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Original Article

Immunoenhancing effect of Lactobacillus reuteri on immunized mice intestine using cholerae toxin subtype B

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ABSTRACT

The immunoenhancing effects of probiotic Lactobacillus reuteri have assessed via mouse intragastric inoculation. The number of immunoglobulin A-positive cells, cathelicidin, and beta-defensin in the small intestine in response to adhesion molecule stimulation (CTB v cholera) increased in mice fed L. reuteri. Intestine mass also decreased in the small intestine of mice fed Lactobacillus reuteri. No differences were found in body weight, food intake, and clinical signs between mice fed Lactobacillus reuteri and the control group. Results indicated that Lactobacillus reuteri is a probiotic with immunoenhancing properties via IgA and beta-defensins and cathelicidin.

Keywords: Lactobacillus reuteri, Beta-defensin, Cathelicidin, IgA, Probiotic.

Introduction

Many factors contribute to health such as nutrition, physical activity, level of stress, personality, and behavior [1, 2]. Various medicinal plants have been used traditionally to treat diseases all over the world [3]. The practice of indigenous medicine has repeatedly focused on herbs for their innate antimicrobial activity against a plethora of bacteria and molds since ages [4]. Microbial resistance can be intrinsic or acquired [5]. Probiotics are widely known had increased the host cellular immune response against pathogens. In the gastrointestinal tract, probiotics modulate intestinal epithelial cells to produce antimicrobial peptides (AMP) to enhance the defense system of the intestinal mucosa [6]. Moreover, recent studies have shown that probiotics can produce AMP. AMP had been known had a significant role in protecting host, especially bacterial infections. AMP such as beta-defensins and cathelicidin has an important role as innate immunity to prevent the docking of pathogenic microbes on the mucosa epithelium [7]. Beta-defensin was one of the AMP that plays a role in the lysis of pathogenic microbes that adheres to the intestinal surface. This peptide was cationic, measuring 3-5 kDa, expressed by various types of epithelial cells in innate and adaptive immune responses. Human beta-defensin 2 (HBD-2) was an antimicrobial peptide that has broad-spectrum antibacterial and antifungal activity [8, 9]. HBD-2 is generally produced by epithelial cells, but it can also be produced by monocytes, macrophages, and dendritic cells. If there is a decrease in defensory secretion by Paneth cells to the intestinal mucosa, the mouse intestine is unable to develop inflammation, and so the mice are susceptible to bacterial infections given orally. Cathelicidin and defensins were two main families of antimicrobial peptides that play an important role in immune cells on the surface of epithelial cells. Several defensin lines can be tested on humans and mice, namely; α-defensin, human α-defensin-5 (HD-5) and HD-6, β-defensin, human β-defensin-1-

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4 (HBD-1-4), while cathelicidin was only one strain, LL-37 [10].

Probiotics, in addition to its role in modulating cellular immunity, also play a role in stimulating the humoral immune system via antibody immunoglobulin A (IgA). Some probiotic genus of lactobacillus or bifidobacteria strains can induce mucosal IgA production [11, 12]. These bacteria induce IgA production by developing gut-associated lymphoid tissue (GALT) in the large intestine and small intestine. IgA plays a role as a barrier in capturing pathogens in the mucous layer through the ability of mucin protein to bind to antigens. Some probiotic bacteria such as lactobacillus and bifidobacteria could increase the induction of intestinal mucosal IgA production [13, 14]. Other mechanisms were competitive adhesion of the mucosa and epithelium, strengthen the intestinal epithelial barrier, and interact with Paneth cells to produce AMP (defensins, lysozymes) [15, 16]. Probiotics that are gaining in popularity recently were Lactobacillus reuteri. L. reuteri was reported to be able to produce various antimicrobial substances such as lactic acid, hydrogen peroxide, reuterin, and reutericyclin and has beneficial effects on many hosts. L. reuteri strain could inhibit the growth of enteric pathogens in vitro, including Escherichia coli, Salmonella Typhimurium, Staphylococcus epidermidis, S. aureus, Helicobacter pylori, and rotavirus [17]. Also, L. reuteri produces vitamin B12 and could synthesize L-lysine, and folic acid [18]. Animal and human studies show that oral administration of L. reuteri can reduce the incidence and severity of diarrhea, prevent colic and necrotic enterocolitis, maintain mucosal barrier function, and immunomodulate [19, 20].

The effects of probiotics such as L. reuteri in increasing immunity in the digestive tract are well known, however, the underlying mechanism is still poorly understood. In this research, the mechanism of L. reuteri probiotics in increasing the intestinal immune system will be explored more deeply by analyzed multiple variables i.e intestinal IgA, protein beta-defensins, and cathelicidin.

Materials and Methods

This research is experimental laboratory research. Ethical clearance for this study was obtained from the Ethical Committee of the School of Medicine, Brawijaya University Malang Indonesia with the number 275 / EC / KEPK-S3 / 11/2018. In this study, the research subjects were divided into 3 treatment groups and 1 control group with each group of 5 mice. In group I, each mouse was taken orally by L. reuteri live at a dose of 1 x 1010 CFU / ml/mouse (250 µl) dissolved in Mc Broth media as much as 0.5 ml given daily (once) for 28 days. Group II each mouse was fed orally of 38 kDa.V. cholera conjugated CTB protein adhesions with a dose of 250 µg dissolved in PBS as much as 0.5 ml carried out on days 1, 7, 14 and day 21. Group III each mouse was rounded orally L. reuteri live at a dose of 1 x 1010 CFU / ml/mouse (250 µl) dissolved in Mc Broth media as much as 0.5 ml given every day (once) for 28 days and the next hour after every 30 minutes of mice protein adhesion pili 38 kDa. V.cholerae CTB conjugation with a dose of 250 µg dissolved in PBS 0.5 ml was performed on days 1, 7, 14, and day 21. Group IV was the control of every mouse in 0.5 ml PBS rounds starting from day 1 to 28.

The procedure for making CTB conjugated protein vaccine using the following method. The 37.8 kDa adhesion protein collection results were taken as much as 6 mg in 1.5 ml PBS, while CTB as much as 0.230 mg in 1.5 ml PBS. Then stir in the fume hood and add 2% Glutaraldehyde 3 cc, incubated at room temperature for 1 hour with stirring slowly. Glucine pH 7.2 was added as much as 2.24 ml (MB = 75.07) incubated at room temperature for 1 hour, stirring slowly, after that it was dialed out with PBS overnight 4 times, then stored at -20oC. One week after the last immunization, the animal is terminated by the decapitation method. Measurement of sIgA levels in intestinal mucosal samples by ELISA method. Supernatant stored at -70°C is taken and placed at room temperature before use. Prepare all reagents, standard solutions, and selected samples. Bring all the reagents to the workroom (room temperature) before the work begins. Determine the number of strips needed for testing. Insert the strip to use. Unused strips are stored at -80°C. Add 50 µl of standard solution to the standard well. Add 40 µl of sample to the sample well and add 10 µl of anti-sIgA antibody to the sample well, then add 50 µl of streptavidin HRP to the sample well and standard well. Cover the plate with a translucent cover. Incubation for 60 minutes at 37°C. The cover is removed and the plate is washed 5 times with washing buffer. Add 0.35 ml of washing buffer to each well and leave it for 1 minute each wash. Automatic in washing, aspirate all wells and washed 5 times with washing buffer. Wipe the plate with absorbent paper or tissue. Add 50 µl of a cover solution to each well, the blue color will immediately turn yellow. Determine the optical density (OD value) for each well immediately by using a microplate reader set between 450 nm to 30 nm after adding a stop solution (Bioassay Technology Laboratory, Mouse Secretory immunoglobulin A ELISA Kit, No Cat E0380MO). Calculation of V.cholerae colonization using Colony Counter, all V.cholerae were isolated in TCBSA selective medium from mice intestinal samples after the treatment stage. Colonies were counted by the sampling method using 5 squares of 1 cm2, all colonies attached to the left and top lines of the box were counted, for colonies that were attached to the right and bottom lines of the next counted box. After calculating the 5 boxes the results are divided by 5 and multiplied by the petri dish area (π r2 / 3.14 X 42). The final results are calculated by the colony Forming Unit (CFU) / ml sample. To calculate the intestinal mass weight of Wistar rats, after the diarrhea test treatment of Wistar intestinal samples before and after shaking, incubation 370C for 3 hours incubation. With petri dish and electric scales, all intestines resulting from treatment were weighed both the 0-hour pre-shaking incubation and the 3rd hour after shaking incubation. the resulting difference from the calculation is recorded and concluded as the effect of the diarrhea test. Statistical analysis

uses the ANOVA test to see differences between groups and Tukey tests to test differences between groups.

Results and Discussion

The concentration of anti-hemagglutinin sIgA pili V.cholerae using the ELISA method showed the highest s-IgA levels in the group of mice immunized with Ag + CTB **(Figure 1)**. One Way Anova s-IgA statistical test shows that from the results of Tukey's analysis the evaluation of s-IgA levels of the L reuteri-induced mice group or those of rats exposed to 37.8 kDa pili adhesion protein V.cholerae Conjugation CTB showed a significant increase in value when compared with a control group of mice each with a significance value of $\alpha \leq 0.05$ ie 0.007 and 0.021, while for the treatment of mice induced a combination of both L reuteri and 37.8 kDa V. protein cholera conjugation, showed no significant results when compared to controls. L. reuteri-induced mice treatment group. Observation of the process of increasing the activity of V.cholerae bacterial colonization in mice intestine as indicated by intestinal distension and an increase in mice intestinal mass weight. Based on the weighing data showed that the control group's intestinal rats were a significant increase in mass weight compared to the treated mice group either the L. reuteri addition group or 37.8 kDa V. adhesion protein V. cholera conjugated CTB or a combination of both, each with a significance value of 0.0010, 0.009 and 0.0021. This data is also strengthened from the calculation of the total population of V.cholerae bacteria on the plates so that TCBSA is cultured from mice intestinal mucosal scrapings.

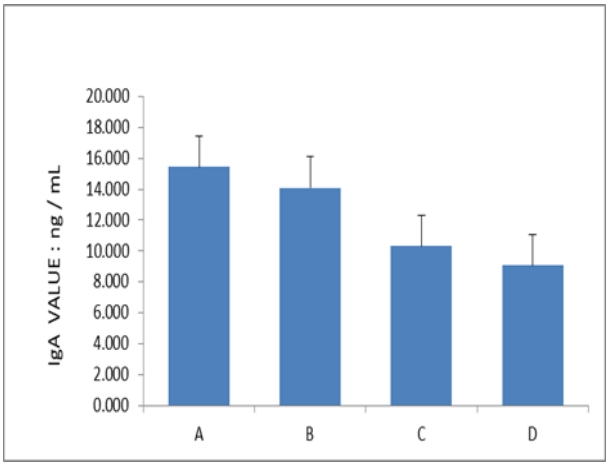

Figure 1. Results of measurement of sIgA concentration in mouse intestinal mucosa scrapes in control and treatment. (A) intestinal mucosa induced L. reuteri, (B) Intestinal mucosa induced pili adhesin protein 37.80 V. cholerae conjugated CTB, (C) Intestinal mucosa induced L. reuteri and pili adhesin protein 37.80 kDa V. CT conjugate cholera, (D) Intestinal mucosa Control mice.

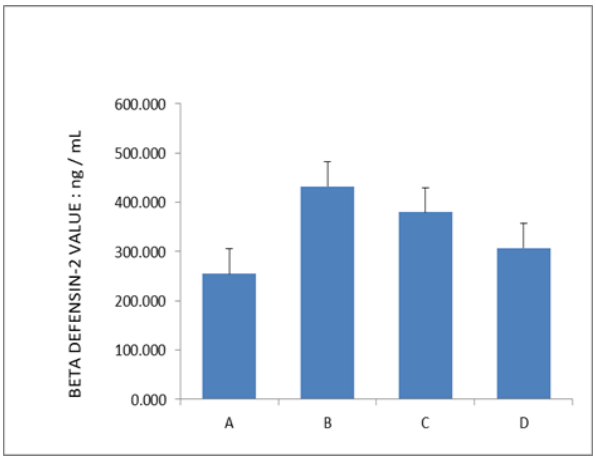

Figure 2. Results of β-defensin-2 concentration in mouse intestinal mucosa scrapes in control and treatment. (A) intestinal mucosa induced L. reuteri, (B) Intestinal mucosa induced pili adhesin protein 37.80 V. cholerae conjugated CTB, (C) Intestinal mucosa induced L. reuteri and pili adhesin protein 37.80 kDa V. CT conjugate cholera, (D) Intestinal mucosa Control mice.

L reuteri probiotics and immunization with pili adhesion protein 37.8 kDa V.cholerae conjugated CTB can increase the productivity of Cathelicidin specific antimicrobial peptide (AMP) LL-37 strain. Evidenced by a significant increase in the treatment group of rats immunized with 37.8 kDa V. cholerae conjugate protein adhesion CTB conjugated compared to the group of mice without immunization (control) with a significance value of $\alpha \leq 0.05$ ie 0.038. combination of L. reuteri and immunized with 37.8 kDa pili adhesion protein V.cholerae conjugated CTB, the results of Cathelicidin LL-37 analysis also showed significantly different results when compared to groups of mice without both exposure (Control) with significant significance $\alpha \leq 0.05$, which is 0.01. Both of these results can answer the hypothesis that the administration of L. reuteri combination and immunized with 37.8 kDa pili adhesion protein V.cholerae conjugated CTB is indeed effective in increasing the productivity of antimicrobial peptide (AMP) specific to the LL-37 strain of Cathelicidin.

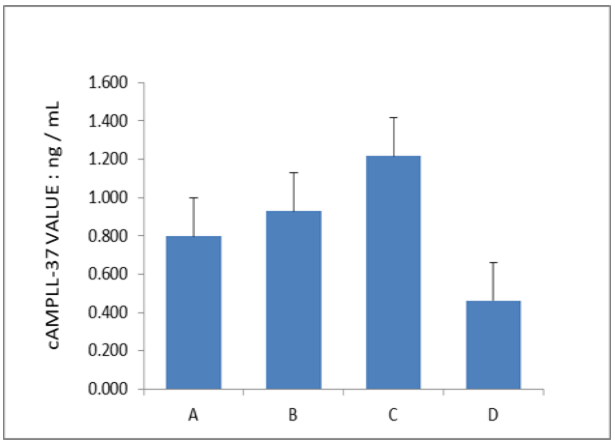

Figure 3. Cathelicidin concentration, LL-37Cluosa Mucosa Control intestine, and treatment, (A) Mice intestinal mucosa induced L. reuteri, (B) Mice intestinal mucosa induced pili adhesion protein 37.80 V. Cholera conjugated CTB, (C)

Mucosa intestine of L. reuteri and pili adhesion protein 37.80 kDa V.cholera conjugated CTB, (D) Control Mouse Mucosa.

Measurement Results of Control and Treatment of the Intestine Mass of the mice treatment group called L. L.uteri. Observation of the process of increasing the activity of bacterial colonization of V, cholerae in mice intestine as indicated by intestinal distension and an increase in mice intestinal mass weight. Based on the weighing data showed that the control group's intestinal rats had a significant increase in mass weight compared to the treated mice group both the addition of L. reuteri or 37.8 kDa of adhesion protein or a combination of both, each of which had a significant value of α50.05, ie 0, 0010, 0.009 and 0.0021 This data is also strengthened from the results of the calculation of the total population of V.cholera bacteria on the plates so that TCBSA is cultured from mice intestinal mucous scrapings.

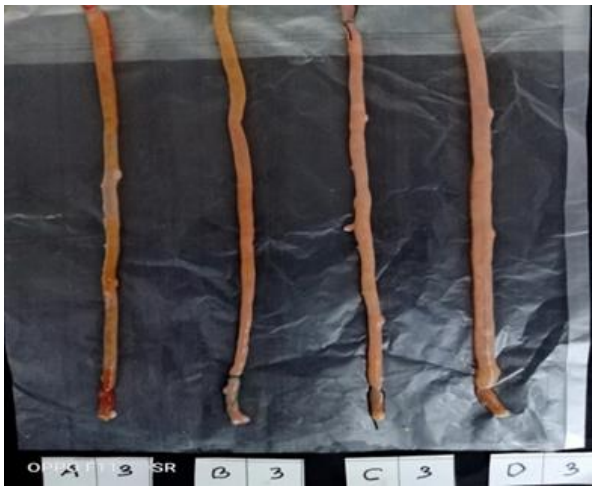

Figure 4. Morphology of V. cholerae Colony Isolated from BALB / C Mice Intestine Pili Adhesin Protein 37.80 kDa V.cholerae Conjugated CTB and L.reuteri Then Infected with V.cholerae Incubation 37 0 C 150 rpm for 3 Hours.

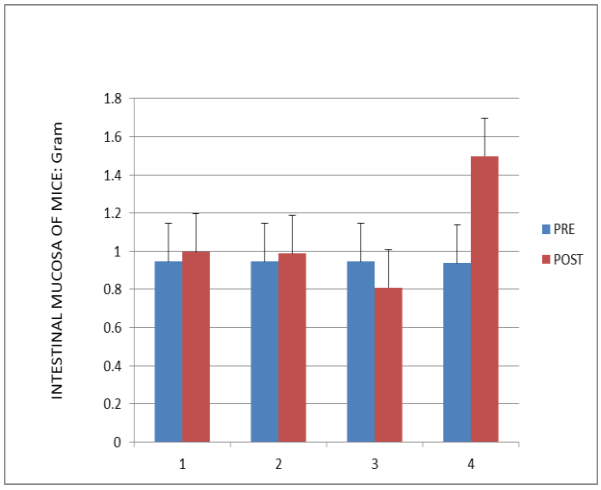

Figure 5. Weighing Results of the Control and Treatment of the Intestine Mass Weight, (A) Mice Mucosa induced L. reuteri, (B) Mucous Mucosa and 37,80 kDa V.cholera conjugate pili adhesion protein, (D) Mucosa of the Intestinal Mouse.

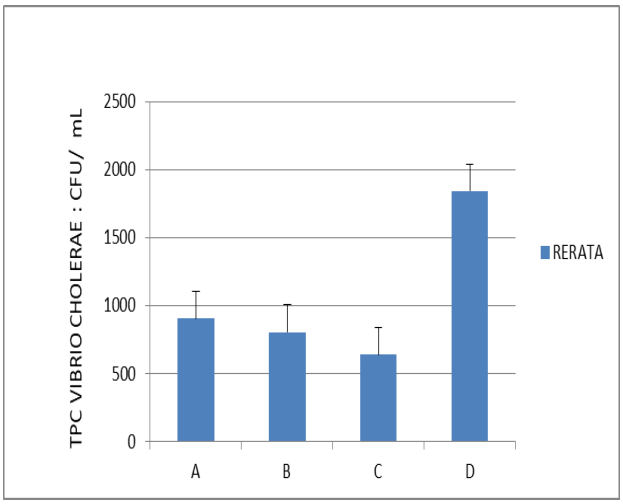

Figure 6. Colony Count Results on TCBSA Medium From Mice Mucosa Fluid Control and Treatment, (A) Mice Mucosa Mucosa was induced by L. reuteri, (B) Mucous Mole Mucosa was induced by adhesion pili protein 37,80 V. conjugate cholera, (C) Mucosa Lactated mice intestine L. reuteri and pili adhesion protein 37.80 kDa V.cholera conjugate, (D) Mucosa of the Control Mouse Intestine.

It has been agreed by scientists that probiotics act as a barrier to the gastrointestinal can also maintain health through mucosal modulation and systemic immunity so that the health of the host remains in optimum condition from pathogen invasion [21]. Studies in vitro and in vivo have shown that certain species and strains of bacteria produce extracellular glycosidases that degrade epithelial glycoproteins and stimulate mucus secretion [22]. Biochemical changes caused by probiotics can inhibit the adhesion of pathogenic bacteria in intestinal cell culture in vitro and also maintain health through mucosal modulation and systemic immunity [23]. In the intestine, probiotic bacteria carry out colonization related to several health effects, including to reduce the danger of certain bacteria, reduce or prevent diarrhea, reduce lactose intolerance, and relieve inflammatory bowel disease (IBD) [21]. Although the mechanisms underlying the benefits of probiotics are not yet fully understood, it has recently shown the ability to regulate immune responses through their effects on dendritic cells (DC) of intestinal mucosa [24]. Intestinal dendritic cells (DC) captures food antigens and bacteria that are in the intestinal lumen then secrete specific cytokines [25, 26]. Cytokines released by dendritic cells (DC) can polarize directly CD4-naïve T cells against different effectors or subsets of regulatory T cells [27]. Probiotics such as Lactobacillus Plantarum 299v, L. reuteri DSM 12246, and L. johnsonii, can induce T cell differentiation and cytokine production [28]. The intestinal immune system has a tolerance mechanism that is controlled by Th1, Th2, or Th17 effector responses to self-antigens and intra-luminal antigens. Lactobacillus reuteri can induce pro-inflammatory effects through Tregs that affect Th17 cells [29]. Research on the type of lactobacillus reuteri through IL-6 and TGF-β which has the effect of maintaining the balance of Th17 has not been much studied [30, 31].

V. cholerae enters a person's body through contaminated food or drink. If the V. cholerae reaches the intestine, it can immediately become infected. The infection mechanism generally consists of 2 stages. In the first stage, the bacteria will make an initial attachment by the pili to the host cell which is anchoring in nature, after that it is followed by sticking through the docking outer membrane. After sticking, V. cholerae will multiply accompanied by the production of bacterial metabolic materials that can harm host cells. In the digestive tract, V. cholerae colonizes and excretes enterotoxins namely; cholera toxin (CT) and coregulated pilus toxin (TCP), as the main virulence factors most responsible for diarrheal disease [32]. To be able to adhere to the surface of the host cell, V. cholerae has a cell adhesion protein molecule that can cause adhesion to the surface of the host epithelial cell. Adhesion protein molecules are molecules on the outer surface of cells that bind to other cells or extracellular matrix. Cell adhesion molecules affect important functions, including the entry of toxins, immune cells into host cells. Antigenic adhesion protein molecules are good antigens to cause specific antibodies, so it is expected to be developed as a vaccine candidate. To prevent cholera outbreak, prevention is done. One way to prevent cholera transmission is to provide adjuvants to the public so they are not easily exposed to cholera. Vaccines are products made from viruses/bacteria (pathogens) that are attenuated or killed that function to enhance one's immunity/immunity against these pathogens. Active immunity to a disease so that it can prevent or reduce the effect of infection by pathogenic organisms. Vaccines can be in the form of weakened viruses or bacteria so that they do not cause disease. Vaccinations have been formally effective for controlling cholera and preventing cholera [33]. Two types of cholera vaccine have been developed, namely a killed oral vaccine (WC / rBS, whole-cell/recombinant B subunit, Dukoral) and a live attenuated oral vaccine (CVD 103-HgR, Alcohol). Both vaccines have not been used as a whole in marginal circles, due to limited costs and licenses. With the availability of low-cost options, the cholera vaccine can be considered for use in endemic countries during an outbreak [34]. To get a low-cost cholera vaccine, various studies have been carried out. Currently, various studies on the use of CTB as a vaccine candidate, bearing in mind the toxin plays a role in modulating the mucosal immune system. Cholera B toxin subunit (CTB) is a component of cholera toxin that is not toxic [35]. These subunits are non-covalently assembled into homopentamer structures, can interact with GM-1 ganglioside receptors on the surface of mammalian cells. Oral administration of CTB can induce a strong antibody response to the mucosal immune system so that it can neutralize the holotoxins released by bacteria. CTB, the most described mucogenic immunogenic to date [36], because these proteins can prepare attractive vaccine platforms for the induction of antibody responses to heterologous antigens. Based on the results of studies, CTB has unique anti-inflammatory activity against immunopathological conditions in allergic and inflammatory diseases [35, 37]. Oral administration of CTB can reduce Crohn's disease in humans [38]. Collectively, CTB is a multifunctional mucosal immunomodulatory protein not only as a cholera vaccine antigen but also as a molecular shell for mucosal vaccines and new immunotherapy [39]. One response is caused by the presence of CTB in the intestine, which stimulates the intestinal surface to produce mucin produced by intestinal epithelial cells (IEC). The mucus acts as a barrier, so pathogens cannot invade and colonize the intestine. Based on the results of the study of Faisal *et al*. (2010) that the adhesion protein molecule V.cholerae 01 pili 37.8 kDa conjugated with CTB was proven to increase the induction of the S-IgA immune response. This is in line with the results of Sumarno et el (2011) study that 37.8 kDa V. cholerae protein adhesion can induce an immune response by increasing s-IgA content and protection against fluid secretion in the small intestine. 37.8 kDa adhesion protein based on the results of these studies has a function as an adhesion molecule. The 37.8 kDa adhesion protein is a monomer of the outer membrane protein of a 76 kDa molecular weight. The molecular weight protein 37.8 kDa at the tip of V. cholerae is identical to 37.8 kDa which is a monomer of the outer membrane of V. cholerae protein. In increasing the mucosal defense, based on the results of recent studies can be improved by the use of probiotics.

Conclusion

Lactobacillus reuteri is a probiotic with immunoenhancing properties via modulation of IgA secretion, beta-defensins, and cathelicidin.

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Conflict of interest: None

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Ethics statement: None

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