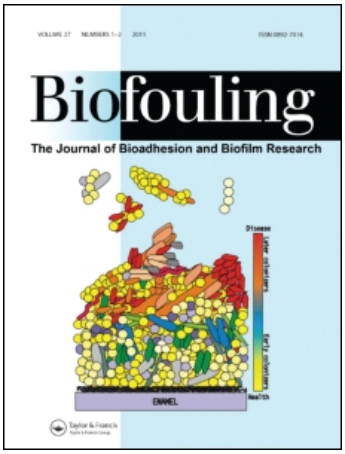

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Biofouling in the oral cavity often causes serious problems. The ability of *Streptococcus mutans* to synthesize extracellular glucans from sucrose using glucosyltransferases (gtfs) is vital for the initiation and progression of dental caries. Recently, it was demonstrated that some biological compounds, such as secondary metabolites of probiotic bacteria, have an anti-biofouling effect. In this study, *S. mutans* was investigated for the anti-biofouling effect of *Lactobacillus fermentum (L.f.)*-derived biosurfactant. It was hypothesized that two enzymes produced by *S. mutans*, glucosyltransferases B and C, would be inhibited by the *L.f.*-biosurfactant. When these two enzymes were inhibited, fewer biofilms (or none) were formed. RNA was extracted from a 48-h biofilm of *S. mutans* formed in the presence or absence of *L.f.* biosurfactant, and the gene expression level of *gtfB*/*C* was quantified using the real-time polymerase chain reaction (RT-PCR). *L.f.* biosurfactant showed substantial anti-biofouling activity because it reduced the process of attachment and biofilm production and also showed a reduction in *gtfB*/*C* gene expression (P value < 0.05).

Keywords: biofouling; biosurfactant; gene expression; *Lactobacillus fermentum*; *Streptococcus mutans*

Introduction

Dental caries are one of the most prevalent chronic human infectious diseases affecting both children and adults worldwide (Petersen et al. 2005; Dye et al. 2008). Colonization of the teeth by mutans streptococci has been associated with the etiology and pathogenesis of dental caries in humans (Loesche 1986; Beighton 2005). The ability of these organisms, particularly *Streptococcus mutans*, to synthesize extracellular glucans from sucrose using glucosyltransferases (Gtfs) is a major virulence factor of this bacterium (Yamashita et al. 1993; Rozen et al. 2001). The Gtfs secreted by *S. mutans* (particularly GtfB and GtfC) provide specific binding sites for either bacterial colonization of the tooth surface or attachment of bacteria to each other, modulating the formation of tightly adherent biofilms, the precursor of dental caries (Paes Leme et al. 2006; Vacca-Smith et al. 2007; Jae-Gyu et al. 2009; Koo et al. 2010; Murata et al. 2010; Xiao and Koo 2010). However, the ability of *S. mutans* to adhere to the tooth surface is vital for the initiation and progression of dental caries (Caglar et al. 2005; Tam et al. 2006). α-(1-3)- and α-(1-6)-linked glucan polymers are encoded by the genes *gtfB, gtfC*, and *gtfD*. *In vitro* studies have indicated that *gtfB* and *gtfC* are essential for the sucrose-dependent attachment of *S. mutans* cells to hard surfaces (Aoki et al. 1986), but *gtfD* is dispensable (Yoshida and Kuramitsu 2002; Yoshida et al. 2005). Therefore, these genes have become a potential target for protection against dental caries (Chia et al. 1991).

Antifouling is the process of removing the accumulation or preventing the accumulation. This process can be used to control biofouling. Numerous anti-plaque agents available in the market have been tested for their ability to interfere with dental biofilm formation or metabolism. However, due to several undesirable side effects associated with these agents, the search for alternate agents is necessary. One suggested approach is to use inexpensive, effective, stable, novel, and natural products as anti-biofouling agents (Briand 2009; Sendamangalama et al. 2011). Therefore, the natural product *Lactobacillus fermentum*-derived biosurfactant (*L.f.* biosurfactant), one of the secondary metabolites of *L.f.*, was investigated.

Lactobacilli, as probiotic agents, are believed to interfere with pathogens through different mechanisms (Rodrigues et al. 2006a). One of its mechanisms is biosurfactant production. Biosurfactants, a

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structurally diverse group of surface-active molecules synthesized by microorganisms, have attracted attention in recent years because they have several advantages over synthetic surfactants, such as low toxicity, inherent efficient biodegradability and ecological acceptability (Vater et al. 2002). The use of biosurfactants of probiotic bacteria as antimicrobial and/or anti-adhesive (anti-biofouling) agents has been studied previously, and their ability to inhibit the adhesion of various microorganisms isolated from explanted voice prostheses has been demonstrated (Rodrigues et al. 2004).

The present study focused on the influence of *L.f.*-derived biosurfactant on the gene expression of *gtfB* and *gtfC* in *S. mutans* biofilm cells using real-time RT-PCR.

Materials and methods

Bacteria and culture conditions

S. mutans ATCC35668 and dental plaque-isolated *S. mutans* 22, which had a high capacity for biofilm formation, were used in this study. *S. mutans* strains were cultured on blood agar and *Mitis salivarius* agar medium and incubated in a CO_2-enriched atmosphere at 37°C. Strain identification was performed using standard biochemical tests, a rapid identification kit for streptococci (Rap ID STR kit) and PCR. *L.f.* ATCC 9338 as a probiotic source was cultured in de Man, Rogosa and Sharpe (MRS) broth or agar.

Biosurfactant production

L.f. cultured overnight (15 ml) was inoculated into 600 ml of MRS broth and incubated for 24 h. The cells were harvested using centrifugation at 10,000 x *g* for 5 min at 10°C, washed twice in demineralized water, and resuspended in 100 ml of PBS. The lactobacilli were incubated at room temperature for 2 h with gentle stirring for biosurfactant production. Subsequently, the bacterial cells were pelleted using centrifugation, and the supernatant was filtered through a 0.22-μm filter (Millipore). A portion of the supernatant (10 ml) 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 6000 to 8000 kDa; Spectrum Medical Industries, Inc.) and freeze-dried as described previously (Velraeds et al. 1996).

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 as follows: extracted biosurfactant (25 μl) was pipetted as a droplet onto 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 (Kuiper et al. 2004; Tugrul and Cansunar 2005).

Fourier transform infrared spectroscopy

Freeze-dried biosurfactants (2 mg) were ground with 100 mg of KBr and compressed at 7500 kg for 3 min to obtain translucent pellets. Infrared absorption spectra were recorded with a Nicolet Impact 400 instrument (the spectral resolution and wave number accuracy were 4 and 0.01 cm^{-1}, respectively). A KBr pellet was used as the background reference. Quantification of a spectral region of interest was determined by normalizing the area under the absorption bands relative to the area of the CH absorption band at approximately 2932 cm^{-1} (Velraeds et al. 1996; Rodrigues et al. 2006c).

Antimicrobial activity of extracted biosurfactant

The growth inhibition test was performed as described by Rodrigues et al. (2006c). Briefly, an overnight culture of *S. mutans* was harvested and diluted to a concentration allowing confluent growth when plated on agar with a cotton swab. Agar plates were dried for 20 min at room temperature, and 5 μl of several concentrations of the extracted *L.f.* biosurfactant (ranging from 2.5 to 100 mg ml^{-1} according to previous studies) (Rodrigues et al. 2004, 2006c, 2006d) was spotted onto the surface of a brain heart infusion (BHI) agar plate. After overnight incubation, the plates were screened for growth inhibition zones around the spots.

Biofilm formation assay: glass slide method

To generate *S. mutans* biofilms on a glass slide, 1 ml of *S. mutans* cultured overnight (10^8 CFU ml^{-1}) was inoculated into a flask containing 100 ml of sterile BHI broth supplemented with 1% sucrose and two slides with and without *L.f.* biosurfactant. Sucrose was selected as a suitable substrate based on previous results (Tahmourespour et al. 2010). The used biosurfactant was the extracted biosurfactant. The glass slides were washed in detergent solution, rinsed twice in distilled water, air dried, and autoclaved at 121°C for 15 min before use. The flasks were incubated in an orbital incubator

(100 rpm) at 35–37°C for 18–20 h. Then, the glass slides were removed from the flasks and rinsed twice with 10 ml of PBS to remove unattached cells. Glass slides were then stained with 2% crystal violet for 5 min, washed, air dried, and photographed under an optical microscope with a digital camera (Nikon, Eclipse, E200, Japan) (Bos et al. 1999).

Biofilm formation assay: 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% CO_2. After incubation for 18 h, 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 an 18-h 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 ml^{-1} of freeze-dried biosurfactant. After incubation for 4 h, the cells of the biofilms were dislodged and transferred to tubes containing 2 ml of PBS solution and vortexed (Tam et al. 2006).

Extraction of total RNA

The prepared biofilm cells on microtiter plates (*S. mutans* ATCC 35668 and *S. mutans* 22 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 μl 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 μl) containing 50 ng of random hexamers, 2 μg of total RNA sample and up to 12 μl of DEPC-treated water was incubated at 70°C for 5 min to remove any secondary structure and placed on ice. Then, 5X RT buffer (4 μl), 20 U μl^{-1} of ribonuclease inhibitor (1 μl), and 10 mM dNTP mix (Cinagen) were added to each reaction mixture. After incubation for 5 min at 37°C, 1 μl of reverse transcriptase was added. Then, the mixture was incubated at 42°C for 60 min. The reaction was terminated by heating the mixture at 70°C for 10 min, and the cDNA samples were stored at −20°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 (Applied Biosystems, USA) instrument and SYBR Green PCR Master Mix (Qiagen). The relative quantification of *gtfB* and *gtfC* genes was performed using 16S rRNA as a reference gene. All primers and their locations are summarized in Table 1 (Tam et al. 2006). The reaction mixture (20 μl) contained 1X SYBR Green PCR Master Mix (Qiagen), the appropriate forward and reverse PCR primers and 1 μl of the cDNA sample. The PCR program consisted of an initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. 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.

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

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, as predicted.

Fourier transform infrared spectroscopy

The molecular composition of the biosurfactant used in this study was analyzed using Fourier transform infrared spectroscopy (Figure 2). The most important bands were located at 2930 cm^{-1} (CH band: CH2—CH3 stretching), 2248 cm^{-1} (probably related to N=N or C≡N), 1660 cm^{-1} (AmI band: C=O stretching in proteins), 1543 cm^{-1} (AmII band: NH bending in proteins), and 1079 cm^{-1} (PII band: polysaccharides). Therefore, one of the components of the *L.f.* biosurfactant appeared to be protein (Figure 2).

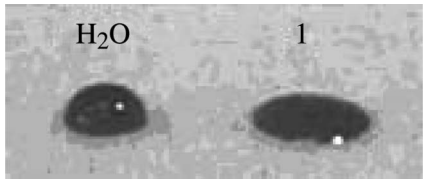

Figure 1. Drop collapse assay. Collapsed droplet (1) is the *L.f.*-biosurfactant and distilled water.

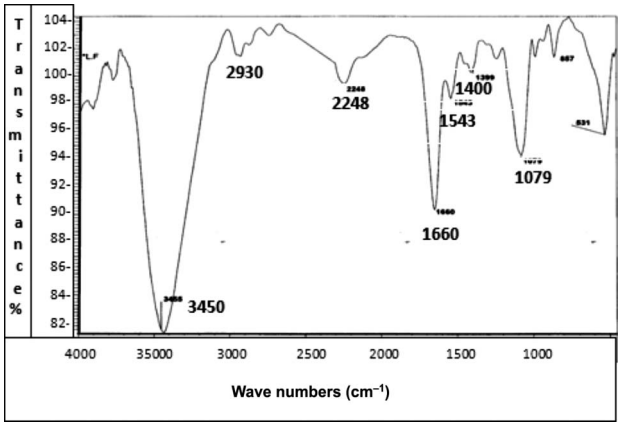

Figure 2. The spectrum of the freeze-dried biosurfactant released from *L.f.* ATCC9338.

Antimicrobial activity

The antimicrobial activity of the purified *L.f.* biosurfactant at several concentrations revealed that there was no direct antimicrobial activity under the experimental condition.

Biofilm formation

As shown in Figure 3A and B, 2.5 mg ml^{-1} of extracted *L.f.* biosurfactant showed substantial anti-biofouling activity because it reduced the process of attachment and biofilm production (Figure 3A and B). The attached bacterial cells were collected from the wells of microtiter plates and used for RNA extraction.

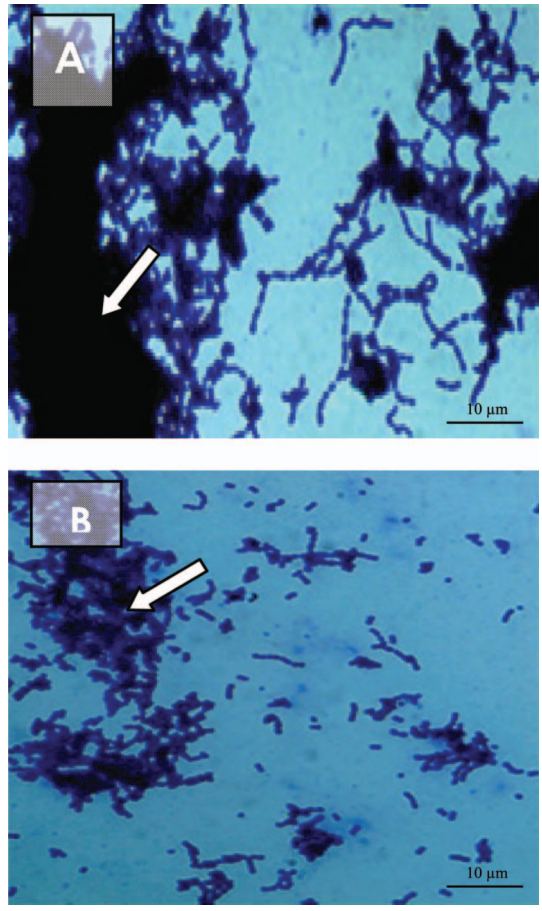

Figure 3. *S. mutans* biofilm formation A = a control group (in the absence of biosurfactant). B = an experimental group (in the presence of *L.f.*-biosurfactant). The arrows show the biofilm depth on a glass slide. The magnification of both images is ×1000.

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

Real-time RT-PCR was used to quantify the effect of *L.f.* biosurfactant on *gtfB* and *gtfC* gene expression in biofilms of *S. mutans* ATCC35668 and *S. mutans* 22 (Figure 4A and B). The 16S rRNA gene was used as an internal reference. In the biofilm environment, biosurfactant significantly reduced *gtfB* and *gtfC* gene expression (P value < 0.05). However, the effect of biosurfactant on the two genes was not identical, and a more significant decrease in *gtfB* gene expression was found. The extent of the decrease in *gtfB* and *gtfC* expression levels in *S. mutans* 22 was the same as the standard strain (P value > 0.05).

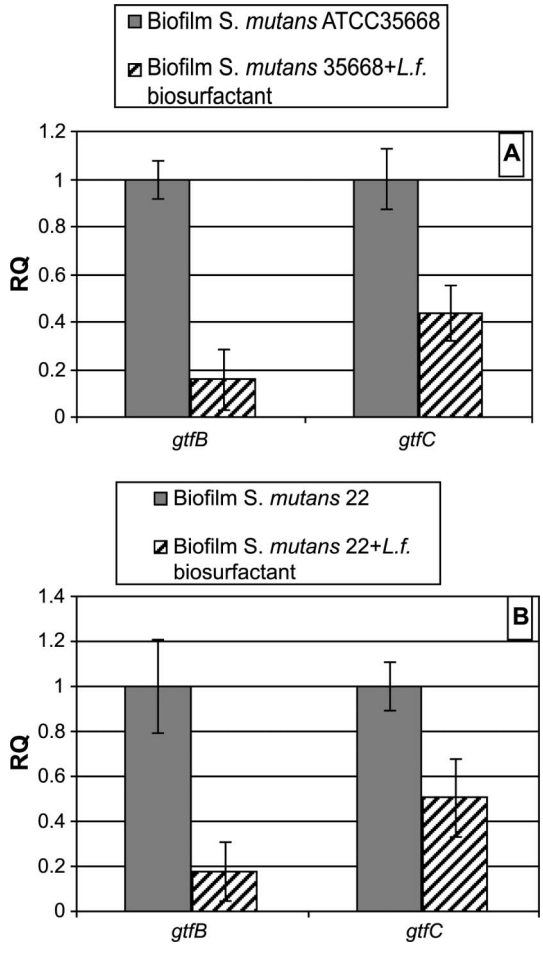

Figure 4. The effect of *L.f.*-derived biosurfactant on *gtfB/C* in immobilized biofilm. A = *S. mutans* ATCC 35668; B = *S. mutans* 22. The mRNA expression levels were calibrated relative to the control group (in the absence of biosurfactant). The results are expressed as the means and standard errors of duplicate experiments using primers specific for *gtfB/C* and 16S rRNA (normalizing gene).

Discussion

Because of its involvement in caries there is a need to more effectively control *S. mutans* in the oral environment. Increasing problems of resistance to synthetic antimicrobials have encouraged research into alternative natural products (Allakera and Douglas 2009), such as probiotic bacteria and their products, since lactic acid bacteria produce biosurfactant and one of the major roles known for surfactants is their negative effect on other microbial species (Velraeds et al. 1996; Rodrigues et al. 2005, 2006c; Uehara et al. 2006). In the present study biosurfactant extracted from *L.f.* as a potential control for *S. mutans* was investigated. Biosurfactant activity was demonstrated by the collapse drop technique.

FTIR analysis of freeze-dried biosurfactant derived from *L.f.* ATCC9338 was compared with the reference compounds albumin, salivary glycoprotein, dextran, lipoteichoic acid and other FTIR spectra of biosurfactants (Velraeds et al. 1996). Based on these results, it was concluded that the biosurfactant from *L.f.* had more protein than polysaccharide and phosphate, similar to *L. acidophilus*-derived biosurfactant (Tahmourespour et al. 2011). As seen in Figure 3A and B, the biosurfactant reduced the adhesion of *S. mutans* to a glass slide. Velraeds et al. (1996) demonstrated that the biosurfactants from *L. acidophilus* RC14 and *L.f.* 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 a biosurfactant, *L.f.* interfered in the adhesion and biofilm formation of *S. mutans* to a glass slide (Figure 3B). van Hoogmoed (2000) and van Hoogmoed et al. (2004) reported that the release of biosurfactant from *S. mitis* BMS could interfere with the adhesion of the cariogenic *S. mutans* to glass in the presence and absence of a salivary conditioning film. Other studies have also confirmed that biosurfactants have an inhibitory effect on bacterial adhesion and biofilm formation (Heinemann et al. 2000; Schooling et al. 2004; Rodrigues et al. 2006a, 2006b, 2006c, 2006d).

However, the precise mechanisms of such effects have not yet been elucidated, and they seem to be highly dependent on the biosurfactant type and the properties of the target bacteria. The simplest way to explain biosurfactant anti-adhesion and anti-biofouling activities would be through direct antimicrobial action. However, antimicrobial activity of biosurfactants has not been observed in all cases (Rodrigues et al. 2006c; Walencka et al. 2008), and it is pH dependent in the case of mutans streptococci (Soldering et al. 2010).

Walencka et al. (2008) also 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 important in overcoming the initial electrostatic repulsion barrier between the microorganism cell surface and its substratum. Surfactants may affect both cell-to-cell and cell-to-surface interactions. Their results support the idea that lactobacillus-derived agents have an effect on these interactions (Walencka et al. 2008).

The effect of *L.f.* biosurfactant on *gtfB* and *gtfC* gene expression levels was also investigated in this study. The expression of these genes 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 that are favorable to the organisms for the persistent colonization of tooth surfaces (Schilling and Bowen 1992). 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 (Paes Leme et al. 2006). Several environmental factors can influence the 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 (Banas and Vickerman 2003; Wen and Burne 2004; Wen et al. 2010).

The results suggest that either the *L.f.* biosurfactant itself or a putative signaling molecule in the extract down-regulated the expression level of genes that play an important role in the process of *S. mutans* attachment and biofilm formation. In addition to down-regulating *gtfB* and *gtfC* (genes involved in insoluble glucan production), it may also have an effect on converting gtf activity from producing insoluble glucans to water-soluble glucans, hence accounting for reduced *S. mutans* biofilm adherence, and this should be studied 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 (Tamwsada and Kawabata 2004; Huang et al. 2008). The ability of *S. mutans* to produce extracellular polysaccharides from dietary carbohydrates has been demonstrated to significantly enhance its cariogenicity. Thus, the less extracellular polysaccharide produced, the lower the cariogenicity of *S. mutans*. Tomita et al. (1998) 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 (Tomita et al. 1998).

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 (Ofek et al. 2003). It is proposed that the secondary metabolite of the probiotic bacterium *L.f.* decreases the expression level of gtf genes and therefore may be useful for the control of *S. mutans* and possibly other species.

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