

Effects of biosurfactant produced by *Lactobacillus casei* on *gtfB*, *gtfC*, and *ftf* gene expression level in *S. mutans* by real-time RT-PCR

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Abstract

Background: The *Streptococci* are the pioneer strains in plaque formation and *Streptococcus mutans* are the main etiological agent of dental plaque and caries. In general, biofilm formation is a step-wise process, which begins by adhesion of planktonic cells to the surfaces. Evidences show that expression of *glucosyltransferase B and C* (*gtfB* and *gtfC*) and *fructosyltransferase* (*ftf*) genes play critical role in initial adhesion of *S. mutans* to the tooth surface which results in formation of dental plaques and consequently caries and other periodontal disease.

Materials and Methods: The aim of this study was to determine the effect of biosurfactants produced by a probiotic strain; *Lactobacillus casei* (ATCC39392) on gene expression profile of *gtfB/C* and *tft* of *S. mutans* (ATCC35668) using quantitative real-time PCR.

Results: The application of the prepared biosurfactant caused dramatic down regulation of all the three genes under study. The reduction in gene expression was statistically highly significant (for *gtfB*, $P > 0.0002$; for *gtfC*, $P > 0.0063$, and for *ftf*, $P > 0.0057$).

Conclusion: Considerable downregulation of all three genes in the presence of the prepared biosurfactant comparing to untreated controls is indicative of successful inhibition of influential genes in bacterial adhesion phenomena. In view of the importance of *glucosyltransferase* gene products for *S. mutans* attachment to the tooth surface which is the initial important step in biofilm production and dental caries, further research in this field may lead to an applicable alternative for successful with least adverse side effects in dental caries prevention.

Key Words: Biosurfactants, *Lactobacillus casei*, oral streptococci, probiotics

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INTRODUCTION

Dental plaque as a microbial biofilm is defined as the multi-species community of micro-organisms formed on the tooth surfaces, in a protective shelter of an extra cellular matrix of host and microbial polymers.

Dental caries are one of the most prevalent chronic human infectious diseases widely spread in all age

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groups.^[1,2] Colonization of the teeth by *S. mutans* is a main etiology of dental caries in human beings.^[3,4] These organisms acquired an evolutionary adaptability to enzymatic conversion of sucrose to extracellular glucans by a set of enzymes, *glucosyltransferases (gtfs)*, produced by the bacterium.^[5,6] The *gtfs* secreted by *S. mutans* (particularly GtfB and GtfC) may act in two ways; either bacterial colonization of the tooth surface or attachment of bacteria to each other, modulating the formation of tightly adherent biofilms, which is a main prerequisite of dental caries.^[7-12] It is now a well-known fact that *S. mutans* adhesion to the tooth surface is vital for the initiation and progression of dental caries.^[13,14] *In vitro* studies have indicated that *gtfB* and *gtfC* are essential for the sucrose-dependent attachment of *S. mutans* cells to hard surfaces,^[15] but *gtfD* is dispensable.^[16,17] Therefore, these enzymes being the main component in dental plaques and caries created much interest to be targeted in anti-caries therapeutic strategies.^[18]

A variety of antiplaque agents are developed and tested for their ability to interfere with dental biofilm formation or metabolism. However, due to their inefficiency or for presence of some harmful side effects, efforts toward finding alternative efficient and safe antiplaque agents are in continued demands. Like all other areas of medical therapeutics that ultimately natural resources proved to be the best, it appears logical to look for a natural product to prevent bacterial biofilm production on teeth surfaces.^[19,20]

Lactobacilli, as probiotic agents, are believed to interfere with pathogens through different mechanisms.^[21] One of its proposed mechanisms is production of biosurfactants. These secondary products are structurally diverse group of surface-active molecules synthesized by some of the biosurfactant producing microorganisms, have received much attention in recent years because of their superiority over synthetic surfactants, such as low toxicity, biodegradability, and environmentally safe.^[22] However, there are some extensive *in vitro* studies as well as clinical trials regarding the application of bacteriotherapy in caries prevention with several proposed mechanisms through which probiotics interfere with the cariogenic bacteria.^[23] Since there are a number of inherent problems in application of direct live bacteria, it seems using probiotics byproducts would be much more desirable and safe.

Here, we investigated effects of biosurfactants produced by *Lactobacillus casei (L.c.)* on *gtfB*, *gtfC*, and *ftf* gen expression level in *S. mutans (ATCC35668)* using real-time RT-PCR.

MATERIALS AND METHODS

Bacteria and culture conditions

S. mutans strain ATCC35668 was cultured on blood agar and Mitis salivarius agar medium and incubated in a CO₂-enriched atmosphere at 37°C. *Lactobacillus casei (ATCC39392)* as a probiotic source was cultured in the Man, Rogosa and Sharpe (MRS) broth or agar.

Biosurfactant production

L. casei cultured overnight (15 ml) was inoculated into 600 ml of MRS broth and incubated for 24 hours. The cells were harvested using centrifugation at 10,000 × g for 5 minutes at 10°C, washed twice with demineralized water, and resuspended in 100 ml of PBS. The lactobacilli were incubated at room temperature for 2 hours with gentle stirring for biosurfactant production. Subsequently, the bacterial cells were pelleted using centrifugation, and the supernatant was filtered through a 0.22-mm filter (Millipore). Then dialyzed against distilled water at 4°C in a Spectrapor membrane tube (molecular weight cutoff 6000 to 8000 Da; Spectrum Medical Industries, Inc.) and freeze-dried as described previously.^[24]

Drop collapse method

To test whether the biosurfactant produced could decrease the surface tension between water and hydrophobic surfaces, its ability to collapse a droplet of water was tested. Briefly, extracted biosurfactant (25 µl) was pipetted as a droplet onto a piece of parafilm, and the flattening and spreading of the droplet on the parafilm surface was followed over seconds or minutes. Subsequently, methylene blue was added to the water and supernatants for photographic purposes. The droplet was allowed to dry, and the diameter of the dried droplet was recorded.^[25,26]

Biofilm formation assay by microtiter plate method

To generate biofilms on microtiter plates, 20 µl of an overnight culture of *S. mutans* were inoculated in each well of a 24-well polystyrene plate and cultivated in 2 ml BHI broth supplemented with 1% sucrose. The plates were incubated at 37°C in an atmosphere enriched with 5% CO₂. After incubation for 18 hours, the spent medium was aspirated, and the wells were washed with PBS to remove unattached cells. The biofilm was incubated again in fresh BHI with 1% sucrose; after 18-hour incubation, the spent medium was aspirated again. The cells were washed, and the biofilm was incubated again in fresh BHI broth with 1% sucrose supplemented with or without 2.5 mg per ml of freeze-dried biosurfactant. After incubation for 4 hours, the cells of the biofilms were dislodged and transferred to tubes containing 2 ml of PBS solution and vortexed.^[14]

Extraction of total RNA

The prepared biofilm cells on microtiter plates (*S. mutans* ATCC 35668 with and without biosurfactant in three replicates) were used for RNA extraction. Cells were disrupted using a ribolyser instrument (Hybaid, UK) and the supplied kit according to the manufacturer's instructions. Briefly, RNA containing supernatant from the ribolyser tube was transferred to a new RNase-free microtube, centrifuged, treated with 300 μ l of chloroform-isoamyl alcohol, vortexed, and centrifuged again. Then, total RNA was recovered by precipitation with isopropanol and dried under appropriate sterile conditions. Quantitative and qualitative evaluations of extracted RNA were performed using a spectrophotometer (Biophotometer, Eppendorf, Rs 232-C, Germany) and agarose gel electrophoresis.

Reverse transcription

Each reverse transcription reaction mixture (20 μ l) containing 50 ng of random hexamers, 2 μ g of total RNA sample, and up to 12 μ l of RNase-free water was incubated at 70° C for 5 minutes to remove any secondary structure and placed on ice. Then, 5X RT buffer (4 μ l), 2 U of ribonuclease inhibitor, and 10 mM dNTP mix were added to each reaction mixture. After incubation for 5 minutes at 37°C, 1 μ l of reverse transcriptase was added. Then, the mixture was incubated at 42°C for 60 minutes. The reaction was terminated by heating the mixture at 70°C for 10 minutes, and the cDNA samples were stored at -70°C for further manipulation.

Real-time quantitative RT-PCR

The amplification of the synthesized cDNAs was first optimized using conventional PCR. Real-time quantitative RT-PCR was performed using the ABI-step I plus (Applied Biosystems, USA) instrument and SYBR Green PCR Master Mix (Qiagen, Germany). The relative quantification of *gtfB*, *gtfC*, and *ftf* genes was performed using 16S rRNA as a reference gene. Three gene-specific primers for *gtfB* gene (F- AGCAATGCAGCCAATCTACAAAT and R-ACGAACTTTGCCGTTATTGTCA), *gtfC* (F-CTCAACCAACCGCCACTGT, R-GGTTTAACGTCAAAATTAGCTGTATTAG), *ftf* (F-AAATATGAAGGCGGCTACAACG, R-CTTACCAGTCTTAGCATCCTGAA), and 16SrRNA (F- CCTACGGGAGGCAGCAGTAG, R-CAACAGAGCTTTACGATCCGAAA) as an internal control were used in quantitation of genes under study.^[14] The reaction mixture (20 μ l) contained 1X SYBR Green PCR Master Mix (Qiagen), the appropriate forward and reverse PCR primers and 1 μ l of the cDNA sample. The PCR program consisted of an initial denaturation at 95°C for 5 minutes,

40 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. Appropriate negative and positive controls were included. Using the two-step protocol described above, all primer pairs were evaluated for primer dimer formation without the addition of a template. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was performed without reverse transcriptase, to determine whether the RNA samples were contaminated with residual genomic DNA.

The critical threshold cycle (Ct) was defined as the cycle at which fluorescence became detectable above the background fluorescence and was inversely proportional to the logarithm of the initial number of template molecules.

RESULTS

Drop collapse assay

In the drop collapse assay, the biosurfactant droplets resulted in a collapsed droplet [Figure 1], indicating the effect of the biosurfactant on the reduction of surface tension. No activity was detected for distilled water.

Effects of the biosurfactant on gene expression profiles

For evaluation of the effect of *L. casei* biosurfactant on *gtfB*, *gtfC*, and *ftf* gene expression in *S. mutans* ATCC35668, Real-time RT-PCR was used.

The 16S rRNA gene was used as an endogenous reference [Figure 2]. The biosurfactant significantly reduced expression of all three genes under study in comparison to the reference gene. The *P* values obtained for each of the three genes (for *gtfB*, *P* > 0.0002; for *gtfC*, *P* > 0.0063; and for *ftf*, *P* > 0.0057), is indicative of the high efficiency of the produced biosurfactant in downregulation of these adhesive promoting genes of *S. mutans*.

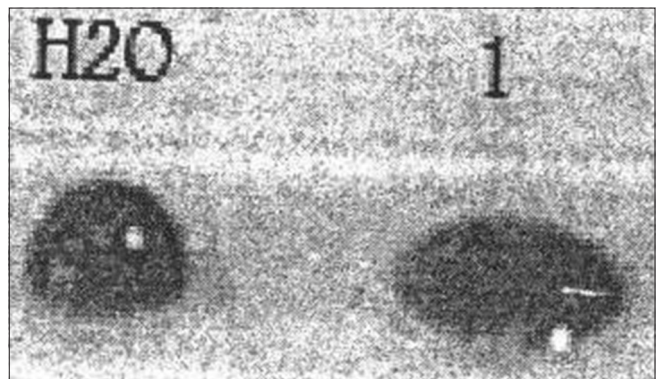


Figure 1: Drop collapse assay. Collapsed droplet (1) is the *L.c.*-biosurfactant and distilled water

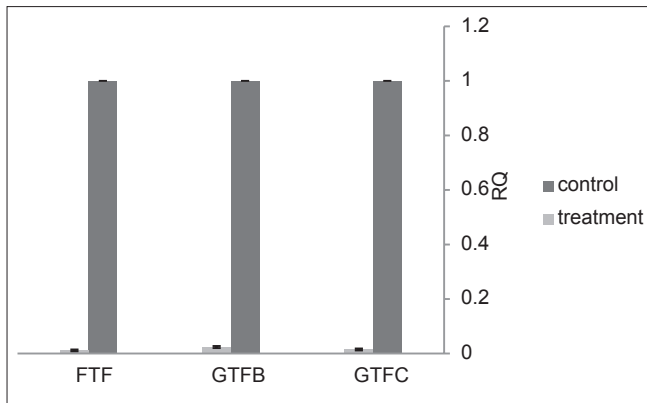


Figure 2: The effect of L.c.-derived biosurfactant on *gtfB/C* and *ftf* gene expression level in *S. mutans* ATCC35668

DISCUSSION

Dental caries are the worldwide serious problems involving all age groups in developing countries as well as developed ones. Since after incidence of dental caries treatments are involving high cost and successful eradication is almost impossible, prevention is most desirable option and recommended strategy. Frequent reports on development of resistance to various synthetic antimicrobials, prompted searching for suitable natural products,^[27] such as herbicidal extractions or probiotic bacteria and their products. Lactic acid bacteria produce biosurfactant and one of the major roles known for surfactants is their inhibitory effect on other microbial species.^[24,28-30] Here, we investigated the effects of biosurfactant extracted from *L. casei* on gene expression profile of standard biofilm producing strain of *S. mutans* ATCC35668. The selected genes studied here are all essential for initiation of bacterial adhesion and plaque formation.

The expression of *glucosyltransferases* like *gtf B* and *C* by biofilm producing bacteria and the production of insoluble extracellular glucans mediate the attachment of *S. mutans* not only to surfaces but also to other active types of bacteria and microorganisms which are in favor of persistent colonization of tooth surfaces by organisms.^[31] Additionally, *gtf* genes are known virulence factors associated with the pathogenesis of dental caries and a high content of insoluble glucans in dental plaque, which is related to an elevated risk of biofilm cariogenicity in humans.^[7] Several environmental factors can influence the expression and activity of the *gtf* enzymes. The existence of at least three enzymes acting on sucrose for production of glucans in addition to factors that involve post-translational modifications of the GTF enzymes, have traditionally complicated the understanding of regulatory studies.^[32-34]

It is assumed that either the *L. casei* biosurfactant itself or a putative signaling molecule in the applied surfactant act upon gene expression level in the treated bacteria and hence reducing the process of *S. mutans* attachment and biofilm formation. Another alternative hypothesis could also put forward stating that biosurfactant may interfere with *gtf* enzyme activity from insoluble glucan production to water-soluble glucans, hence accounting for reduced *S. mutans* biofilm adherence. This notion needs confirmation through further studies in the future.

Most studies have focused on the production and gene regulation of virulence factors, such as *gtfs*, which play an important role in biofilm formation by *S. mutans*, for controlling dental caries.^[35,36] The ability of *S. mutans* to produce extracellular polysaccharides from dietary sucrose has been demonstrated to significantly enhance its cariogenicity. Thus, the less extracellular polysaccharide produced, the lower the cariogenicity of *S. mutans*. It is demonstrated that chemical surfactants exerted different effects on the synthesis of *glucosyltransferases* in *S. mutans*; Tween 80 significantly increased the level of *gtfs*, while Triton X-100 decreased *gtf* levels.^[37] In general, the data suggest that biosurfactant treatment can provide an option for controlling biofilm development and also influence the adhesive ability of bacterial pathogens.^[38]

Apart from biosurfactants, much efforts were employed to evaluate effects of different natural or synthetic products like different polyphenols,^[39] cocoa polyphenols,^[40,41] plant extracts,^[42] Lectins,^[43] ursolic acid (UA)-containing composites,^[44] Cranberry A-type proanthocyanidins,^[45] mushroom, and chicory extracts,^[46] chitosan containing composite resin.^[47] However, compared to our study which is providing an insight on mode of inhibitory effect of the applied substances through gene expression profile study, the aforementioned works although proved the inhibitory effects on *S. mutans* adhesion or biofilm production but providing no information on detail about the underlying molecular mechanism through which this inhibitory effect is exerted.

In view of the results obtained in the present study, it is proposed that *L. casei* biosurfactant is highly effective in downregulating three proadhesive genes in *S. mutans*. Therefore, it would be an efficient alternative to the present therapeutics options generally considered for dental caries treatment. However, more elaborated experiments would be necessary to not only elucidate the molecular basis of biosurfactant mechanism in downregulation of the

genes under study but also to make a suitable ground for moving toward clinical application of this strategy.

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