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Original Article

THE EXPRESSION OF TLR-2 AND NOD-2 IN GINGIVAL EPITHELIUM OF RAT AFTER PROBIOTIC *Lactobacillus reuteri* SUPPLEMENTATION TO INHIBIT *Streptococcus mutans* GROWTH

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ABSTRACT

Lactobacillus reuteri is probiotic from Gram positive bacteria which has specific molecular structure, consisted of peptidoglycan (PG) and lipotheihoic acid (LTA). These structure have potential in pattern recognition receptors (PRRs) activation, such as TLR-2 and NOD-2 that are the up stream of beta defensin-2 (BD-2) signaling cascade. BD-2 is antimocrobial peptides naturally produced in mouth cavity that can against *Streptococcus mutans* effectively. This study was aimed to prove that probiotic *L. reuteri* supplementation can increase the expression of TLR-2 and NOD-2 in gingival epithelium. Experimental design in this study was randomized control group post test only design. Study was carried on 24 white rat (*Rattus norvegicus*) Wistar strain which divided into 4 groups. Positive control was rats that induced with *S. mutans*, while rats in negative control group were not induced. Group I was rats that suppelemented with *L. reuteri* for 14 days (day 1-14) and induced with *S. mutans* for 7 days (day 8-14). Group II was rats that supplemented with *L. reuteri* and induced with *S. mutans* simustaneously for 14 days (day 1-14). Concentration of bacterial suspension was 10^8 cfu/ml for *L. reuteri* and 10^{10} cfu/ml for *S. mutans*. Both of these two bacteria was given orally to rats. TLR-2 and NOD-2 expressions were evaluated with immunohistochemistry technique. Significant differences of protein expression between each treatment groud was analyzed with ANOVA (p=0.001). TLR-2 nd NOD-2 expressions were higher than negative control. It can be conclude that *L. reuteri* supplementation as probiotic could increase the expression of TLR-2 and NOD-2 in gingival epithelium of rat.

Keywords: *Probiotic, L. reuteri, TLR-2, NOD-2, caries, S. mutans*

INTRODUCTION

Dental caries remain as national issue in Indonesia nowdays. According to National Basic Health Research institution (Riskesdas) of Indonesia Ministry of Health, 76% of children in East Java have been diagnosed with dental caries. There were several efforts have been done in order to prevent dental caries such as chemoprophylactic agents utilization, dental health education (DHE), and vaccine formulation in animal models (Fu Chen and Dong Wang, 2010). However, there were no effective resulted from these efforts.

Bacteria that form biofilm in dental plaque become more resistence, including to flour and chlorhexidine (Scheie, 2004). This condition has encouraged people prefer natural treatment such as probiotic consumption for balancing the normal flora inside body. World Health Organization (WHO) defined probiotic as live microorganism, which, when administered in adequate amounts, confer a health benefit to the host (Wallace et al., 2011).

The etiology of dental caries was multifactorial. There are 3 main factors that cause dental caries, carbohydrate diet (esspecially sucrose), *S. mutans* infection, and specific response from host (innate immunity) (Tao et al., 2005). One of component of innate immunity is antimicrobial peptides (AMP) that keep body in homeostasis (Abbas et al., 2010). The first identified antimicrobial peptide in oral cavity is beta defensin (BD) that can be found as small peptide in gingival epithelium, buccal mucosa, saliva gland, saliva ductus, and saliva (Dale et al., 2006; Gursoy and Kononen, 2012). There are 3 kind of beta defensin (BD) that secreted by oral keratinocyte in different conditions. Beta defensin-1 (BD-1) will expressed constitutively in by oral keratinocytes, while BD-2 and BD-3 are expressed when there is stimulation by bacterial antigen product and proinflamatory stimuli such as interleukin Iβ (IL-

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1β),tumor necrosis factor α (TNF- α) and interferon-γ (IFN-γ) (Yoshihiro et al., 2003).

L. reuteri is commensal bacteria which means it is an excellent inducer for BD-2 (Dale and Fredericks, 2007). There are 2 species of bacteria that have been known as main dental caries cause, *Streptococcus mutans* and *Streeptococcus sobrinus.* Both of these two bacteria are sensitive to BD-2 (Nishimura et al., 2004). There is specific structure in cell wall of probiotic bacteria that called as microorganism-associated molecular patterns (MAMPs). It can be found as peptidoglycan (PG) or lipoteichoic acid (LTA) that will be recognized by pattern recognition receptors (PRRs) (Lebeer et al., 2010). The interction of MAMP and PRR will induce the activation of nuclear factor-kB (NF-kB) signaling cascade as the inhibitor of NF-kB kinase (IkBK) and mitogen-activated protein kinase (MAPK).

There are some factors that can induce BD-2 activation specifically, such as TLR-2 and NOD-2 as the up stream of BD-2 signaling cascade. Toll-like receptors (TLRs) are transmembrane proteins expressed by cells of the innate immune system, which recognize invading microbes and activate signaling pathways that launch immune and inflammatory responses to destroy the invaders. TLR2 recognizes a variety of microbial components. These include lipoproteins/lipopeptides from various pathogens, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol anchors from Trypanosoma cruzi, a phenol-soluble modulin from Staphylococcus epidermis, zymosan from fungi and glycolipids from Treponema maltophilum (Takeda and Akira, 2005). Nucleotide-binding oligomerization domain (NOD) proteins are members of a large family of proteins named "NOD-like receptors" whose functions are essential in innate/adaptive host responses to various commensal and pathogenic bacteria.The NOD proteins act as intracellular sensors of bacterial infection. NOD-2 is able to act as a general sensor of bacterial infection (Sun and Jobin,2014; Murphy,2008). This study evaluated the ability of *L. reuteri* to induce the expression of TLR-2 and NOD-2 that related to prevention of dental caries formation.

METHODS

Research Design and Animal Model

This research ethical clearance has been approved by the Commission of Ethical Feasibility of Health Research-Faculty of Dentistry, Universitas Airlangga. Animal models were obtained from The Integrated Research and Testing Laboratory Jogjakarta, (LPPT). Rats that have been used in this research were 24 albino rat wistar strain which divided into 4 groups. Positive control was rats that induced with *S. Mutans*, while rats in negative control group were not induced. Group I was rats that suppelemented with *L. reuteri* for 14 days (day 1-14) and induced with *S. mutans* for 7 days (day 8-14). Group II was rats that supplemented with *L. reuteri* and induced with *S. mutans* simustaneously for 14 days (day 1-14). Concentration of bacterial suspension was 4 x 10^8 cfu/ml for *L. reuteri* and 10^{10} cfu/ml for *S. mutans* (Valeur et al., 2004; Ooshima et al.1988). Each control group was consisted of 7 rats, while treatment group consisted of 5 rats. Analysis unit of this research was the gingival epithelium of rat models. Rats of all groups were euthanized on day 15. Gingival epithelium tissue of rats were taken for immunohistochemistry preparation.

L. reuteri suspension preparation

L. reuteri Prodentis starter contained in tablet x (DSM 17938+ATCC PTA 5289) was cultured in BHI broth then incubated for 24h at 37°C anaerobically in gas generating kit (Oxoid). Bacteria cell mass will appear as white sediment in tube. Bacteria then cultured into MRS medium agar (DeMan-Rogosa-Sharpe; Merck GmbH, Darmstadt, Germany) by streak method and incubated for 2x24h at 37°C. Bacteria single cell colony was taken and reincubated into BHI medium for 24h at 37°C to obtain OD=10^8 cfu/ml (λ=625) nm (Sutton, 2011).

S. mutans suspension preparation

S. mutans serotype c was cultured from freeze-dried stock. Bacteria colony was taken with oose then cultured into BHI broth then incubated for 24h at 37°C. Cultured bacteria was streak into blood agar medium and incubated for 24h. Bacteria single cell colony was taken and reincubated into BHI medium for 24h at 37°C to obtain OD=10^{10} cfu/ml (λ=625) nm (Oshima et al., 1988)

Immunohistochemistry

Histopathology preparation was referred to Humason (1972) in Sudiana (2005). Immunohistochemistry results calculation was based on Soini et al., 1998; Pizem and Cor, 2003). Rat gingival tissues were fixed in 4% paraformaldehyde for 4 h at 4^0C. After being washed in PBS, tissues were embedded in OCT compound then frozen immediately. Six-micrometer thick frozen tissue sections were incubated with anti TLR-2 primary antibody and anti NOD-2 primary antibody (Lab Vision) overnight at 4^0 C. Subsequently, tissue sections were incubated with rat monoclonal anti OPG secondary antibody (Lab Vision) and HRP kit overnight at 4^0C. The chromogen that used in this study was 3.3´-diaminobenzidine tetrahydrochloride. Tissue sections were then counterstained with hematoxylin.

Statistical Analysis

Significant differences of protein expression between each treatment groud was analyzed with ANOVA (p=0.001).

RESULTS

The expression of TLR-2 was different significantly for eah group (p=0.001). ANOVA test result shows that TLR-2 expression in gingival epithelium of treatment groups and positive control were higher compared to

negative control (Table 1). Positif control group (20.600) TLR-2 expression was higher significantly than negative control (5.400). However, this expression decreased in group I from 15,143 to 10,429.

Table 1. ANOVA analysis of TLR-2 expression in all groups

Group	Mean	Standard Deviation	Significance *)
Control (-)	5.400	1.94936	p=0.001
Control (+)	20.600	3.36155	
Group 1	15.143	4.94734	
Group 2	10.429	2.93582	

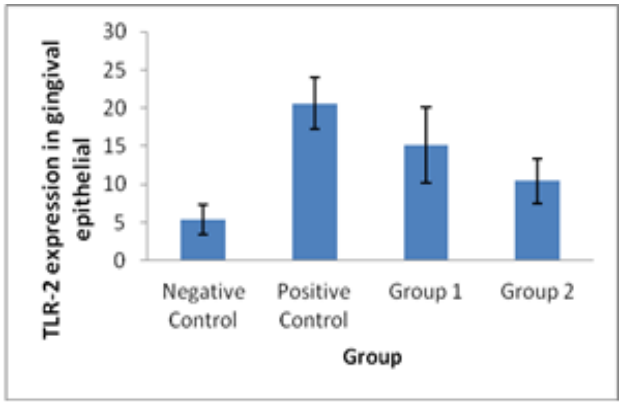

Figure 1. TLR-2 expression level in gingival epithelium after probiotic *L. reuteri* supplementation.

HSD significance different test results show that negative control was different significantly with positive control and group I. However, the comparison between negative control to group II and group I to group II results were different but not significant.

The distribution of cells that expressed NOD-2 in gingival epithelium induced with probiotic *L. reuteri* for each group can be seen in Table 3. There were significant differences of NOD-2 expression in each group (p=0.001). NOD-2 expression was higher in positive control (6.000) compared to negative control (5.200).

Table 3. ANOVA analysis of NOD-2 expression in all groups

Group	Mean	Standard Deviation	Significance*)
Control (-)	5.200	2.588	p=0.001
Control (+)	6.000	2.121	
Group 1	14.000	4.000	
Group 2	21.428	5.309	

NOD-2 expression in gingival epithelium of negative control group was lower than the other groups (Figure 2). However, there was no signifiant difference between positive control and negative control. The expression of NOD-2 in gingival epithelium of group II was higher than group I.

DISCUSSION

Gingival epithelium cell is the first defense mechanism against bacterial infection. It is the barrier of host millieu barrier with microbes in gingival pocket. The epithelial cells act as censor in bacterial indentification.

This patterns can be seen on Figure 1. There was increase of TLR-2 expression in gingival epithelium of all groups, except negative control (p=0.001). However, the expression of TLR-2 decreased in group I and group II.

Table 2. HSD significance difference test of all groups

Group	Negative control	Positive control	Group 1	Group 2
Control (-)	-	0.000*	0.000*	0.117
Control (+)		-	0.073*	0.000*
Group 1			-	0.101
Group 2				-

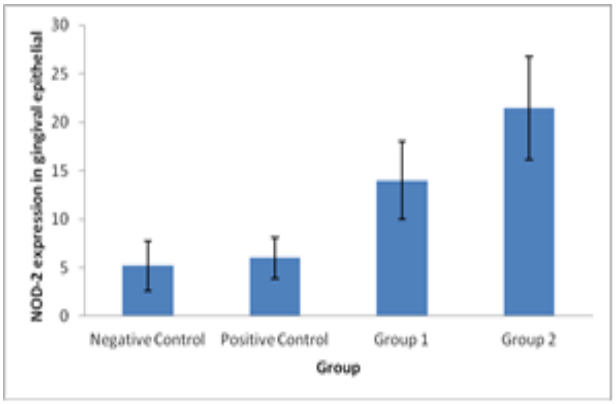

Figure 2. NOD-2 expression level in gingival epithelium after probiotic *L. reuteri* supplementation.

Direct contact of oral mucosal surface with bacteria induce the expression of various proteins as immune response mediators to epithelial cells that will give innate immunity response (Beklen et al, 2009). This immune response is designed to recognize the specific molecular structure possesed by microbes that known as pathogen-associated molecular patterns (PAMPs), consist of lipo-polysaccharide from cell walls of Gram negative bacteria, peptidoglycan and lipotechoic acid from cell walls of Gram positive bacteria, manosa, bacterial DNA, N-formilmetionin found in bacterial protein, RNA double helix from viruses, lipoarabinomannan from mycobacterium, and mannan from yeast (Ma'at, 2009). Main group of pithelial cell receptor that recognize PAMPs is Toll-like receptors (TLRs). It act as membrane signaling receptor in transmembrane. It plays an important role in host response against Gram positive and negative bacteria.

Probiotic *L. reuteri* is a Gram positive bacteria that has specific molecular structure, such as PG and LTA. These 2 structures are ligand from a surface cell receptor (PRRs), namely Toll-like receptor-2 (TLR-2). Interaction of TLR-2 and its ligand will induce signalling as the probiotic effect. The lower expression of TLR-2 was on gingival epithelium of negative control or rats that have not treated with *L. reuteri* or *S. mutans*. Without any contact with these bacteria, there were no bacteria censor mechanism in gingival epithelial cells. As the result, there was no interaction of PRR and PAMP. TLR-2 expression depends on specific stimuli. Cell manifestation of TLR-2 on cell surface is generally low and varies. This manifestation can also be cathegorized as limited (Ma'at

2009. This result also supported by Athman (2004) that stated epithelial cells in normal condition are unresponsive or hyper-responsive to microbial stimulation to avoid inflammation caused by excessive microflora.

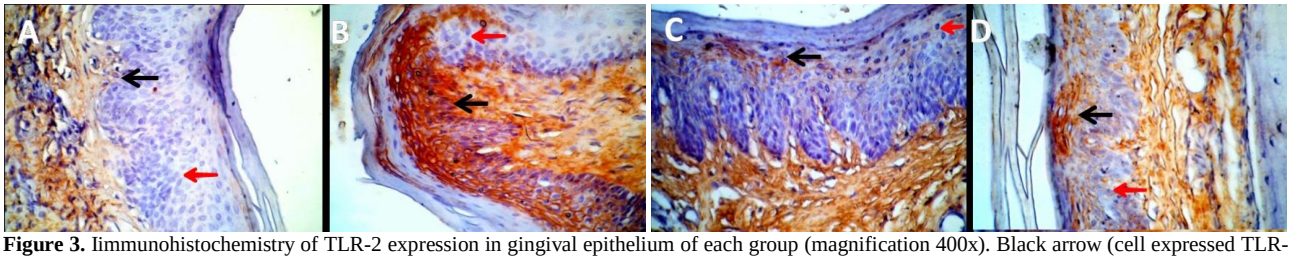

Figure 3. Iimmunohistochemistry of TLR-2 expression in gingival epithelium of each group (magnification 400x). Black arrow (cell expressed TLR-2). Red arrow (cell unexpressed TLR-2). Expression of TLR-2 gingival epithelium in negative control group (A); positive control group (B); group I (C); group II (D).

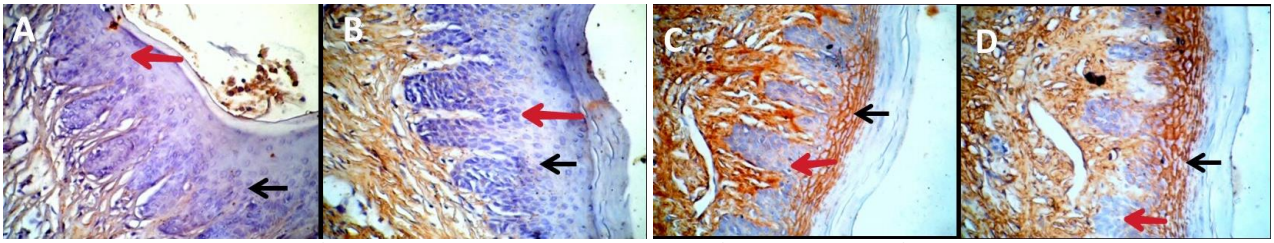

Figure 4. Iimmunohistochemistry of NOD-2 expression in gingival epithelium of each group (magnification 400x). Black arrow (cell expressed TLR-2). Red arrow (cell unexpressed NOD-2). Expression of TLR-2 gingival epithelium in negative control group (A); positive control group (B); group I (C); group II (D).

There are two different mechanisme of immune system, innate and adaptive immune system. Innate immune system is the first defense from pathogenic microbes infection. It is mediated by phagocyte cells such macrophages and dendritic cells. Innate immunity identifies microorganisms through PRRs. PRRs will recognize microbial components as PAMPs. TLRs and Nod-like receptors (NLRs) are the representations of PRRs (Jeon et al., 2011). PRRs that recognize PAMPs will allow cell to identify the infection and stimulate innate immune response by increase the production of inflamatory cytokines, natural antimicrobials and stimulate adaptive immune response (Mitchell et al., 2007).

One member of the Nod-like receptors (NLRs) that has an important role in innate immunity family is NOD-2 (CARD15) (Holly et al., 2012). Nucleotide-binding oligomerization domain (NOD) consists of cytosolic protein PRRs. Both NOD-1 and NOD-2 can detect peptidoglycan from bacteria. NOD-1 detects meso-diaminopimelic acid (iE-DAP) that commonly found in Gram negative bacteria, while NOD-2 responds with muramyl dipeptide (MDP), that can be found in both Gram-positive and Gram-negative bacteria.

The expression of NOD-2 in all of treated groups were higher than negative control. These patterns were shown in Table 3 and Figure 2. Rats in negative control group were not treated with *L. reuteri* and *S. mutans*. It caused there was no interaction between PAMP and PRR. The induction of BD-2 by probiotic bacteria *L. reuteri* in gingival epithelium needs NOD-2 to mediate Rick and signal line IKK - IkB - NF-kB.

This research cannot explain the reason of there were no differences between TLR-2 and NOD-2 expression pattern in gingival epithelium cells. However, there is probability of sinergic effect from NOD1 / NOD-2 with TLR agonic in producing PGRPs (Peptidoglycan Recognition Proteins) and BD-2 in epithelial cells in oral cavity through NF-kB pathway. On the other hand, Co-stimulation of NOD-1 / NDO-2 and TLR ligand has no effect on the production of proinflammation citokynes (IL-6, IL-8 and monocyte chemoattractant protein-1). It shows that the combination of TLR and NOD signaling in innate immune responses against microbes will produce synergic activation from antibacterial responses in epithelial cells in oral cavity (Uehara and Takada, 2008). It can be concluded that probiotic *L. reuteri* supplementation could increase the expression of TLR-2 and NOD-2 in gingival epithelium.

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