Research Article

Anti-inflammatory effect of *Lactobacillus casei* on human epithelial cell responses to oral pathogens

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ABSTRACT

Objective: The objectives of this study were to analyze the anti-inflammatory effects of the *Lactobacillus casei* strain Shirota on epithelial cell expression of interleukin (IL)-8 and human beta-defensin 2 (hBD-2) in the presence of pathogenic bacteria *in vitro*. **Methods:** HaCaT epithelial-cells (1×10^5cell/mL) were exposed to heat-killed *Streptococcus mutans* or *Porphyromonas gingivalis* (1×10^7 colony-forming units/mL) and then challenged with the probiotic *L. casei* train Shirota for 3, 6, and 24 h. The mRNA transcription levels of IL-8 and hBD-2 were analyzed by reverse transcription-polymerase chain reaction. In addition, cell viability was analyzed using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay. Data statistically evaluated by one-way analysis of variance test. **Results:** *L. casei* does not effect the HaCaT epithelial cells viability (101.3%). IL-8 and hBD-2 transcription on HaCaT cells was increased after exposed to the heat-killed bacteria. The addition of *L. casei* reduced the transcription levels of IL-8; in contract, probiotics challenges increased the transcriptional expression of h-BD2 mRNA in the HaCaT cells ($P < 0.05$). IL-8 expression significantly reduced ($P < 0.05$) after the *L. casei* challenge. **Conclusion:** *L. casei* conveyed a beneficial immune modulation, reducing IL-8 levels, and elevating production of hBD-2. The epithelial cells showed no cytotoxic effects from *L. casei*. Further studies are needed to investigate the molecular processes related to this probiotic's anti-inflammatory properties.

KEY WORDS: Cytokine, Gene expression, Human beta-defensins-2, Interleukin-8, *Lactobacillus casei*

INTRODUCTION

Inflammation in the oral cavity often occurs due to an increase in the normal oral flora, such as *Streptococcus mutans*, a primary pathogenic species that caused dental caries.[1] Prolonged accumulation of plaque on the gingival margin and the subgingival region leads to larger colonization of anaerobic bacteria, such as *Actinomyces* spp., *Porphyromonas gingivalis*, and *Treponema denticola*.[2,3] Bacterial colonization around the periodontal tissue causes increased production of interleukin (IL), a cytokine involved in the regulation of immune responses and inflammatory reactions.[4,5]

During infections, epithelial cells provide the primary defense against pathogenic invasion, rapidly expressing and upregulating pro-inflammatory cytokines, such as IL-8, as part of an instant innate immune response.[6] Most epithelial cells, including keratinocytes and human oral epithelial cells,[7] are rich in IL-8 during inflammation.[8] In addition to cytokines, the epithelium also expresses defensins, small cationic antimicrobial peptides which have antimicrobial activity against positive and negative Gram bacteria, candida, and viruses.[9,10] Human beta-defensin 2 (hBD-2) protein is present in the gingival epithelium, saliva, and crevicular gingival fluid, leading the immune system's defense in the oral cavity.[11] The expression of hBD-2 increases during inflammation.[12]

Probiotics have important roles in maintaining immunological function. Probiotics are needed to develop the immune system, protecting the body from disease-causing bacteria. Microflora classified as probiotics are primarily lactic acid-producing bacteria of the genus *Lactobacillus* and bifidobacteria.[5] Probiotic bacteria can improve oral health. Consuming probiotic yogurt containing *Bifidobacterium lactis* is shown to reduce *S. mutans* level in the saliva.[13] Many studies have been conducted to assess the probiotics anti-inflammatory properties. Several *in vitro* studies have used human dendritic cells, intestinal T cells, intestinal porcine epithelial cells, monocyte-derived

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dendritic cells, and peripheral blood mononuclear cells. Chemical inflammation induction of animal models or human patients has also involved in many *in vivo* studies.[14-16]

Different strains of Lactobacilli promote different host cell responses;[17] therefore, the results from one strain cannot be generalized to others.[18] *Lactobacillus casei* Shirota as probiotic possesses specific biological activity in the human body and has been used widely as yogurt drinks and dairy food in Japan and Europe.[16] *L. casei* Shirota is considered safe and it is thought to activate the host's immune systems[19] by inducing cytokines such as interferon-γ, ILs, and Tumor necrosis factor (TNF)-α,[16] also T-cells and natural killer cells.[20] This study explored how *L. casei* strain Shirota affects HaCaT epithelial cell lines, specifically focusing on the *in vitro* expression of chemical mediators related non-specific and specific immune responses, IL 8, and human beta-defensin 2.

MATERIALS AND METHODS

Cell Lines, Bacterial Strains, and Infections

Commercial probiotic drinks containing *L. casei* strains Shirota were cultured in de Mann, Rogosa, and Sharpe (MRS) agar for 24 h at 37°C in a medium containing 30 mg glucose. An inoculum comprising 1% of the culture was re-cultured into MRS broth and incubated for 16 h. For bacterial harvesting, the solution was centrifuged at 4000×g and rinsed using 50 mL phosphate-buffered saline (PBS) (pH 7.5). *L. casei* was confirmed using polymerase chain reaction (PCR).

S. mutans ATCC 25175 cultured in brain-heart infusion (BHI) broth and incubated in a CO_2-enriched atmosphere at 37°C. *P. gingivalis* ATCC 33277 was cultured in BHI broth using the GasPakjar system. Both of them were purchased from DIPA lab. The *L. casei* strain Shirota (10^6 colony-forming units [CFU]/ml) (isolated above) cultured in MRS broth at 37°C for 48 h under anaerobic conditions.

HaCaT cells, derived from human epithelial cells, were purchased from Oral biology laboratory, International Islamic University Malaysia. HaCaT cells were cultured in Dulbecco's Modified Eagle medium (Gibco, USA) added with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 1% Fungizone, 100 uG/mL streptomycin, and 10 uG/mL penicillin. The cells were incubated in tissue culture flasks at 37°C in a humidified atmosphere (RH 96%) with 5% CO_2. The HaCaT cells were centrifuged (Hermle, USA) at 2000 rpm for 10 min at 4°C, suspended in DMEM complete medium, and plated into culture flasks. Cell enumeration was performed with a hemocytometer with two flasks that were counted per time point and growth condition.

The confluent culture of HaCaT cells (10^5 cell/mL) [Figure 1] was exposed to heat-killed (by heating to 80°C) *S. mutans* ATCC 25175 (10^7 CFU/mL) or *P. gingivalis* ATCC 33277 (1×10^7 CFU/mL) for 24 h at 37°C and 5% CO_2. Subsequently, probiotic *L. casei* strain Shirota (10^7 CFU/mL) was added to the cell cultures, incubated for 3, 6, and 24 h' time period. After 1 h of infection, cells were washed in PBS, and the RNA extraction is carried out.

Analysis of Cell Viability using a Metabolic Assay

Cell viability was assessed using the metabolic assay based on the reduction of (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium) (MTT) by the mitochondrial dehydrogenase of intact cells to form a purple formazan product. HaCaT cells (10^5 cells per well) plated in a 96-well plate and treated with *L. casei* (1×10^7 CFU/mL). After incubations of 15 and 30 min, 1 h, 3 h, 6 h, and 24 h, MTT (1 mg/ml in PBS) (VWR Life Science, USA) was added to the wells, and cells were incubated for 3 h. The formazan amount was analyze by measuring the absorbances at 490 nm using a microplate-reader (SAFAS, Monaco). The cell viability was measured as a percentage using the formula: cell death (%) = (control cells optical density [OD]–sample cells OD)/control cells OD × 100%. The assay was performed in triplicate.

RNA Extraction

IL-8 and hBD-2 mRNA expression levels were determined by reverse transcription (RT) –PCR (RT-PCR). RNA extraction was performed using TRIzol reagents (Invitrogen/Thermo Fisher Scientific, Waltham, Massachusetts, USA). Approximately 1 mL of TRIzol was added into the cells, followed by incubation for 5 min at room temperature. Then, 200 µL of chloroform was added. Next, the suspension was transferred into a new 1.5 mL microtube, and the tube was flipped for 15 s to homogenize the mixture. After that, the suspension was incubated for 3 min at room temperature and then was centrifuged at 12,000 × g for 15 min 4°C. The upper layer (aqueous phase) was taken out and then transferred into a new 1.5 mL microtube. About 100% isopropanol at the amount of 0.5 mL was added into the tube and then incubated at room temperature for 10 min. The tube was then centrifuged at 12000 × g for 10 min at 4°C. The supernatant was discarded, and 75% ethanol at amount of 1 mL was added into the tube. The mixture was homogenized using a vortex mixer and then was centrifuged again at 7500 × g for 5 min at 4°C. The supernatant was removed, and the pellet was allowed to air-dry for 10 min at room temperature. The RNA-containing pellet was then resuspended again by adding 20 µL of ddH_2O, and incubated in a thermoblock machine (Biosan, Riga, Latvia) at 57°C for 15 min. After that, the pellet was stored at −70°C. A spectrophotometer was used to determine RNA concentration. The isolated RNA was taken out (approximately 2 µL),

and diluted in 498 µL ddH_2O. Then, RNA solution was fed into a glass cuvette, and the cuvette was inserted to the spectrophotometer. The RNA concentrations were determined using a spectrophotometer (SAFAS, Monaco).

RT from RNA to cDNA and Quantitative (q)PCR

After the RNA concentration was measured, RT-PCR was performed to obtain the cDNA sequences. We used Thermo Fisher Scientific's GeneAmp Gold RNA PCR Reagent Kit with a random oligo (dT) primer and 1 µg of RNA template (Thermo Fisher Scientific, USA). Total reaction (25 µL) containing 12.5 µL of SYBR-Green PCR MasterMix, forward and reverse primers (300 nM), nuclease-free water (11 uL), and cDNA sample (1.5 µL). The initial denaturation step was set for 1 min at 95°C and amplified for 40 cycles (15 s) at 95°C and 60 s at 60°C. All primers used are listed in Table 1 for IL-8[8] and hBD-2,[21] as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[8] IL-8 and hBD-2 levels were normalized to the GAPDH expression level. qPCR method was performed in triplicate, with an StepOne™. RT-PCR System (Thermo-Fisher Scientific, USA) and the specific PCR product amplification were detected using the SYBR Green PCR Master Mix (Applied Biosystem, USA). The DNA quantification of each sample was done using the $2^{-\Delta\Delta Ct}$ formula, where the Ct value represents the threshold cycle of PCR at which the amplified product was detected. All treatments were performed in triplicate.

Statistical Analysis

The one-way analysis of variance tests was applied to reveal significant differences in IL-8 and hBD-2 mRNA expressions in HaCaT cells exposed to *S. mutans*, *P. gingivalis,* and *L. casei* for a range of treatment times in three independent experiments. The value of $P < 0.05$ was considered as statistically significant difference. The SPSS Statistics for Windows software version 20 (IBM, USA) was used for statistical calculations.

RESULTS

MTT Assay to Assess *L. casei* Cytotoxicity on Epithelial Cells (HaCaT)

L. casei cytotoxicity on the HaCaT epithelial cells was assessed using the MTT assay to measure cell viability according to ISO 10993-5. Results showed that the HaCaT cell viabilities were in the range of 91.8–106.7%. The result indicates that *L. casei* does not cause toxicity in human epithelial cells [Figure 2].

L. casei Reduced the HaCaT Expression Levels of IL-8 after Incubation with *Streptococcus mutans* or *P. gingivalis*

IL-8 mRNA expression levels were obtained during the initial incubation of the HaCaT cells with the heat-killed bacteria and during the cells' subsequent incubation with *L. casei* for 3 h, 6 h, and 24 h. IL-8 expression levels increased significantly after exposing the cells to *S. mutans* ($P = 0.000$) or *P. gingivalis* ($P = 0.000$) [Figure 3a and b]. The addition of *L. casei* could inhibit both *S. mutans* and *P. gingivalis* IL-8 mRNA expression of HaCaT cells at 3 h, 6 h, and 24 h of treatment ($P = 0.000$). Significant differences were observed when *S. mutans*-induced IL-8 mRNA expressions in *L. casei* for 3 hand 24 h ($P = 0.000$) were compared. Significant differences were also observed when *P. gingivalis*-induced IL-8 mRNA expressions in *L. casei* for 3 h and 24 h ($P = 0.000$) [Figure 3a and b] were compared.

L. casei Increased the HaCaT Expression Levels of hBD-2 after Incubation with *S. mutans*- or *P. gingivalis*

hBD-2 expression levels increased after exposing the cells to *S. mutans* or *P. gingivalis* [Figure 4a and b]. In contrast to IL-8, the hBD-2 mRNA expression increased significantly in HaCaT cells exposed to *S. mutans*, *P. gingivalis* after *L. casei* treatment in 3 and 6 h period ($P = 0.000$). The hBD-2 mRNA expressions in the HaCaT cells exposed to *S. mutans or P. gingivalis* and *L. casei* were significantly decreased after 24 h of treatment ($P = 0.000$) [Figure 4a and b].

DISCUSSION

Host cell immune responses can vary depending on the type of invading pathogen. In this study, heat-killed bacteria were used to avoid bacterial overgrowth in the medium and the overproduction of immune responses in the cultured cells. The heat-killed Gram-positive *S. mutans* and Gram-negative *P. gingivalis* both successfully induced the expression of IL 8 and hBD-2 *in vitro* during the initial incubation period. We also noticed a slight increase in IL-8 and hBD-2 mRNA expression immediately after *L. casei* was

Table 1: Primers used for RT-PCR

Primers	Sequence (5'-3')
IL-8 forward primer	TCTCTTGGCAGCCTTCCT
IL-8 reverse primer	ACTGAACCTGACCGTACATGTCTTTATGCACTGACATCT
hBD-2 forward primer	GGTGTTTTTGGTGGTATAGGC
hBD-2 reverse primer	AGGGCAAAAGACTGGATGACA
GAPDH forward primer	CTGAGTACGTCGTGGAGTC
GAPDH reverse primer	ACTGAACCTGACCGTACACAGAGATGATGACCCTTTTG

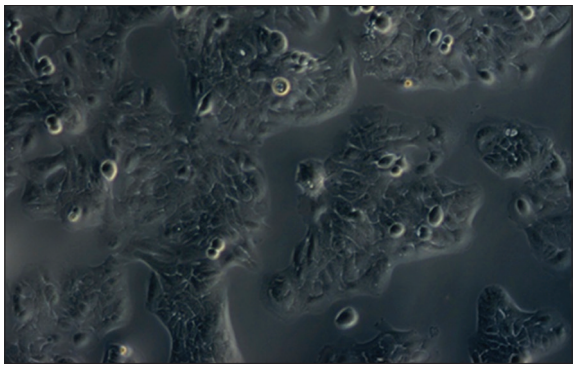

Figure 1: Confluent HaCaT cell culture seen under an inverted microscope

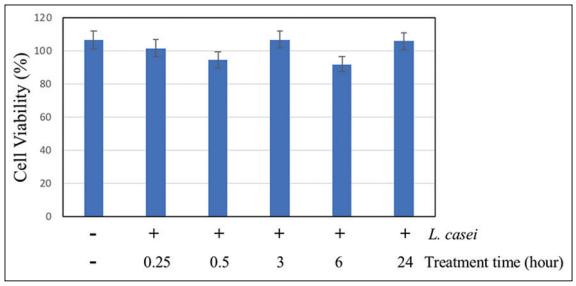

Figure 2: The percentage of viable HaCaT cells after treatment with *Lactobacillus casei.* HaCaT cells were seeded in a 96-well plate at a density of 10^4 cells/well. Cells were treated with 10^6 CFU/mL of *L. casei* for 15 min, 30 min, 1 h, 3 h, 6 h, or 24 h. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) determined the percent of cell viability. Each treatment was done in triplicate

added to the cells. This was likely due to the presence of lipoteichoic acid in the *L. casei* cell wall. The cell walls of *Lactobacilli* and other Gram-positive bacteria contain peptidoglycans and lipoteichoic acid.[22] These components can modulate the pro-inflammatory cytokines production such as IL 8 through the signaling pathways activation, primarily through transcriptional nuclear factor (NF)-kB, the phosphatidylinositol-3-kinase-Akt pathway,[23] the mitogen-activated protein kinase (MAPK) pathway, and Toll-like receptors (TLRs).[24,25]

Most epithelial cells produce cytokines in response to inflammation. IL-8 is the most predominant cytokine found in keratinocytes. Although IL-8 is required to eliminate invading pathogens from inflammation sites, prolonged IL-8 expression is considered dangerous to the host cells because it can lead to cell apoptosis and tissue destruction due to massive neutrophil infiltration.[23] Thus, host cell IL-8 production is both dose and time-dependent.[26] *Lactobacilli* strains have been proved to suppress the production of IL-8 in bacteria-derived lipopolysaccharides stimulated intestinal epithelial cells.[27] *L.casei* modulates IL 8 expression by inhibiting NF-kB activation at a terminal step in the signaling pathway.[28]

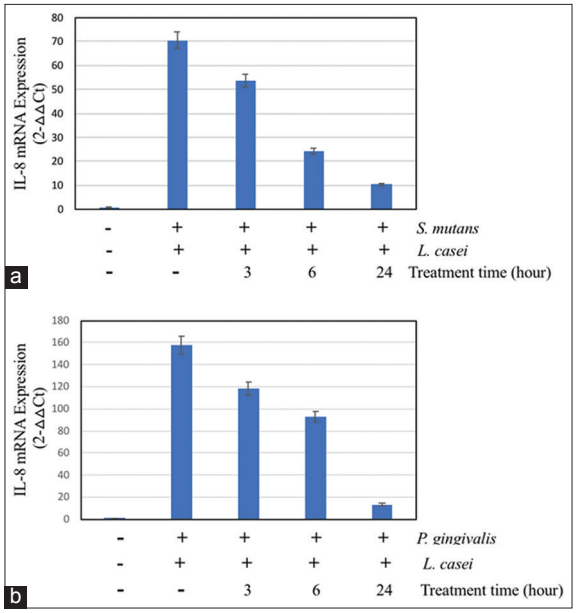

Figure 3: Interleukin-8 mRNA expression of HaCaT cells exposed to heat-killed *Streptococcus mutans* (a) or *Porphyromonas gingivalis* (b) and treated with *Lactobacillus casei.* One hundred thousand HaCaT cells were exposed to 10^7 colony-forming units (CFU) of preheated *S. mutans* or *P. gingivalis* for 24 h and then treated with 10^7 CFU of *L. casei* for 3, 6, or 24 h. Each treatment was done in triplicate

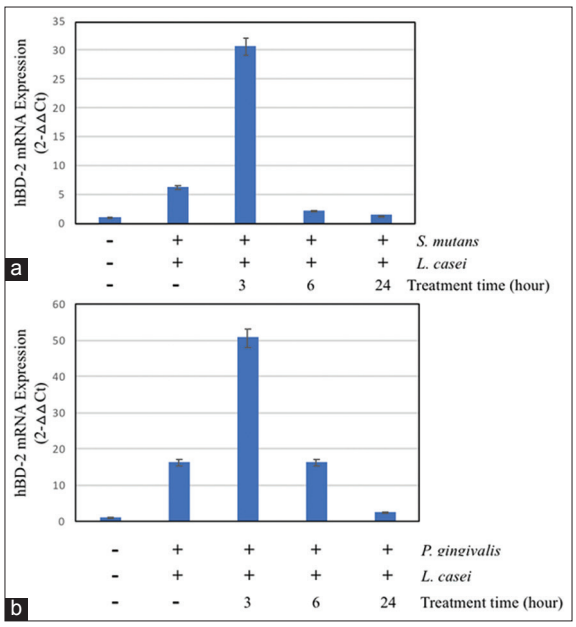

Figure 4: Human Beta-Defensin-2 mRNA expression of HaCaT cells exposed to heat-killed *Streptococcus mutans* (a) or *Porphyromonas gingivalis* (b) and treated with *L. casei.* One hundred thousand HaCaT cells were exposed to 10^7 colony-forming units (CFU) of preheated *S. mutans* or *P. gingivalis* for 24 h and then treated with 10^7 CFU of *L. casei* for 3, 6, or 24 h. Each treatment was done in triplicate

A study of cultured epithelial cells, which were infected with *Shigella flexneri* and then challenged with *L. casei.L. casei* downregulated the transcription of cytokines, chemokines, and adherence molecules

as pro-inflammatory factors. This anti-inflammatory effect appeared to be mediated by inhibition of the NF-κB pathway, particularly through stabilization of I-κBα.[28] Study concerning the ability of Lactobacillus to suppress the production of pro-inflammatory cytokine from colonic epithelial cells that have been stimulated by Clostridioides difficile, *L. casei* L39 isolate, along with another two *Lactobacillus rhamnosus* isolates (*L. rhamnosus* L35 and *L. rhamnosus* L34) have significantly suppressed approximately 50% of IL-8 production compared to the control through in activation of transcription factors without inhibiting toxin production *or C. difficile* growth.[29] Another study showed that *Lactobacillus reuteri* with glycerol supplementation significantly reduced the expression of IL Figure 4: Human Beta-Defensin-2 mRNA expression of HaCaT cells exposed to heat-killed *Streptococcus mutans* (a) or *Porphyromonas gingivalis* (b) and treated with *L. casei.* One hundred thousand HaCaT cells were exposed to 10^7 colony-forming units (CFU) of preheated *S. mutans* or *P. gingivalis* for 24 h and then treated with 10^7 CFU of *L. casei* for 3, 6, or 24 h. Each treatment was done in triplicate-8 on infected epithelial cells.[30]

In this study, the transcription levels of hBD-2 were increased after exposed to *S. mutans* or *P. gingivalis* and *L. casei*. This result is in concordance with the previous study of Niyonsaba *et al.*, which reported that epithelial tissues contain high amounts of hBDs at the sites of inflammation, although the average concentrations were not precisely known.[31] Another study showed increased levels of beta-defensin-2 expression in the epithelial parotid glands after the addition of *L. reuteri*.[32] Expression of hBD-2 can be induced by various pro-inflammatory agents, including TNF-α, Gram-negative bacterial lipopolysaccharides, Gram-positive bacteria, and yeast infections.[26,33] In our study, the increase of hBD-2 expression peaked in the first stages (at 3 and 6 h) after the introduction of the heat-killed bacteria. According to Yoon *et al.*, in a study using intestinal epithelial cells, increased hBD-2 mRNA expression was first noted 2 h after stimulation and peaked at 6 h post-stimulation before decreasing to the baseline. This data reveal the time-dependent manner of epithelial cell hBD-2 expression.[34]

The expression of hBD-2 in the epithelial cell is primarily regulated by signaling pathways through TLRs,[26] MAPK, NF-kB, or activator protein (AP)-1.[34-36] The hBD-2 gene contains promoter with binding sites for NF-kB and AP-1.[34,37] Lactic acid bacteria are known to induce hBD expression in host cells through the transcription factors NF-kB and AP-1.[38]

Reports have also indicated that hBD scan encourage wound healing by defense in-stimulated cell-proliferation and increased wound closure *in vitro*, thus benefiting tissue regeneration.[39,40] Similarly, preliminary studies have examined the wound-healing effects of the *L. casei* strain Shirota used in probiotic drinks. Results from animal experiments have shown that *L. casei* may help heal oral cavity ulcers and speed up epithelization.[41] These properties would be useful for periodontal treatments[42] and post-extraction management.[43] Interest in probiotic research has grown in recent decades; however, little information about the use of probiotic bacteria in the oral cavity has been reported. The anticancer, antiinflammatory, and antifungal properties of probiotics as well as their immune-boosting benefits are beyond doubt.[38,44]

CONCLUSION

This study indicated that the *L. casei* strain Shirota does not possess cytotoxicity against HaCaT epithelial cells. *L. casei* conveyed a beneficial immune modulation, reducing IL-8 levels, and elevating the production of hBD-2. Further studies are encouraged to investigate this probiotic's anti-inflammatory properties at the molecular level.

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