



## **Probiotics *Lactobacillus reuteri* increase levels of $\beta$ -Defensin1, sIgA and decrease number of *Staphylococcus aureus* bacteria colonies in vaginal mucosa on puerperal mice model infected with *Staphylococcus aureus***

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### **Abstract**

Puerperal infection is a bacterial infection in the genitalia and female reproduction system, which cause the highest mortality among women who post give birth in recent years. The causative agent of this infection is *Staphylococcus aureus* (*S. aureus*). Alternative therapy using probiotics such as *Lactobacillus reuteri* was developed to reduce the increasing incidence of antibiotic resistance. This article studies the effect of probiotics *L. reuteri* increasing  $\beta$ -defensin1 levels, sIgA levels, and decreasing the number of bacteria *S. aureus* colonies in the vaginal mucosa on puerperal mice model that induced with *S. aureus* bacteria. Mice BALB/c was used as an animal model and divided into four different groups. The measurement  $\beta$ -defensin1 levels, sIgA levels, and the number of bacteria *S. aureus* colonies were conducted in one and three days postpartum. Enzyme-Linked Immunosorbent Assay (ELISA) method was to measure performed for  $\beta$ -defensin1 levels, and sIgA levels, Total plate count was used for the quantity of bacteria *S. aureus* colonies.  $\beta$ -defensin1 and sIgA levels showed a similar pattern and elevated significantly in all treated group compared to control. The highest value was obtained from a group that administered with *L. reuteri* and induced with *S. aureus* in all measurements. The levels of  $\beta$ -defensin1 and sIgA in three days postpartum were higher than that day one. The number of *S. aureus* colonies was lower in the treated group compare that of the positive control. The average number of bacteria in one day postpartum was higher than three days. The administration of *L. reuteri* and or induction of *S. aureus* could increase the level of  $\beta$ -defensin1 and sIgA but reduced decrease in the number of bacteria *S. aureus* colonies.

**Keywords:** *Lactobacillus reuteri*; *Staphylococcus aureus*;  $\beta$ -defensin1; sIgA; bacteria *S. aureus* colonies

Infeksi puerperal adalah infeksi bakteri pada alat kelamin dan sistem reproduksi wanita yang menyebabkan kematian tertinggi pada wanita pasca melahirkan dalam beberapa tahun terakhir. Agen penyebab infeksi ini adalah *Staphylococcus aureus* (*S. aureus*). Terapi alternatif menggunakan probiotik seperti *Lactobacillus reuteri* dikembangkan untuk mengurangi peningkatan kejadian resistensi antibiotik. Penelitian ini mengkaji pengaruh probiotik *L. reuteri* meningkatkan kadar  $\beta$ -defensin1, kadar sIgA, dan

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menurunkan jumlah koloni bakteri *S. aureus* di mukosa vagina pada model mencit nifas yang diinduksi dengan bakteri *S. aureus*. Tikus BALB/c digunakan sebagai hewan model dan dibagi menjadi empat kelompok berbeda. Pengukuran kadar  $\beta$ -defensin1, kadar sIgA, dan jumlah koloni bakteri *S. aureus* dilakukan dalam satu dan tiga hari pasca melahirkan. Metode Enzyme-Linked Immunosorbent Assay (ELISA) digunakan untuk mengukur kadar  $\beta$ -defensin1, dan kadar sIgA. Total plate count digunakan untuk mengukur jumlah koloni bakteri *S. aureus*. Kadar  $\beta$ -defensin1 dan sIgA menunjukkan pola yang sama dan meningkat secara signifikan pada semua kelompok perlakuan dibandingkan dengan kontrol. Nilai tertinggi diperoleh dari kelompok yang diberikan *L. reuteri* dan diinduksi dengan *S. aureus* di semua pengukuran. Kadar  $\beta$ -defensin1 dan sIgA pada tiga hari pasca melahirkan lebih tinggi dibandingkan hari pertama. Jumlah koloni *S. aureus* lebih rendah pada kelompok perlakuan dibandingkan dengan kontrol positif. Rata-rata jumlah bakteri dalam satu hari pasca melahirkan lebih tinggi dari tiga hari. Pemberian *L. reuteri* dan atau induksi *S. aureus* dapat meningkatkan kadar  $\beta$ -defensin1 dan sIgA tetapi menurunkan jumlah koloni bakteri *S. aureus*.

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**Kata Kunci:** *Lactobacillus reuteri*; *Staphylococcus aureus*;  $\beta$ -defensin1; sIgA; koloni bakteri *S. aureus*

## Introduction

Puerperal infection is the leading cause of mortality among mothers at 48,17% (Depkes Jatim, 2012). This infection is an inflammation of all genitalia organs after birth periods caused by aerobic and anaerobic bacteria, one of which is *Staphylococcus aureus* (*S. aureus*). The inflammation process is through perineum and endometrium surface injury (Cunningham, MacDonald, & Gant, 2006). *S. aureus* produces a polysaccharide capsule or thin membrane that plays a role in the virulence of this bacteria (Jawetz et al., 2014). Some study is the source or provides trauma or injury such as an open wound from mucosa a medium for de entry of *S. aureus* infection (Li, Peres, Damian, & Madrenas, 2015). The incubation period in *S. aureus* 1-8 hours; however, in case of toxic shock syndrome occurs in 5 days (Brooks, Butel, & Morse, 2005).

Probiotics are commonly consumed orally, the antigen produced by probiotic go through barriers that exist in the gastrointestinal tract consisting of mucus. in Plaque Peyer (ileum) the antigen binds to APC will be presented by MHC II,

and activate all CD4+ T cells into Th1, Th2, Th17, Treg cells. The activated immune cells enter the blood circulation and undergo homing to mucosal tissues throughout the body, such as mucosal tissue gastrointestinal tract, genitourinary tract, upper and lower respiratory tract, and the gland duct mammae dan lymphoid tissues (spleen and lymph nodes) (Baratawidjaja & Rengganis, 2014).

Pathogenic bacteria can invade traktus genitalis by attacking the epithelial cells that is the early barrier defense mechanism by secreting nonspecific antimicrobial peptides such as defensin, cathelicidin, lactoferrin, and lysozyme that are capable of killing bacteria gram-positive and gram-negative. Then the non-specific defense system failed, the next defense mechanism is the adaptive immunity consisting of cellular and humoral immunity will be activated. Cellular immunity in the genitalia tract consists of differentiation of T cells into Th1, Th2, Treg, Th17, while humoral immunity composed of the primary sIgA, IgG, and IL 17, and IL 23. (Hardy, Harris, Lyon, Beal, & Foey, 2013).

Treatment for infection caused by *S. aureus* is using antibiotics; however, a recent study found that *S. aureus* isolated from hospital generally showed against resistance mostly used antibiotics. Such as oxacillin and more than 85% of patients encounter a resistance toward this antibiotic (Karska-Wysocki, Bazo, & Smoragiewicz, 2010). Therefore, natural medicine such as probiotics offers an alternative therapy to reduce the use of antibiotics that are widely studied (Karska-Wysocki et al., 2010).

Administration of *L. reuteri* against the non-specific immunity increase production of mucin activates natural killer (NK) cells, and activates macrophages and phagocytosis consequently. Probiotics also affect specific immunity by increasing production cytokines as IL-2, IL-6, TNF- $\alpha$ , and levels of sIgA (Saavedra, 2007). We reported the effect of *L. reuteri* administration to the concentration of  $\beta$ -defensin1 and sIgA, and the number of bacteria *S. aureus* on the puerperal mice model were induced with *S. aureus*.

## Methods

### Animal preparation

Pregnant mice strain BALB/c was used in this study; they were purchasing from Biotech Laboratory, State Islamic University, Malang. Mice were acclimated for three days and divided into four different groups. Without treatment (C); induction with *L. reuteri* only (L); infected with *S. aureus* only (S); and induction with *L. reuteri* and infected with *S. aureus* (LS). Each group contained eight mice in which four mice were used for one day postpartum measurement, and the

other four was used or three days postpartum measurement.

### Induction with *L. reuteri*

In this study, *L. reuteri* strain (ATCC 6475) 20108 USA was employed as probiotic. Bacteria were grown in MRS broth media and administered through oral gavage with dose  $1 \times 10^{10}$  CFU for 250  $\mu$ L every day. Bacteria culture was distributed starting from day 13th of gestation age until one day postpartum and three days postpartum.

### Injection of *S. aureus*

*S. aureus* bacteria was obtained from Microbiology Laboratory, Medical Faculty, University of Brawijaya. Bacteria were grown in nutrient broth media and injected intravaginal using 1 mL syringe in which the needle was replaced by surflo catheter. The injection dose was  $5 \times 10^7$  CFU for 200  $\mu$ L. Injection was conducted between 0-12 hours postpartum.

### Sample preparation

The liquid sample was taken from vaginal mucous mice with vaginal lavages technique. The sample was collected and kept in the refrigerator until used for analysis.

### Cytokines

Measurement of  $\beta$ -defensin1 and sIgA were done using Method for ELISA (Bioassay Technology Laboratory). The number of bacteria *S. aureus* colonies were calculated using total plate count method. The procedure was conducted according to the protocol in every kit.

### Statistical analysis

All data was shown as mean of measurement. Data was analyzed using independent sample T-test for parametric analysis and Mann Whitney test for nonparametric analysis with was considered as  $P < 0.05$ .

### Ethical clearance

All materials and methods for this experiment Ethical Committee approved the present of health research Politeknik Kesehatan Kemenkes Malang, East Java, Indonesia (Reg No. 301/KEPK-POLKESMA/2016).

## Result and Discussion

### The level of $\beta$ -defensin1 in one day and day three postpartum

The measurement of  $\beta$ -defensin1 levels in day one and days three postpartum showed a similar pattern (Figure 1). The level of  $\beta$ -defensin1 elevated significantly in every treatment compared to control. The highest level of  $\beta$ -defensin1 was obtained on groups treated with *L. reuteri* and *S. aureus* in one day ( $56.23 \pm 7.76$  ng/mL) but not in three days postpartum ( $55.74 \pm 14.47$  ng/mL). The average of  $\beta$ -defensin1 level in three days postpartum of each group was higher than one-day postpartum measurement (Figure 1).

Figure 1 shows the measurement of  $\beta$ -defensin1 level in the puerperal mice model that was induced with *L. reuteri* and infected with *S. aureus* in day one and days three postpartum. Without treatment (C); induction with *L. reuteri* only (L); infected with *S. aureus* only (S); and induction with *L. reuteri* and infected with *S. aureus* (LS) Significant  $P \leq 0.05$ .

### The level of sIgA levels of each group in day one and days three postpartum

In line with the results of  $\beta$ -defensin1 levels, the results of sIgA levels measurement was also shown an increase in every treatment group (Figure 2). On day one postpartum, the level of sIgA has reached the highest level in group with induced *L. reuteri* ( $33.68 \pm 8.20$  ng/mL), then followed by group treated with both *L. reuteri* and *S. aureus* ( $27.85 \pm 5.49$  ng/mL) and group injected with *S. aureus* ( $25.11 \pm 2.93$  ng/mL). In order hand in days three postpartum, the highest level of sIgA was also obtained from the group with *L. reuteri* and *S. aureus* ( $32.54 \pm 1.80$  ng/mL). Although there was a similar pattern in sIgA measurement between one day and three days postpartum, overall, three days postpartum showed a higher average concentration in control groups and group with *L. reuteri* and *S. aureus*.

Figure 2 shows the measurement of sIgA level in the puerperal mice model that was induced with *L. reuteri* and infected with *S. aureus* in day one and days three postpartum. Without treatment (C); induction with *L. reuteri* only (L); infected with *S. aureus* only (S); and induction with *L. reuteri* and infected with *S. aureus* (LS) Significant  $P \leq 0.05$ .

Figure 3 shows the measurement of the number of bacteria colonies in the puerperal mice model was induced with *L. reuteri* and infected with *S. aureus* in day one and days three postpartum. Without treatment (C); induction with *L. reuteri* only (L); infected with *S. aureus* only (S); and induction with *L. reuteri* and infected with *S. aureus* (LS) Significant  $P < 0.05$ .

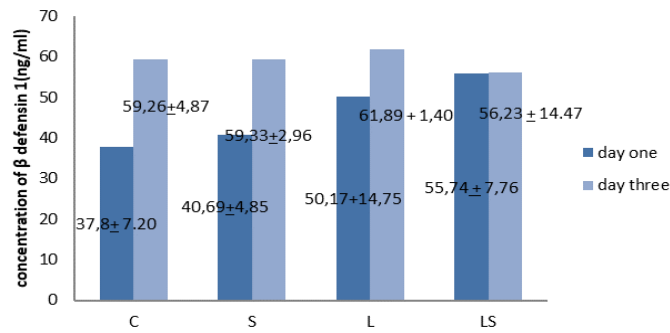


Figure 1.

The measurement of  $\beta$ -defensin1 level in puerperal mice model that induced with L. reuteri and infected with S. aureus in day one and days three postpartum

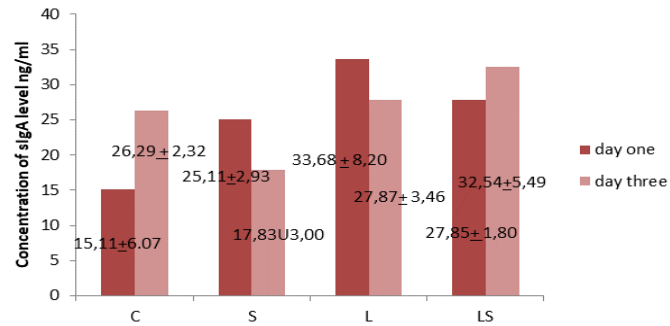


Figure 2.

The measurement of slgA level in puerperal mice model that induced with L. reuteri and infected with S. aureus in day one and days three postpartum.

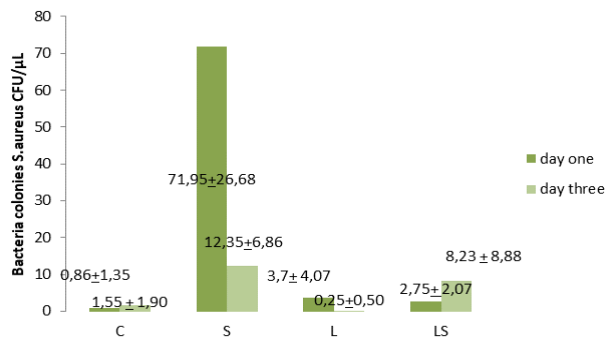


Figure 3.

The measurement of the number of bacteria colonies in puerperal mice model that induced with L. reuteri and infected with S. aureus in day one and days three postpartum.

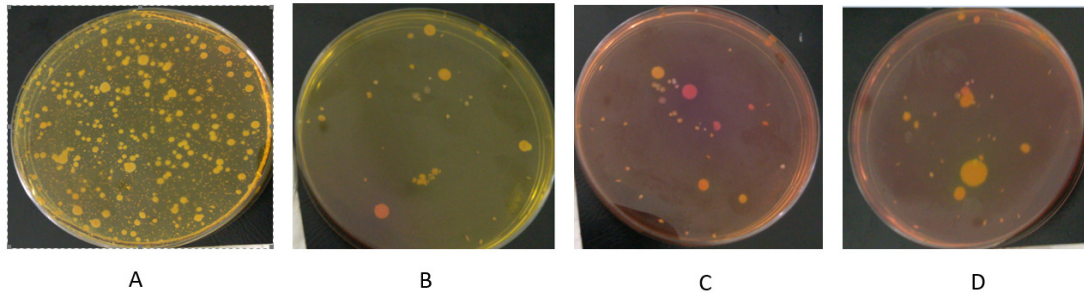


Figure 4.

The number of bacteria colonies in the puerperal mice model that induced with *L. reuteri* and infected with *S. aureus* in one day postpartum.

Figure 4 shows The Number of bacteria colonies in the puerperal mice model that was induced with *L. reuteri* and infected with *S. aureus* in one day postpartum. Infected with *S. aureus* in day one (A), infected with *S. aureus* in day three (B) induction with *L. reuteri* and infected with *S. aureus* in day one (C), induction with *L. reuteri* and infected with *S. aureus* in day three (D)

#### **Comparison between the number of bacteria *S. aureus* colonies in one day and three days postpartum**

The counting of the number of bacteria *S. aureus* colonies indicated that the number of *S. aureus* in the treatment group was lower than that is control (Figure 3). On day one postpartum, the number bacteria *S. aureus* colonies reached it decreased in group induced *L. reuteri* (3.7 CFU/ $\mu$ l) then followed by group induced with *L. reuteri* and infected with *S. aureus* (2.75 CFU/ $\mu$ l). The highest number in a group infected with *S. aureus* (71,76 CFU/ $\mu$ l). A similar profile was shown in days three postpartum, where the

highest average bacteria colonies were also obtained from the group with infected *S. aureus* (12,35 CFU/ $\mu$ l) and followed by group treated with *L. reuteri* and induced *S. aureus*. Still, overall, one day, postpartum showed a higher average bacteria colonies in positive control groups, and group with *L. reuteri* compare to one day postpartum.

In the present study, we explored the role of *L. reuteri* as probiotic against infection of *S. aureus* in the puerperal mice model. The level of  $\beta$ -defensin1 increased significantly on all treated group, in which the highest level was obtained on group administered with *L. reuteri* only and slightly lower when the mice were injected with *S. aureus*, whether in one or three days postpartum.  $\beta$ -defensin1 is an important non-specific immunity that provides the first-line barrier the body in against pathogenic microorganisms. In humans,  $\beta$ -defensin1 (hbd-1) expressed in a genital and urinary tract, mucous the mouth, pancreas, kidneys, colon, pulmonary, trachea, skin, ear, intestines smooth/the small intestine). In contrast, mice  $\beta$ -defensin1 (mbd-1)

expressed in pulmonary, kidneys, heart, intestines, macrophages alveoli, and the female reproductive organs (Kaiser & Diamond, 2000).

Pathogenic and commensal bacteria can induce immune response both innate immunity and adaptive immunity that will activate the synthesis pro-inflamasi cytokines or anti-inflamasi cytokines, lymphoid cells in the immune system also produced il-17 and il-22 that mostly found in the mucosa of digestive tract and play a role in mucous defense response. These cells do not express TCR but a subset of the cell similar a subset of T helper cells. The cytokine secreted strengthen the defense system of the intestinal mucosa and the gastrointestinal tract that stimulate production tympanic and defensin (Abbas, Lichtman, & Pillai, 2011). Strains lactobacillus demonstrates the ability to modulate microbiota commensal composition and metabolism, compete with pathogens, therefore hindering from adhesion of pathogens. Probiotics bacteria were also able to induce production mucin on the digestive tract (Caballero-Franco, Keller, De Simone, & Chadee, 2007). The third day after delivery, mice is not in the puerperium phase but still in the reproduction metestrus phase, where the *copus luteum* secrete hormone progesterone, although hormone estrogen low concentration still detected. This phase takes place around six hours. In line with the theory that epithelial cell and mucous of women reproductive organs is greatly affected by hormonal status, estradiol, and the menstrual cycle hence will influence on the synthesis of defensin. The high concentration of estradiol/estrogen hinders production antimicrobial

peptide, so defensin is produced. Research conducted by Hickey, Patel, Fahey, and Wira (2011) showed during the menstrual cycle where levels of estrogen go up, and production of defensin will be repressed so that in this condition allowing the infections to (Hickey et al., 2011).

Measurement of sIgA also resulted in a similar pattern with  $\beta$ -defensin1 measurement. The level of sIgA was increase in all treated groups, in which the highest level was obtained on the group administered with *L. reuteri*. A key intestinal strategy to generate immune protection in non-inflammatory / manner is the production of immunoglobulin A (IgA). This sIgAs may block or sterically hinder microbial component involve in ephitelial attachment, mediates intraepithelial neutralization of incoming pathogens and microbial inflammatory product. (Cerutti, Chen, & Chorny, 2011). Data from animal studies have indicated that probiotic lactic acid bacteria can significantly influence the immune response of host animals in promoting the productions of the secretory Ig (sIgA), enhancing phagocytosis. They were altering the balance of Th1 and Th2 and a cytokine production profile (Kekkonen, 2008).

Rautava, et al., 2006 suggested that lactobacillus GG, bifidobacterium lactis BB-12, saccharomyces boulardii can increase the production of IgA in mucous gastrointestinal. Probiotics induces expression of an epithelial cell that is related to cytokine, TGF  $\beta$ , IL-6, as well as IL-10 that stimulate of producing the IgA through B cells maturation and the switching process (Hardy et al., 2013).

Saavedra (2007) also proved that the influence probiotics induce the non-specific immunity system by increasing the production of mucin, natural killer cell (NK) activation, macrophages, and phagocytosis activation. Probiotics also affect specific immunity due to increasing cytokines production such as IL2, IL6, TNFa, and the sIgA (Saavedra, 2007).

Probiotics *L. reuteri* can reduce the number of *S. aureus* bacteria colonies on the vagina mucosa puerperal mice model infected with bacteria *S. aureus* on day one after birth. *S. aureus* is one of the bacterial pathogens in the vagina that can cause infections. The colonization of bacteria *S. aureus* on the vagina mucous may lead to toxic septic shock (Guta, 2013; Mitchell, Gottsch, Liu, Fredricks, & Nelson, 2013). When lactic acid bacteria form colonization in mucous membranes of the vagina that serves to maintain vagina acidity, these pathogenic bacteria is a source of infections in genitalia and urinary tract and sexually transmitted diseases. In contrast, lactobacillus probiotics and bifidobacteria that are administered through an oral or vaginal could hinder the growth of *S. aureus* bacteria in animals (Lazarenko et al., 2012).

Baratawidjaja and Rengganis (2014) described that induction of immune response by antigens from gastrointestinal bacteria stimulates lymphocytes to other mucous such as respiratory tract, mammae, or the reproductive tract that in turn will to the local antigen. That is why the administration of *L. reuteri* by orally can increase the immune system in the mucous vagina (homing), consequently inhibits the growth of *S. aureus* bacteria (Hardy et al., 2013). The mechanism of

probiotics *L. reuteri* in the vaginal mucosa in the case genitourinary infections allegedly involves many factors such as lactic acid production, antimicrobial, and hydrogen peroxide that will influence immune response. Antimicrobial compound that may protect from pathogenic microorganisms in epithelial, the ability to form specific molecules against pathogenic bacteria virulence have been demonstrated in some research in vivo and in vitro (Lazarenko et al., 2012). In accordance with the number of *S. aureus* bacteria colonies on vagina mucosal of puerperal mice model on the one day after birth. However, this is not the case on days three also that increased after induction with *L. reuteri* and infected with *S. aureus*, and one explanation should be that the normal flora composition of the mucous vagina is unusually influenced by stimulation of reproductive hormone such as estrogen. The change in the balance of flora normal is due to changes in the vagina pH in order to trap that invade vagina (Abbas et al., 2011). The number of *S. aureus* bacteria colonies on vagina mucosa is influenced by the establishment of microbes commensal and several factors, including hormonal status, estradiol, and pro-inflammatory cytokines (Cribby, Taylor, & Reid, 2008). *S. aureus*, which administered the vagina, will across the vagina epithelial barrier that has strengthened by elevated levels of  $\beta$ -defensin1 and sIgA antibodies as a response to *S. aureus* so that the extracellular bacteria will be eliminated by the non-specific and adaptive immune system. The mechanisms an immune response against bacteria extracellular product such as activation complement mechanism, and the response of phagocytosis inflammatory.



Peptidoglycan on gram-positive bacteria and lipopolysaccharide (LPS) on gram-negative bacteria activate complement through alternative routes, while the process of phagocytosis the bacteria is conducted through antibodies opsonization. Antimicrobial peptidases also activities toll-like receptors (TLR) that will, in turn, phagocytosis microbes (Abbas et al., 2011).

## Conclusion

The administration induction with *L. reuteri* and infected with *S. aureus* in the puerperal mice model could increase the level of  $\beta$ -defensin1 and sIgA; however, it decreases the number of bacteria *S. aureus* colonies.[]

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