

The Probiotic Bacterium Isolated from Bekasam (Traditional Fermented Food), *Lactobacillus Sp.* Induces Activation of Gut Mucosal Immune System in Rat

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Abstract

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BACKGROUND: Bekasam is one of the traditional foods in South Sumatra, Indonesia, a mixture of fermented fish containing Lactic Acid Bacteria (LAB), *Lactobacillus sp.* Non-commensal bacteria and probiotics can induce intestinal mucosal immune responses.

AIM: This pilot study aimed to see the efficacy of *Lactobacillus sp.* to the immune response of the intestinal mucosa by assessing the levels of IgA in the intestinal fluid and markers of T cell populations, such as CD4 and CD8 in the intestinal mucosa.

METHODS: This study was an in vivo experimental study. As many as 30 rats were grouped into 3 treatment groups (doses 107, 108, and 109 CFU/rat/day, for 7 days) and 2 groups of controls (negative control, 10% non-fat milk, and positive control, *Lactobacillus casei* 108 CFU/rat/day for 7 days). At the end of the treatment, the intestinal mucosa was taken to examine the levels of IgA, CD4 and CD8 using the Enzyme-Linked Immunosorbent Assay (ELISA) method, according to the manuals of each ELISA kit. All displays of research data were presented with means ± SD. T-test was used to assess the significance of differences.

RESULTS: Secretion of Ig A increased with the addition of *Lactobacillus sp.* from bekasam. Administration of *Lactobacillus sp.* yielded no effect on helper T cell level (CD4 markers), as well as on cytotoxic T cell levels (CD8 markers).

CONCLUSION: *Lactobacillus sp.* probiotic from bekasam improved the intestinal mucosal immune system by increasing the production of Ig A, but exhibited no effect on T lymphocyte cells.

Introduction

The immune system plays a role in the body's defence against the threat of invasion or infection from various pathogenic microorganisms. The immune system consists of innate and adaptive, both of which will induce a systemic and mucosal immune response. In the intestinal mucosa, the innate immune response acts not only as an initial defence of microorganisms but also induces activation of the adaptive immune response. Non-commensal bacteria and probiotics can induce intestinal mucosal immune responses [1].

Macrophages and dendritic cells play a major role in initiating innate immune responses, which will

specialise into phagocytic cells. Phagocytic cells possess receptor patterns able to recognise pathogenic molecules found on the surface of pathogenic cells. These receptors will be activated by pathogens [2].

One family receptor that operates in the introduction of pathogen molecules is the Toll-like receptor (TLR). TLR plays an important role in the body's defence warning system against the presence of pathogenic material [3]. TLR activates innate immune responses, especially inflammation, before the activation of the adaptive response system [4], [5]. TLR-2 can recognise microbial components such as lipoprotein/lipopeptides from various pathogens and able to detect the presence of peptidoglycan and

lipoteichoic acid from gram-positive bacteria. TLR-2 can recognise lipopolysaccharide (LPS) from enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pylori* [6], [7].

Another family that operates in the introduction of molecular pathogens is the mannequin receptor family. CD-206 is a mannose family receptor that plays a role in the clearance of self-antigens such as endogenous proteins, myeloperoxidase, lysosomal hydrolase and several hormones that contain groups of sulfated carbohydrate [8]. This receptor binds to a group of carbohydrates containing mannosyl/fucosyl residues so that it can recognise proteoglycans from microbial origins [9].

Bekasam is one of the traditional foods in South Sumatra, Indonesia. This food is a mixture of fermented fish. Bekasam contains Lactic Acid Bacteria (LAB), *Lactobacillus sp.* [10]. Several studies show the role of probiotic, especially LAB, as a major component in maintaining health and preventing various diseases. Consumption of probiotics is useful in managing diarrhoea, including antibiotic-induced diarrhoea and diarrhoea in children commonly caused by rotavirus [11], [12], [13]. Innate immune components interplay with intestinal epithelial cells and bacteria [14]. Probiotics had also been proven to benefit in urogenital health [15], improvement of the periodontal condition [16], chronic colitis [17], inflammatory bowel disease [18], [19], and even to prevent or reduce the incidence of oral and respiratory tract infections in paediatrics [20].

Probiotic microorganisms are supplements of living bacteria to maintain the balance of bacteria in the intestine. *Lactobacillus sp.* is a microorganism commonly used as probiotics. Probiotics can stimulate the secretion of immunoglobulin (Ig) A in the intestine. Also, probiotics can increase the secretion of proinflammatory cytokines such as tumour necrosis factor (TNF) alpha and increase regulatory inflammatory cytokines such as interleukin (IL)-4 and IL-10 [21].

This pilot study aimed to see the efficacy of *Lactobacillus sp.* to the immune response of the intestinal mucosa by assessing the levels of IgA in the intestinal fluid and markers of T cell populations, such as CD4 and CD8 in the intestinal mucosa.

Material and Methods

This study was an in vivo experimental study. As many as 30 rats (age 10 weeks, body weight 180-200 gr) *Rattus norvegicus* were used in this study. Each treatment and control group consisted of 6 rats. Each rat was supplied with food and drink in ad

libitum. This study was approved by the research ethics committee of the Faculty of Medicine, Universitas Sriwijaya-RSMH (kpt fkunsri-rsmh no. 113/2018). Furthermore, rats were grouped into 3 treatment groups (doses 10^7 , 10^8 , and 10^9 CFU / rat/day, for 7 days) and 2 groups of controls (negative control, 10% non-fat milk, and positive control, *Lactobacillus casei* 10^8 CFU / rat/day for 7 days). Probiotics were administered by 1 mL of sterile 10% non-fat milk.

Bekasam was composed by mixing 1 kg of fish with 3% salt and 250 grams of rice. Furthermore, the mixture was wrapped with plastic tightly and left for 8 days at room temperature (28-34°C). Bacteria derived from samples were grown into MRSA media. Of these bacterial cultures, colonies that grew dominant with different morphology, both in terms of color, shape as observed from the top, and shape of the protrusion were taken into the media of Man Rogosa Sharpe Agar (MRSA) and morphology the cell was observed. At the end of the treatment, the rat was killed by anesthesia, and the intestine was evacuated. Furthermore, the intestinal mucosa was taken to examine the levels of IgA, CD4 and CD8 using the Enzyme-Linked Immunosorbent Assay (ELISA) method, according to the manuals of each ELISA kit (Cloud-Clone Corp®, Texas, USA).

Statistical analysis was conducted with SPSS for Windows (SPSS Inc., Chicago, Illinois, USA). All displays of research data were presented with means \pm SD. T-test was used to assess the significance of differences between treatment and control groups.

Results

Secretion of Ig A increased with the addition of *Lactobacillus sp.* from bekasam. Efficacy of *Lactobacillus sp.* in increasing levels of Ig A at 10^9 CFU dose was greater than the positive control group with *Lactobacillus casei* at a dose of 10^8 CFU. In the group with *Lactobacillus sp.* at a dose of 10^8 CFU possessed the effect of increased Ig A production almost comparable to the control group with *Lactobacillus casei* at a dose of 10^8 CFU, although statistically, the increase in the positive control group was slightly greater than the treatment group receiving *Lactobacillus sp.* at dose of 10^8 CFU. Table 1 showed that *Lactobacillus* probiotic administration elevated Ig A secretion in the intestinal mucosa.

Table 1: Level of IgA in Intestinal Mucosa

Group	Level (ng/mL)	p-Value
Treatment 10^7 CFU	94.18 \pm 6.21	0.021 [*] , 0.027 [#]
Treatment 10^8 CFU	124.83 \pm 8.55	0.001 [*] , 0.043 [#]
Treatment 10^9 CFU	189.11 \pm 10.17	0.001 [*] , 0.001 [#]
Negative control	91.23 \pm 3.45	0.001 [#]
Positive control	125.23 \pm 9.23	0.001 [*]

*Independent T test VS negative control; # Independent T test VS positive control.

Lactobacillus sp. probiotic possessed no effect on T cells in the intestinal mucosa (Table 2). Administration of *Lactobacillus sp.* yielded no effect on helper T cell level, which was characterised by CD4 markers, as well as no effect on cytotoxic T cell levels, which was marked by CD8 markers.

Table 2: Level of CD4 and CD8 in Intestinal Mucosa

Group	Level CD4 (ng/mL)	Level CD8 (ng/mL)	p-Value CD4	p-Value CD8
Treatment 10 ⁷ CFU	23.18 ± 8.28	22.18 ± 6.28	0.17*, 0.11 [#]	0.37*, 0.31 [#]
Treatment 10 ⁸ CFU	23.63 ± 7.15	24.83 ± 7.15	0.11*, 0.32 [#]	0.21*, 0.31 [#]
Treatment 10 ⁹ CFU	23.91 ± 7.76	23.11 ± 8.16	0.15*, 0.18 [#]	0.11*, 0.21 [#]
Negative control	21.43 ± 8.01	21.23 ± 6.01	0.14 [#]	0.21 [#]
Positive control	22.73 ± 6.29	22.23 ± 5.29	0.21*	0.16*

*Independent T test VS negative control; [#]Independent T test VS positive control.

Discussion

Epithelial surface of the intestine is the area contacting with intestinal commensal microorganisms, which play a role in optimising the immune system. The surface of the gastrointestinal epithelium also always confronts various external microorganisms. This condition causes a significant role of the intestinal mucosa as a defence against invasion from various microorganisms. An effective defence mechanism is in dire need to maintain the homeostasis of the intestinal mucosa. One defence mechanism in the intestinal mucosa is through the production of IgA antibodies. Whereas, protective microflora will protect the intestinal mucosa from the invasion of pathogenic microorganisms. Protective microflora in the intestinal mucosa will stimulate the proliferation of epithelial cells and the development of the mucosal immune system [6], [14].

Intestinal epithelial cells are the first defence against various bacterial invasions and various products of pathogenic bacteria, especially in apical epithelial cells. Whereas at the basolateral side of epithelial cells, immune cells are present. Epithelial cells are capable of introducing components of bacterial structures that are in contact with the intestine so that they will be able to recognise these bacteria as pathogens or non-pathogens [3]. Non-pathogenic probiotic bacteria originating from food can affect the mucosal immune system in the intestine. Contact between these microorganisms and epithelial cells as well as immune cells in Peyer's patches of the intestine will trigger immune cells such as monocytes/macrophages and dendritic cells to initiate an innate immune response and antigenic stimulation. The basic functions of the mucosal immune system include protection from various pathogens, preventing penetration of various foreign antigens, inducing oral tolerance to various antigens and maintaining mucosal homeostasis. The main difference between the mucosal immune system and the adaptive

immune system is in the presence of innate immunity and the activation of B cells rather than T cells [22].

Probiotic species alone do not result in a clinical effect; rather, they facilitate modulation of the gut microbiota composition and metabolic activity, thereby influencing the immune response [23]. Probiotic bacteria interact with the intestinal epithelial cells (IECs) or immune cells associated with the lamina propria, through Toll-like receptors, and induce the production of different cytokines or chemokines. Macrophage chemoattractant protein 1, produced by the IECs, sends signals to other immune cells leading to the activation of the MIS, characterised by an increase in immunoglobulin A+ cells of the intestine, bronchus and mammary glands, and the activation of T cells. Specifically, probiotics activate regulatory T cells that release IL-10. Interestingly, probiotics reinforce the intestinal barrier by an increase of the mucins, the tight junction proteins and the Goblet and Paneth cells. Another proposed mechanism of probiotics is the modulation of intestinal microbiota by maintaining the balance and suppressing the growth of potentially pathogenic bacteria in the gut [24].

This study was the first study to observe the efficacy of *Lactobacillus sp.* from typical fermented foods of South Sumatra, Indonesia (Bekasam) to the optimisation of the mucosal immune system in the intestine. This study showed *Lactobacillus sp.* probiotic elevated Ig A production in the intestinal mucosa but possessed no effect on T lymphocyte cells, which was characterised by no significant effect of *Lactobacillus sp.* probiotic to the expression of CD4 and CD8 in the intestinal mucosa. The administration of *Lactobacillus casei* in this study also exhibited a similar result to the administration of *Lactobacillus sp.* from bekasam. They improved the mucosal immune system by increasing the secretion of IgA but exhibited no effect on T lymphocyte cells.

In conclusion, *Lactobacillus sp.* probiotic from bekasam improved the intestinal mucosal immune system by increasing the production of Ig A. Efficacy of *Lactobacillus sp.* in increasing levels of Ig A was comparable to *Lactobacillus casei*. *Lactobacillus sp.* probiotic possessed no effect on T cells in the intestinal mucosa. Administration of *Lactobacillus sp.* yielded no effect on helper T cell level, which was characterised by CD4 markers, as well as no effect on cytotoxic T cell levels, which was marked by CD8 markers.

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