The Anti-inflammatory Effects of Glycerol-supplemented Probiotic Lactobacillus reuteri on Infected Epithelial cells In vitro

Abstract
Background: One of the most interesting effects of probiotics is their ability to modulate the immune system through the induction of cytokines and to enhance the host immune response. Aims: The purpose of this study was to evaluate the anti-inflammatory effect of glycerol-supplemented Lactobacillus reuteri on the transcription level of interleukin (IL)-8 and human-beta-defensin (hBD)-2 expressed by epithelial cells after exposure to bacteria. Materials and Methods: The confluent-cultured HaCat cell line (10⁵ cells/mL) was exposed to Streptococcus mutans ATCC-25175 and Porphyromonas gingivalis ATCC-33277 (10⁷ colony-forming units [CFU/mL]) for 24 h and challenged with probiotic L. reuteri ATCC-55730 (10⁷ CFU/mL) supplemented with glycerol. Subsequently, the transcription levels of IL-8 and hBD-2 in HaCat cells were analyzed using reverse-transcription polymerase chain reaction (RT-PCR). In addition, cell viability was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All the obtained data were statistically analyzed using the one-way analysis of variance test, with P < 0.05 set as the level of significance. Results: The MTT assays confirmed no cytotoxic effects of glycerol-supplemented L. reuteri on HaCat cells (viability >90%). mRNA expression of IL-8 and hBD-2 increased after exposure to both bacteria. The presence of glycerol-supplemented L. reuteri significantly reduced the expression of IL-8 and hBD-2 on HaCat cells (P < 0.05). Conclusion: Glycerol-supplemented L. reuteri reduced the expression of IL-8 and hBD-2, and the results may be proof of principle for a probiotic approach to combating inflammation. However, further studies are needed to validate this probiotic effect.

Keywords: Anti-inflammation, beta-defensin-2, glycerol, interleukin-8, Lactobacillus reuteri, probiotic

Introduction
Oral diseases represent a considerable public health problem worldwide. Dental caries and periodontal disease are caused by bacteria, such as Streptococcus mutans and Porphyromonas gingivalis, and they are characterized by increases in pathogenic microorganisms, demineralization, inflammation, and high levels of inflammatory markers. The major factor that induces inflammation of the gingival tissue is the presence of bacterial biofilm (dental plaque) on the teeth/gingival interfaces. The products of biofilm bacteria, such as lipopolysaccharide (LPS), are known to initiate a chain of reactions, leading to a host response, as well as a destructive process. As a result, some inflammatory factors increase, most prominently, interleukin (IL)-1β, IL-1α, IL-6, and IL-8, prostaglandins, and tumor necrosis factor (TNF)-α. In addition to the inflammatory factors, antimicrobial peptides, such as human-β-defensins (hBDs) and calprotectin, are produced as the innate immune response of epithelial cells.

Bacteriotherapy is an alternative and promising way to combat infections using harmless bacteria to displace pathogenic microorganisms. Probiotics have been associated with gut health, and most clinical interest has focused on the prevention or treatment of gastrointestinal infections and diseases. However, several investigators have also suggested the use of probiotics for oral health purposes. Bifidobacterium and Lactobacillus as probiotics may also prove useful for the prevention of oral diseases, including caries and periodontal disease. A recent study showed that probiotic yogurt containing Bifidobacterium lactis BB-12 consumption can reduce the levels of S. mutans as pathogenic bacteria...
in the saliva.[9] Among these bacteria, probiotics are widely used.[1] *Lactobacillus reuteri*, as a probiotic bacterium, also has been proven to enhance oral health. *L. reuteri* is efficacious in reducing both gingivitis and dental plaque in patients with moderate-to-severe gingivitis.[9]

Some *in vivo* experiments have shown that probiotic *Lactobacillus* may modulate the immune response.[10] *L. reuteri* ATCC-55730 and ATCC PTA 5289 were reported to decrease the levels of inflammatory factors in the gingival crevicular fluid.[11] However, as for hBDs and ILs, the role of *L. reuteri* is not yet clearly known. Therefore, the current study was conducted to investigate the effect of the glycerol-supplemented probiotic *L. reuteri* on the IL-8 and hBD-2 mRNA expression of *S. mutans* and *P. gingivalis*-infected HaCat cells.

**Materials and Methods**

This study is an *in vitro* experimental design. HaCat cells were kindly provided by Dr. Solachuddin Jauhari Arief (Faculty of Dentistry, International Islamic University, Malaysia). The microorganisms used in the study were standard strains of *S. mutans* ATCC 25175 and *P. gingivalis* ATCC 33277. They were obtained from the Microbiology Laboratory of Dipa Pharmalab Intersains (PT Dipa Healthcare) in Jakarta, Indonesia.

**Microbial strains and growth conditions**

*S. mutans* ATCC 25175 was cultured in brain–heart infusion (BHI) broth (Thermo Scientific, Waltham, MA, USA) and incubated in anaerobic conditions under CO₂ at 37°C. *P. gingivalis* ATCC 33277 was cultured in BHI broth and incubated in a GasPak jar system (Becton Dickinson, Franklin Lakes, NJ, USA). *L. reuteri* ATCC 55730 was cultured in de Man, Rogosa, Sharpe broth (Thermo Scientific) and incubated at 37°C under anaerobic conditions. Before exposing the HaCat cells, *S. mutans* and *P. gingivalis* were killed by heating them at 80°C for 30 min.

**HaCat cell culture**

HaCat cells were cultured in Dulbecco’s Modified Eagle Medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum, 1% fungizone, 100 μg/mL penicillin, and 10 μg/mL streptomycin (Gibco, Gaithersburg, MD, USA). The cells were maintained in tissue culture flasks in a humidified 5% CO₂ incubator at 37°C.

**3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium assay**

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium (MTT) assay was conducted based on the reduction of tetrazolium MTT dye by the mitochondrial dehydrogenase of the intact cells to a purple formazan product. Briefly, treated HaCat cells were added to phosphate-buffered saline with 1 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 3 h. The amount of formazan was determined by measuring the absorbance at 490 nm using an AccuReader Microplate Reader (Metertech, Taipei, Taiwan). The assay was performed in triplicate.

**Reverse-transcription and quantitative polymerase chain reaction**

The confluent-cultured HaCat cell line (10⁵ cells/mL) was exposed to *S. mutans* ATCC-25175 and *P. gingivalis* ATCC-33277 (10⁷ colony-forming units [CFU]/mL) for 24 h under 5% CO₂, at 37°C and challenged with probiotic *L. reuteri* ATCC-55730 (10⁵ CFU/mL) supplemented with glycerol and incubated for 3- and 6-h periods. To observe the expression levels of IL-8 and hBD-2 mRNAs, quantitative real-time-polymerase chain reaction (RT-PCR) was carried out. Total mRNA from treated HaCat cells was isolated with TRIzol reagent (Invitrogen, Carlsbad, USA). Then, RT was performed with a GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, Foster City, USA), followed by quantitative PCR (qPCR) with the StepOnePlus™ Real-Time PCR System (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems) for 40 cycles. All primers used are listed in Table 1 for IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as well as hBD-2.[12] The IL-8 and hBD-2 levels were normalized to that of GAPDH. The RT-qPCR was performed in triplicate; moreover, quantification was carried out using the formula of the 2−ΔΔCT method.

**Statistical analysis**

The Shapiro–Wilk test was used to test for normality, and Levene’s test was used to test for homogeneity of variance. One-way analysis of variance test was applied to reveal significant differences of IL-8 and hBD-2 mRNA expressions in the HaCat cells exposed with *S. mutans*, *P. gingivalis*, and *L. reuteri* with/without glycerol supplementation for different treatment times in three independent experiments. Differences were considered

| Table 1: Primers used for interleukin-8, human-beta-defensin-2, and glyceraldehyde-3-phosphate dehydrogenase |
|-------------|---------------------------------------------------------------|
| **Type of primer** | **Sequence (5’-3’)** |
| IL-8 | **Forward** TCT CTT GGC AGC CTT CCT  <br> **Reverse** ACT GAA CCT GAC CGT ACA TGT  <br> CTT TAT GCA CTG ACA TCT |
| hBD-2 | **Forward** GGT GTT TTT GGT GGT ATA GGC  <br> **Reverse** AGG GCA AAA GAC TGG ATG ACA |
| GAPDH | **Forward** CTG AGT ACG TCG TGG AGT C  <br> **Reverse** ACT GAA CCT GAC CGT ACA CAG  <br> AGA TGA TGA CCC TTT TG |

**IL-8**: Interleukin-8; **hBD**: Human-beta-defensin; **GAPDH**: Glyceraldehyde-3-phosphate dehydrogenase
statistically significant if $P < 0.05$. Statistical calculations were performed with SPSS Statistics for Windows software version 20 (IBM, USA).

**Results**

**Lactobacillus reuteri did not affect HaCat cell viability**

Under treatment with *L. reuteri* supplemented with glycerol for different treatment times, the HaCat cell viabilities were in the range of 89.7%–119.2%. As shown in Figure 1, *L. reuteri* with glycerol supplementation for different times caused only slight cell viability differences in the HaCat cells. Moreover, the glycerol supplementation did not affect HaCat cell viability in a time-dependent manner.

**Glycerol-supplemented Lactobacillus reuteri reduced Streptococcus mutans- or Porphyromonas gingivalis-induced interleukin-8 mRNA expressions of HaCat cells**

IL-8 mRNA expression of HaCat cells increased significantly after exposing the cells to *S. mutans* ($P = 0.000$) or *P. gingivalis* ($P = 0.000$) [Figure 2a and b]. The *S. mutans*-induced IL-8 mRNA expression was significantly reduced by the addition of *L. reuteri* for 6 h ($P = 0.000$) [Figure 2a]. The glycerol-supplemented *L. reuteri* could inhibit both *S. mutans* and *P. gingivalis*-IL-8 mRNA expression of HaCat cells at 3 h and 6 h of treatment ($P = 0.000$). Glycerol supplementation accelerated the *L. reuteri* inhibition activity. Significant differences were observed when *S. mutans*-induced IL-8 mRNA expressions in *L. reuteri* with and without glycerol supplementation for 3 h ($P = 0.000$) and 6 h ($P = 0.018$) were compared. Significant differences were also observed when *P. gingivalis*-induced IL-8 mRNA expressions in *L. reuteri* with and without glycerol supplementation for 3 h ($P = 0.000$) and 6 h ($P = 0.000$) [Figure 2b] were compared.

**Streptococcus mutans, Porphyromonas gingivalis, and Lactobacillus reuteri with/without glycerol supplementation affected human-beta-defensin-2 mRNA expressions of HaCat cells**

Via exposure to *S. mutans* or *P. gingivalis*, hBD-2 mRNA expression of HaCat cells increased significantly ($P = 0.000$) [Figure 3a and b]. hBD-2 mRNA expressions were observed in HaCat cells exposed to *S. mutans*, *P. gingivalis*, and *L. reuteri* for 3 h. The hBD-2 mRNA expressions were then reduced in HaCat cells exposed to *S. mutans*, *P. gingivalis*, and *L. reuteri* for 6 h ($P = 0.000$). With glycerol supplementation, the hBD-2 mRNA expressions in the HaCat cells exposed to *S. mutans* and *L. reuteri* for both 3 and 6 h were diminished [Figure 3a]. With glycerol supplementation, the hBD-2 mRNA expressions in the HaCat cells exposed to *P. gingivalis* and *L. reuteri* for 6 h were also diminished ($P = 0.000$) [Figure 3b].

**Discussion**

The cell response to microbial pathogens varies depending on the specific pathogen or microbial product, its...
Anti-inflammatory effect of probiotic gene. The gingivalis-induced IL-8 mRNA expressions were addition, and Contemporary Clinical Dentistry | Volume 9 | Issue 2 | April-June 2018 mutans-reuteri L. Widyarman, et al.: Anti-inflammatory effect of probiotic for 6 h. The reduction was also or gingivalis can potentially suppress proinflammatory gene is essential mutans. On hundred thousand HaCat cells were seeded, and treated with glycerol-supplemented is a Gram-positive bacterium. In through the conversion -induced (b) for 24 h, and treated with 's effectiveness in modulating inflammation. strain ATCC 55730 stimulated gingivalis and expression reuteri reuteri -specific, modulates the expression of a gene cluster L. reuteri reuteri addition (3 h to of 54x61 IL-8 in cells can cause a massive influx of neutrophils, a hallmark of inflammation, especially at the early stage of bacterial colonization.[17] After incubation with L. reuteri for 6 h, both S. mutans- and P. gingivalis-induced IL-8 mRNA expressions were significantly reduced, compared with the first 3 h of incubation. These results were considered beneficial to the epithelial cells since prolonged production of IL-8 can cause cell destruction due to the accumulation of neutrophils. In general, the activation of a central regulator of the epithelial innate immune response, nuclear factor (NF)-κβ, is induced by bacterial infection. Translocation of NF-κβ to the nucleus can give rise to many inflammatory mediators, such as IL-8 and hBD-2.[17] Live L. reuteri can upregulate an unusual anti-inflammatory molecule, nerve growth factor (NGF), and inhibit NF-κB translocation to the nucleus, thereby resulting in a decreased level of IL-8 expression.[16] L. reuteri can potentially suppress proinflammatory cytokines such as human TNF by converting a dietary component, the amino acid L-histidine, into an immunoregulatory signal, the biogenic amine histamine. Histamine suppresses mitogen-activated protein (MAP) kinase activation and cytokine production by signaling via histamine receptor type 2 (H2) on the cell. One such gene was found to be a regulator of genes involved in histidine-histamine metabolism by the L. reuteri-specific immunoregulatory (rsiR) gene. The rsiR gene is essential for human TNF suppression by L. reuteri and expression of the histidine decarboxylase gene cluster on the L. reuteri chromosome. The presence of the regulatory gene, rsiR, modulates the expression of a gene cluster known to mediate immunoregulation by probiotics at the transcriptional level.[18,19] In the current results, after 3 h of L. reuteri addition, the level of hBD-2 mRNA expression induced with P. gingivalis was not reduced. This was correlated with the hBD-2 innate immune response against the resulting IL-8 mRNA expression. Substantial evidence has shown that the anti-inflammatory signaling pathway, called the phosphatidylinositol-3-kinase-Akt (PI3K/Akt) pathway, was activated by epithelial cells to suppress the detrimental effect of IL-8 overexpression after prolonged bacterial infection. In contrast, the PI3K/Akt pathway activation has no significant effect on hBD-2 expression, which is needed to defend the cells against bacterial infection continuously.[17] The hBD-2 mRNA expression was reduced after glycerol supplementation, which may have accelerated L. reuteri’s effectiveness in modulating inflammation. Meanwhile, S. mutans- or P. gingivalis-induced hBD-2 mRNA expression was also reduced significantly by addition of L. reuteri for 6 h. The reduction was also accelerated significantly by glycerol supplementation. These results were possibly related to the suppression of human TNF-α by L. reuteri through the conversion concentration, and duration of exposure.[11] The present findings illustrate the potential differences in the expression patterns of IL-8 and hBD-2 from HaCat cells in response to two different types of bacteria, namely Gram-positive and Gram-negative bacteria. In addition, differences were noted in the expression patterns after treatment with L. reuteri at different incubation times.

In this study, IL-8 and hBD-2 mRNA expressions were increased with exposure to S. mutans or P. gingivalis. Oral bacteria can trigger chronic inflammatory responses in the host, resulting in tissue destruction, with inflammatory expression by epithelial cells.[14] However, at the early stage of L. reuteri addition (3 h of incubation time), the level of IL-8 mRNA also slightly increased in both S. mutans- and P. gingivalis-induced IL-8 mRNA. L. reuteri is a Gram-positive bacterium. In the absence of LPS, the secreted factors of the formed biofilms of live L. reuteri strain ATCC 55730 stimulated TNF-α production,[15] which modulated the expression of cytokines, including IL-8.[16] The increased level of IL-8 in cells can cause a massive influx of neutrophils, a
of L-histidine to histamine that suppresses MAP kinase activation and cytokine production by rsIR gene.[17] Since the expression of hBD-2 in keratinocyte cells is induced by proinflammatory cytokines, such as IL-1β and TNF-α, the suppression of TNF-α can reduce the expression of hBD-2 in keratinocytes.[19]

HaCat cells are a spontaneously immortalized human keratinocyte line that has been widely used for the studies of epithelial cell mechanisms, such as differentiation and inflammatory responses related to infection.[20] The keratinocyte is the primary type cell in most gingival epithelial tissues, and it has been used to study the constant challenge of oral bacteria and oral diseases, including caries and periodontitis.[21] However, most oral keratinocyte cells, such as primary gingival epithelial cells, have a more finite lifespan and are more difficult to grow.[22] HaCat cells are a suitable substitute for human oral keratinocytes, such as human gingival epithelial cells, because these cells have a longer lifespan and can easily be grown, maintained, and passaged indefinitely in vitro.[23]

Keratinocytes are a rich source of IL-8, a proinflammatory cytokine that has a direct effect on immune cells,[24] and are considered one of the most important factors involved in the initiation and maintenance of many immune and inflammatory reactions. IL-8 attracts and activates neutrophils, which are the first line of immune cells recruited to the infected sites. Moreover, IL-8 has been found to be expressed by human oral epithelial cells following infection with periodontal pathogens.[25] In addition to cytokines, most epithelial cells also express antimicrobial peptides, called defensins, in the infected area, which activate the adaptive immune system.[26] Defensins are small, cationic antimicrobial peptides that comprise an important component of the mammalian innate immune defense. The α-defensins can be found in neutrophils and the intestine, while the three β-defensins (hBD-1, 2, and 3) are primarily expressed in epithelial cells.[27] Specifically, these β-defensins are induced in gingivitis[28] and periodontal diseases.[29] In general, it has been observed that hBD-2 and 3 are induced in vitro in gingival and buccal epithelial cells in response to most microbial pathogens.[30]

The antimicrobial and anti-inflammatory activities of L. reuteri were assessed by examining supernatants of planktonic cells. Planktonic cells and biofilms of L. reuteri produced reuterin although differences in reuterin production were evident among the strains.[31] In the presence of glycerol, L. reuteri can synthesize 3-hydroxypropionaldehyde, which is excreted into the medium, where it forms a dynamic multicomponent equilibrium (HPA system: reuterin) in conjunction with the hydrate and dimer.[32] Maximum reuterin production by L. reuteri occurs in late log and stationary phase cultures.[18] This study showed that probiotic L. reuteri requires the presence of glycerol over a certain period to produce reuterin to develop the beneficial capability of inhibiting oral pathogenic bacteria[33] and acting as an anti-inflammatory agent to reduce inflammatory mediators, such as TNF-α and IL-8.

L. reuteri suppresses the activation of the activator protein-1 transcription factor, which regulates the expression of proinflammatory cytokine genes in response to the activation of Toll-like receptors.[33] L. reuteri can also target specific signaling pathways and immune responses; these bacterial strains may represent future therapeutic agents that could serve to suppress chronic inflammation.[34] In a clinical study, L. reuteri-containing tablets were found to decrease the proinflammatory factors IL-1β, IL-6, and IL-8 in the peri-implant mucositis.[35] This result suggests that L. reuteri is a possible option where probiotics may affect oral health through enhanced host immune response and reduced proinflammatory cytokine. This probiotic may influence local and systemic immune responses that cause a reduction of inflammation and tissue destruction.

Conclusion

The results of this study suggested that L. reuteri supplemented with glycerol suppresses the expression of IL-8 and induces the hBD-2 inflammatory gene response to S. mutans and P. gingivalis infection. Reduction of IL-8 and hBD-2 may be proof of principle for a probiotic approach to combating inflammation. No cytotoxic effects of L. reuteri on the epithelial cells were detected. However, this result should be confirmed with in vivo studies.

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Conflicts of interest

There are no conflicts of interest.

References

1. Reddy RS, Swapna LA, Ramesh T, Singh TR, Vijayalaxmi N, Lavanya R. Bacteria in oral health — Probiotics and prebiotics: A review. Int J Biol Med Res 2011;2:1226-33.
2. Caglar E, Kargul B, Tanboga I. Bacteriotherapy and probiotics’ role on oral health. Oral Dis 2005;11:131-7.
3. Saha S, Tomaro-Duchesneau C, Rodes L, Malhotra M, Tabrizian M, Prakash S, et al. Investigation of probiotic bacteria as dental caries and periodontal disease biotherapeutics. Benef
Anti-inflammatory effect of probiotic Lactobacillus reuteri suppresses TNF via modulation of PKA and ERK signaling. PLoS One 2012;7:e31951.

Widyarman, et al.: Anti-inflammatory effect of probiotic

Microbes 2014;5:447-60.

4. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. Periodontol 2000 1994;5:78-111.

5. Okada H, Murakami S. Cytokine expression in periodontal health and disease. Crit Rev Oral Biol Med 1998;9:248-66.

6. Dale BA, Kimball JR, Krishnapraporkitt S, Roberts F, Robinovitch M, O’Neal R, et al. Localized antimicrobial peptide expression in human gingiva. J Periodontal Res 2001;36:285-94.

7. Kohlgraf KG, Pingel LC, Dietrich DE, Brogden KA. Defensins as anti-inflammatory compounds and mucosal adjuvants. Future Microbiol 2010;5:99-113.

8. Widyarman AS, Yunita ST, Prasetyadi T. Consumption of yogurt containing probiotic *Bifidobacterium lactis* reduces *Streptococcus mutans* in orthodontic patients. Scientific Dental Journal 2018;2:19-25.

9. Krasse P, Carlsson B, Dahl C, Paulsson A, Nilsson A, Sinkiewicz G, et al. Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*. Swed Dent J 2006;30:55-60.

10. Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: Impact on the gut immune system and allergic reactions. J Leukoc Biol 2011;89:685-95.

11. Twetman S, Derawi B, Keller M, Ekstrand K, Yucel-Lindberg T, Huang FC. Differential regulation of interleukin-8 and human beta-defensin-3 and interleukin-8 receptors on the migration of human keratinocytes, the role of PLC-γ and potential clinical implications. Exp Ther Med 2012;3:231-6.

12. Oshima AL, Walker SL, Roberts CH, Hagge DA, Neupane KD, Khadge S, et al. Human beta-defensin 3 is up-regulated in cutaneous leprosy type 1 reactions. PLoS Negl Trop Dis 2012;6:e1869.

13. Feezor RJ, Oberholzer C, Baker HV, Novick D, Rubinstein M, Moldawer LL, et al. Molecular characterization of the acute inflammatory response to infections with gram-negative versus gram-positive bacteria. Infect Immun 2003;71:5803-13.

14. Hasturk H, Kantarci A, Van Dyke TE. Oral inflammatory diseases and systemic inflammation: Role of the macrophage. Front Immunol 2012;3:118.

15. Kang MS, Oh JS, Lee HC, Lim HS, Lee SW, Yang KH, et al. Inhibitory effect of *Lactobacillus reuteri* on periodontopathic and cariogenic bacteria. J Microbiol 2011;49:193-9.

16. Ma D, Forsythe P, Bienenstock J. Live *Lactobacillus rhamnosus* [corrected] is essential for the inhibitory effect on tumor necrosis factor alpha-induced interleukin-8 expression. Infect Immun 2004;72:5308-14.

17. Huang FC. Differential regulation of interleukin-8 and human beta-defensin 2 in *Pseudomonas aeruginosa*-infected intestinal epithelial cells. BMC Microbiol 2014;14:275.

18. Hemarajata P, Gao C, Pflughoeft KJ, Thomas CM, Saulnier DM, Spinder JK, et al. *Lactobacillus reuteri*-specific immunoregulatory gene *rsiR* modulates histamine production and immunomodulation by *Lactobacillus reuteri*. J Bacteriol 2013;195:5567-76.

19. Thomas CM, Hong T, van Pijkeren JP, Hemarajata P, Trinh DV, Hu W, et al. Histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA and ERK signaling. PLoS One 2012;7:e31951.

20. Wilson VG. Growth and differentiation of HaCaT keratinocytes. Methods Mol Biol 2014;1195:33-41.

21. Plegueuzuelos O, Dainty SJ, Kapas S, Taylor JJ. A human oral keratinocyte cell line responds to human heat shock protein 60 through activation of ERK1/2 MAP kinases and up-regulation of IL-1beta. Clin Exp Immunol 2005;141:307-14.

22. Moffatt-Jauregui CE, Robinson B, de Moya AV, Brockman RD, Roman AV, Cash MN, et al. Establishment and characterization of a telomerase immortalized human gingival epithelial cell line. J Periodontal Res 2013;48:713-21.

23. Moharamzadeh K, Van Noort R, Brook IM, Scutt AM. Cytotoxicity of resin monomers on human gingival fibroblasts and HaCaT keratinocytes. Dent Mater 2007;23:40-4.

24. Jiang WG, Sanders AJ, Ruge F, Harding KG. Influence of interleukin-8 (IL-8) and IL-8 receptors on the migration of human keratinocytes, the role of PLC-γ and potential clinical implications. Exp Ther Med 2012;3:231-6.

25. Huang GT, Haake SK, Kim JW, Park NH. Differential expression of interleukin-8 and intercellular adhesion molecule-1 by human gingival epithelial cells in response to *Actinobacillus actinomycetemcomitans* or *Porphyromonas gingivalis* infection. Oral Microbiol Immunol 1998;13:301-9.

26. Dale BA. Periodontal epithelium: A newly recognized role in health and disease. Periodontol 2000 2002;30:70-8.

27. Diamond G, Ryan L. Beta-defensins: What are they really doing in the oral cavity? Oral Dis 2011;17:628-35.

28. Offenbacher S, Barros SP, Paquette DW, Winston JL, Biesbrock AR, Thomason RG, et al. Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans. J Periodontol 2009;80:1963-82.

29. Lu Q, Jin L, Darveau RP, Samaranayake LP. Expression of human beta-defensins-1 and -2 peptides in unresolved chronic periodontitis. J Periodontal Res 2004;39:221-7.

30. Diamond DL, Kimball JR, Krishnapraporkitt S, Ganz T, Dale BA. Detection of beta-defensins secreted by human oral epithelial cells. J Immunol Methods 2001;256:65-76.

31. Jones SE, Versalovic J. Probiotic *Lactobacillus reuteri* biofilms produce antimicrobial and anti-inflammatory factors. BMC Microbiol 2009;9:35.

32. Vollenweider S, Lacroix C. 3-hydroxypropionaldehyde: Applications and perspectives of biotechnological production. Appl Microbiol Biotechnol 2004;64:16-27.

33. Lin YP, Thibodeaux CH, Peña JA, Ferry GD, Versalovic J. Probiotic *Lactobacillus reuteri* suppress proinflammatory cytokines via c-Jun. J Inflamm Bowel Dis 2008;14:1068-83.

34. Thomas CM, Versalovic J. Probiotics-host communication: Modulation of signaling pathways in the intestine. Gut Microbes 2010;1:148-63.

35. Flichy-Fernández AJ, Ata-Ali J, Alegre-Domingo T, Candel-Marti E, Ata-Ali F, Palacio JR, et al. The effect of orally administered probiotic *Lactobacillus reuteri*-containing tablets in peri-implant mucositis: A double-blind randomized controlled trial. J Periodontal Res 2015;50:775-85.