhesin of 51.74 kDa (zoom of 1000 X).

Adherence data of *E. coli* that can be inhibited by *Bifidobacterium* sp cell wall adhesin of 51.74 kDa were listed on Table 2. Data on Table 2 are normally distributed and their variance are also homogenous with $p > 0.05$. Therefore, the data can be analyzed parametrically by applying one way Anova and followed by LSD posthoc test. It was obtained, that the 51.74 kDa adhesin exactly inhibit adherence of *E. coli* on enterocyte as indicated on Table 3.

Table 2
Adhesion of *E. coli* on enterocyte

| No | Adherence of <i>E. coli</i> on 100 enterocyte due to inhibition of 51.74 kDa adhesin | | | | | |
|---------|--|-----------|------------|------------|------------|------------|
| | 0 μ l | 5 μ l | 10 μ l | 15 μ l | 20 μ l | 25 μ l |
| 1 | 1570 | 1310 | 724 | 495 | 321 | 293 |
| 2 | 1490 | 1169 | 710 | 558 | 309 | 207 |
| 3 | 1495 | 1230 | 715 | 579 | 355 | 317 |
| 4 | 1482 | 1322 | 825 | 498 | 321 | 216 |
| 5 | 1497 | 1311 | 621 | 478 | 432 | 207 |
| 6 | 1419 | 1217 | 790 | 415 | 298 | 105 |
| 7 | 1410 | 1210 | 790 | 410 | 290 | 100 |
| 8 | 1400 | 1200 | 700 | 400 | 200 | 90 |
| Average | 1470. | 1246.1 | 734.3 | 479.1 | 315.7 | 190.5 |
| | 38 | 3 | 8 | 3 | 5 | 8 |

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. Compositon of guanine and sitosine DNA of the bacteria are between 54 and 67% per molecule, and the bacteria are sacarolityc.⁹⁻¹²

Table 3

Resume of the different between Adhesin towards adhesion of *E. coli* on enterocyte

| LSD | Variable | Mean | Standar error | p |
|----------------------|----------|----------|---------------|-------|
| Conc. 0 (Control) | Conc. 5 | 224.00* | 33.79 | 0.001 |
| | Conc. 10 | 736.00* | 33.79 | 0.001 |
| | Conc. 15 | 991.25* | 33.79 | 0.001 |
| | Conc. 20 | 1154.63* | 33.79 | 0.001 |
| | Conc. 25 | 1278.50* | 33.79 | 0.001 |
| Conc. 5 | Conc. 0 | -224.00* | 33.79 | 0.001 |
| | Conc. 10 | 511.75* | 33.79 | 0.001 |
| | Conc. 15 | 767.00* | 33.79 | 0.001 |
| | Conc. 20 | 930.38* | 33.79 | 0.001 |
| | Conc. 25 | 1054.25* | 33.79 | 0.001 |
| Conc. 10 | Conc. 15 | 255.25* | 33.79 | 0.001 |
| | Conc. 20 | 418.65* | 33.79 | 0.001 |
| | Conc. 25 | 542.50* | 33.79 | 0.001 |
| Conc. 15 | Conc. 20 | 163.38* | 33.79 | 0.001 |
| | Conc. 25 | 287.25* | 33.79 | 0.001 |
| Conc. 20 | Conc. 25 | 123.88* | 33.79 | 0.001 |

Remarks: Conc = concentration in ppm

*consider significant

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 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 adherence 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 intestinal 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 appropriate 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 *E. coli* adhesion on mice enterocyte as depicted on Figure 4, adhesion models observed is localized adhesion indicated by rod shape of *E. coli* adherence on enterocyte receptor. There should be an exposure and adherence of *E. coli* to enterocyte before invation of the bacteria as indicated on Figure 4. Adhesion of *E. coli* is a prerequisite for colonization and infection in gastrointestinal tract and becoming a major factor for occurrence 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 m² that can be exposed with external environment. The content of the GIT mucosal are epithelial cells, immune cells, and inhabitanace of natural microorganism.²⁰ Adhesion ability of *E. coli* 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 *E. coli* into epithelial barrier.²² Host cytoskeleton is taken by enteric microbial pathogens, including *Salmonella typhi* as a media during cells penetration. In other words, this cytoskeleton 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 *E. coli* on enterocyte. Meanwhile, as can be seen on Figure 4, *E. coli* itself has an ability to adhere on enterocyte, proven by its index adhesion of 1470 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 defence 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 metabolites and leading to inappropriate 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 exclusion effect.¹⁸

Inhibition of 51.75 kDa Adhesin towards E. coli on Mice Enterocyte

Table 2 reveals that *Bifidobacterium* sp cell wall adhesin of 51.74 kDa inhibit adhesion of E. coli on enterocyte, indicated by data on column 1, inwhich there is a significant difference of 224.00 bacteria between control (protein concentration of 0 µL) with protein of = 5 µL with $p < 0.00$. Moreover, for the following data, the same trend was also observed inwhich there is a significant difference between control and protein with concentration of 10 µL. Therefore, it can be considered that increase of protein concentration enhance adhesion ability of the protein to inhibit adhesion of E. coli on enterocyte. In other words, an increase of protein concentration leads to decrease of E. coli adhesion on enterocyte.

CONCLUSSION AND FUTURE WORKS

Conclusion

1. Bifidobacterium sp cell wall adhesin of 51.74 kDa isolated from infant feces has an ability to inhibit adhesion of E. coli on mice enterocyte.
2. Increase concentration of Bifidobacterium sp cell wall adhesin of 51.74 kDa isolated from infant feces reduced adhesion of E. coli on enterocyte.

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