Original Article

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

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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 (10^5 cells/mL) was exposed to *Streptococcus mutans* ATCC-25175 and *Porphyromonas gingivalis* ATCC-33277 (10^7 colony-forming units [CFU]/mL) for 24 h and challenged with probiotic *L. reuteri* ATCC-55730 (10^7 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*,[1,2] and they are characterized by increases in pathogenic microorganisms, demineralization, inflammation, and high levels of inflammatory markers.[3] 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.[4] As a result, some inflammatory factors increase, most prominently, interleukin (IL)-1β, IL-lα, IL-6, and IL-8, prostaglandins, and tumor necrosis factor (TNF)-α.[5] 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.[6,7]

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

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in the saliva.[8] 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 CO_2 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% CO_2 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 (10^5 cells/mL) was exposed to *S. mutans* ATCC-25175 and *P. gingivalis* ATCC-33277 (10^7 colony-forming units [CFU]/mL) for 24 h under 5% CO_2, at 37°C and challenged with probiotic *L. reuteri* ATCC-55730 (10^7 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 $2^{-\Delta\Delta Xt}$ 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
Reverse	ACT GAA CCT GAC CGT ACA TGT CTT TAT GCA CTG ACA TCT
hBD-2	
Forward	GGT GTT TTT GGT GGT ATA GGC
Reverse	AGG GCA AAA GAC TGG ATG ACA
GAPDH	
Forward	CTG AGT ACG TCG TGG AGT C
Reverse	ACT GAA CCT GAC CGT ACA CAG 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.

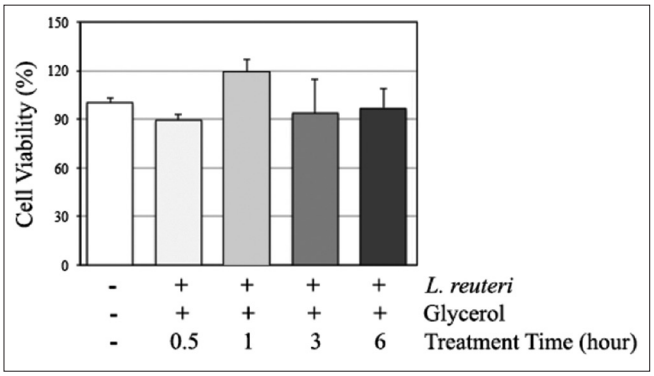

Figure 1: Percentage of viable HaCat cells after treatment with glycerol-supplemented *Lactobacillus reuteri*. HaCat cells at a density of 10^4 cells/well were seeded in a 96-well plate. Cells were treated with 10^6 colony-forming units/mL of *Lactobacillus reuteri* supplemented with 300 mM/mL of glycerol for 0.5, 1, 3, or 6 h. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was carried out as described in the Materials and Methods section

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

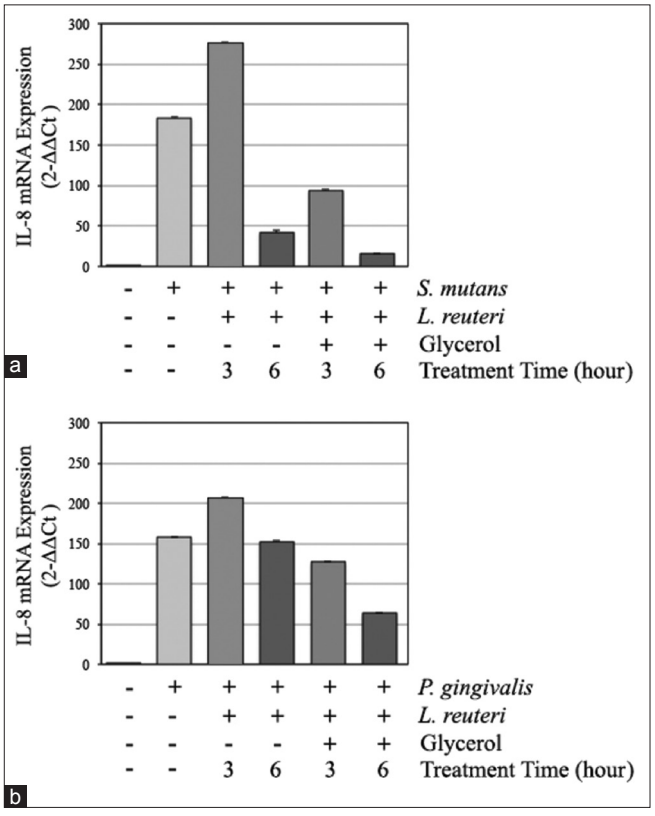

Figure 2: Interleukin-8 mRNA expression of HaCat cells exposed to *Streptococcus mutans* and treated with glycerol-supplemented *Lactobacillus reuteri*. One hundred thousand HaCat cells were seeded, exposed to 10^7 colony-forming units of preheated *Streptococcus mutans* (a) or *Porphyromonas gingivalis* (b) for 24 h, and treated with 10^7 colony-forming unit of *Lactobacillus reuteri* with/without 300 mM/mL glycerol for 3 or 6 h. Following this, the total mRNAs of the treated cells were isolated, and reverse-transcription quantitative polymerase chain reaction was carried out for interleukin-8 as described in the Materials and Methods section

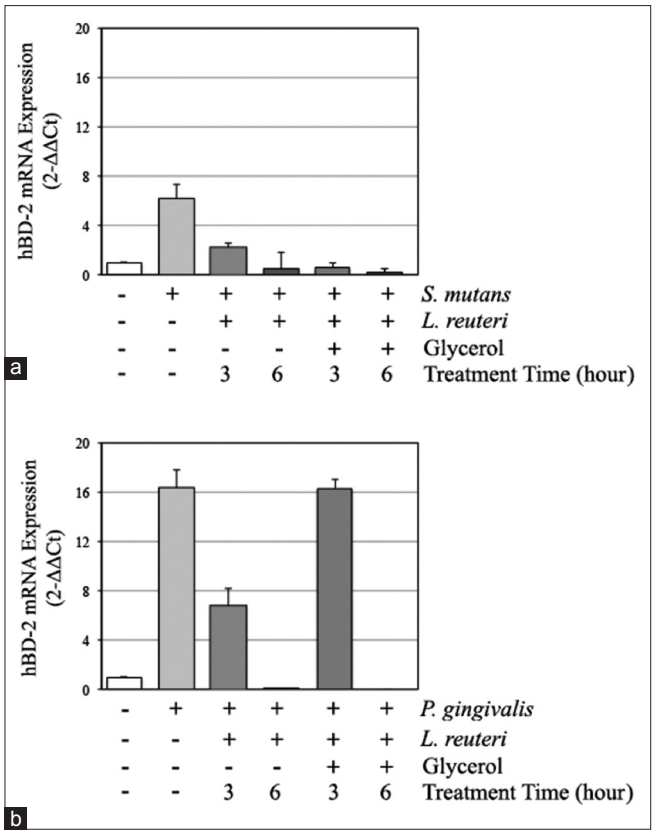

Figure 3: Human-β-defensin-2 mRNA expression of HaCat cells exposed to *Streptococcus mutans* and treated with glycerol-supplemented *Lactobacillus reuteri*. On hundred thousand HaCat cells were seeded, exposed to 10^7 colony-forming units of preheated *Streptococcus mutans* (a) or *Porphyromonas gingivalis* (b) for 24 h, and treated with 10^7 colony-forming unit *Lactobacillus reuteri* with/without 300 mM/mL glycerol for 3 or 6 h. After that, total mRNAs of treated cells were isolated, and reverse-transcription quantitative polymerase chain reaction was carried out for human-beta-defensin-2 as described in the Materials and Methods section

concentration, and duration of exposure.[13] 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 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

of L-histidine to histamine that suppresses MAP kinase activation and cytokine production by *rsi*R 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 cell type 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[15] 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 how 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.

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