

Original article

The effect of combination probiotic *Lactobacillus brevis* and *Leuconostoc mesenteroides* on $\text{tgf-}\beta$ and il-12 expression in ileumPujiati^{1,2,*}, Dono Indarto^{3,4}, Susilorini⁵, Diani Retno Widayati⁶, Haneda Ilzafira Damayanti⁶, Reviono^{3,7}, Soetrisno^{3,8}**Abstract**

Background: Asthma is a disease of the respiratory tract in the form of chronic inflammation. Chronic inflammation is one of them characterized by the *remodeling* of the airways mediated by the anti-inflammatory cytokine TGF- β . In addition there are also several immune cells that play a role such as macrophages, dendritic, neutrophils as producers of IL-12. The presence of *the gut-lung axis* allows the spread of inflammatory cytokines from the lungs to the intestines and vice versa. *Lactobacillus brevis* and *Leuconostoc mesenteroides* have the potential to modulate the immune system through its colonization of the gut. The study aimed to look at the effect of probiotics combined *Lactobacillus brevis* and *Leuconostoc mesenteroides* on the expression of TGF- β and IL-12 in the asthma model mouse ileum.

Method: This experimental *post-test only control group design* study used 18 Sprague-Dawley mice. The mice were divided into 3 groups at random: control group (KI), asthma treatment (KII), asthma treatment with a combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* (KIII). Sensitization of asthma with OVA through intraperitoneal injection on days 0 and 14 and through inhalation on days 21 -63. Administration of combination probiotics per oral per day on days 21-63 after inhalation of OVA. The ileum network was taken on the 64th day and measured the expression of TGF- β and IL-12 with immunohistochemical methods and analyzed the percentage proportion of TGF- β and IL-12. Data analysis were conducted by One way ANOVA test and continued *post hoc* tests.

Results: The percentage calculation of the proportion of TGF- β in the control group (KI), asthma group (KII), and the combination group of *L. brevis* and *L. mesenteroides* (KIII) is 22.4 ± 15.27 ; 1.6 ± 2.12 ; 19.4 ± 6.65 . While the percentage calculation of il-12 proportion in each group consecutively were 24.3 ± 19.67 ; 64.63 ± 9.90 ; 51 ± 16.62 . One way ANOVA Test results on the percentage proportion of TGF- β have a p value of 0.013 ($p < 0.05$) which means there is a significant difference in the entire group. Furthermore, the *Post Hoc Games-Howell* test was conducted and obtained a p value of 0.003 ($p < 0.05$) between the asthma group (KII) and the combination group of *L. brevis* and *L. mesenteroides* (KIII) which means there are significant differences between the two groups. One way ANOVA Test results on the percentage proportion of IL-12 have a p value of 0.011 ($p < 0.05$) which means there is a significant difference in the entire group. Furthermore, the *Post Hoc LSD* test was conducted and obtained a p value of 0.001 ($p < 0.05$) between the control group Post Hoc analyses was conducted and obtained a p value of 0.002 ($p < 0.05$) with number of errors was 11.353 between the control group (KI) and asthma group (KII) which means there are significant differences between the two groups.

Conclusion: The probiotic administration of *Lactobacillus brevis* and *Leuconostoc mesenteroides* had no effect on the expression of TGF- β and had no effect on il-12 expression in the asthma model mouse ileum. It is necessary to do research on mice with asthma using combinations with other bacteria in order to get maximum effect.

Keywords: asthma, TGF- β , IL-12, *Lactobacillus brevis*, *Leuconostoc mesenteroides*, *dysbiosis*, *gut-lung axis*

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Asthma is an allergic inflammatory disease of the respiratory tract. Asthmatics have an excessive

immune response to allergens around which are usually harmless. Some of the symptoms include hyperresponsive bronchi, reversible obstruction,

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and excessive mucus production due to immune system stimulation mainly due to inhalant allergens. This disease often affects children and is associated with a family history of atopy. Asthma that is not due to atopic history is more commonly found in adulthood². Asthma is closely related to the involvement of a complex immune system. Some of the dominant immune cells include IgE, Th2 lymphocytes, cytokines, macrophages, eosinophils, and mast cells³. There are currently about 50 different mediators involved in asthma progressivity. IL-12 is a heterodimeric cytokine produced by monocytes activated by *macrophages*, *neutrophils*, and *dendritic* (DC) cells. IL-12 plays a role in encouraging the response of cytokines Th1 and inhibiting cell differentiation Th2. In addition IL-12 plays a direct role in inhibiting a number of effectors that play an important role in allergic diseases, namely the production of IgE and mast cell activation. In chronic asthma, the airway remodeling process is mediated by cytokine TGF- β as a profibrosic cytokine. TGF- β is one of the anti-inflammatory cytokines produced by T-regulator⁶ cells. Currently asthma treatment focuses on inflammatory processes in the respiratory tract whereas according to research by Marsland et al (2015) in chronic lung disease one of which is asthma shows changes in the composition of the intestinal microbiota (*dysbiosis*). This indicates the presence of a *gut-lung axis* thus allowing endotoxins, microbial metabolites and inflammatory cytokines to spread from the lungs to the intestines and vice versa. The microbiota in the gut plays an important role as a regulatory T cell forming. If the amount of good microbiota in the intestine decreases, the airway is susceptible to allergens. *Lactobacillus sp.* *Leuconostoc sp.* is a type of lactic acid bacteria that is often used as a probiotic. Treatment of asthma with probiotics is expected to reduce dysbiosis thereby lowering the risk of asthma and minimizing side effects from corticosteroid treatment.

Research conducted by the *Global Initiative for Asthma* (GINA) estimates that there are about 300 million people worldwide who suffer from asthma and according to estimates by 2025 the number will increase to 400 million people. It is estimated that about 8.4% of the population in the United States has asthma. The average prevalence of asthma is highest in children (9.5%), while in adults (7.7%). The prevalence of this disease is more common in people with blacks. The number of asthma cases is reported to be increasing in the low economic rate population^{of}

¹⁰. Indonesia asthma is not a rare disease, based on the results of Riskesdas in 2018 there are 6 provinces that experience an increase in the prevalence of asthma sufferers, namely the Provinces of YOGYAKARTA, East Java, Banten, South Sulawesi, Bengkulu, and Riau Islands. The proportion of asthma recurrence in a year in Indonesia is also quite high, which is about 57.5%¹¹.

In asthma pathogenesis, immunity plays an important role. Previous research conducted by Abrahamsson (2014), found a link between low diversity of gut microbiota in neonates and the development of asthma in children aged 7 years (8 years). According to Qian's research (2017), proving that a high diversity of gut microbiota can reduce the risk of developing asthma through a balance of T-helper 1 and T-helper 2. The gut microbiota contributes to the formation of T-regulator cells, so if the number is less, the lungs will be more susceptible to allergens. This indicates that there is an axiom between the intestinal and the lungs (*Gut-Lung Axis*). In the presence of the *Gut-Lung Axis* this allows inflammatory cytokines, hormones, and other materials in the lungs to spread to the intestinal or vice versa. This spread through blood vessels and lymph vessels⁸.

Treatment for asthma is growing with the times. Research using probiotics is widely done as there have been previous studies with oral administration of *Lactobacillus brevis* HY7401 in mice induced ovalbumin (OVA) obtained a decrease in levels of IgE and IgE specific OVA. In addition, there is also an increase in the levels of cytokine IFN- γ as an inhibitor of allergic response produced by T-helper cells 1/Th1¹². Research by Bermudez et al (2012) in mice given *Salmonella* showed an interaction between probiotics (*Lactobacillus paracasei*) and APCs primarily through TLR led to a decrease in pro-inflammatory mediators, while anti-inflammatory mediators such as TGF- β had an increase of¹³. Previous studies have proven that *Lactobacillus casei* strains of *Shirota* (LcS) can promote the development of Th1 cells from naïve CD4+ T cells through the secretion of IL-12 by macrophages. IL-12 is thought to play an important role in probiotic function, especially in improving Th1 response and cellular immunity. The ability of *lactobacillus* strains to induce IL-12 can be an index of immunostimulated^{activity}. Previous research has found that the combination of *Leuconostoc mesenteroides* with *Streptococcus thermophilus* can induce maximum IL-12 secretion compared to *lactobacillus sp* strains.¹⁵.

Asthma management currently uses corticosteroids one of them to prevent recurrence. However, if long-term corticosteroid consumption will arise some side effects such as hypertension, increased blood sugar levels, unstable emotions, increased liver enzymes, osteoporosis, fractures and stunted growth for example in^{children} 16. Treatment of asthma with probiotics is expected to reduce dysbiosis thereby lowering the risk of asthma and minimizing side effects from corticosteroid treatment.

But so far there is still little research evidence that shows the benefits and success of probiotics, especially the combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* in improving a person's immunity, especially in asthma. Thus, further research was conducted to find out the effect of probiotics containing a combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* on the expression of TGF- β and IL-12 in the ileum of mice with asthma.

Research Methods

Design and Research Subjects

The study used experimental studies with *post-test only control group* design. The samples in the study used 18 Sprague-Dawley mice. The mice were randomly divided into 3 groups: the control group (KI), asthma treatment (KII), asthma treatment with a combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* (KIII). Inclusion criteria in this study were healthy female Sprague-Dawley mice with bright eyes, healthy coat, active and good appetite; body weight \pm 30 grams; age 6-8 weeks. While the exclusion criteria in this study are mice with congenital abnormalities or physical disabilities.

Ethical clearance obtained from medical research bioethics commission, Faculty of Medicine, Sultan Agung Islamic University, Semarang, number 816/X/2019.

Preparation of *Lactobacillus brevis* dan *Leuconostoc mesenteroides*

L. brevis and *L. mesenteroides* bacteria were obtained from Shrimp Culture Biotechnology Research Center Japan. Both bacteria are plated using MRSA and CaCO₃ 1% media on petri dish and incubated in incubators at 37°C for 48 hours. After the incubation is done the calculation of the number of bacterial colonies are ready to be administrated.

Lactobacillus brevis and *Leuconostoc*

mesenteroides treatment into asthma model mice with ovalbumin

In this study, mice were first adapted for one week in the laboratory. Each group of mice were placed in a cage. The day after the adaptation was completed the mice were randomly divided into three groups, namely the control group (KI) group that did not received any treatment; the asthma group (KII) with OVA exposure, the treatment group (KIII) which immediately after OVA exposure, also received *L. brevis* and *L. mesenteroides* peroral.

To make the asthmatic mice, the mice were desensitized using intraperitoneal OVA solution (i.p) on day 0 and repeated again to day 14. Exposure with OVA aerosol (inhalation using nebulizer) is performed on the 21st to 63rd days of the animal's inhalation of 1% OVA in normal saline (NaCl 0.9%). Aerosol for 30 minutes every 3 times / week for 6 weeks. The treatment to make asthmatic mice model were performed for group I and group III.

Group III in addition to OVA inhalation were also given *L. mesenteroides* and *L. brevis* peroral treatment which is given daily as soon as the OVA exposure process is completed. Twenty-four hours after the mice were in the nebulizer and given probiotics (day 64) the mice were terminated by ketamine 200 μ g/g intraperitoneal injection (Locke et al., 2007). The ileum tissue is used to measure of IL 12 and TGF- β expressions by immunohistochemicals painting.

Isolation of Immunochemical Ileum

The ileum tissue that has been extracted is fixated in a formalin buffer solution for \pm 3 hours. After that rehydration is conducted by soaking in alcohol 70% overnight, then in alcohol 80% for 3 times each 30 minutes, alcohol 90% as much as 3 x each - 30 minutes each and alcohol 96% as much as 2 times each 15 minutes. After that clearing by soaking the ileum tissue in Xylol for 15 minutes. Embending is done by soaking ileum tissue in xylol paraffin mixture overnight at room temperature, in 4 x liquid paraffin, 15 minutes each. The final stage of embending is the creation of an ileum network in the form of blocks, i.e., by inserting the ileum network in liquid paraffin and left until the paraffin freezes so that a hard block is formed and easy to slice with microtomes. paraffin blocks were cut to a thickness of 4-5 microns. Placed on poly-L-lysine slides are then incubated at 37°C for 1 night (to make it more attached to the slides).

Deparaffination is done by soaking in xylol, alcohol,

and washed with aquadest. Then endogenous peroxidylized methanol H₂O₂ 3% were dripped for 20 minutes. Wash with running water for 5 minutes. Wash again with aquadest for 5 minutes followed by washing with PBS for 2 X 5 minutes. Retrieval antigen is done in a microwave oven with Tris EDTA pH 9 at high temperature until boiled then continued at low temperature to 10.a) After cold wash with PBS for 2 X 5 minutes Drip with serum blocking for 10 minutes. Drain, then drip with the antibodies that have been prepared. Incubation at 4°C for 18 hours. Wash with PBS for 2 X 5 minutes Drip with biotin for 15 minutes. Wash with PBS for 2 X 5 drops with streptavidin for 10 minutes. Wash with PBS for 2 X 5 minutes. Administration of Enzin peroxidizing substrate: DAB for 3-5 minutes. Wash with running water for 10 minutes. Drip with hematoxylin for 4 minutes. Wash with running water for 10 minutes. Mounting, cover with deckglass. Reading of preparation under microscope light with magnification of 10x and 40x

Statistical Analysis

The results of the data will be conducted using statistical tests to determine the normality using Shapiro wilk and homogeneity by levene tests in each group. Parametric tests were then conducted for IL-12 and TGF-β groups using One Way ANOVA

due to the homogeneous and normality tests were met. Then a *post hoc* test done to see the differences between groups.

Results

In this study, the expression TGF-β and IL-12 in the ileum were painted in brown in the cytoplasm of the mucosa as in figures 1 and 2. Table 1 shows the percentage proportions in each of the TGF-β and IL-12 groups. The description of the data was determined using the median values for TGF-β and the mean for IL-12 is illustrated in figure 3. Data analysis using One way ANOVA parametric test because the normal data distribution is displayed in table 2. In the *One way ANOVA* test, p for TGF-β and IL-12 was 0.013 and 0.011 (p<0.05, meaning that there was a significant percentage difference in the proportion of TGF-β and IL-12 between all groups. A *Post Hoc* test was conducted to find out which groups had significant differences. In table 3 of the TGF-β analysis showed a significant difference with a p value of 0.003 (p<0.05) between the asthma group (KII) and the combination administration group *L.brevis* and *L.mesenteroides* (KIII). In the IL-12 analysis, there was a significant difference between the control group (KI) and the asthma group (KII) and between the control group (KI) and the combination of *L.brevis* & *L.mesenteroides* (KIII).

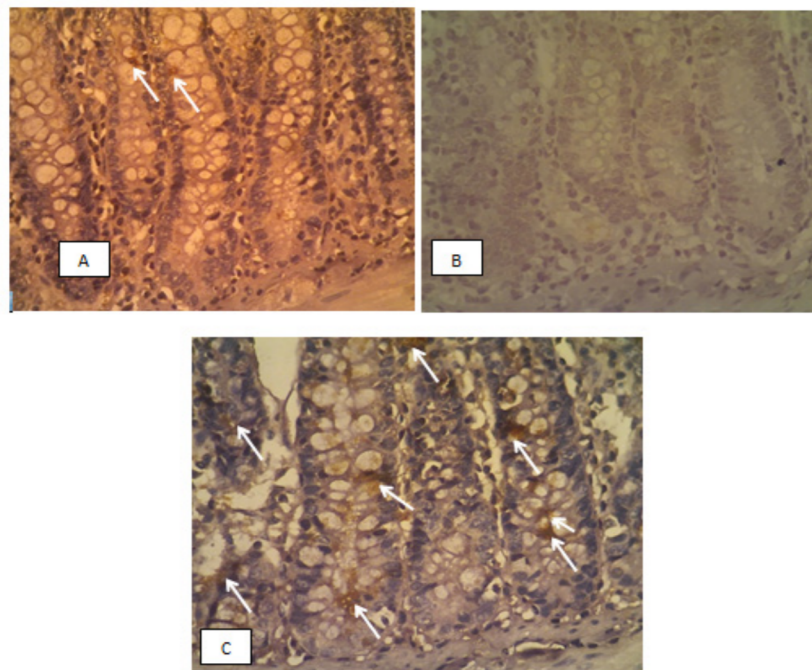


Figure 1 Histology Image of Mice Ileum Mucosa Painted using Immunoohistochemicals with 400x magnification Description: White arrow showing the expression TGF-β A: Group I, B: Group II, C : Group III

Figure 1 shows the brown-painted expression of TGF- β on the cytoplasm of each group's mucosa. In group I there are several expressions of TGF- β with a weak intensity. Group II has no TGF- β expression. While group III there are several expressions of TGF- β with moderate degree of intensity.

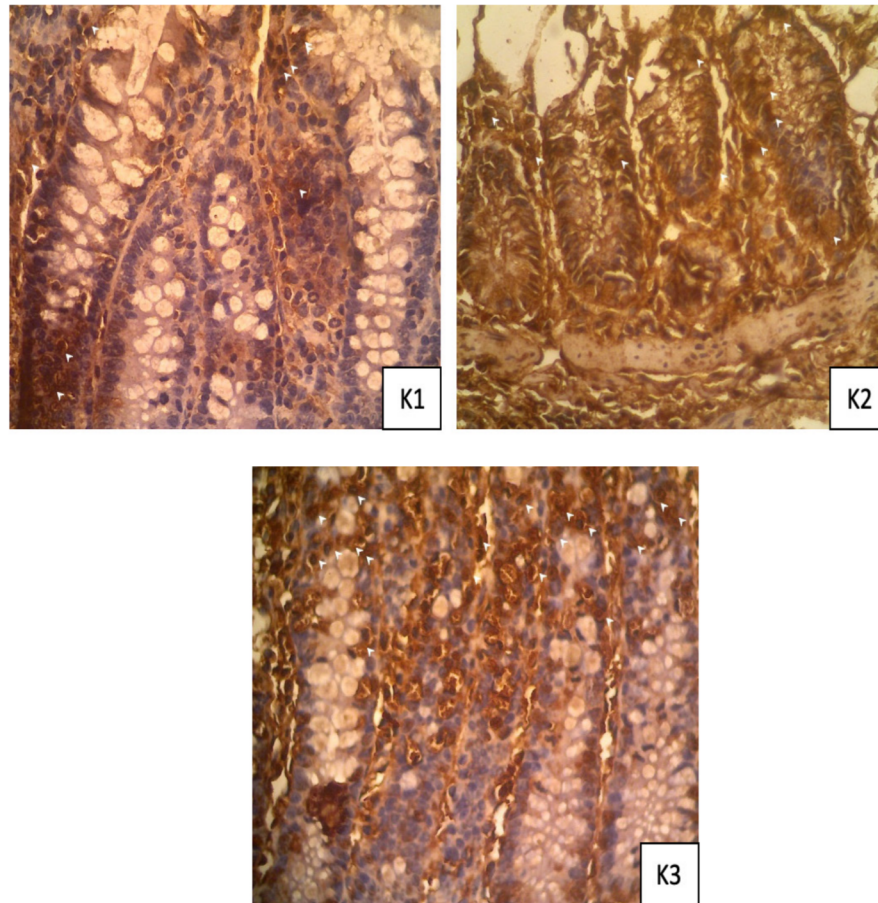


Figure 2 Histology Image of Mice Ileum Mucosa Painted using Immunohistochemicals with 400x Magnification

Description: The white arrows indicate the expression IL-12.

Figure 2 shows the results of the histopathological ileum in each group. In the K1 group with no treatment presenting the expression of IL-12 with weak

degrees, in the K2 group with ovalbumin showed IL-12 strong degree expression, and in the K3 group with ovalbumin treatment and a combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* showed IL-12 moderate degree expression.

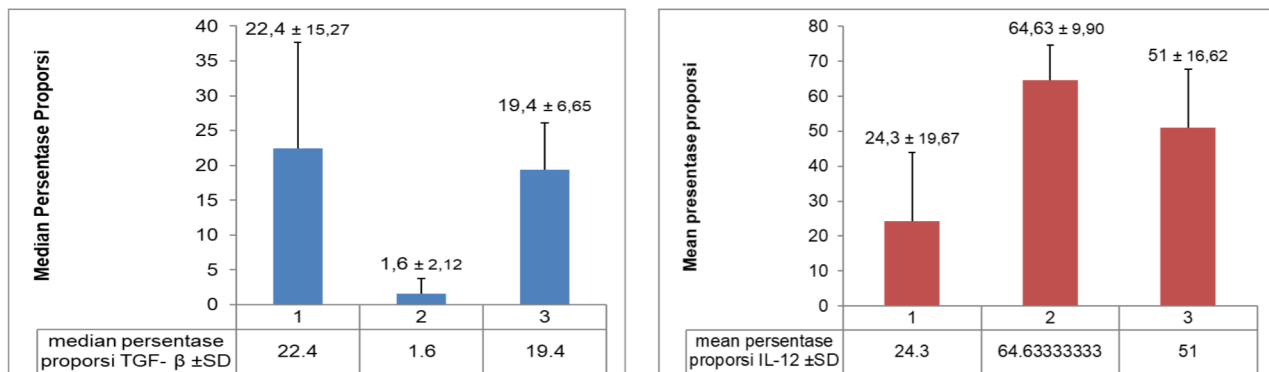


Figure 3 TGF- β and IL-12 Median Percentage Diagram

Table 2 One way ANOVA result

Parameter	Group	Median ±SD	p-value
IL-12	K1	24,3 ± 19,67	One way Anova 0,011
	K2	64,63 ± 9,90	
	K3	51 ± 16,62	
TGF- β	K1	22,4 ± 15,27	One Way Anova 0,013
	K2	1,6 ± 2,12	
	K3	19,4 ± 6,65	

Table 3 Post Hoc test result

TGF- β				IL-12			
Uji Post Hoc	KI	KII	KIII	Uji Post Hoc	KI	KII	KIII
KI	-	0,136	0,887	KI	-	0,001*	0,027*
KII	0,136	-	0,003*	KII	0,001*	-	0,241
KIII	0,887	0,003*	-	KIII	0,027*	0,241	-

Description: *Mean difference between two groups are significant

One way Anova Test results on the percentage proportion of IL-12 have a F value 4.105 between groups and p value of 0.011 (p<0.05) which means there is a significant difference in the entire group. Post Hoc LSD analyses was conducted and obtained a p value of 0.002 (p<0.05) with number of errors was 11.353 between the control group (KI) and asthma group (KII) which means there are significant differences between the two groups.

One way Anova Test results on the percentage proportion of TGF-β have a F value 6.425 between groups and p value of 0.013 (p<0.05) which means there is a significant difference in the entire group. Post Hoc analyses using Games-Howell was conducted and obtained a p value of 0.003 (p<0.05) with number of errors was 7.45 between the asthma group (KII) and the combination group of *L.brevis* and *L.mesenteroides* (KIII) which means there are significant differences between the two groups.

Discussion

TGF-β Analysis Results

This study used 3 groups of mice with different treatments. One way ANOVA test results percentage

proportion of TGF-β obtained p value of 0.013 (p<0.05) which means there is a significant difference in the entire group. The percentage proportion of the control group was 22.4 ± 15.27. Normally the surface of the intestinal mucosa has a very high immune tolerance because it is always exposed to components that can damage the intestinal barrier so that to maintain its tolerance the intestinal mucosa provides susceptibility to invasion of pathogens and infections by creating an anti-inflammatory environment.¹⁷.

The asthma group (KII) had the lowest number of positive cells. This is in line with previous research that in mice models of asthma with ovalbumin induction there is an imbalance of the immune system due to failure of tolerance characterized by a decrease in the number of T-regulator cells. Therefore, TGF- β as one of the anti-inflammatory cytokines produced by T-regulator cells will decrease as well.¹⁸.

The Games-Howell analysis between the asthma group (KII) and the combination of *L.brevis* and *L. mesenteroides* (KIII) had a p value of 0.003 (p<0.05) which meant there were significant differences between the two groups. In accordance with previous research that states that probiotics such as *Lactobacillus sp.* can modulate the immune system directly by increasing macrophage activity, increasing barriers in the intestinal epithelium and modulating inflammatory cytokines. Its role as an immunomodulator is known through its colonization process mediated by TLR(*Toll-Like Receptor*) which then increases the activation of the *Caspase 8* gene and induces the secretion of TGF-β 13..

Probiotic combinations often used as a treatment, such as the combination of *Lactobacillus sp.* with *Bifidobacterium sp.* in previous studies using mice with IBD showed that the combination of *L. acidophilus* with *B. lactis* led to a significant reduction in pro-inflammatory cytokines in the digestive tract compared to individual administration.²⁰.

As in previous research *Leuconostoc sp.* is known to induce the immune system by producing exopolysaccharides (EPS) which then increase the secretion of IgA as a mucosal immune defense system. In addition, anti-inflammatory cytokines such as TGF-β also experienced an increase brokered by T dependent cells²¹. Other studies by Zubaidah showed that the combination of *Leuconostoc mesenteroides* with sauerkraut modulated the immune system higher than the administration of *Leuconostoc* individual²². Previous studies of the combination of

Leuconostoc mesenteroides with *Bacillus subtilis* provided a more maximal growth-inhibiting effect of *V. cholerae* compared to individual administration.²³.

IL-12 Analysis Results

In the control group (K1) obtained the lowest mean value between groups, which is $24.37 \pm 19,671$. In the group with asama treatment (K2) had the highest mean value of $64.60 \pm 9,905$. In the asthma treatment group with a combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* (K3) bacteria showed a higher mean than the control group of $51.00 \pm 16,620$.

In the Anova test with Post Hoc, significant results were obtained so that there were mean difference between the two significant groups between the Control group (K1) and the Asthma group (K2). While in the group with a combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* (K3) did not indicate significant results so there are no significant difference between the K1 group and other groups.

IL-12 expression increased in K2 group on mice given OVA treatment. This is in line with research conducted by Meyts et al., 2006 which mentioned that mice desensitized with OVA showed increased levels of IL-12 significantly. IL-12 appears as an early pro-inflammatory cytokine in the immune response to pathogen²⁴. IL-12 is a pro-inflammatory cytokine that plays an important role in the regulation and activation of Th1 and Th2 cells. IL-12 acts in stimulating the Th1 response and inhibiting the development of IL-4-producing Th2. Through its ability to induce Th1 allows the cytokine to be able to decrease the pathological response of increased Th2 cells in asthma.²⁵.

In the K3 group with probiotic combinations, there were increase in the control group but not as high as the asthma group. Probiotics are associated with their ability to reduce allergic reactions through regulation of cell response Th2. Most of probiotics have anti-inflammatory and immunomodulatory properties through inhibition of various signaling pathways such as the NF-kB pathway. Probiotics also have the potential to inhibit the attachment of lipopolysaccharides to CD14 receptors, thereby reducing the activation and production of pro-inflammatory cytokines.²⁶.

Lactobacillus brevis may increase cytokine Th1 and lower cytokine Th2. Furthermore, there was the highest increase in TLR-2 expression in the group

with *Lactobacillus brevis* administration. Activation of TLR-2 is said to play a role in inhibition of Th2 cell response and IgE production.²⁷. Some studies have proven that TLR-2 is needed by some strains of *Lactobacillus* to activate the immunomodulatory effects of these bacteria. It has also been proven that whole peptidoglycan in *Lactobacillus* can inhibit the production of Interleukin 12 through signaling by TLR-2. *Lactobacillus* has a cell wall-building component called *Lipotechoic Acid* (LTA) which is able to improve the integrity of the intestinal epithelial barrier by activating the protein kinase C in the TLR2 signaling pathway.²⁸.

Exopolysaccharides (EPS) produced by lactic acid bacteria are safe immunomodifiers to induce mucosal immune responses. EPS produced by *Leuconostoc mesenteroides* bacteria can induce antigen-specific antibody response when administered intranasally in mice. EPS stimulation leads to a strong increase in the production of the pro-inflammatory cytokine IL-12. *Leuconostoc mesenteroides* increase the expression of IL-6, IL-10 and IL-12²⁹. In other studies mentioned that *Leuconostoc mesenteroides* is the most powerful inducing cytokine IL-12.³⁰.

The combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* showed insignificant results, this is possible due to the different effects of these two bacteria, *Lactobacillus brevis* works by inhibiting the production of IL-12 while in *Leuconostoc mesenteroides* is a strong induced cytokine IL-12.

Limitations in this study cause high standard deviation values, including researchers can not observe the success of probiotic colonization, no examination of clinical signs after probiotic administration, limited study time so that probiotic administration was conducted for short period of time.

Conclusions And Suggestions

The result of this study is probiotics combination of *Lactobacillus brevis* and *Leuconostoc mesenteroides* affect the expression of TGF- β in the ileum of asthmatic mice. The percentage proportion of TGF- β expression in the control group (KI) were 22.4 ± 15.27 . The percentage proportion of expression of TGF- β in the asthma group (KII) were 1.6 ± 2.12 . The percentage proportion of TGF- β expression in the combination of *L.brevis* and *L.mesenteroides* (KIII) group were 19.4 ± 6.65 . While the administration of probiotics combined *Lactobacillus brevis* and

Leuconostoc mesenteroides had no effect on the expression of IL-12 in the ileum of asthmatic mice. The percentage proportion of IL-12 expression in the control group (KI) were 24.3 ± 19.67 . The percentage proportion of IL-12 expression in the asthma group (KII) were 64.63 ± 9.90 . The percentage proportion expression of IL-12 in the combination of *L.brevis* and *L.mesenteroides* group (KIII) were 51 ± 16.62 . It is necessary to do research on mice with asthma using combinations with other bacteria to get more maximum effect.

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The authors declared that there is no conflicts of interest.

Contribution of Authors:

Data gathering and idea owner of this study and study design are written by Pujiati, Dono Indarto, Susilorini, Reviono, and Soetrisno. Pujiati, Haneda Ilzafira, Diani Retno are participated in writing and submission of manuscript. All of the authors participated in editing and approval of the final draft.

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