LACTOBACILLUS ACIDOPHILUS-DERIVED BIOSURFACTANT EFFECT ON GTFB AND GTFC EXPRESSION LEVEL IN STREPTOCOCCUS MUTANS BIOFILM CELLS

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ABSTRACT

Streptococcus mutans (*S. mutans*), harboring biofilm formation, considered as a main aetiological factor of dental caries. *Gtf* genes play an important role in *S. mutans* biofilm formation. The purpose of this study was to investigate the effect of *Lactobacillus acidophilus*–derived biosurfactant on *S. mutans* biofilm formation and *gtfB/C* expression level (*S. mutans* standard strain ATCC35668 and isolated *S. mutans* strain (22) from dental plaque).

The Lactobacillus acidophilus (L. acidophilus) DSM 20079 was selected as a probiotic strain to produce biosurfactant. The FTIR analysis of its biosurfactant showed that it appears to have a protein-like component. Due to the release of such biosurfactants, L. acidophilus was able to interfere in the adhesion and biofilm formation of the S. mutans to glass slide. It also could make streptococcal chains shorter. Using realtime RT-PCR quantitation method made it clear that gtfB and gtfC gene expression were decreased in the presence of L. acidophilus–derived biosurfactant fraction.

Several properties of *S. mutans* cells (the surface properties, biofilm formation, adhesion ability and gene expression) were changed after *L. acidophilus-* derived biosurfactant treatment. It is also concluded that biosurfacant treatment can provide an optional way to control biofilm development. On the basis of our findings, we can suggest that the prepared biosurfactant may interfere with adhesion processes of *S. mutans* to teeth surfaces, provided additional evaluation produce satisfactory results.

Key words: Biofilm formation, biosurfactant, gtfB/C, Lactobacillus acidophilus, real-time RT PCR, *Streptococcus mutans*

INTRODUCTION

Streptococcus mutans (S. mutans), harbouring the dental biofilm, is one of the etiological factors of dental caries (16). Its ability to adhere to the teeth surface is vital for the initiation and progression of dental caries (3, 6, 31). The *S. mutans* adhesion mechanism is related to synthesis of both extracellular enzymes, glucosyl transferase (GTF) and fructosyl transferase (FTF) (7, 17, 26). These enzymes are responsible for the synthesis of extra cellular polysaccharides such as glucans and

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fructans. These polymers mediate adherence of *S. mutans* to the tooth surfaces and surfaces of bacteria. They also play an important role in the formation of plaque, the precursor of dental caries (13, 27).

α-(1-3) and α-(1-6)-linked glucan polymers are encoded by the genes *gtfB*, *gtfC* and *gtfD*. Recently, in vitro studies indicated that *gtfB* and *gtfC* are essential for the sucrose-dependent attachment of *S. mutans* cells to hard surfaces (2) but *gtfD* is dispensable (8, 43). Therefore, these genes have become a potential target for protection against dental caries (7).

Lactobacilli, as probiotic agents, are believed to interfere with pathogens by different mechanisms (22, 30). One of its mechanisms is biosurfactant production.

Biosurfactants, a structurally diverse group of surface active molecules synthesized by microorganisms, have attracted attentions in recent years. Because the reason, they had several advantages on synthetic surfactants, such as low toxicity, inherent good biodegradability and ecological acceptably. Biosurfactants include unique amphipathic properties derived from their complex structures, which include a hydrophilic moiety and a hydrophobic portion (37). The use of biosurfactants from probiotic bacteria as antimicrobial and/or anti-adhesive agents has been studied before and their ability to inhibit adhesion of various microorganisms isolated from explanted voice prostheses has been demonstrated (20).

The present research focused on the influence of *Lactobacillus acidophilus* (*L. acidophilus*)–derived biosurfactant on the *gtfB* and *gtfC* genes expression level in *S. mutans* biofilm cells by real time RT PCR for the first time.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *S. mutans* strains used in this study were *S. mutans ATCC35668* and dental plaque isolated *S. mutans 22,* with high ability of biofilm forming.

S. mutans strains were cultured on blood agar and mitis salivarius agar media and were incubated in a CO_2 enriched, 37 °C atmosphere. The identification of strains was done by usual biochemical tests and rapid identification kit of Streptococci (Rap ID STR kit) and by PCR. *L. acidophilus DSM 20079* as a probiotic source was cultured in MRS broth or Agar.

Biosurfactant production

15 mL of *L. acidophilus* cultured overnight was inoculated to 600-ml of MRS broth and incubated for 24 hours. The cells were harvested by centrifugation at 10,000 ✗ *g* for 5 min at 10 °C, washed twice in demineralized water, and were resuspended in 100 ml of PBS. The lactobacilli were incubated at room temperature for 2 hours with gentle stirring for biosurfactant production.

Subsequently, the bacteria were removed by centrifugation, and the remaining supernatant liquid was filtered through a 0.22 mm-pore-size filter (Millipore). A 10-ml portion of the supernatant was used immediately in the adhesion assay, and the remainder was dialyzed against demineralized water at 4 °C in a Spectrapor membrane tube (molecular weight cutoff, 6'000 to 8'000; Spectrum Medical Industries, Inc.), and was freeze-dried as in method of Velraed *et al* (38).

Drop-collapse method

In order to test whether produced biosurfactant was able to decrease the surface tension between water and hydrophobic surfaces, the ability to collapse a droplet of water was tested as follows: 25 μL of extracted biosurfactant was pipetted as a droplet onto parafilm; the flattening of the droplet and the spreading of the droplet on the parafilm surface was followed over seconds or minutes.

Subsequently, methylene blue (which had no influence on the shape of the droplets) was added to the water stain and supernatants for photographic purposes. The droplet was allowed to dry and the diameter of the dried droplet was recorded (14, 34).

Fourier transform infrared spectroscopy

Freeze-dried biosurfactants (2 mg) were ground with 100 mg KBr and compressed at 7'500 kg for 3 min to obtain translucent pellets. Infrared absorption spectra were recorded

with a model (Nicolet Impact 400) instruments (the spectral resolution and wave number accuracy were 4 and 0.01 cm^{-1}, respectively). KBr pellet was used as the background reference.

Quantity of a spectral region of interest was determined by normalizing the area under the absorption bands regarding to the area of the CH absorption band around 2'932 cm^{-1} (25, 38).

Biofilm formation assay

Glass slide method: In order to generate *S. mutans* biofilm on the glass slide, 1 mL of *S. mutans* over night cultured (10^8 CFU/ml) was inoculated into a flask containing 100 mL of sterile BHI broth supplemented with 1% sucrose and two slides with and without *L. acidophilus*–derived biosurfactant. The glass slides were washed in detergent solution, rinsed in distilled water twice, then air dried and autoclaved at 121 °C for 15 minutes before use. The flasks were incubated in an orbital incubator (100 rpm) at 35-7 °C for 18-20 h. Then, the glass slides were removed from the flasks and rinsed twice with 10 ml of PBS solution in order to remove unattached cells. Removed glass slides were stained with 2% crystal violet for 5 minutes, washed, air dried and photographed under an optical microscope with digital camera (Nicon, Eclipse, E200, Japan)(5).

Microtiter plate method: In order to generate biofilm on microtiter plate wells, 20 μL of overnight cultured *S. mutans* was placed in each wells of a 24 wells polystyrene multidishes and was cultivated with 2 mL of BHI broth supplemented with 1% sucrose. The plates were incubated at 37 °C in an atmosphere enriched with 5% CO_2. After 18 hours of incubation, the spent medium was aspirated and wells were washed with PBS solution in order to remove unattached cells. The biofilm was incubated again in fresh BHI with 1% sucrose; after another 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 and without 2.5 mg/ml of freeze-dried biosurfactant. After 4 hours of incubation, the cells of the biofilms were dislodged into tubes containing 2 mL PBS solution and vortexed (31).

Extraction of total RNA

The prepared biofilm cells on microtiter plates (*S. mutans ATCC 35668* & *S. mutans 22* with and without biosurfactant, in 3 replicate) were used for RNA extraction. Cells were disrupted using ribolyser instrument (Hybaid, UK.) and the supplied kit according to the manufacturer's instruction in which RNA-containing supernatant from ribolyser tube was transferred to new RNase free microtube, centrifuged and treated with 300 μL of chloroform–isoamyl alcohol, vortexed and centrifuged. Then total RNA was recovered by precipitation with isopropanol and dried under appropriate sterile conditions. Quantitative and qualitative evaluations performed on the extracted RNA by spectrophoto-metrically (biophotometer, Eppendorff, Rs 232-C, Germany) and Agarose-gel electrophoresis.

Reverse transcription

A reverse transcription (RT) reaction mixture (20 μL) containing 50 ng of random hexamers, 2 μg of total RNA sample and up to 12 μL DEPC-treated water was incubated at 70 °C for 5 min to remove any secondary structure and was placed on ice. Then 5X RT buffer (4 μL), 20 U/μL Ribonuclease Inhibitor (1 μL) and 10 mM dNTPs mix (Cinagen) were added to each reaction mixture, after 5 minutes incubation in 37 °C, 1 μL 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 -20 °C to be used later.

Real-time quantitative RT-PCR

PCR amplification of synthesized cDNAs were first optimized by conventional PCR. Real time quantitative RT-PCR was performed using the ABI-step I (Applied Biosystems, USA) instrument and SYBR Green PCR Master Mix (Qiagene).

The relative quantitation of *gtfB/C* genes were made against 16s rRNA as a reference gene. All primers and their location summerised in Table 1 (31).

The reaction mixture (20 µL) contained 1X SYBR Green PCR Master Mix (Qiagene), the appropriate forward, reverse PCR primers (1 µM) and 1 µL of the cDNA sample. PCR program consisted of an initial denaturation at 95 °C for 5 minutes, then 40 cycles of amplification applied as follow: denaturation at 95 °C for 15 second 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 checked for primer–

dimer formation without the addition of template. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out without reverse transcriptase in order to identify whether the RNA samples were contaminated by residual genomic DNA. The critical threshold cycle (Ct) was defined as the cycle in which fluorescence becomes detectable above the background fluorescence and was inversely proportional to the logarithm of the initial number of template molecules.

Table 1. Nucleotide sequences of primers

Sequence (5$'$ - 3$'$)	Fragment location	Accession number	Primer
gtfB – F	AGCAATGCAGCCAATCTACAAAT	1150-1172	M17361
gtfB – R	ACGAACTTTGCCGTTATTGTCA	1224-1245	M17361
gtfC – F	CTCAACCAACCGCCACTGTT	434-453	M22054
gtfC – R	GGTTTAACGTCAAAATTAGCTGTATTAG	496-524	M22054
16S- rRNA-F	CCTACGGGAGGCAGCAGTAG	243-262	X58303
16S – rRNA-R	CAACAGAGCTTTACGATCCGAAA	321-343	X58303

RESULTS

Drop collapse assay

In drop collapse assay, no activity was detected for distilled water as predicted. The biosurfactant droplets do result in a collapsed droplet (Figure 1), indicating their effects on reduction of surface tension.

Fourier transform infrared spectroscopy

The molecular composition of the biosurfactant used in this study was analysed by Fourier transform infrared spectroscopy (Figure 2). The most important bands were located at 2'933 cm^{-1} (CH band: CH2- CH3 stretching), 1'653 cm^{-1} (AmI band: CAO stretching in proteins), 1'480 cm^{-1} (AmII band: NOH bending in

proteins), 1'248 cm^{-1} (PI band: phosphates), and 1'099 cm^{-1} (PII band: polysaccharides). Therefore one of the components of *L. acidophilus* DSM 20079 biosurfactants appeared to be protein (Figure 2).
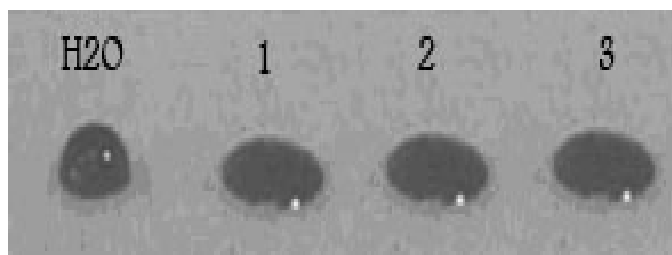

Figure 1. Drop collapse assay. Collapsed droplets (1, 2 & 3) are the *L.acidophilus*-derived biosurfactant
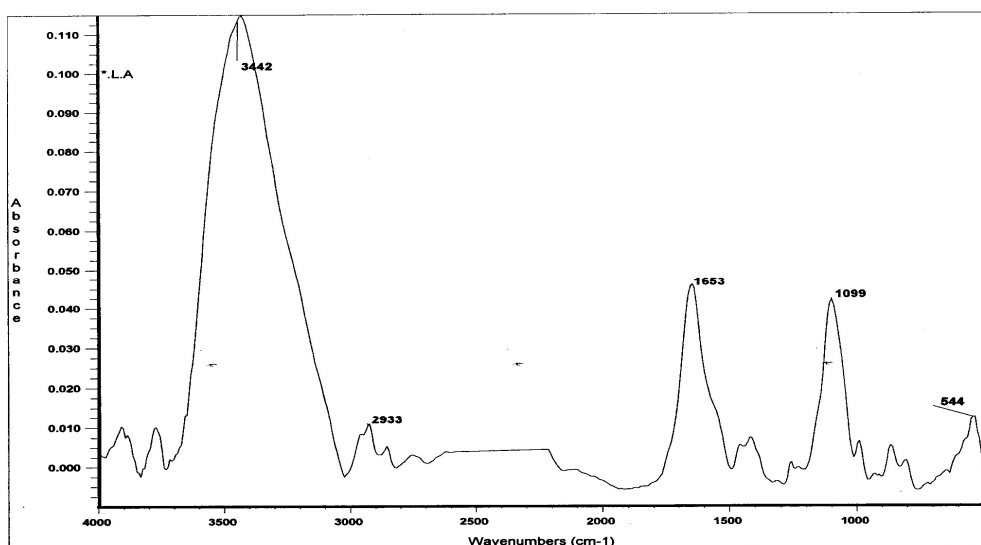
Figure 2. The spectrum of the freeze-dried biosurfactant released from *Lactobacillus acidophilus*

Biofilm formation

As it is shown in Figure 3A/B, the presence of 2.5mg/ml L. acidophilus derived biosurfactant, dramatically reduced the process of attachment and biofilm production .It could also make chain shortening.

The attached bacterial cells were collected from the wells of microtiter plates (Figure 4A) and used for RNA extraction by mentioned method (Figure 4B).

The effect of biosurfactant on *gtfB* and *gtfC* expression

Real-time RT-PCR was used to quantify the effect of *L. acidophilus*–derived biosurfactant on *gtfB* and *gtfC* gene

expression in biofilm of *S. mutans ATCC35668* and *S. mutans 22* (Figures 5A and B). As an internal reference 16srRNA gene was used.

In the biofilm environment, tested biosurfactant significantly reduced *gtfB* and *gtfC* gene expression ($p < 0.05$). However, the effect of biosurfactant on two genes was not identical with more pronounced gtfB gene expression suppression. Regarding *S. mutans 22*, the extent of reduction of gtfB expression level was more than the standard strain (p,0.05), but it's effect on gtfC expression level was the same as the standard strain.

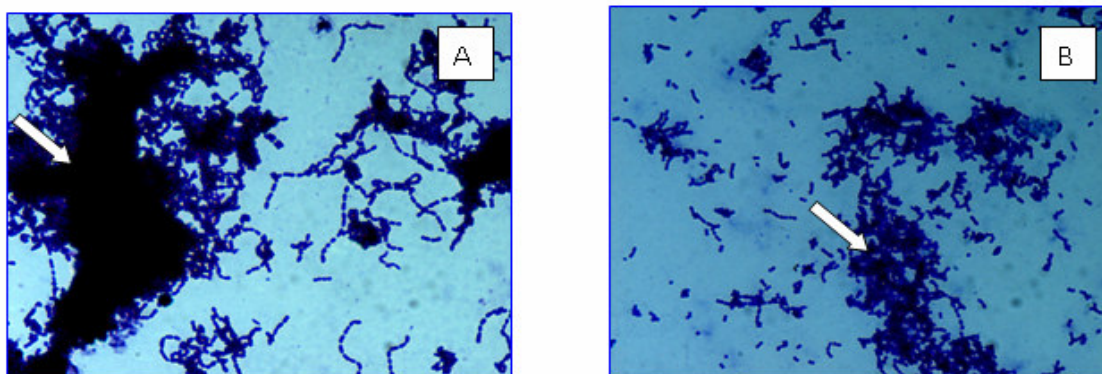**Figure 3.** *S.mutans* biofilm biofilm formation on glass slide. A:control group (in the absence of biosurfactant). B: Experimental group(in the presence of *Lactobacillus acidophilus*-derived biosurfactant) (The arrows show depth of biofilm.)
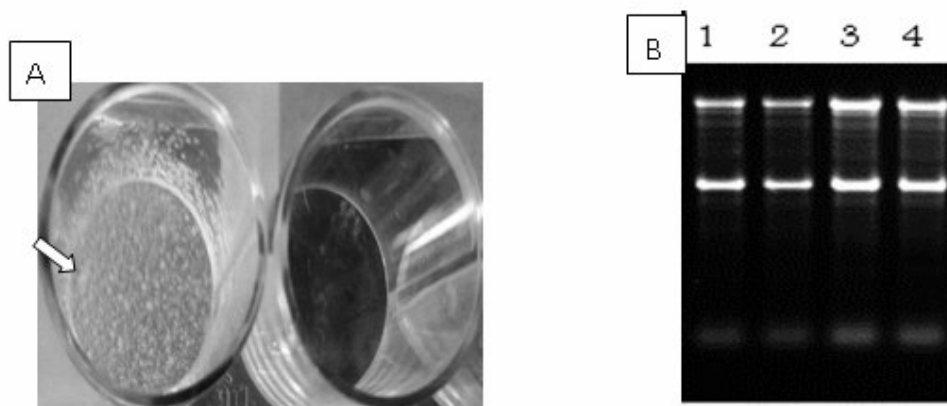
Figure 4. A: Attached cells to PVC microtiter plate well. B: total RNA extracted RNA from biofilm producing cells on PVC micrtiter plate. (lane1,2: Total RNA from *S.mutans 22* in the abscence and presence of biosurfactant, respectively. lane 3,4 Total RNA from standard strain in the abscence and presence of biosurfactant, respectively.)
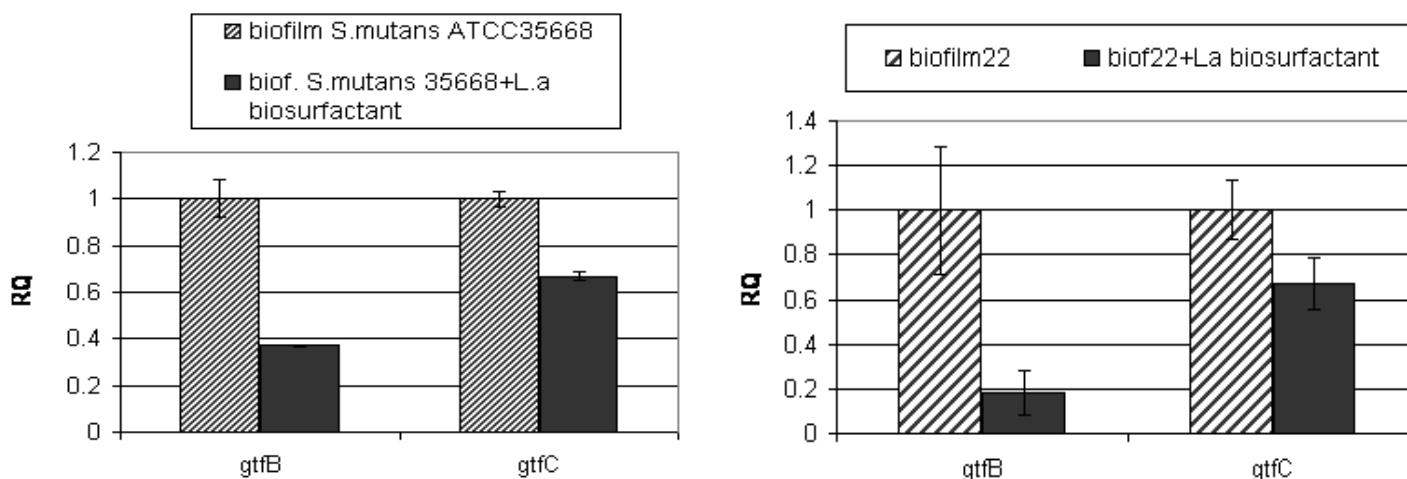

Figure 5. The effect of *Lactobacillus acidophilus*-derived biosurfactant, on *gtfB/C* in biofilm immobilized. A: S. mutans ATCC 35668; B: S. mutans 22 (isolated from dental plaque).

The mRNA expression levels were calibrated relative to the control group (in the absence of biosurfactant). The results were expressed as the means and standard errors of dublicate experiments using primers specific for *gtfB/C* and 16S rRNA (normalizing gene).

DISCUSSION

Oral infection constitute some of the most prevalent and expensive forms of human infection. The disease is widespread in all age groups with least success to control the actual infection. Dental caries is a multi factorial disease tightly related with the presence of cariogenic bacteria, *Streptococcus mutans*, which are embedded in the dental plaque biofilm.

As *S. mutans* is involved in dental caries, it must be inhibited. Increasing problems of resistance to synthetic antimicrobials have encouraged the researchers to research on alternative natural products (1) such as probiotic bacteria and

their products.

Some of microorganisms such as lactic acid bacteria were found to be biosurfactant producing strains (21, 25, 35, 38). One of the major roles known for biosurfactants is their negative effect on other microbial species.

In this study, 24-h culture of *L. acidophilus* was used to release biosurfactant, because Velraed *et al.* (38) and Rodrigues *et al.* (21) showed that the 24-h cultures exhibited the largest and most rapid decreases in liquid surface tension.

The biosurfactant activity showed that we had used real surfactant preparations, since the force or interfacial tension between the drop containing the surfactant and the parafilm surface was reduced and resulted in the spread of the drop. Rodrigues *et al.* (25), Kuiper *et al.* (14) and Walencka *et al.* (39) also showed that surface tension was reduced by biosurfactants.

The dialyzed (molecular weight cutoff 6,000 to 8,000) and freeze-dried biosurfactant derived from *L. acidophilus DSM20079* was examined by Fourier transform infrared spectroscopy and was compared with the reference compounds albumin, salivary glycoprotein, dextran, lipoteichoic acid and other FTIR spectra of biosurfactants (38).

On the basis of the results, it was concluded that the biosurfactant from *L. acidophilus* had more protein than polysaccharide and phosphate. According to figure 3-A and 3-B, it is also clear that, the biosurfactant could reduce the adhesion of *S. mutans* to glass slide. Velraeds *et al.* (38) demonstrated that, the biosurfactants from *L. acidophilus* RC14 and *L. fermentum* B54 were richer in protein and also contained less polysaccharide and phosphate than the biosurfactants from *L. casei* subsp. *rhamnosus* 36 and ATCC7469.

Due to the release of such biosurfactant, *L. acidophilus* could interfere in the adhesion and biofilm formation of the *S. mutans* to glass slide. It also could make streptococcal chains shorter (Figure 3b).

Van Hoogmoed *et al.* (10, 11) reported that, the release of biosurfactant from *S. mitis BMS* could interfere in the adhesion of the cariogenic *S. mutans* to glass in the presence and absence of a salivary conditioning film. Others also confirmed that

biosurfactants had inhibitory effect on bacterial adhesion and also biofilm formation (9, 22-25, 29).

However, the precise mechanisms of such effects have not yet been explained. It seems to be highly dependent on biosurfactant type and the properties of the target bacteria. The simplest way to explain biosurfactant antiadhesion and anti-biofilm activities would be their direct antimicrobial action. However, the antimicrobial activity of biosurfactants have not been observed in all cases (25, 39).

Thus, Walencka *et al.* (39) reported that the way in which surfactants influenced bacterial surface interactions appeared to be more closely related to the changes in surface tension and bacterial cell-wall charge. These factors are very important in overcoming the initial electrostatic repulsion barrier between the microorganism cell surface and its substrate. Surfactants may affect both cell-to-cell and cell-to-surface interactions. Their results support the idea that lactobacilli-derived agents remarkably have an effect on these interactions (39).

In this study, we made an effort to investigate the effect of *L. acidophilus*–derived biosurfactant on *gtfB* and *gtfC* genes expression level. The expression of these genes and the production of insoluble extracellular glucans mediate the attachment of *S. mutans* not only to the surfaces but also to other active form of bacteria which are favorable to the organisms for the persistent colonization of tooth surfaces (28).

Additionally, *gtf* genes are known virulence factors associated with the pathogenesis of dental caries and high content of insoluble glucans in dental plaque which is related to elevated risk of biofilms cariogenicity in humans (19). Vacca-Smith *et al.* (36) mentioned that GtfB levels in saliva were correlated with presence of caries in humans.

Several environmental factors can influence on expression and activity of the GTF enzymes. The existence of various enzymes in the process of carbohydrate metabolism and transport, glucan synthesis and secretion, and degradation in the oral streptococci, in addition to factors that involve post-translational modifications of the GTF enzymes, have traditionally complicated the understanding of regulatory studies (4, 41).

Multiple regulatory networks that incorporate external signals, such as two-component regulatory systems, are necessary for biofilm formation. *S. mutans* also possesses a LuxS-mediated signaling pathway that affects biofilm formation. LuxS is the enzyme that catalyzes the reactions leading to the production of the AI-2 signal molecule (40, 42). In addition, a number of other gene products, such as BrpA (a cell surface-associated biofilm regulatory protein), have also been shown to play critical roles in environmental stress responses and biofilm development by *S. mutans* (40, 41). While much effort has been dedicated to understanding the molecular mechanisms of adherence, biofilm development and virulence gene expression by *S. mutans* in pure cultures, there are large gaps in our knowledge of how this cariogenic bacterium acts in response to inter-generic interactions with bacteria normally found in the supragingival plaque (41).

Our results showed that the L. acidophilus-derived biosurfactant, or a putative signaling molecule in the extract, down regulate the expression level of genes playing important role in the process of S. mutans attachment and biofilm formation.

Clearly, 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 the purpose of controlling dental caries (12, 32).

The ability of *S. mutans* to produce extracellular polysaccharides from dietary carbohydrates has been demonstrated to significantly enhance its cariogenicity. Thus, the less these extracellular polysaccharides produced, the cariogenicity of *S. mutans* can be less.

Tomita *et al* (33) demonstrated that chemical surfactants exerted different effects on the synthesis of glucosyltransferases in *S. mutans*. So that the presence of Tween 80 in media significantly increased in glucosyltransferases (GTFs), while Triton X-100 decreased it.

These results indicated that several properties of bacterial cells (the surface properties, gene expression, etc) were also changed after biosurfactant treatment. The situation in natural systems is obviously more complex and requires the consideration of many additional parameters (15, 39).

In general, biosurfacant treatment can provide an optional way for controlling biofilm development and also influence the adhesion ability of bacterial pathogens (18).

Currently, further investigations are being done in our laboratory on the effect of other probiotic biosurfactant such as *L. fermentum* and *L. rhamnosus* on *gtfB*, *gtfC* and *ftf* expression in *S. mutans* biofilm.

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