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Research Report

The increasing of beta-defensin-2 level in saliva after probiotic *Lactobacillus reuteri* administration

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ABSTRACT

Background: *Commesal bacteria is an excellent inducer for beta defensin-2 (BD-2). Probiotics bacteria Lactobacillus reuteri (L. reuteri) as commensal bacteria may play the same role as an excellent inducer for BD-2. Beta defensin is natural antimicrobial peptides widely expressed in oral cavity, including in epithelium salivary gland. Streptococcus mutans (S. mutans) as the main of bacteria causing caries are sensitive to BD-2.* ***Purpose:*** *This research was aimed to determine whether administration of probiotic L. reuteri can increase salivary BD-2 level in Wistar rats.* ***Methods:*** *This research can be considered as a laboratory experimental research with a randomized control group post test only design. Twenty-four male Rattus norvegicus Wistar strain rats aged 3 months were used. They were randomly divided into four groups, namely two control groups (negative control group that was not induced and positive control group induced with S. mutans), and two treatment groups (K1: induced with L. reuteri for 14 days and S. mutans for 7 days, and K2: induced with L. reuteri and S. mutans simultaneously for 14 days). L. reuteri culture at a concentration of 10^8 CFU/ml and S. mutans culture at a concentration of 10^{10}CFU/ml were induced into the oral cavity of Wistar rats. An examination of BD-2 level was then conducted by using Elisa techniques.* ***Results:*** *There was significant difference of salivary BD-2 level among those treatment groups (p=0.001). BD-2 level in saliva was increased after the administration of L. reuteri.* ***Conclusion:*** *L. reuteri probiotic can increase salivary BD-2 level in Wistar rats.*

Keywords: *Probiotic; L. reuteri; level of BD-2; S. mutans; caries*

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INTRODUCTION

Dental caries is a disease mostly found in oral cavity, especially in children. Similarly, Riset Kesehatan Dasar conducted by the Indonesian Ministry of Health in 2007 showed that 76% of child population in East Java suffered with dental caries, and according to data from Dinas Kesehatan Kota Surabaya, it is also known that among 61,214 students, 4,359 students had dental caries.[1,2]

Etiology of dental caries are multifactorial. There are three main factors causing dental caries: carbohydrate diet factors, especially sucrose, bacterial factors, especially *Streptococcus mutans* (*S. mutans*), and response factors of the host, especially innate immunity.[3] In the oral cavity, innate immunity is a part of immune system participating in defense process against pathogen. One of innate immunity, which has an important contribution in maintaining a balance between healthy and sick tissues, is antimicrobial peptides (AMP).

Antimicrobial peptides first identified in the oral cavity are beta defensins (BDs), which have antimicrobial activities against Gram positive and Gram negative bacteria as well as against fungi and viruses.[4] BDs are antimicrobial peptides, which are small cationic peptides widely expressed in the oral cavity, including gingival epithelium, buccal mucosa, salivary glands, salivary duct and saliva.[5] The family of beta defensins consists of four peptides, namely BD-1, BD-2, BD-3, and BD-4, but only BD-1, BD-2, and BD-3 are found in oral cavity. BD-1 is expressed constitutively, while BD-2 and BD-3 are induced by bacteria.[5,6] On the

other hand, microorganisms playing an important role in the etiology of dental caries are oral streptococci, especially *S. mutans* and *S. sobrinus*.[7] It is known that those two bacterial species are sensitive to BD-2.[6]

Probiotic administration as a preventive treatment against dental caries has currently been evaluated. According to the WHO, probiotics can be defined as live microorganisms which when consumed in sufficient quantities, they would be beneficial to health.[6] Commensal bacteria is excellent inducer for *BD-2* in epithelial cells of the oral cavity. It means that probiotic *Lactobacillus reuteri (L. reuteri)* as commensal bacteria can possibly act as an inducer for secreting BD-2 possibly detected in saliva samples.[7]

Thus, this research was aimed to know whether the administration of probiotic *L. reuteri* into the oral cavity of Wistar rats can increase *BD-2* level in the saliva inoculated with *S. mutans.*

MATERIALS AND METHODS

This research can be considered as a true experimental laboratory research with randomized control group post test only design. This research was approved by the Commission on Health Research Ethics Airworthiness of Faculty of Dental Medicine, Universitas Airlangga. Experimental units in this research were twenty-four white rat *Rattus norvegicus* Wistar strain provided by Integrated Research and Development Laboratory (LPPT) Yogyakarta. Those Wistars were used as animal models for dental caries.[9] Those rats were divided into four groups: negative control group not induced either by *S. mutans* or *L. reuteri*, positive control group induced by *S. mutans*, treatment group 1 induced by *L. reuteri* from day 1 to day 14 (for 14 days) and by *S. mutans* from day 8 to day 14 (for 7 days), treatment group 2 induced by *L. reuteri* and *S. mutans* from day 1 to day 14 (simultaneously for 14 days).

The sample of this research, furthermore, was saliva. The concentration of *L. reuteri* used as inducer was 4 x 10^8 CFU/ ml.[10] There are several steps to make *L. reuteri* culture. Tablet "X" which contains probiotic *L. reuteri* Prodentis (DSM 17 938 + ATCCPTA 5289) was put in liquid BHI, and then incubated for 1 x 24 hours in anaerobic gas generating kit (Oxoid). After removed from the incubator, it would show the presence of turbidity and sediment showing *L. reuteri* growth. Then it was cultured to MRS (De Man, ROGOSA and Sharpe) agar plates (Oxoid) by scratching, and put it into the incubator again for 2 x 24 hours. Some colonies were cultured in liquid BHI media, and then incubated again for 1 x 24 hours. To know whether the bacterial density had reached 4 x10^8 CFU/ ml, it needs to be tested by using a spectrophotometer with a wavelength λ= 625, identical to the Mc Farland 0.5.[11] The concentration of *S. mutans* inoculated in this research was 10^{10} CFU/ ml.[12] *S. mutans* used was *S. mutans* serotype c in the form of freeze dry taken about one oasis and then put into liquid BHI medium incubated for 1 x 24 hours.

Further culturing was conducted in the same way with the making of *L. reuteri* culture. On day 15, saliva samples (whole saliva) were taken after the stimulation of salivary secretion by inducing 1cc of ketamine HCl 100 mg/ cc and 1cc of Diazepam (Stezolid) 5 mg/ml into thigh area.[4] After 2-3 minutes, the saliva was taken about 50 mL by using a micropipette, and then inserted into 1.5 ml Eppendorf tubes (Figure 1). After centrifuged at 6000 rpm for 10 min at 4^0 C, the supernatant was taken with a micropipette and then stored at -80^0 C. Elisa procedures were then conducted based on manual kit RnD System. Then 20 µl of saliva samples was added with 80 µl of blocking buffer (0.20% Triton X-100 and 5% BSA), put into microplate polycarbonate previously been coated with Ab capturing of anti BD-2 (rat monoclonal antibody) (Santa-Cruize), and then incubated at 4^0 C for 24 hours. After then it was washed 3 times with wash solution (0.15 M NaCl + 0.05% Triton X-100 + 0.02 g NaN3 in 1 liter of distilled water).

Next, it was added with Ab detection (secondary Ab) labeled with biotin, and incubated at room temperature for 2 hours. It was then washed 3 times with wash solution (0.15 M NaCl + 0.05% Triton X-100 +0.02 g NaN3 in 1 liter of distilled water). Afterwards, each well was added with 100 µl of Ab detection (anti BD-2) labeled with HRP, and incubated at room temperature for 1 hour. It was then washed 3 times with wash solution (0.15 M NaCl + 0.05% Triton X-100 + 0.02 g NaN3 in 1 liter of distilled water). Next, it was added with 50 µl of TMB substrate (Tetra Methyl Bensidine), then incubated at room temperature for 40 minutes, and stooped by adding 1 N H2SO4. The results of optical density was read by using microplate reader (Bio-Rad Model 680) at 450 nm wavelength. Anova/

Figure 1. Collecting saliva of Wistar rat with a micropipette.

SPSS test was conducted to find the difference of salivary BD-2 level among groups.

RESULTS

The examination conducted by Elisa test was aimed to determine BD-2 level in saliva after induced with probiotics *L. reuteri* in each group as shown in Table 1. The results of Anova test showed that there was significant difference in the level of salivary BD-2 in Wistars among treatment groups (p=0.001). It can also be seen that the mean level of salivary BD-2 in the positive control group (induced with *S. mutans*) was declined compared to that in the negative control group, from 11.14 to 9.50. On the other hand, the mean level of salivary BD-2 in Group 2 (induced with probiotics *L. reuteri* for 14 days and also induced with *S. mutans* for 14 days) was increased compared to group 1 (induced with *probiotics L. reuteri* for 14 days and induced with *S. mutans* for 7 days), from 12.53 to 14.96.

Based on all of the results, it can be said that the difference of BD-2 levels was significant. BD-2 level in the negative control group was significantly different from that in the treatment group 2, whereas BD-2 level in the positive control group was significantly different from that in the treatment group 2. However, the difference of BD-2 levels among the other groups was not significant.

Table 1. Mean values and standard deviations BD-2 levels in the saliva of Wistar rats (ng/ mL) at each treatment group

Group	Mean	Standard deviation	Significant*)
Negative control	11.14	0.32	
Positive control	9.50	1.10	
Group 1	12.53	1.37	
Group 2	14.96	4.13	P = 0.001

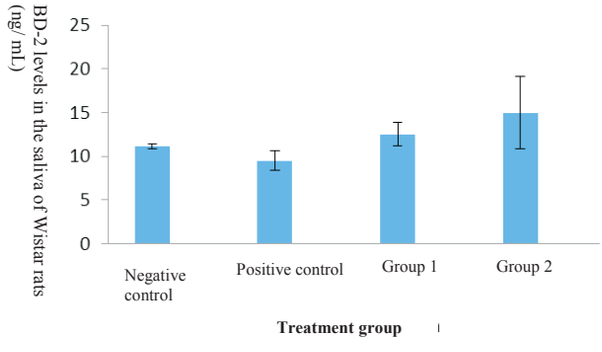

Figure 2. Mean BD-2 levels in the saliva of Wistar rats after diindukssi with probiotics *L. reuteri* in each treatment group.

DISCUSSION

BDs will universally be expressed in all epithelial cells. The epithelial cells can be found in the gingival salivary gland in the oral mucosa of the oral cavity. It is also known that Beta defensin has effective and broad antimicrobial activities against Gram positive and Gram negative bacteria, such as *S. mutans*, *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*.[5]

BDs is secreted in biological fluids, including urine, bronchial fluid, saliva and gingival crevicular fluid (GCF). This peptide shows specific expression pattern. BD-1 is expressed constitutively, while BD-2 and BD-3 expressions, which have bacterial components and inflammatory mediators, such as interleukin-1β (Il-1β), tumor necrosis factor α (TNF-α) and interleukin-17 (Il-17) can be induced by bacteria.[13] Although BD-2 is induced and expressed only as long as there is inflammation in the epithelial tissue, BD-2 can actually be expressed in healthy epithelial tissues in the oral cavity (gingival tissue is not clinically inflamed).[14]

Based on Table 1 and Figure 2, it is known that the induction of probiotic *L. reuteri* bacteria into the oral cavity of Wistar rats can increase BD-2 level since BD-2 is potentially against *S. mutans*. It is expected that the number of *S. mutans* bacteria can be reduced, then resulting the reducing of the occurrence of dental caries in rats. It is because *L. reuteri* are probiotic bacteria, which active biological molecules are on the surface of their cell wall, called microorganism-associated molecular patterns (MAMPs) such as peptydoglican (PG) and lipoteichoic acid (LTA) potentially activating surface cell receptors, namely pattern recognition receptors (PRRS) of the host.[15]

Pattern recognition receptors (PRRS), such as Toll-like receptor-2 (TLR-2) and nucleotide-binding oligomerization-domain protein-2 (NOD-2) in cytoplasm, furthermore, can recognize a variety of microbial components. Peptydoglican (PG) and lipoteichoic acid (LTA) derived from the cell wall of *L. reuteri* function as ligands of TLR-2.[16] Interaction between peptydoglican (PG) and lipoteichoic acid (LTA) with TLR-2 and NOD-2 induces a signaling cascade involving nuclear factor-kB (NF-kB) and inhibitor of NF-kB kinase (IkBK). With PG and LTA of *L. reuteri*, the phosphorylation, ubiquitination, and degradation of IkB proteins occur, which cause NF-kB translocation into the nucleus, then resulting the activation of NF-kB and activating the promoter of BD-2.

The levels of BD-1 and BD-2 in human saliva actually varies from undetectable to 39 ng/ mL for BD-1 and 33 ng/ mL for BD-2.[13] It is known that the average level of BD-2 in the saliva samples from healthy people (n = 60) is 9.5 (1.2 to 21) mg/ L.[17] Most of protein material in the saliva is produced by acini cells in the salivary gland, so beta defensin does not represent a major component of saliva. Beta defensin secreted by the salivary ducts may act locally to attack bacteria, viruses and fungi using salivary gland ducts as their invasion route.[18]

Table 2. Significance difference test between the study groups using HSD

Group	Negative control	Positive control	Group 1	Group 2
Negative control	_	0,765	0,825	0,024*
Positive control		_	0,113	0,001*
Group 1			_	0,214
Group 2				_

The inoculation of *S. mutans* in the oral cavity of those rats was aimed to make the oral cavity of those rats similar to the human oral cavity since *S. mutans* was not found in the normal oral cavity of Wistar rats. In addition, this research was also aimed to know whether *S. mutans* can induce BD-2 in saliva. Based on Table 1 and Figure 2, it is known that BD-2 level in the positive control group only induced by *S. mutans* was the lowest one. It may indicate that *S. mutans* is not strong to induce *BD-2*. It may also indicate that *S. mutans* in the oral cavity of Wistar rats can be considered as pathogenic bacteria so that the host will use BD-2 to fight the infection. Therefore, in the salivary examination of BD-2 level by using Elisa method, it is known that the mean concentration of BD-2 in the positive control group was the lowest one, about 9.50 ng/ ml, but this concentration can already be active because defensins can be active at the concentration of 1 to 10 ng/ ml. [19]

Based on Table 2, the average level of BD-2 in the negative control group was different from that in the treatment group 2 ($p = 0.024$). In other words, it is known that the average level of BD-2 in the negative control group was significantly increased compared to that in the treatment groups 2, from 11:14 ng/ mL to 14.96 ng/ mL. The increasing of BD-2 level can be considered due to the strong induction of probiotic *L. reuteri* for 14 days. Meanwhile, the level of BD-2 in the control group positive induced with *S. mutans* for 14 days was only 9.5 ng/ mL.

Based on the results of this study, it can be concluded that the administration of probiotic *L. reuteri* can increase BD-2 level in the saliva of Wistar rats inoculated with S. *mutans*. Further research is needed to know the effect of the administration of probiotic *L. reuteri* on BD-2 and BD-3 levels in preschool students to get dental caries prevention.

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