Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2016 March; 49(1): 50–54

Research Report

Beta-defensins-2 expressions in gingival epithelium cells after probiotic *Lactobacillus reuteri* induction

Tuti Kusumaningsih
Department of Oral Biology
Faculty of Dental Medicine, Universitas Airlangga
Surabaya-Indonesia

ABSTRACT

Background: *Beta-defensins (BD) are antimicrobial peptides that play a role in defense against pathogens. Beta-defensins (BD) are expressed by a variety of epithelial cells, including gingival epithelium, salivary glands, saliva and salivary duct. BD-1 is expressed constitutively, while BD-2 and BD-3 expressions can be induced by commensal bacteria. Probiotics are commensal bacteria, thus L. reuteri as probiotic bacteria may act as "inducer" for BD-2 in epithelial gingiva. S. mutans is the main bacteria causing dental caries and sensitive to BD-2.* ***Purpose:*** *This study was aimed to prove that the administration of probiotic L. reuteri may improve BD-2 expressions in the gingiva epithelium.* ***Method:*** *This study was conducted in vivo using twenty-four male Rattus norvegicus Wistar strains aged 10-12 weeks and weighed 120-150 g. Those rats were randomly divided into four groups, namely negative control group (not induced with L. reuteri or S. mutans), positive control group (induced with S. mutans for 14 days), treatment group 1 (induced with L. reuteri for 14 days and S. mutans for 7 days), and treatment group 2 (induced with L. reuteri and S. mutans for 14 days concurrently). The concentration of L. reuteri used was $4x10^8$cfu/ml, while the concentration of S. mutans was 1x 10^{10}cfu/ml. 0.1 ml of each was dropped in the region of the mandibular incisors. BD-2 expression was calculated using immunohistochemical method. The difference of BD-2 expressions in gingival epithelial cells in the respective groups was analyzed by Anova/SPSS.* ***Results:*** *There were significant differences in BD-2 expressions in gingival epithelial cells in each group based on the results of Anova test (p=0.001).* ***Conclusion:*** *The administration of probiotic L. reuteri is able to increase BD-2 expressions in gingival epithelial cells.*

Keywords: *beta defensins-2 expression; gingival epithelium; probiotic; L. reuteri; S. mutans*

Corespondence: Tuti Kusumaningsih, Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga. Jl. Mayjend. Prof. Dr. Moestopo no. 47 Surabaya 60132, Indonesia. E-mail: tutikusumaningsih@yahoo.com

INTRODUCTION

Gingival epithelium is a defense against bacteria in oral cavity, not only physically but also chemically. Defense function of the gingival epithelium is characterized by their unique structure and integrity of the anti-microbial peptide (AMP), such as human beta defensins (hBDs).[1] Defensins are antimicrobial peptides first discovered in mammals. Defensins in humans consist of two sub-families, namely alpha and beta-defensins.

Alpha-defensins are produced by polymorphonuclear leukocytes and panet cells, while beta-defensins are produced by epithelial surface of skin, intestine, trahea, and oral cavity.[2] Defensins have broad activities against bacteria, fungi, and viruses. In the optimal conditions, antimicrobial activities of defensins work at low concentration of 1-10mg /mL.[3] Human beta-defensins (hBDs) are widely expressed in tissues of the oral cavity, including gingival epithelium, salivary glands, saliva and salivary duct. These peptides are involved in defense against bacteria that colonizes in the oral cavity.

Defensins in the oral cavity have an important role to protect the structure of the teeth from bacteria causing caries. Human beta-defensins have broad antimicrobial activity against oral microorganisms, such as *Streptococcus mutans, Porphyromonas gingivalis,* and *Actinobacillus actinomycetemcomitans*.[3] Changes in lifestyle and diet can affect the composition and amount of the normal flora in someone's oral cavity. Several factors causing the

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 56/DIKTI/Kep./2012.

DOI: 10.20473/j.djmkg.v49.i1.p49-53

occurrence of dental caries are physical factors, biological factors, environmental factors, habits, and life style.[4]

Dental caries is an example of a disease that one of causes is too often consuming sucrose being offset by the improper process of oral cavity cleaning. Dental caries is an infectious disease caused by an imbalance of homeostasis between host and microbe.[5] *Streptococcus mutans (S. mutans)* and *Streptococcus sobrinus (S. sobrinus)* are the main causes of dental caries.[5-7] Excessive amount of *S. mutans* in the oral cavity can be considered as an early occurrence of dental caries.

Recently, there have been many outstanding supplement products containing probiotics. According to the WHO / FAO, probiotics are life micro-organisms which, when administered in adequate amount, Confer a health benefit on the host. Meanwhile, International Life Science Institute (ILSI) Europe defines probiotics as a life of microbial food ingredient that, when ingested in sufficient quantities, exerts health benefits on the consumer.[8] Based on both definitions, it can be concluded that probiotics are live microorganisms causing an effect on health. One of the many probiotics often used in dentistry is *Lactobacillus reuteri (L. reuteri).* Probiotics including *L. reuteri* are commensal bacteria. Commensal bacteria are excellent inducers to BD-2 in epithelial cells of the oral cavity, so it is possible for probiotic bacteria to act as an inducer for BD-2.[9] There are two species of bacteria causing dental caries, namely *S. mutans* and *S. sobrinus*, sensitive to BD-2.[10]

Thus, it indicates that BD-2 can be used as an alternative material for preventive and therapeutic potential against dental caries. Therefore, this research was aimed to determine whether the administration of probiotic *L. reuteri,* induced into gingival mice could increase BD-2 expressions in the gingival epithelium.

MATERIALS AND METHOD

This research was approved by Komisi Kelaikan Etik Penelitian Kesehatan-KKEPK (the Commission on Health Research Ethics Airworthiness), Faculty of Dental Medicine, Universitas Airlangga.

Twenty-four male rats (*Rattus norvegicus* wistar strain) aged 10-12 weeks with body weight of 120-150 grams were divided into four groups, namely negative control group, positive control group, treatment group 1, and treatment group 2. There were five rats in the negative control group, which were not induced by *S. mutans* or *L. reuteri*. In the positive control group, there were five rats induced with *S. mutans* for 14 days. In the treatment group 1, furthermore, there were seven rats induced with *L. reuteri* from day 1 to day 14 (14 days), and *S. mutans* from day 8 to day 14 (7 days). On the other hand, in the treatment group 2, there were seven rats induced with *L. reuteri* and *S. mutans* from day 1 to day 14 simultaneously. On day 15, those rats were sacrificed, and then their jaws and gingival tissue were cut for the preparation of paraffin blocks for immunohistochemical examination (IHC).

The concentration of the bacterial suspension used was 4 x 10^8 cfu/ml of *L. reuteri*, and 1 x 10^{10} cfu / ml of *S. mutans*.[9] DSM 17 938 + ATCC PTA 5289 was added to brain heart infussion (BHI) liquid, and then incubated in anaerobic condition for 1x24 hours using gas generating kit with Oxoid brand. After removing from the incubator, turbidity and sediment will appear, indicating *L. reuteri* growth. Planting to MRS agar (De Man, ROGOSA and Sharpe; Merck GmbH, Damrstadt, Germany) was performed, and then incubated for 2x24 at a temperature of 37^0 C. After that, several colonies were taken and planted into a liquid BHI medium, and then incubated for1 x 24 hours at a temperature of 37^0 C. After removing from the incubator, bacterial density were observed to know whether it had reached 4 x 10^8cfu / ml, and then examined using spectrophotometry with a wavelength lambda of 625 nm and an optical density of 0.08- 0.10 identical to the Mc Farland 0.5.[9]

S. mutans used in this research were *S. mutans* serotype c taken from the stock in the form of freeze dry. *S. mutans* were taken about 1 ose, added to liquid BHI medium, and then incubated for 1 x 24 hours at a temperature of 37^0C. After removing from the incubator, turbidity and sediment will appear, indicating *S. mutans* growth. Planting to a blood agar medium was performed, and then incubated for 1x24 at a temperature of 37^0 C. After that, several colonies were taken and planted into a liquid BHI medium, and then incubated for1 x 24 hours. After removing from the incubator, bacterial density were observed to know whether it had reached 1 x 10^{10} cfu / ml, and then examined using spectrophotometry with a wavelength lambda of 625 nm and an optical density of 0.08- 0.10 identical to the Mc Farland 0.5.[9]

Examination of beta-defensin-2 (BD-2) expressions was performed. There are several preparation stages of histopathology conducted in this research as stated in Humason's method cited in Sudiana, 2005. Calculating method of the results of immunohistochemical staining, according to Soini et al.[11,12] consists of several stages. First, gingival tissue was fixated in paraformaldehid 4% for 4 hours at a temperature of 4^0C. After washed in PBS, the tissue was planted in OCT compound, and then immediately frozen. Then the tissue was cut into a thickness of 6 µm, and then incubated with primary antibody anti BD-2 (Lab Vision) for 24 hours at a temperature 4^0C. The tissue sections were incubated with secondary antibody rat monoclonal, anti OPG (Lab Vision), and HRP kid for 24 hours at a temperature 4^0C. Chromogenics used in this research were 3.3 - diaminobenzidine tetrahydrochloride, and then counterstained with HE.[9] Immunohistochemical staining with indirect method was carried out to show cells expressing BD-2 protein. The cells expressing BD-2 were shown with a brown cytoplasm, and then observed with a light microscope with a magnification of 400 x.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 56/DIKTI/Kep./2012.

DOI: 10.20473/j.djmkg.v49.i1.p49-53

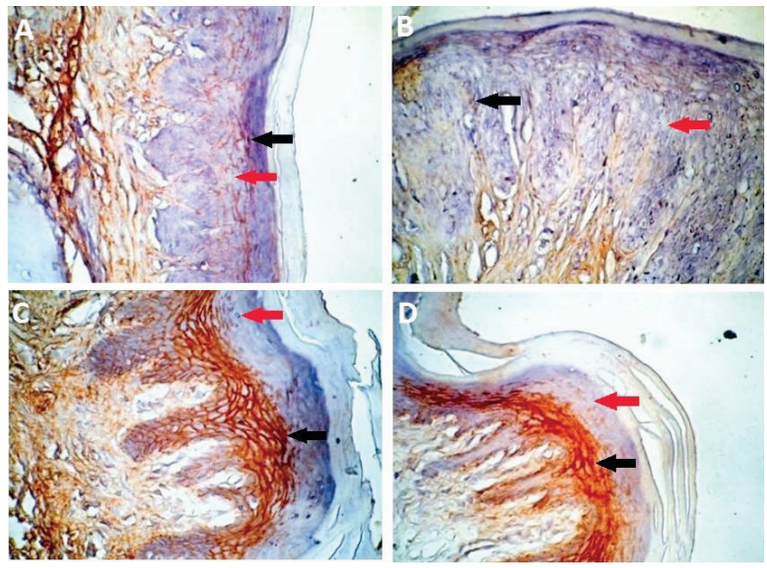

Figure 1. Immunohistochemical examination results of BD-2 expressions in the gingival epithelium in each group (400x magnification); Black arrow (cells expressing BD-2); Red arrows (cells not expressing BD-2). A) BD-2 expressions in the gingival epithelium in the negative control group; B) BD-2 expressions in the gingival epithelium in the positive control group; C) BD-2 expressions in the gingival epithelium in the treatment group 1; D) BD-2 expressions in the gingival epithelium in the treatment group 2.

Anova test, was performed to determine the differences in *BD-2* expressions in the gingival epithelium of each group. HSD test was conducted to determine the significance of the differences between groups.

RESULTS

Immunohistochemical examination was performed to determine the distribution of cells expressing BD-2 in the gingival epithelium after induced with probiotics, *L. reuteri* bacteria, in each group (Table 1).

Based on Table 1, the results show that there was a significant difference of BD-2 expression in the gingival epithelium of *Wistar rats* among the treatment groups ($p = 0.001$). A decline in the mean of BD-2 expressions in the gingival epithelium was found in the positive control group (group induced by *S. mutans*), compared to the negative control group, namely from 15.80 into 4.80. An increase in the mean of BD-2 expressions in gingival epithelium was found in the treatment group 2 (induced by probiotic *L. reuteri* for 14 days and *S. mutans* for 14 days), compared to the treatment group 1 (induced with probiotic *L. reuteri* for 14 day and *S. mutans* for 7 days), namely from 24.00 into 27.00

The overall BD-2 expressions were significantly different among the groups. Based on the results of HSD test, it is known that there was a significant difference in BD-2 expressions between the negative control group and the positive control group. There were also significant differences in BD-2 expressions between the negative control group and the treatment group 1 as well as between the negative control group and the treatment group 2. Similarly, there were also significant differences in BD-2 expressions between the positive control group and the treatment group 1 as well as between the positive control group and the treatment group 2. However, there was no significant difference between the treatment group 1 and the treatment group 2.

DISCUSSION

The administration of *L. reuteri* as probiotics in this research could increase the expressions of BD-2 at gingival epithelial cells (Table 1 and Figure 1). Gingival epithelium is stratified squamous (flat stratified epithelium) which serves as a defense against pathogenic bacteria. Epithelial tissues in the oral cavity are continuously in contact with various kinds of bacteria, even so most individuals can maintain the balance of their health. Epithelial tissues of the oral cavity play a role in protecting the host not only with the physical defense, but also through the *innate immune responses* in the form of antimicrobial peptides. BDs are small antimicrobial peptides, which form cautions produced by epithelial cells playing an important role in mucosal defense and skin.[13] Based on Figure 2, the results of immunohistochemical examination for BD-2 expressions in the gingival epithelium showed that BD-2 is expressed in stratum spinosum and stratum granulosum. The results is in line with the results of a research conducted by Weinberg et al.,[14] stating that within the gingival tissue, *mRNA* from

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 56/DIKTI/Kep./2012.

DOI: 10.20473/j.djmkg.v49.i1.p49-53

Table 1. The mean and standard deviation of BD-2 expressions in gingival epithelium after induced with Probiotic *L. reuteri* in each group.

Group	Mean	Standard Deviation	Significance *)
Negative control	15.80	2.28	
Positive control	4.80	1.30	
Treatment group 1	24.00	2.94	
Treatment group 2	27.00	2.58	P=0.001

Table 2. The results of HSD test among the groups

Group	Negative control	Positive control	Treatment group 1	Treatment group 2
Negative control	_	0.000*	0.000*	0.000*
Positive control		_	0.000*	0.000*
Treatment group1			_	0.334
Treatment group 2				_

SW-1 and SW-2 is located in the suprabasal stratified epithelium, while peptide BD-2 is detected in the upper epithelial layers.[15]

Gingiva, hard palate, and the dorsal part of the tongue is the keratinized epithelium of the oral cavity, while the floor of the mouth and the buccal area are not keratinized epithelium. The results of immunohistochemical examination conducted to observe BD-2 expressions in the keratinized epithelial in oral cavity (including gingiva) showed a weak positive result, while in the non-keratinized epithelial showed a negative result. This situation suggests that keratinization in the epithelium of the oral cavity plays an important role for the retention of peptides. [14] The results found in the negative control group in this research can be associated to the results found in normal epithelium condition as stated by Abiko Y *et al*. since the group was not treated. BD-2 expressions in the gingival epithelium in the negative control group were 15.80, while BD-2 expressions in the treatment group 1were 24.00, and 127.00 in the treatment group 2 (Table 1). It indicates that BD-2 expressions increased after the induction of probiotic bacteria, namely *L. reuteri* and *S. mutans* bacteria.

The induction of probiotics in the treatment group 1 was assumed as a precaution. In this treatment group, *L. reuteri* bacteria were first induced from day 1 to day 14, whereas *S. mutans* bacteria causing caries were induced from day 8 to day 14. On the other hand, the induction of probiotics in the treatment group 2 was assumed as a therapeutic action. In the treatment group 2, *L. reuteri* and *S. mutans* were administered together from day 1 to day 14.

In Table 1, the number of gingival epithelial cells expressing *BD-2* in the negative control group the group not induce *L. reuteri* and *S. mutans* bacteria was lower than in the treatment groups. This condition showed that BD-2 expressions were still expressed both at the gingival epithelium in normal circumstances and at the gingival epithelium in inflammation circumstances. In contrast, in most other epithelia, such as skin epithelium, tracheal epithelium, and intestinal epithelium, BD-2 is expressed only in inflammation circumstances. [16] This statement is supported by the results of a research conducted by Whasun & Dale, stating that in most tissues, BD-2 is induced and expressed only in inflammation circumstances, but in epithelial tissues of the oral cavity, BD-2 is expressed only in normal circumstances (without inflammation) since the oral mucosa is continuously exposed to a variety of bacteria.[17]

The lowest number of BD-2 expressions was found in the positive control group (Table 1). The positive control group is a group only induced with *S, mutans*. Thus, it can be said that *S. mutans* is not able to induce BD-2. A research conducted by Wehkamp et al showed that *E. coli Nissle 1917* strains can induce BD-2, while the other 40 isolates of *E. coli* cannot induce BD-2. The results of the isolation and purification of flagellant protein derived from *E. coli Nissle 1917* are able to induce BD-2, while *Flagellin deficient mutans* derived from the bacteria are not able to induce defensins. LPS of *E. coli Nissle 1917* even cannot stimulate the production of *BD-2*. Therefore, it indicates that flagellant protein is decisive *MAMP* in *E. coli Nissle 1917* probiotics that can stimulate BD-2 excessively.[17]

Based on these results, it can be said that the administration of probiotics, *L. reuteri* bacteria, for 14 days with a concentration $4x10^8$CFU/ml could increase BD-2 expression significantly (p=0.001). This result can be seen in Table 1 and Figure 2 which show that the number of BD-2 expressions found in gingival epithelial cells in the treatment group 2 induced with probiotics, *L. reuteri* bacteria, for 14 days, was the highest one. The reason is that the active molecule on the cell wall of the probiotic bacteria, peptidoglycan, activates NOD-2 receptors in the cytoplasm. Activation of signaling through NOD-2 can trigger a recruitment of protein adaptor (RICK). RICK then will be activated, resulting in phosphorylating complex IKK. NF-kB is in the inactive form, which binds to IKB, inhibitor protein in the cytoplasm. This stimulus will result in phosphorylation, ubiquitination, and degradation of IKB proteins that will cause the translocation of NF-kB into the nucleus and the activation of NF-kB.[18,19] It will activate target genes for synthesis of BD-2 protein. In conclusion, probiotics, *L. reuteri,* can improve BD-2 expressions in the epithelial cells of the gingiva.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 56/DIKTI/Kep./2012.

DOI: 10.20473/j.djmkg.v49.i1.p49-53

REFERENCES

1. Ji S, Shin JE, Kim YS, Oh JE, Min BM, Choi Y. Toll-like receptor 2 and NALP2 mediate induction of human beta-defensins by fusobacterium nucleatum in gingival epithelial cells. Infect Immun 2009; 77(3): 1044-52.
2. Gomes PS, Fernandes MH. Defensin in the oral cavity: distribution and biological role. J Oral Pathol Med 2010; 39(1): 1-9.
3. Barrera GJ, Tortolero GS, Rivas A, Flores C, Gonzales JE. Increased expression and levels of human β defensins (hBD2 and hBD3) in adults with dental caries. J Health Sci 2013; 3(2): 88-97.
4. Karpiński TM, Szkaradkiewicz AK. Microbiology of dental caries. J Biol Earth Sci 2013; 3(1): M21-4.
5. Negrini TC, Duque C, Hofling JF, Goncalves RB. Fundamental mechanisms of immune response to oral bacteria and the main perspectives of a vaccine against dental caries: A brief review. Rev. odonto cienc 2009; 24(2): 198-204.
6. Forssten SD, Bjorklund M, Ouwehand AC. Streptococcus mutans, caries and stimulation models. Nutriens 2010; 2(3): 290-8.
7. Li L, Guo L, Lux R, Eckert R, Yarbrough D, et al. Targeted antimicrobial therapy against Streptococcus mutans establishes protective non-cariogenic oral biofilm and reduces subsequent infection. Int J Oral Sci 2010; 2(2): 66-73.
8. Bhardwaj A, Bhardwaj SV. Role of probiotics in dental caries and periodontal disease. Arch Clin Exp Surg 2012; 1(1): 45-9.
9. Kusumaningsih T. The increasing of beta-defensin-2 level in saliva after probiotic *Lactobacillus reuteri* administration. Dent J (Majalah Kedokteran Gigi) 2015; 48(1): 31-4.
10. Nishimura E, Eto A, Kato M, Hashizume S, Imai S, Nisizawa T, Hanada N. Oral streptococci exhibit diverse susceptibility to human β-defensin-2: Antimicrobial effects of hBD-2 on oral streptococci. Curr Microbiol 2004; 48(2): 85-7.
11. Soini Y, Paakko P, Lehto VP. Histopathological evaluation of apoptosis in cancer. Am J Pathol 1998; 153(4): 1041-51.
12. Sudiana K. Tehnologi Ilmu Jaringan dan Imunohistokimia. Jakarta: Sagung Seto; 2005. p. 4-20.
13. Chung WO, Dale BA. Innate immune response of oral and foreskin keratinocytes: Utilization of different signaling pathways by various bacterial species. Infection and Immunity 2004; 72(1) : 352-8
14. Weinberg A, Jin G, Sieg S, McCormick TS. The yin and yang of human Beta-defensins in health and disease. Front Immunol 2012; 3: 294.
15. Abiko Y, Nishimura M, Kaku T. Defensin in saliva and the salivary glands. Med Electron Microsc 2003; 36(4): 247-52.
16. Reddy S, Sumanthi J, Anuradha Ch, Shekar Ch, Chandra K, Reddy BVR. Oral Defensin - The Antimicrobial Polypeptides. J Orofac Sci 2010; 2(2): 64-70.
17. Schlee M, Wehkamp J, Altenhoefer A, Oelschlaeger TA, Stange EF, Fellermann K. Induction of human beta-defensin 2 by the probiotic Escherichia coli Nissle 1917 is mediated through flagellin. Infect Immun 2007; 75: 2399-407.
18. Tripathi P, Aggarwal A. NF-kB transcription factor : a key player in the generation of immune response. Curr Sci 2006; 90(4): 519-31.
19. Carpenter S, O'Neill LA. How important are Toll-like receptors for antimicrobial responses? Cell Microbiol. 2007; 9(8): 1891-901.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 56/DIKTI/Kep./2012.

DOI: 10.20473/j.djmkg.v49.i1.p49-53