Original Article

Effects of *Lactobacillus reuteri*-derived biosurfactant on the gene expression profile of essential adhesion genes (gtfB, gtfC and ftf) of *Streptococcus mutans*

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Abstract

Background: Streptococci are the main causative agents in plaque formation and mutans streptococci are the principle etiological agent of dental plaque and caries. The process of biofilm formation is a step-wise process, starting with adhesion of planktonic cells to the surfaces. It is now a well known fact that expression of *glucosyltransferases (gtfs)* and *fructosyltransferase (ftf)* genes play a critical role in the initial adhesion of *Streptococcus mutans* to the tooth surface, which results in the formation of dental plaques and consequently caries and other periodontal diseases.

Materials and Methods: In the present study, we have determined the effect of biosurfactants purified from *Lactobacillus reuteri* (DSM20016) culture on gene expression profile of *gftB/C and fft* of *S. mutans* (ATCC35668) using quantitative real-time polymerase chain reaction.

Results: The application of biosurfactant caused considerable down-regulation of the expression of all three genes under study. The reduction in gene expression was statistically very significant ($P > 0.0001$ for all three genes).

Conclusions: Inhibition of these genes by the extracted *L. reuteri* biosurfactant shows the emergence of a powerful alternative to the presently practicing alternatives. In view of the importance of these gene products for *S. mutans* attachment to the tooth surface, which is the initial important step in biofilm production and dental caries, we believe that the biosurfactant prepared in this study could be considered as a step ahead in dental caries prevention.

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

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INTRODUCTION

Dental plaque represents an important example of microbial biofilm harboring a very complex microbial species. Dental plaque formation, which begins with the adhesion of oral bacteria to the tooth surface, is believed to be the main underlying cause of two important oral diseases: Dental caries and periodontal disease.[1]

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These prevalent diseases result from the complex interactions between virulent microorganisms, their products, host salivary composition and frequency of carbohydrates consumption.[2-4] Broad-spectrum microbicides (e.g. chlorhexidine) application to control the infection is not favorable because of the indiscriminate eradication of all species present in the oral cavity, including those with a beneficial role to oral health.[5-7] Even approaches like removing dental plaque is not a desirable approach because dental plaque is made of large numbers of commensal bacteria with a limited number of oral pathogens; therefore, the "remove all or kill all" approach creates open, non-competitive surfaces for pathogens to propagate in the oral cavity. Therefore, novel therapies that target the ability of virulent species to assemble and maintain biofilms on tooth surfaces are very effective and safe alternatives to the conventional antibiotic therapy.

Sucrose being frequently ingested as a regular component of our diets can serve as the catalyst for caries development as it is a substrate for both the production of acid (through fermentation) and exopolysaccharide (EPS) synthesis. EPS are important structural and protective matrix components of dental biofilms, acting both as a supportive framework and barrier to diffusion.[2-4,8] Production of acids cause damage to the tooth enamel, consequently leading to localized decalcification, cavity formation and breakdown of calcified dental tissue.[9]

Streptococcus mutans, a gram positive oral bacterium, is efficient to synthesize extracellular glucans, which is the key virulence factor contributing to the pathogenesis of dental caries in humans.[10-12] There are three distinct *gtf* genes in the genome of *S. mutans* with glucosyltransferase (GTF) activity.[13] Two of these genes, *gtfB* and *gtfC,* which are arranged in an operon-like array, encode enzymes that, in a sucrose-dependent process, produce water-insoluble glucans with α-(1→3) bonds. However, the *gtfD* gene, which is not located in the *gtfB/C* locus, encodes an enzyme that synthesizes water-soluble with α-(1→6)-linked glucans.

In addition to oral diseases, this bacterium is also known to cause infective endocarditis, which may cause significant morbidity and mortality.[3] Oral streptococci can enter the bloodstream via bruises in the oral cavity and attach to platelet–fibrin matrices, formed on injured endothelial tissue. The adherence of *S. mutans* to damaged heart tissue is an important event in the pathogenesis of chronic infective endocarditis.[14]

So far, many efforts have been employed to find an efficient alternative with least undesirable side-effects to suppress *S. mutans* adhesion to surfaces without adverse effects on the natural mouth microbiata. The adherence of bacterial cells to the tooth surface is of great importance to the development of carious lesions, and interference with some of the mechanisms of adherence can prevent dental caries.[15] In this context, varieties of alternatives have been tested and there are a number of reports on the results achieved, among which application of plant polyphenols,[16,17] probiotic therapy[18,19] and herbal extracts.[19]

In the present study, the aim was to evaluate the effects of bisurfactants produced by *Lactobacillus reuteri* (DSM20016) on the gene expression profile of adhesion essential genes, gftB/C and *ftf*, in *S. mutans (*ATCC35668).

MATERIALS AND METHODS

The *S. mutans* standard strain (*ATCC35668*) was cultured on blood agar and mitis salivarius agar media and incubated in a CO_2-enriched 37°C atmosphere. *L. reuteri* (DSM20016) as a probiotic source was cultured in Man, Rogosa, Sharpe (MRS) broth or agar.

Fifteen milliliters of *L. reuteri* cultured overnight was inoculated into 600 mL of MRS broth and incubated for 24 h. The cells were harvested by centrifugation at 10,000 g for 5 min at 10°C, washed twice in demineralized water and resuspended in 100 mL of phosphate-buffered saline (PBS). The lactobacilli were incubated at room temperature for 2 h 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, MA, USA), dialyzed against demineralized water at 4°C in a Spectrapor membrane tube (molecular weight cut-off, 6'000 to 8'000 Da; Spectrum Medical Industries Inc. CA, USA) and was freeze-dried as described previously.[20]

In order to test whether the 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 the extracted biosurfactant was pipetted as a droplet on to a piece of 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 supernatant for photographic purposes. The droplet was allowed to dry and the diameter of the dried droplet was recorded.[21,22]

In order to generate a biofilm on the microtiter plate wells, 20 µL of overnight cultured *S. mutans* was placed in each well of a 24-well polystyrene plate and was cultivated with 2 mL of Brain heart infusion (BHI) broth supplemented with 1% sucrose. The plates were incubated at 37°C in an atmosphere enriched with 5% CO_2. After 18 h of incubation, the spent medium was aspirated and wells were washed with PBS solution in order to remove the unattached cells. The biofilm was incubated again in fresh BHI with 1% sucrose; after a further 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 and without 2.5 mg/mL of freeze-dried biosurfactant. After 4 h of incubation, the cells of the biofilms were dislodged into tubes containing 2 mL PBS solution and vortexed.[23]

The prepared biofilm cells on the microtiter plates (*S. mutans ATCC 35668* with and without biosurfactant, in three replicates) were used for RNA extraction. The cells were disrupted using a ribolyser instrument (Hybaid, Cambridge, UK) and the supplied kit according to the manufacturer's instruction. Briefly, RNA containing supernatant from the ribolyser tube was transferred to a 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 were performed on the extracted RNA by spectrophotometry (biophotometer, Eppendorff, Rs 232-C, Germany) and agarose gel electrophoresis.

A reverse transcription 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 were added to each reaction mixture after 5 min of incubation in 37°C, 1 µL reverse transcriptase (RT) 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 to be used later.

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

The relative quantitation of *gtfB/C* genes and *ftf* were made against 16s rRNA as a reference gene. All primers and their location are summarized in Table 1.[23] The reaction mixture (20 µL) contained 1X SYBR Green PCR Master Mix (Qiagen), the appropriate forward and reverse PCR primers (10 pM) and 1 µL of the cDNA sample. The PCR program consisted of an initial denaturation at 95°C for 5 min, then 40 cycles of amplification applied as follows: 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 checked for primer–dimer formation in the template-free reaction. As an additional control for each primer pair and each RNA sample, the cDNA synthesis reaction was carried out without RT 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.

RESULTS

In the drop collapse assay, no activity was detected for distilled water as predicted. The biosurfactant showed appearance of collapsed droplet [Figure 1], indicating their effects on reduction of surface tension.

Real-time RT-PCR was used to quantify the effect of *L. reuteri*–derived biosurfactant on *gtfB/C* and *ftf* gene expression in the biofilm of *S. mutans* (*ATCC35668*) [Figure 2]. As an internal reference, 16srRNA gene was used. In the biofilm environment, application of the extracted biosurfactant significantly reduced expression of all three genes under study ($P > 0.0001$ for all three genes).

DISCUSSION

Dental caries is widespread in all age groups, and is a world-wide serious threat to human health. In spite of much efforts and considerable research works

Table 1: Nucleotide sequences of primers

Genes	Primer sequences (5'-3')
gtfB-F	AGCAATGCAGCCAATCTACAAAT
gtfB-R	ACGAACTTTGCCGTTATTGTCA
gtfC-F	CTCAACCAACCGCCACTGTT
gtfC-R	GGTTTAACGTCAAAATTAGCTGTATTAG
Ftf-F	AAATATGAAGGCGGCTACAACG
Ftf-R	CTTCACCAGTCTTAGCATCCTGAA
16S-F	CCTACGGGAGGCAGCAGTAG
16S-R	CAACAGAGCTTTACGATCCGAAA

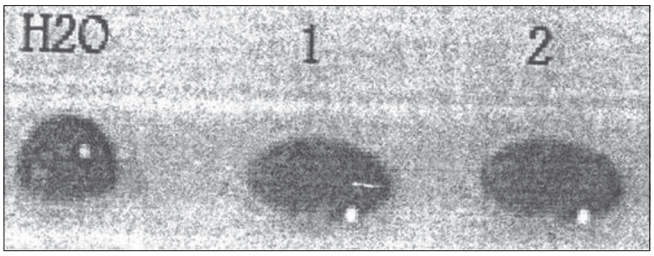

Figure 1: Drop collapse assay. Collapsed droplet (1 and 2) is the L. *reuteri*-biosurfactant and distilled water

being performed in this field, we still have far ahead to achieve success. Dental caries is a multi-factorial disease tightly related with the presence of the cariogenic bacteria, *S. mutans*, which are embedded in the dental plaque biofilm. The challenges ahead for *S. mutans* control by conventional antimicrobial drugs is the development of resistance to synthetic antimicrobials and their adverse effects on the beneficial bacteria in the oral cavity. This situation has encouraged the researchers to divert their attention to natural products, such as probiotic bacteria and herbicides, as a potential alternative.[24] It is already a well-known fact that some of the microorganisms such as lactic acid bacteria are biosurfactant-producing strains.[25-28]

One of the major roles known for biosurfactants is their inhibitory effects on other microbial species. Based on the model presented, Gtfs play a central role in carieogenic properties of *S. mutans*.[29] The Gtfs (particularly GtfC) secreted by *S. mutans* are incorporated into the pellicle as well as on bacterial surfaces (mainly GtfB). Incorporation also takes place on the surface of other microorganisms that do not produce Gtfs (e.g. Actinomyces spp.). Furthermore, salivary α-amylase is also incorporated into the pellicle, taking part in hydrolyzing starch that contributes to the formation of sucrose/starch-derived glucans, which in turn can also bind Gtfs. After this initial incorporation of Gtfs on *S. mutans* and pellicle surfaces, dietary sucrose is rapidly utilized to synthesize insoluble and soluble glucans *in situ*. The glucan molecules provide binding sites on the surfaces for *S. mutans* (and other microorganisms), mediating tight bacterial accumulation and adherence to the tooth enamel. Furthermore, Gtf-adsorbed bacteria become actually glucan producers binding to the tooth and microbial surfaces by the same mechanisms.

This model could explain the rapid formation and accumulation of highly cohesive-adherent plaque in the presence of sucrose (and possibly starch) even if the number of *S. mutans* is relatively low. After the establishment of a glucan-rich biofilm

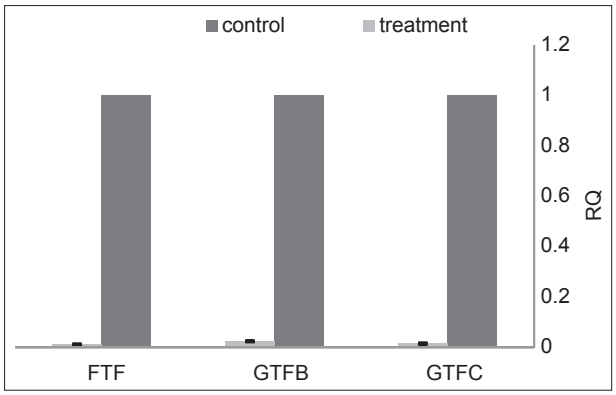

Figure 2: The effect of *L. reuteri*-derived biosurfactant on *gtfB/C* and *ftf* gene expression levels in *S. mutans ATCC35668*. Note that in biosurfactant-treated bacteria, the gene expression level is dramatically dropped down

matrix, ecological pressure (e.g. pH) will determine which bacteria may survive and dominate within plaque under frequent sucrose (or other fermentable carbohydrate) exposure.

Based on the above-mentioned model, it is well illustrated that *glucosyltranferases* are the main players of the dental caries scene. Because of the complex composition of oral microbiota that are harboring more beneficial species than harmful ones, the strategy based on "remove all or kill all" is not advisable to be adopted.[5] The selective suppression of *glucosyltranferases* sounds very promising if it comes to reality. In this study, we made an effort to investigate the effect of *L. reuteri*–derived biosurfactant on *gtfB, gtfC* and *ftf* gene 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 forms of bacteria that are favorable to the organisms for the persistent colonization of tooth surfaces.[30] 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 an elevated risk of biofilm formation and cariogenicity in humans.[31]

The mechanism of adhesion prevention by probiotic bacteria may be through gene expression manipulation of different virulent bacterial genes as well as interfering with the salivary protein composition. Van Hoogmoed *et al*.[32] reported that a biosurfactant generated by *S. mitis* strains BA and BMS cells was able to decrease the adhesion of *S. mutans* as well as several other periodontopathogens. Interestingly, probiotics have been shown to inhibit adhesion by altering the protein composition of the binding site. In this connection, Haukioja *et al*.[33] have shown

that some probiotic strains change the salivary pellicle protein composition by omitting an important adhesion protein, salivary agglutinin gp340, which plays an important role for the adhesion of *S. mutans*. This resulted in a lower colonization efficiency of *S. mutans*. However, some of the studies clearly demonstrated that biosurfactants from certain Lactobacillus sepsis downregulate glucan-producing genes of *S. mutans* and consequently attenuate the biofilm production property of this pathogenic bacterium.[15,34]

Based on our results, we could clearly demonstrate that the *L. reuteri*-derived biosurfactant is a potent inhibitor of the *glucosyltransferases* and *fructosyltransferase* in the standard strain of *S. mutans* (*ATCC35668*). This suppression is particularly desirable in the sense that the action is very selective and do not interfere with the microbial ecosystem of the mouth.

However, we need to gather more basic information to shed light on the underlying molecular mechanism of this gene suppression by the applied biosurfactant in order to take additional steps toward considering the clinical application of biosurfactants as a safe and selective therapeutic option for dental caries prevention.

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