

In Vivo Studies of Combined Probiotics on IFN- γ , Ig-E and Bronchial Muscular Layer of Rats with Allergic Asthma

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ABSTRACT

Background: *Leuconostoc mesenteroides* and *Lactobacillus brevis* are being used as probiotic to improve the immune system. The effect of probiotic containing combination of the 2 bacteria on enhancing innate and adaptive immune responses is not fully understood. **Objective:** This was conducted to analyse the effect of probiotic containing in single or combination content of *L. mesenteroides* (Lm) and *L. brevis* (Lb) on IFN- γ concentration, Ig-E concentration, and histopathology of bronchial muscular layer of ovalbumin-induced allergic asthma. **Materials and Methods:** A total of 40 male Sprague Dawley rats (6–8 weeks, 200-300 g) were randomly divided into 5 groups: NC (non-induced control group); NgC (OVA-induced control group), Lm (OVA+probiotics containing *L. mesenteroides*), Lb (OVA+probiotics containing *L. brevis*), and Lm + Lb (OVA+probiotics containing combination of *L. mesenteroides* and *L. brevis*). On 64-day, concentration of IFN- γ and Ig-E in serum were measured. Histology of bronchus was performed. **Results:** IFN- γ concentration, Ig-E The administration of single or combined probiotics increased IFN- γ ($p < 0.001$), yet decreased IgE ($p < 0.001$) of all treatment groups, but only a combination of both probiotics reduced the thickness of the bronchial epithelium. **Conclusion:** The combination of Lm and Lb single or combined probiotics improve systemic and local anti-inflammation effects and ameliorate airway remodelling in the ovalbumin-induced chronic asthma rat model.

Key words: Asthma, *Leuconostoc mesenteroides*, *Lactobacillus brevis*, Ig-E, IFN- γ , Bronchus histopathology.

INTRODUCTION

Probiotics is one of food supplements containing microorganisms like bacteria and fungi which can alter the host microbiota.¹ Probiotic enhance innate and adaptive immune responses through binding between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) on cells. Type of bacteria in the probiotics influence its beneficial effects in improving immune responses, especially the levels of produced cytokines.²

Probiotics containing *Lactobacillus* and *Bifidobacterium* is reported as effective treatment for asthma allergy. Consumption of probiotics containing *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Streptococcus*, *Dextrose* was proven to reduce IL4 and IgE levels in children with allergic asthma. However, the mechanism of action of probiotics in increasing levels of IFN- γ , Ig-E and bronchial muscular layer is not known with certainty.³ Administration of probiotic containing *Bifidobacterium* increased the level of IL-10 and Foxp3 expression in lung tissue of ovalbumin (OVA)-induced asthma allergic mice.⁴

Leuconostoc strains is reported to be more potent than *Lactobacillus* strains in inducing the production of IL-12 and IFN- γ . *Leuconostoc mesenteroides* as probiotics increased the amount of IFN- γ which was mediated by the production of IL-12 through NF- κ B, P38, and JNK.⁵ The bacteria maintain Th1/Th2 balance and induce TH1 cytokines. Probiotic containing *Lactobacillus brevis* HY7401 has proven to cause the increasing of Th1 cytokine, reducing Th2 dan IgE.⁶ Therefore,

probiotic containing *Leuconostoc mesenteroides* and *Lactobacillus brevis* is expected can be an effective alternative therapy for asthma allergy. This research has been conducted to analyze the effect of *Leuconostoc mesenteroides* and *Lactobacillus brevis* on the level of IFN- γ , Ig-E and bronchial muscular layer in OVA-induced asthma allergy.

MATERIAL AND METHODS

Animals

A total of 40 male Sprague Dawley rats (6–8 weeks, 200-300 g) were initially acclimatized to standard animal laboratory conditions for one week prior to experiments. During the study, all animals were kept in standard cages with free access to food and water. The experiments were strictly conducted in accordance with the protocols approved by the Ethics Committee of Faculty of Medicine, Sultan Agung Islamic University (No: 418/XII/2020/Komisibioetik).

Experimental Protocol and Design

Rats were randomly divided into 5 groups (n=8/group). NC= normal control, NgC= negative control, Lm (chronic asthma rats + *L. mesenteroides*), Group Lb (chronic asthma rats + *L. brevis*), Group Lm+Lb (chronic asthma rats + combination *L. mesenteroides* + *L. brevis*).

For creating asthma models, rats were sensitized on days 0 and 14 by the IP injection of 10 μ g OVA (Sigma Ovalbumin, A5503-1G) emulsified in 1 mg aluminum hydroxide in NaCl 0.9 %. The level of eosinophils and neutrophils were measured to ensure the OVA-induced asthma allergy. The rats were challenged with 1% OVA aerosol (w/v) in NaCl 0.9

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% for 30 min, 3 times/week on days 21 to days 63. Exposure to the aerosolized solution was done by aerosol nebulizer (CompMisk model 40-105-000, USA) in a closed chamber (27×20×9 cm). Treatments with probiotics were done once a day from the day 21 to 63 at via oral gavage.

IFN- γ , Ig-E and bronchial muscular layer parameters

Serum IFN- γ , Ig-E concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using reagent kits of Elabscience Rat IFN- γ (Interferon Gamma) and Elabscience Rat IgE.

To analyse the histology of bronchial muscular layer, on day-64 the rats were sacrificed under ip anesthesia with ketamine 200 μ g/g, then the bronchus were removed rapidly and fixed in 4% paraformaldehyde for \geq 24 h at room temperature. Then, bronchus tissues were dehydrated in a graded series of ethanol solutions, cleared in xylene, and embedded in paraffin. Histology slices (thickness = 5 μ m) were prepared using a microtome. These slices were stained with hematoxylin and eosin (H&E) and Periodic Acid Schiff (PAS) for measurement of the number of goblet cells and the thickness of the epithelium and smooth muscle cells using a microscope (CX21; Olympus) equipped with a digital camera.

Statistical analysis

ANOVA (Analysis of variance) test was used to assess the differences in the concentration of IFN γ and IgE, the thickness of the epithelium and smooth muscle cells and the number of goblet cells in OVA-induced asthmatic rats. Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 19.0 (SPSS Inc., IL, USA). A probability of P < 0.05 was considered as significantly different.

RESULTS

Ovalbumin sensitization

Ovalbumin is made of chicken's egg white. Ovalbumin is phosphoglycoprotein monomer with the molecular weight of 43-45 Kd. Ovalbumin has proven resulting in asthma to the experimented

animals. The increasing number of eosinophils and neutrophils on Day-14 revealed the success of OVA induction in making asthmatic rat models. The number of eosinophils and neutrophils in the blood by OVA induction was higher, and it showed an insignificant difference (p<0.001) in group NgC, Lm, Lb and Lm+Lb than in the NC group, which was not induced with OVA. The increased number of eosinophils and neutrophils after 14 days of OVA induction indicated the presence of asthma (Figure 1).

The molecular effects of probiotic therapy on IFN- γ and Ig-E levels in chronic asthma model rats

The IFN- γ and Ig-E have been evaluated in this study resulted in a significantly increased (p<0.001) of IFN- γ level and significantly lowered (p<0.001) of Ig-E level in the groups after 24 hours administrated with probiotics *L. mesenteroides*, *L. brevis* and a combination of both. However, both IFN- γ (109.4 \pm 8.4) and Ig-E (57.5 \pm 4.8) levels in the Lb group showed an identical value and insignificantly different results (p>0.05) than the normal control group (113.3 \pm 11.3 pg/mL; 61.5 \pm 5.3 pg/mL), respectively. The immunological result of all groups is shown in (Table 1).

The statistical testing results show that there were level differences of IgE and IFN- γ pre and post treatments of probiotics Lm, Lb and Lm+Lb (Figure 2 and 3). The levels of IgE in groups with probiotics treatments (Lm, Lb and Lm+Lb) were lower and significantly different from the average levels of IgE belonging to groups containing rats induced with OVA and without probiotics treatments (PC) (p<0.001). Based on the result, it was revealed that the administration of probiotics *L. mesenteroides*, *L. brevis* and the combination of probiotics *L. mesenteroides* and *L. brevis* (Lm, Lb and Lm+Lb) decreased the IgE of rat models with chronic asthma.

Conversely, the levels of IFN- γ belonging to groups treated with probiotics (Lm, Lb and Lm+Lb) were higher and significantly different from the average levels of IFN- γ belonging to groups of rats induced with OVA and without treatments of probiotics (PC) (p<0.001). Based on the results, it was revealed that the administration of probiotics *L. mesenteroides*, *L. brevis* and combination of probiotics *L. mesenteroides*

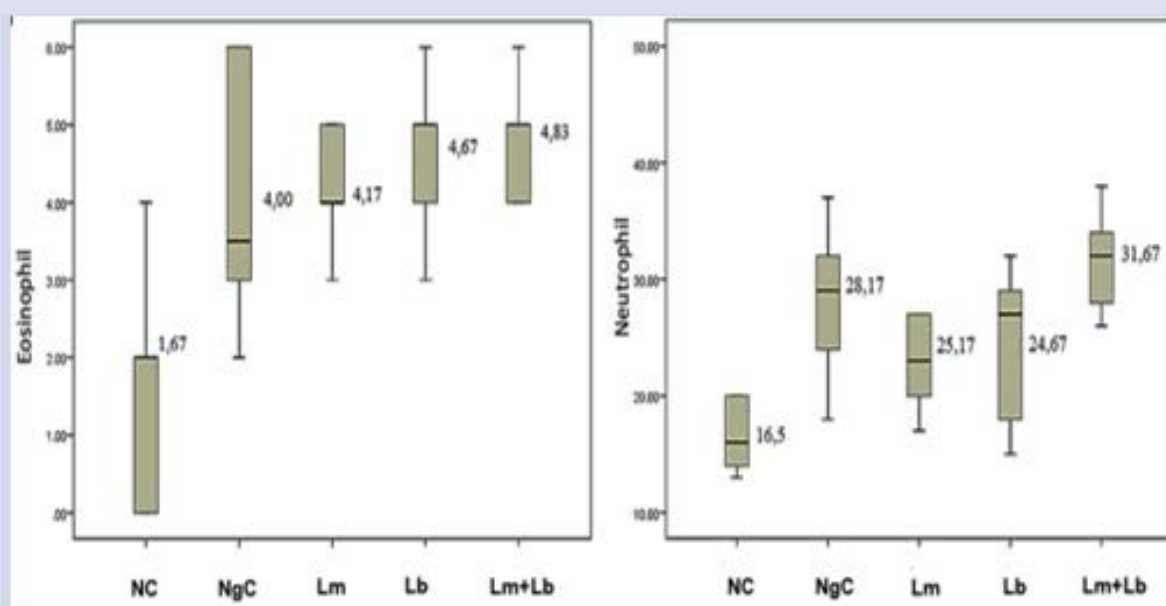


Figure 1: Mean value of eosinophils and neutrophils levels post-induction of ovalbumin.

NC= normal control, NgC= negative control, Lm (chronic asthma rats + *L. mesenteroides*), Group Lb (chronic asthma rats + *L. brevis*), Group Lm+Lb (chronic asthma rats + combination *L. mesenteroides* + *L. brevis*).

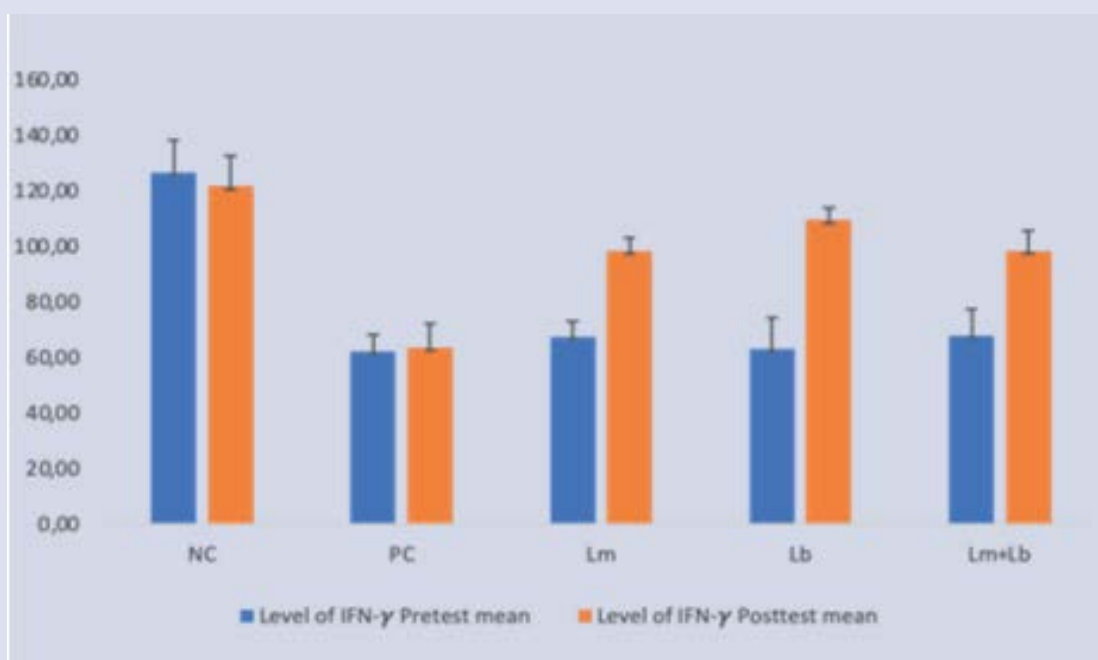


Figure 2: Mean levels of IFN- γ .

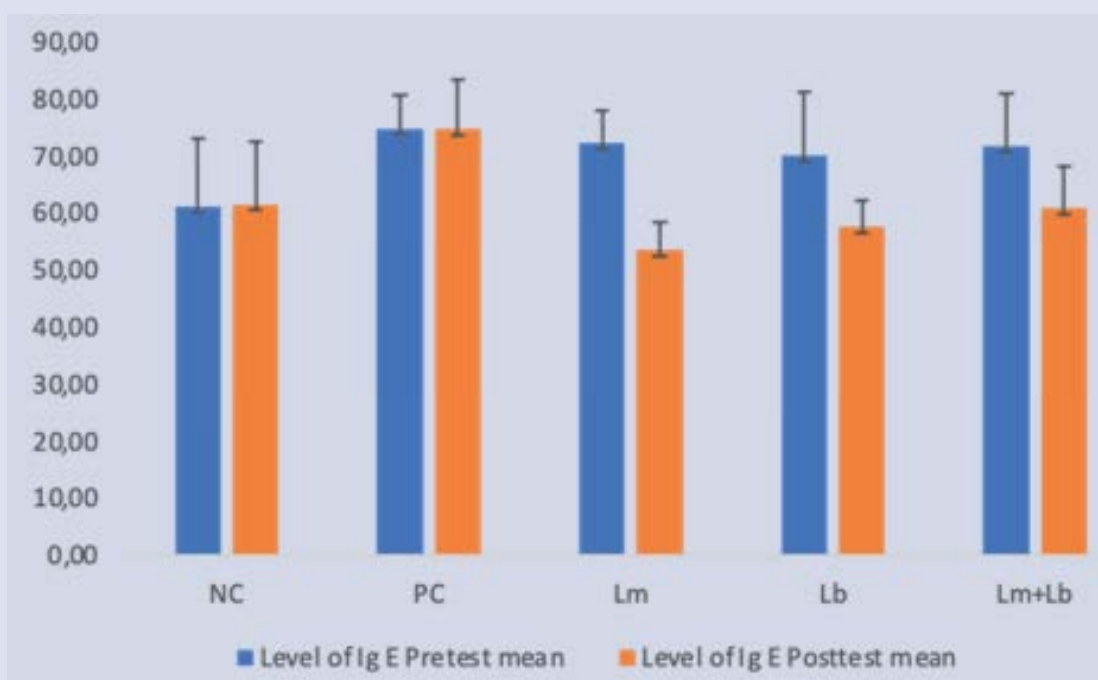


Figure 3: Mean levels of Ig-E.

Table 1: The immunological effects of probiotic therapy on IFN- γ and Ig-E levels in the ovalbumin-induced chronic asthma rats.

Immunological Indicator	Groups				
	NC	NgC	Lm	Lb	Lm+Lb
IFN- γ	113.3 \pm 11.3	63.5 \pm 18.7	98.3 \pm 9.0*	109.4 \pm 8.4*.#	98.5 \pm 8.4*
Ig-E	61.5 \pm 5.3	74.4 \pm 4.1	53.6 \pm 8.3*	57.5 \pm 4.8*.#	60.9 \pm 4.8*.#

Values represented are Mean \pm S.E.M (n=6), p<0.001 (*) compared to the non-induced control group; p>0.05 (#) compare to the non-induced control group. NC= non-induced control group, NgC = PV-induced control group, Lm (chronic asthma rats + *L. mesenteroides*), Group Lb (chronic asthma rats + *L. brevis*), Group Lm+Lb (chronic asthma rats + combination *L. mesenteroides* + *L. brevis*).

and *L. brevis* (Lm, Lb and Lm+Lb) increased the IFN- γ of rat models with chronic asthma. There were no significant level differences of IFN- γ and IgE in the treatment groups (Lm, Lb and Lm+Lb) with $p > 0.05$ except in between PC and Lm+Lb related to their IgE levels. The results show that the dosage of both single and combined probiotics had the same influence on IFN- γ and IgE.

The bronchial histopathological findings

The bronchial histopathology was assessed in this study, including the thickness of the epithelium and smooth muscle cells and the number of goblet cells. The ovalbumin-induced chronic asthma rats administration significantly increased the thickness of smooth muscle cells ($p < 0.001$). On the other hand, the probiotic-treated groups (Lm, Lb, Lm+Lb) showed a decreased mean value in thickness of smooth muscle cells, but it was not substantially different ($p > 0.05$) from the negative control group. The thickness of smooth muscle in all groups can be seen in (Figure 4). Different statistical results were shown in the bronchial epithelium after ovalbumin induction which had thickened but not significant ($p = 1,000$) when compared to the normal control

group ($42.1 \pm 6.1 \mu\text{m}$ vs $41.9 \pm 14.9 \mu\text{m}$). Although these findings suggested that ovalbumin administration enhances bronchiolar epithelial thickness, the increase in thickness is insignificant. Probiotic administration for 24-hours in ovalbumin-induced chronic asthma rats could significantly inhibit bronchial epithelial thickening ($p < 0.001$), compared to the negative control group ($42.1 \pm 6.1 \mu\text{m}$). The mean value is shown as follows: $24.5 \pm 4.0 \mu\text{m}$; $24.4 \pm 4.0 \mu\text{m}$; $24.7 \pm 4.1 \mu\text{m}$ respectively in the Lm, Lb and Lm+Lb group. The mean number of bronchial goblet cells in the ovalbumin-induced chronic asthma (NgC) was higher than in the normal control group ($20.9 \pm 5.8 \mu\text{m}$ vs $8.4 \pm 2.7 \mu\text{m}$). In addition, statistical results showed a significant difference ($p = 0.020$) between the NgC and NC groups, suggesting that administering ovalbumin to the bronchioles can significantly enhance the number of goblet cells. Among the probiotic administrated group, only the *L. brevis* (Lb) group had a substantial decrease ($p = 0.022$) in the number of goblet cells. At the same time, the *L. mesenteriodes* and also the combination *L. mesenteriodes* and *L. brevis* had no significant difference ($p > 0.05$) compared to the negative control group (NC). The histological score of bronchial damage can be seen in (Table 2).

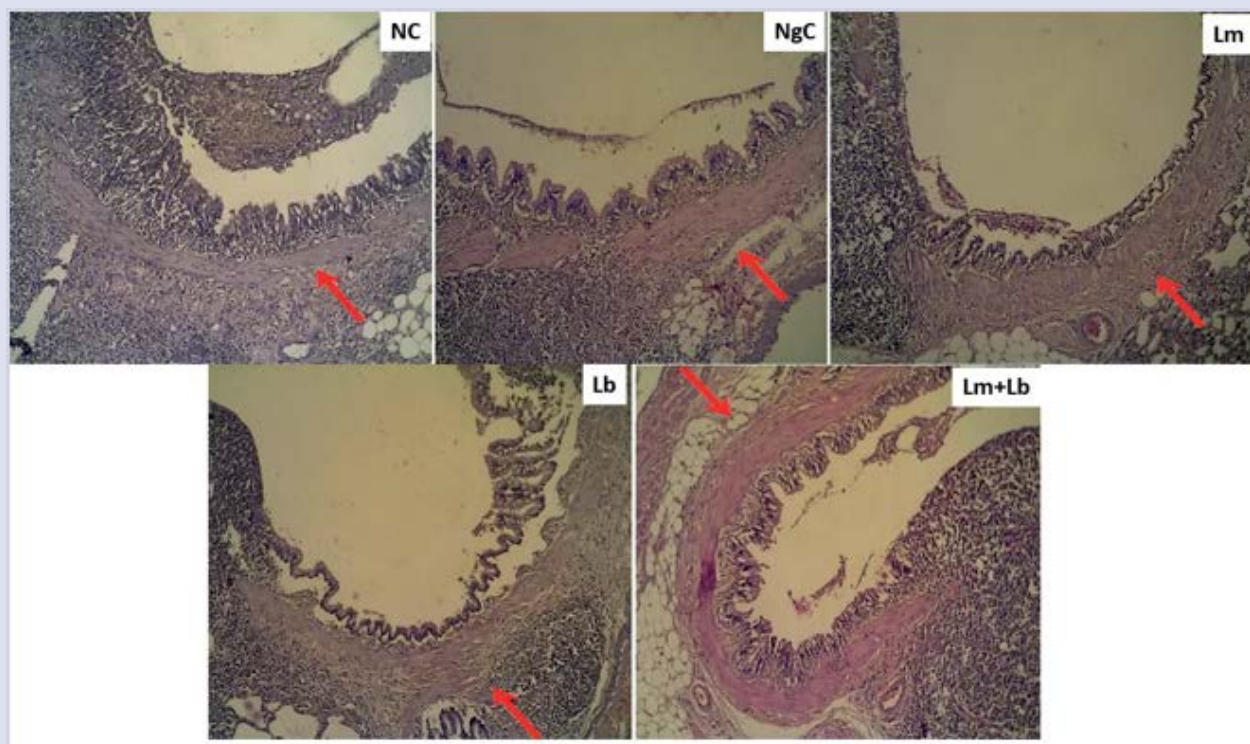


Figure 4: The thickness of the bronchial smooth muscle cells. The arrows showed the thickening of airway smooth muscle cells in all groups. NC= normal control, NgC= negative control, Lm (chronic asthma rats + *L. mesenteriodes*), Lb (chronic asthma rats + *L. brevis*), Lm+Lb (chronic asthma rats + combination *L. mesenteriodes* + *L. brevis*). Hematoxylin-eosin (HE) staining; 100x magnification- Image Optilab Pro 6.1 software.

Table 2: The histological score of bronchial deformities in the ovalbumin-induced chronic asthma rats.

Histological cell	Groups				
	NC	NgC	Lm	Lb	Lm+Lb
SMC	94.4 ± 16.3*	176.1 ± 29.9	141.8 ± 25.8 [#]	153.8 ± 27.7 [#]	144.7 ± 27.4 [#]
BE	41.9 ± 14.9 [#]	42.1 ± 6.1	24.5 ± 4.0*	24.4 ± 4.0*	24.7 ± 4.1*
GC	8.4 ± 2.7*	20.9 ± 5.8	14.8 ± 2.8 [#]	8.7 ± 3.4*	12.1 ± 6.6 [#]

Values represented are Mean ± S.E.M (n=6), $p < 0.001$ (*), $p > 0.05$ (#) compared to the negative control group. SMC= the thickness of smooth muscle cells. BE= the thickness of the bronchial epithelium, GC= the number of goblet cells, NC= normal control, NgC= negative control, Lm (chronic asthma rats + *L. mesenteriodes*), Group Lb (chronic asthma rats + *L. brevis*), Group Lm+Lb (chronic asthma rats + combination *L. mesenteriodes* + *L. Brevis*).

DISCUSSION/ CONCLUSION

This study was aiming to explore the beneficial effects of probiotics containing *L. mesenteroides* and *L. brevis* on the regulation of host immune response through analysis of IgE, IFN- γ and histology of bronchus. Interferon γ (IFN- γ) is the cytokine produced by Th-1 cells and responsible for the immunity against intracellular pathogens,⁷ and recently known that the effector cells responsible for asthma inflammation also cover Treg producing IL-10 and Th-17 producing IL-17.^{8,9} Treg cells regulate Th2, Th17, APC, B-cells and inflammation cells. Treg prevents inflammation development, IgE discharge, mucosal hypersecretion, and airway hyperresponsiveness (AHR).¹⁰ However, allergic inflammation is also characterized by increasing IgE, which activates the mucosal mast cells.¹¹

This study successfully demonstrated that IgE levels decreased with $p < 0.001$, whilst the number of IFN- γ significantly increased, showed that administration of probiotics *L. mesenteroides*, *L. brevis* and combination of *L. mesenteroides* and *L. brevis*, in ovalbumin-induced chronic asthma rats balanced the ratio of Th1/Th2 and Th17. The previous research reported that the administration of probiotics *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Streptococcus* was proven decreasing the levels of IL-4 and IgE in children with allergic asthma,¹² yet the effect on IL-17, IL-10, and airway remodelling was not defined yet. According to this study, the treatment of probiotics in the form of single species or a combination of *L. mesenteroides* and *L. brevis* reduces the thickness of the bronchial epithelium in ovalbumin-induced chronic asthmatic rats. On the other hand, probiotics did not affect the thickness of the smooth muscle or the number of goblet cells.

Some combinations of probiotics, such as *Bifidobacterium longum* and *S. thermophilus*, were known to suppress the production of IL-17, while the combination of *S. thermophilus* and *Leuconostoc* strains more strongly triggered the production of cytokine Th1 consisting of IL-12 and IFN- γ when compared with the probiotics *Lactobacillus* strains which were clinically used recently.¹³ The other study demonstrated that the administration of *L. brevis* HY7401 increased the level of Th1 cytokine, decreased Th2 cytokine and IgE production.¹⁴ This is why *Leuconostoc mesenteroides* (*L. mesenteroides*) and *Lactobacillus brevis* (*L. brevis*) were chosen for this study.

Leuconostoc mesenteroides result from bacteria fermentation, which produces bacteriocin that contains organic acids so that the bacteria can exist in a wide pH range. The bacteria have exopolysaccharide (EPS) on its cell walls to modulate the systemic and local immune responses binding the lipopolysaccharide (LPS) Toll-like receptor (TLR)-4 agonist, controlling the intestinal mucosa's inflammation response. The TLR-4 activation can give the anti-inflammation effect and immunoregulator.¹⁵ *Lactobacillus brevis* is lactate acid in rod-shaped, gram-positive bacteria with 16 different strains. Both *L. mesenteroides* and *L. brevis* can be found in fermented food, such as salted vegetables or fruits and pickles. *L. brevis* is a normal intestinal flora within the humans' intestines, vagina and fesses¹⁵

This study's limitations are that the effects of prebiotic administrations on immune system parameters in the intestinal mucosa were not examined, and the culture to determine whether the probiotics influenced the intestinal growth was not performed.

The results described in this study are important to prove that *L. mesenteroides*, *L. brevis* and combination of *L. mesenteroides* and *L. brevis* affected the immune system and probiotic combination of *L. Mesenteroides* and *L. Brevis* reduced the thickness of the bronchial epithelium of ovalbumin-induced chronic asthma rat models.

SUMMARY

L. mesenteroides, *L. brevis* and combination of *L. mesenteroides* and *L. brevis* affected the immune system especially Ig-E and IFN- γ , probiotic combination of *L. Mesenteroides* and *L. Brevis* also reduced the thickness of the bronchial epithelium of ovalbumin-induced chronic asthma rat models.

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None.

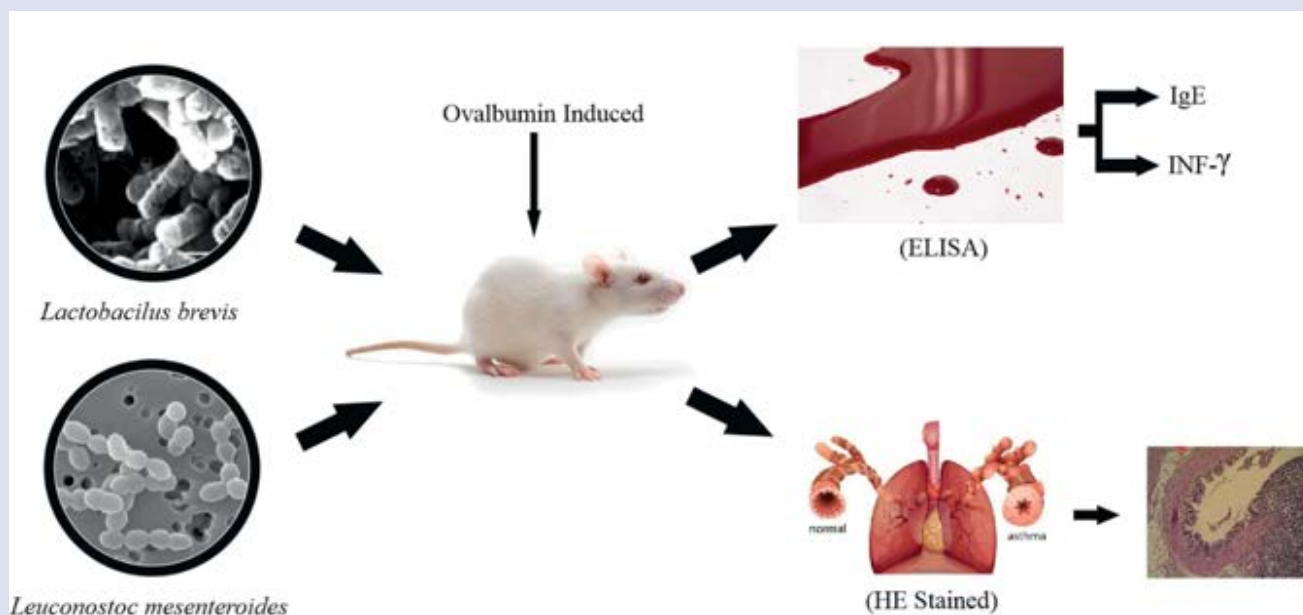
CONFLICTS OF INTEREST

None.

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GRAPHICAL ABSTRACT



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