

The level of beta defensin-2 in saliva and its expression in parotid gland epithelial cells after probiotic (*Lactobacillus reuteri*) induction to inhibit *Streptococcus mutans* in caries

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ABSTRACT

Objective: The aim of this study was to prove that administrating *L. reuteri* probiotics can increase the level of BD-2 saliva and BD-2 expression in the epithelial parotid glands of Wistar rats. **Materials and Methods:** Experimental design in this study was randomized control group post test only. Twenty-four white male *Rattus norvegicus* Wistar strain rats were divided into four groups. The negative control group included rats not induced by *S. mutans* whereas the positive control group included rats induced by *S. mutans*. The two treatment groups are as follows: treatment 1 (T1), the group that is induced for 14 days by *L. reuteri* and 7 days by *S. mutans* and treatment 2 (T2), the group which is induced simultaneously by *S. mutans* and *L. reuteri* for 14 days. *L. reuteri* culture at a concentration of 108 colony-forming unit/ml and *S. mutans* culture at a concentration of 1010 are induced in the oral cavity of the Wistar rats. The Elisa technique is used to examine the salivary level of BD-2, whereas the immunohistochemical technique is used to examine the BD-2 expression in the epithelial salivary glands. **Results:** The study shows the increasing levels of BD-2 and BD-2 expression in the epithelial parotid glands after the administration of *L. reuteri* probiotics. Besides, there is a relationship between the increasing expression of BD-2 in the epithelial parotid glands with the decreasing amount of *S. mutans*. **Conclusion:** Giving *L. reuteri* probiotic scan increases the level of saliva of BD-2 and the expression of BD-2 in the parotid glands.

Key words: Beta defensin-2, caries, *Lactobacillus reuteri* probiotics, *Streptococcus mutans*

INTRODUCTION

Dental caries is a problem that is commonly found in the oral cavity, especially in children. The United States Surgeon General's publication in May 2000 described that dental caries is a chronic disease in childhood.^[1] Based on the Basic Health Research (Riskesdas) in 2007 released by the Ministry of Health, 76% of children in East Java experienced dental caries, whereas in

the Department of Health (Dinkes) of Surabaya, 4359 students among 61,214 students have oral cavities.^[2,3]

Even though the prevention of dental caries has been done, there is no effective way to control it in children. Etiology of dental caries was very multifactorial. The etiology of dental caries was multifactorial.

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There are three main factors that cause dental caries, carbohydrate diet (especially sucrose), *Streptococcus mutans* infection, and specific response from the host (innate immunity).^[4] Innate immunity in the oral cavity is the part of immune system that serves as a front-line defense against pathogens. One of the innate immunities that has an important contribution to keep the body balance is antimicrobial peptides (AMPs). AMP that was first identified in oral cavity epithelium is beta defensins (BDs). These peptides have a wide range of antimicrobial activity capable of against Gram-positive and Gram-negative bacteria as well as against fungi and viruses.^[5]

One of the caries prevention approaches that has been focused on recently is probiotics consumption. According to the WHO, probiotics are living microorganisms which will give benefits for health when consumed in sufficient amount.^[6] Probiotics are commensal bacteria, and it is an excellent inducer for BD-2 in oral cavity epithelial cells.^[7] The aims of this research were to determine the effects of *Lactobacillus reuteri* probiotics induction on BD-2 level on saliva and its expression in parotid gland epithelial cells to reduce *S. mutans* as the cause of dental caries.

MATERIALS AND METHODS

Research design and animal model

Ethical clearance of this research was obtained from the Commission of Ethical Feasibility of Health Research, Faculty of Dentistry, Universitas Airlangga. Experimental design in this study was randomized control group post test only. Wistar Rats are used as animal models in caries research.^[8] Twenty-four male white rats (*Rattus norvegicus* Wistar strain) were divided into four groups: Negative control group, positive control group, treatment group 1 (T1), and treatment group 2 (T2). Negative control group was not induced either with *S. mutans* or *L. reuteri* and positive control group was induced with *S. mutans* for 14 days. T1 was induced with *L. reuteri* for 14 days and *S. mutans* for 7 days, whereas T2 was induced with *S. mutans* and *L. reuteri* simultaneously for 14 days. The analysis unit in this study was saliva and parotid gland epithelial cells tissue. *L. reuteri* concentration that has been used as inducer was 4×10^8 colony-forming unit (CFU)/ml^[9] while the concentration of *S. mutans* was 10^{10} CFU/ml.^[10] On the 15th day, rats' saliva was taken before they were dissected. Rats' saliva production was stimulated by the administration of 1 cc of ketamine HCl (100 mg/cc) and 1 cc of diazepam (Sutezolid) (5 mg/cc) that were

injected intramuscularly through the thigh.^[11] Saliva was taken with micropipette after 2–3 of stimulation and then placed into Eppendorf column for about 100 µl. Centrifugation was done at 6000 rpm for 10 min at 40°C, and supernatants were collected with micropipette and then stored at –80°C–800°C of temperature for Elisa preparation.

Elisa procedures were then conducted based on manual kit RnD system. Then, 20 µl of saliva samples were added with 80 µl 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-Cruz), and then incubated at 4°C for 24 h. After that, it was washed three times with wash solution (0.15 M NaCl + 0.05% Triton X-100 + 0.02 g NaN₃ in 1 L of distilled water). Next, it was added with Ab detection (secondary Ab) labeled with biotin and incubated at room temperature for 2 h. It was then washed three times with wash solution. Afterward, each well was added with 100 µl of Ab detection (anti-BD-2) labeled with horseradish peroxidase and incubated at room temperature for 1 h. It was then washed three times with wash solution. Next, it was added with 50 µl of tetramethylbenzidine substrate, then incubated at room temperature for 40 min, and stopped by adding 1 N H₂SO₄. The results of optical density were read by using microplate reader (Bio-Rad Model 680) at 450 nm wavelength.

The parotid glands were taken out and prepared on paraffin blocks for immunohistochemistry. ANOVA/SPSS (Developed by IBM Corp., Armonk, New York, USA) test was conducted to determine the differences between BD-2 saliva level and BD-2 expression in parotid gland epithelial cells among the groups.

The preparation of *Lactobacillus reuteri* suspension

L. reuteri ProDentis starter contained in tablet x (DSM 17938 + ATCC PTA 5289) was cultured in brain heart infusion (BHI) broth and then incubated for 24 h at 37°C anaerobically in the gas-generating kit (Oxoid). Bacteria cell mass will appear as white sediment in the tube. Bacteria were then cultured into MRS medium agar (DeMan-Rogosa-Sharpe; Merck GmbH, Darmstadt, Germany) by streak method and incubated for 2 × 24 h at 37°C. Bacterial single-cell colony was taken and reincubated into BHI medium for 24 h at 37°C to obtain OD = 10^8 cfu/ml (λ = 625) nm.^[12]

The preparation of *Streptococcus mutans* suspension

S. mutans serotype c was cultured from freeze-dried stock. Bacterial colony was taken with inoculating

loop, cultured into BHI broth, and then incubated for 24 h at 37°C. The cultured bacteria were streaked into blood agar medium and incubated for 24 h at 37°C. Bacterial single-cell colony was taken and reincubated into BHI medium for 24 h at 37°C to obtain OD = 10^{10} cfu/ml ($\lambda = 625$) nm.^[12]

Examination of beta defensin-2 expressions

Observation of BD-2 expression using immunohistochemistry and immunohistochemical staining was done based on previous research.^[13,14]

RESULTS

Beta defensin-2 levels in rats' saliva

Elisa test was conducted to determine the levels of BD-2 in saliva after *L. reuteri* probiotics induction [Table 1]. The levels of BD-2 in the Wistar rats saliva among the treatment groups are significantly different ($P = 0.00$). BD-2 level in saliva of positive control group (induced by *S. mutans*) decreased compared to negative control group (11.14–9.50). Otherwise, BD-2 level in the saliva of the second group (T2) (induced with *L. reuteri* and *S. mutans* for 14 days) increased compared to T1 (12.53–14.96) [Figure 1].

Beta defensin-2 expression in parotid gland epithelial cells

The examination was conducted through immunohistochemistry method. The distribution of BD-2-expressing cells in parotid gland cells in each group after *L. reuteri* probiotics induction is shown in Table 2.

BD-2 expression in parotid gland epithelial cells was significantly different among the treatment groups ($P = 0.001$). Average BD-2 expression of negative control group decreased compared to positive control group (10.00–5.60) whereas this expression in T2 increased when compared to T1 (24.43–33.71) [Figure 1].

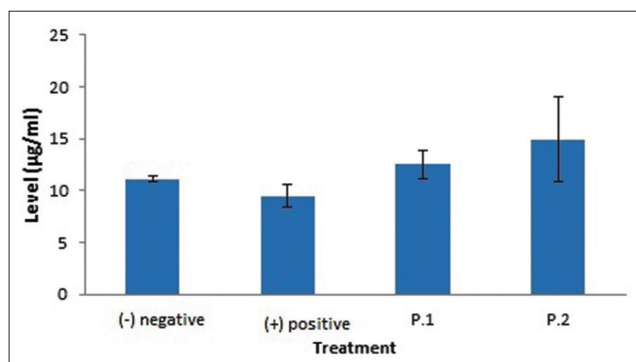


Figure 1: Mean values and standard deviations of beta defensin-2 levels in the saliva of Wistar rats after being induced with *Lactobacillus reuteri* probiotics in each treatment group

DISCUSSION

BDs play an important role in the oral cavity; they are the first defense against bacterial infection. BDs consist of AMPs in the form of small cations. There are three BDs; BD-1, BD-2, and BD-3. They can be detected in salivary glands, gingival, tongue, and buccal mucosa. BDs have a bactericidal activity that can damage bacterial cell membranes. Expressions of BD-2 and BD-3 in oral mucosal epithelial cells are influenced by the existence of bacterial components or inflammatory mediators. Two main species of bacteria that cause dental caries are *S. mutans* and *Streptococcus sobrinus*, which are sensitive to BD-2.^[15] Induction of *L. reuteri* into oral cavity increased the expression of BD-2. It reduces the number of *S. mutans* bacterial population related to dental caries.

BDs expression in salivary glands is a defense mechanism in the oral cavity against infection. BD-2 is a potent antimicrobial peptide effective against a broad spectrum of bacteria, including Gram-positive bacteria, Gram-negative bacteria and fungi. *S. mutans*, *Lactobacillus acidophilus*, *Actinobacillus actinomycetemcomitans*, and *Porphyromonas gingivalis* are bacteria sensitive to BD-2 and are the members of periodontal bacteria.^[16]

The average concentration of defensins in epithelial cells reached 10–100 µg/ml. Peptides' distribution was not equal, so the local concentrations become higher.^[17] Table 1 shows that the lowest concentration of BD-2 saliva was in the positive control group. The host will utilize BD-2 against *S. mutans* as the cause of caries infection. The average BD-2 level examined with Elisa

Table 1: Beta defensin-2 levels in saliva of Wistar rats in each treatment group

Group	Average	SD	Significance*
Negative control	11.14	0.32	P=0.001
Positive control	9.50	1.10	
Treatment Group 1 (T1)	12.53	1.37	
Treatment Group 2 (T2)	14.96	4.13	
SD: Standard deviation			

Table 2: Beta defensin-2 expression in parotid gland epithelial cells

Group	Average	Mean deviation	Significance*
Negative control	10.00	2.12	$P=0.001$
Positive control	5.60	1.67	
Treatment Group 1 (T1)	24.43	3.10	
Treatment Group 2 (T2)	33.71	4.15	

of the positive control group was 9.50 µg/ml. This is the lowest concentration needed for activation. This is the lowest concentration needed for activation, because defensin will be active on 1-10 µg/ml.^[18] Almost all BDs in the oral cavity are expressed by keratinocytes inside the mouth and ductal cells of salivary glands. It supports this study results as shown in Table 2 and Figure 2 which indicate that the highest expression of BD-2 occurred in Group 2. The highest levels of BD-2 also occurred in Group 2 [Table 1 and Figure 1]. Most of the proteins in saliva are produced by salivary gland acini cells. BD secreted from salivary ducts may attack bacteria, viruses, and fungi locally.^[19]

This study proved that *L. reuteri* administration can increase BD-2 expression in epithelial parotid gland [Table 2 and Figure 2]. Parotid gland epithelial cells protect the host not only by providing a physical barrier but also through innate immune responses in the form of antimicrobial peptides.

Table 2 and Figure 2 show that the number of BD-2 expressions in positive control groups is the lowest compared to the other groups. The positive control group is the only group that was induced with *S. mutans*. It can be explained that *S. mutans* was not able to induce BD-2 expression in parotid gland epithelial cells. The research conducted by Schlee *et al.* showed that *Escherichia coli* Nissle 1917 strain can induce BD-2 expression, while the other forty *E. coli* isolates cannot induce it. Isolation and purification results of flagellin protein in *E. coli* Nissle 1917 were able to induce BD-2 expressions, while flagellin-deficient mutants of these bacteria were not able to induce defensins. Flagellin is an MAMP-determining factor in *E. coli* Nissle 1917 probiotic which can remarkably stimulate BD-2 expression.^[20]

The induction of *L. reuteri* probiotics for 14 days with a concentration of 4×10^8 CFU/ml can increase the expression of BD-2 significantly ($P = 0.001$)

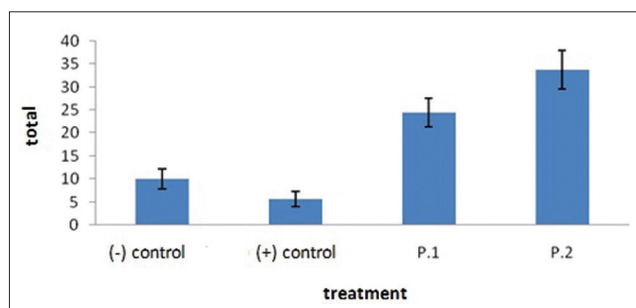


Figure 2: The mean and standard deviations of number of cells of beta defensin-2 expression in the epithelial parotid glands after each group is being induced by *Lactobacillus reuteri* probiotics

[Table 2 and Figures 2, 3]. It indicates that BD-2 expression in epithelial cells of the parotid gland in Group 2 (T2) shows the highest BD-2 expression compared to the other groups. This can be expected that active molecules on *L. reuteri* cell wall and peptidoglycan will activate NOD-2 receptors in the cytoplasm. The signaling activation through Nod2 pathway involves RICK adapter proteins. The signaling activation through Nod2 pathway involves RICK adapter protein and will be activated to phosphorylate IKK complex. Nuclear factor-κB (NF-κB) in inactive form will bound to IκBa protein inhibitor in the cytoplasm. The stimuli will induce phosphorylation, ubiquitination, and degradation of IκB proteins which will lead to NF-κB translocation into the nucleus cores, and then NF-κB becomes active. The stimuli will induce phosphorylation, ubiquitination and degradation of IKB protein which will lead to NF-κB translocation into the nucleus cores, and then NF-κB becomes active to stimulate the target genes.^[21]

CONCLUSION

It can be concluded that the induction of *L. reuteri* probiotics can increase BD-2 level in saliva and its expression in parotid gland epithelial cells.

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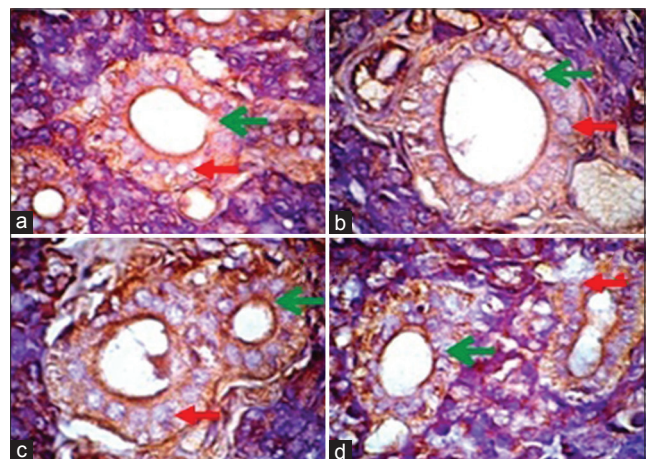


Figure 3: Results of immunohistochemical examination showing beta defensin-2 expression in the epithelial parotid gland in each group magnified (×1000). Green arrow showing cells expressing beta defensin-2. Red arrow showing cells that do not express beta defensin-2. Description: (a) Negative control, (b) positive control, (c) treatment Group 1 (P. 1), (d) treatment Group 2 (P. 2)

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Nil.

Conflicts of interest

There are no conflicts of interest.

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