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The Downregulation of Pro-inflammatory Cytokine Interleukin-6 after Oral Administration of *Lactobacillus plantarum* IS-10506 in Mice Model for Endometriosis

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ABSTRACT

Background: As a multi-functional cytokine, interleukin-6 (IL-6) is advanced in endometriosis. Probiotics are known to be able to modulate/regulate immune responses through downregulation of the pro-inflammatory cytokine IL-6. *Lactobacillus plantarum* IS-10506 (LIS-10506) is a novel probiotics strain derived from curd (a milk product which look like a yogurt) from traditionally made from buffalo milk or usually called by *dadih* from West Sumatra, Indonesia. This investigation intended to comprehend the impact of LIS-10506 administration on IL-6 expression in the endometrial tissue, serum IL-6 levels and endometriosis implant areas in mice model for endometriosis.

Methods: This study conducted at the Animal Laboratory, Professor Nidom Foundation, Surabaya, Indonesia from January to June 2021. This experimental study was conducted on 32 adult female mice 12 weeks old divided into 4 groups in a completely randomized design with a posttest only control group design. LIS-10506 microencapsulation of curd was administered orally at a dose of 10^9 CFU/mice/day for 7 and 21 days. Serum IL-6 levels were assessed using IL-6 ELISA Kit for mice, IL-6 expression was assessed semi quantitatively according to the Remmele method and implant areas were measured by the area of hyperemia in mice's peritoneal wall.

Result: This investigation revealed that the peritoneal IL-6 expression and implant area experienced a decrease. In summary, the administration of LIS-10506 could downregulate pro-inflammatory cytokine IL-6. This present study has demonstrated the effect of LIS-10506 on one of the pathophysiological pathways in endometriosis.

Key words: Animal model, Endometriosis, Interleukin-6, *Lactobacillus plantarum* IS-10506.

INTRODUCTION

Endometriosis is described as the existence of endometrium-like tissue outside the uterus. The reproductive aged women have prevalence level of getting an endometriosis at approximately 10% (Shafrir *et al*., 2018). However, endometriosis' real chance of happening is uncertain if not done properly with the requirement of surgical visualization for definitive diagnosis. Indeed, the chances are ranged from 2 to 11% prevalence for women without symptoms, 5 to 50% prevalence for infertile women and 5 to 21% prevalence for hospitalized women with the symptom of pelvic pain (Zondervan *et al*., 2020).

The occurrence of lesions is figured from originally endometrial fragments shedding during menstruations and reaching peritoneal cavity through Fallopian tubes. A retrograde menstruation, which this occurrence is called, is thought to be the possible cause of endometriosis (Sourial *et al*., 2014). Endometriosis transpires only in the menstruating species, like primates. Thus, this offers a suggestion that the requirement of endometriosis is the occurrence of menstruation (Burney and Giudice, 2012).

For the last decades, the role of macrophages in endometriosis was growingly perceived (Capobianco and Rovere-Querini, 2013). As the master regulators, macrophages will initiate responses to any injuries, infection and neoplastic in the tissues. For example, macrophages are the ones responsible in deciding the proper response in restoring homeostasis, on one hand, consisting both pathogens' recognition and damage and both cells with infection and transformation; on the other hand, containing

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the differentiation, activation and proliferation of precursor/ stem cell and neo-vessel generation.

Furthermore, IL-6 is a multi-functional cytokine advanced in peritoneal fluid (PF) and serum of women suffering from endometriosis (Kang *et al*., 2014). Macrophages are the essential cells to produce IL-6 in PF. A number of previous investigations have shown that the activation of macrophages in the PF of women suffered from endometriosis significantly heightens its numbers along with secreting more cytokines, which, indeed, includes IL-6 (Li *et al*., 2017).

In general, as mentioned by several studies, microbiota a significant role in the treatment advancement of inflammatory illness like the microbiome's essential contribution in modifying inflammatory channels through several and complex identified molecular interactions provoking the safeguarding of the host from any pathogens or exacerbating inflammation in inflammatory diseases (Belkaid and Hand, 2014). In particular, the uncontrolled and exacerbated inflammatory reaction is triggered by the components of dysbiosis microbiota leading to the promotion of deregulation in the induction of pro-inflammatory cytokine and immune cells' differential regulation (Blander *et al*., 2017; Mendes *et al*., 2019).

Crucially, gut microbiota may be engaged in any endometriosis' commencement and development (Laschke and Menger, 2016). In experimental models from a low-grade inflammation condition, probiotics, particularly for *Bifidobacterium* and *Lactobacillus*, exhibit major contribution in determining the immune response by down-regulating the pro-inflammatory cytokines (IL-6 and TNF-α) and up-regulating the anti-inflammatory cytokine (IL-10). The fact that the consequences of probiotics' utilization which are strain specific and the benefits of probiotics' combination themselves may explain any gaps between the scientific investigations related to cytokine levels (Karamese *et al*., 2016).

Thus, this investigation would like to identified the after effect of LIS-10506 administration on IL-6 expression in the endometrial tissue, serum IL-6 levels and endometriosis implant areas in mice model for endometriosis by using the strains of *Lactobacillus plantarum* IS-10506 (LIS-10506), isolated from the Indonesian traditional fermented buffalo milk (*dadih*). Its resilience to acid and bile, its antimicrobial features against pathogenic bacteria and its antimutagenic characteristics became the reason why this investigation invested in the indigenous *dadih* probiotic strains (Collado *et al*., 2007; Surono *et al*., 2014; Kandola *et al*., 2016; Liu *et al*., 2018; Sharif *et al*., 2018).

MATERIALS AND METHODS

Preparation of diets

The identification of probiotic LIS-10506 from *dadih* (milk product which looks alike as yoghurt) was performed by using 16S rRNA gene sequencing as *Lactobacillus plantarum* (GenBank: DQ860148) based on the previous study (Surono *et al*., 2008). The incorporation of microencapsulated bacteria was completed for experimental purposes at a dose of 1×10^9 CFUs/mice/day displaying neither any significant forfeiture of viability or contamination due to storing process during the whole investigation duration. The powder was then diluted in 25 µL bicarbonate buffer. Placebo using distilled water were given in the same quantity.

Animals and administration of probiotic mixture

The ethical approval of this investigation was generated from the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia (Reference number: 2.KE. 063. 06. 2021). This study was conducted at Animal Laboratory, Professor Nidom Foundation, Surabaya, Indonesia from January to June 2021. Thirty-two female mice (*Mus musculus*), 12 weeks old and weighing around 20-30 g, were purchased from veterinary center in Indonesia. Animals were then stored in boxes at the certain temperatures varying between 19°C and 22°C using a standard 12-hour light-dark cycle. The division of mice was done randomly into 4 groups; they were the control group (Group 1), placebo group (Group 2), 7 days LIS-10506 administration group (Group 3), 21 days LIS-10506 administration group (Group 4) (Fig 1). Probiotics were given by oral gavage. Endometriosis modeling process for group 2, 3 and 4 start at day 8 of experiment using standard university protocols.

Sample collection

In collecting the sample, the mice used in this study were first euthanized. The blood was derived from the cardiac ventricle' s apex then centrifuged at 4,000 rpm for 10 minutes. Mouse IL-6 ELISA Kit (Abcam, UK) was utilized to assessed the levels of serum IL-6, while employing ELISA reader to read the results at a wavelength of 450 nm (Wang *et al*., 2021). After obtaining the blood samples, surgery was performed on the stomach of the mice to generate its peritoneal wall. IL-6 expression was then assessed semi quantitatively according to Remmele method and implant areas were calculated by the measuring of hyperemia areas in the peritoneal wall of mice (Remmele *et al*., 2013).

Statistical analysis

The data analysis was completed by employing a statistical software called SPSS version 25.0 (IBM, Chicago, USA) (Costi *et al*., 2021). Further analysis from those continuous variables with parametric distributions was done using the one-way ANOVA test. If p-value was less than 0.05, a significantly difference was obtained and identified between groups statistically.

RESULTS AND DISCUSSION

There were three parameters studied to determine the effect of LIS-10506, expression of IL-6 in endometriosis tissue, the levels of serum IL-6 and the area of endometriosis implants.

Levels of serum IL-6

As the statistically significant test reaching Sig. 0.028, therefore, a significant difference is concluded (Table 1). A post-hoc test as seen in Fig 2 was conducted to determine the comparison between each group, it turned out that the difference was only found in group 1 compared to group 4 obtaining statistically significant differences.

Expression of IL-6 in endometriosis tissue

Histopathological examination (Fig 2) was carried out to determine the expression of IL-6 in each sample and then assessed semi-quantitatively according to Remmele method which had been modified (Remmele *et al*., 2013). As the result showing Sig. <0.05, thus, significantly differences were concluded between groups (Table 2). Moreover, a post-hoc test was applied in determining the comparability between each group. Group 1 expressed a significantly difference with to group 2 and group 3, while it was not significantly different with group 4. Even though with a difference score of 1.42, group 3 is not significantly different with group 4.

Endometriosis implant area

In determining the extent of endometriosis lesions (Fig 3), the area of hyperemia in the peritoneal wall of mice was measured. Table 3 shows the results of measuring area in each group. The results of the data normality test displayed that group 1 data was not normally distributed.

With the finding of no implant area was found in group 1, thus, the difference is obtained. A significant difference was also identified if group 2 was compared to all other groups. However, group 3 is not significantly different with group 4. In addition, the correlation between each parameter was

Table 1: Mean serum IL-6 levels in mice blood.

Group	N	Serum IL-6 level (ng/L)	P values*
Group 1	8	14.9±1.89	0.028
Group 2	8	13.81±2.40	
Group 3	8	12.20±2.25	
Group 4	8	11.72±2.29	

Values are presented as mean±standard error of the mean values. *Sig. value of the Shapiro-Wilk test is >0.05. Oneway ANOVA was applied to compare data.

Table 2: Mean IL-6 expression of endometrial mice tissue.

Group	N	IL-6 expression (IRS)	P values*
Group 1	8	1.83±0.67	<0.001
Group 2	8	8.18±1.27	
Group 3	8	5.66±2.72	
Group 4	8	4.23±3.18	

Values are presented as mean±standard error of the mean values *Sig. value of the Shapiro-Wilk test is >0.05. Oneway ANOVA was applied to compare data.

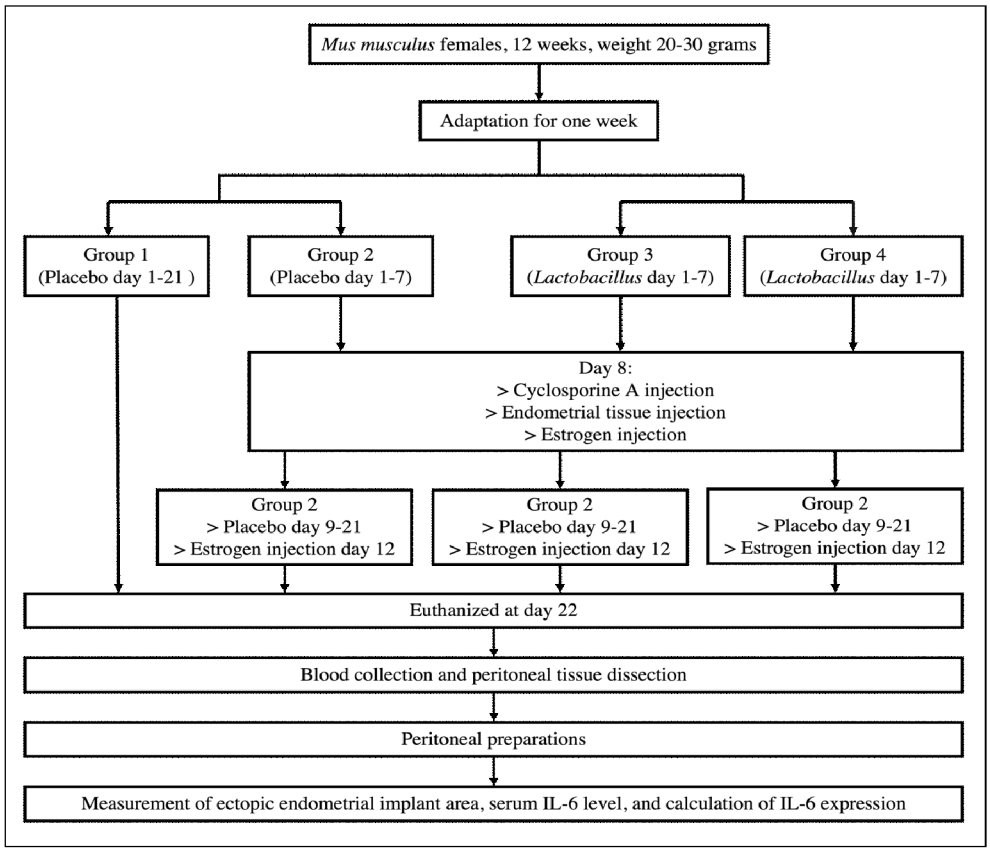

Fig 1: Research flow. Mice were divided into 4 groups, 8 mice each group.

tested with Pearson bivariate correlation analysis was utilized to test the correlation between parameters expressing a discovered correlation between peritoneal IL-6 expression with both IL-6 levels and endometriosis implant area and, on the contrary, a correlation could not be found between the levels of IL-6 with the implant area (Table 4).

Furthermore, American Society for Reproductive Medicine (ASRM) proposed their consideration of endometriosis as a chronic disease requiring lifelong treatment by using as visible as it can get for the medical treatment and evading any aggressive procedures like surgery (Practice Committee of the American Society for Reproductive Medicine, 2014). Additionally, the combination of medical treatment with a pharmacological therapy in endometriosis treatment should be enforced containing of both hormonal and non-hormonal agents. It is expected that the hormonal therapy is able to intervene with ovulation during the post-operative period that is crucial for conception. However, their application for treating endometriosis has been still controversial. Meanwhile, the non-hormonal drugs are under the spotlight these days due to their successful protection to ovulation and its prospects in eradicating lesions or alleviating symptoms (Chen *et al*., 2019).

Meanwhile, a cell wall component from Gram-positive bacteria which contributes greatly in inflammatory reactions by inducting the pro-inflammatory cytokines and affecting the bacterial growth is called by lipoteichoic acid (LTA) (Bachanti *et al*., 2018). Pathogenic bacteria have been the focus of previous structural and functional studies on LTA due to LTA's feature as a key stimulator in both inflammatory and infectious illnesses (Sutariya *et al*., 2020; Geeta *et al*., 2021). On the contrary, different from pathogenic bacteria isolated LTAs, some probiotic LTAs diminish any excessive inflammatory responses by inducting any tolerance against pathogenic ligands. Furthermore, LTA from *Lactobacillus plantarum* K8 (pLTA) reducing the number of lipopolysaccharide (LPS)-induced excessive inflammation by inhibiting TNF-α and IL-6 (Percy and Gründling, 2014). Collado *et al.* (2007) tested probiotic strains in displacing and inhibiting the pathogen adhesion leading to unfavored strain-specific results with its time and pathogen strain dependency. Generally, LIS-10506 exhibited the best ability against pathogen adhesion (Surono *et al*., 2014).

Particularly, the results of this examination displayed that the average levels of IL-6 in the control group without treatment was 14.9 ng/L for its average serum IL-6. Correspondingly, this result is consistent with the previous study that the values of serum IL-6 in mice ranged from 5-20 pg/mL (Stenina *et al*., 2012). Thus, it was concluded that the control group without treatment had the levels of serum IL-6 within normal limits. However, the averages of serum IL-6 levels

Table 3: Mean implant area of endometrial mice tissue.

Group	N	Implant area (mm^2)	P values*
Group 1	8	0	<0.001
Group 2	8	92.86±34.28	
Group 3	8	26.97±20.06	
Group 4	8	9.51±6.78	

Values are presented as mean±standard error of the mean values *Sig. value of the Shapiro-Wilk test is <0.001. Kruskall-Wallis was applied to compare data.

Table 4: Pearson correlation.

	Serum IL-6 levels	IL-6 expression	Implant area
Serum IL-6 levels	-	.411*	.346
IL-6 expression	.411*	-	.458*
Implant area	.346	.458*	-

*Correlate.

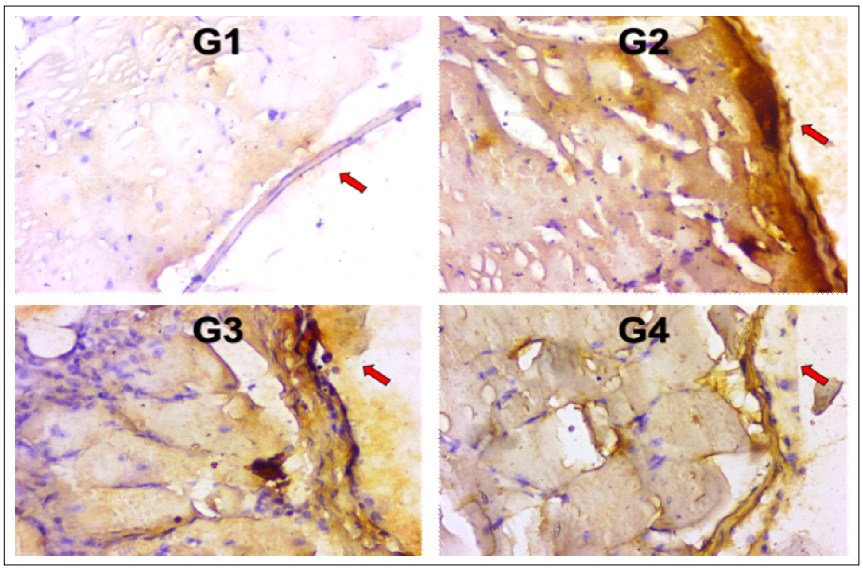

Fig 2: Immunohistochemistry result of IL-6 expression in endometriosis tissue. The red arrow showed IL-6 expression. DAB anti IL-6, 400× magnification.

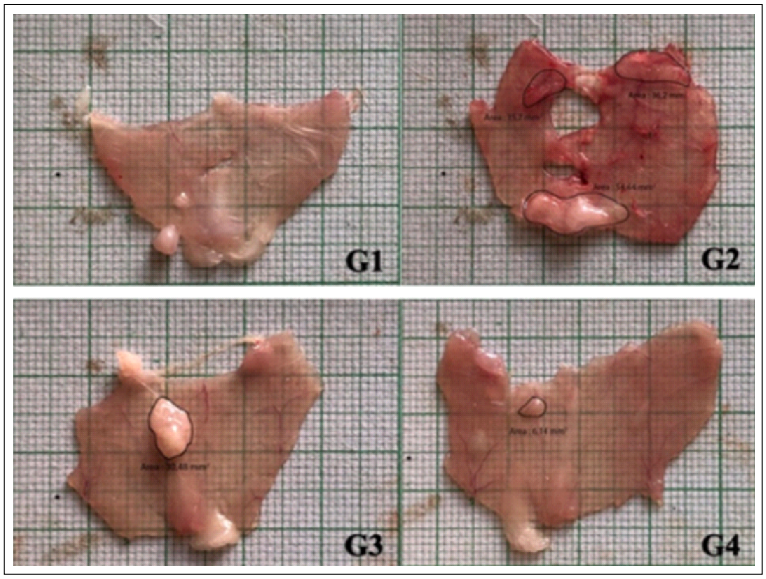

Fig 3: Peritoneal images of animals in the group 1 and hypervascularization images of the endometriosis model groups (Group 2, Group 3 and Group 4).

in the other three treatment groups experienced a decrease, but no statistically significant differences were found.

In a previous investigation conducted by Li *et al.* (2017), a study of IL-6 levels in serum and peritoneal fluid of patients with endometriosis was carried out. A significant increase was retrieved in peritoneal fluid IL-6 levels of endometriosis patients in group A (stage I-II r-AFS) and group B (stage III-IV r-AFS) compared to the control group. Meanwhile, in the measurement of plasma IL-6 levels, a significant difference was identified in the control group and group B, whereas when compared with group A, no significant difference was discovered.

CONCLUSION

In summary, in this investigation, the ability of LIS-10506 in inhibiting IL-6 was concluded which greatly contributes to the pathophysiology of endometriosis. However, further research can be done using LIS-10506 by looking at other parameters to determine its role in the immune mechanism of endometriosis, thus, the expectation that LIS-10506 as an alternative to prevent endometriosis can be achieved.

Conflict of interest: None.

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