

Original Research

Antimicrobial Effects of Postbiotic Mediators Derived from *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* on *Streptococcus mutans*

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Abstract

Background: *Streptococcus mutans* is a major component of dental plaque, contributing to cariogenic biofilm formation and inducing dental caries. Attempts have recently been made to use postbiotic mediators (PMs) to prevent dental caries. This research evaluated the antimicrobial/antibiofilm activity of PMs derived from *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus reuteri* (LR) against *S. mutans* *in vitro*. **Methods:** PMs were obtained from the *Lactobacilli* supernatants. The minimum inhibitory concentration, minimum bactericidal concentration, antibiofilm potential, and metabolic activity of PMs against *S. mutans* were evaluated using CFU/mL, scanning electron microscopy, and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay. The expression of *gtfB* gene as one of the most important genes involved in *S. mutans* biofilm formation was also measured using qRT-PCR. **Results:** CFU score was reduced by both PMs, but the reduction was only significant in LGG ($p = 0.02$). Both PMs caused a significant decrease in the metabolic activity of *S. mutans* compared with the controls ($p \leq 0.002$). *S. mutans* treated with LGG PMs exhibited more destructive effects than LR PMs ($p > 0.05$). *S. mutans* *gtfB* gene expression was significantly downregulated when treated with the PMs obtained from both LGG and LR ($p = 0.01$ for both). **Conclusions:** We showed that PMs isolated from two *Lactobacillus* strains inhibited *S. mutans* biofilm, metabolic activity, and *gtfB* gene expression. Therefore, these derivatives may be a suitable biofilm-destruction agent against *S. mutans*. However, the oral environment is a complex ecosystem that needs further investigation.

Keywords: antibiofilm effects; dental caries; gene expression; postbiotic mediators; *Streptococcus mutans*

1. Introduction

Tooth decay, the most common biofilm-dependent disorder, is a major public health problem that globally affects 2.3 billion adults and 530 million children worldwide [1]. Caries is a multifactorial condition that forms from dysbiosis of host oral microbiota [2]. Dental biofilm, or plaque, constitutes a favorable environment for the growth of many bacteria, among which *Streptococcus mutans* (*S. mutans*) are known to play an important role in developing dental caries. In addition to acidic metabolites, *S. mutans* produces GtfB, GtfC, and GtfD, which are glucosyltransferase (GTF) enzymes that synthesize intracellular and extracellular polysaccharides [3,4]. They are encoded by *gtfB*, *gtfC*, and *gtfD* genes, respectively. Water-insoluble glucans are synthesized by the action of *gtfB*, during the formation of extracellular polysaccharides from sucrose [5]. As a result, large amounts of insoluble glucans promote the

colonization and adherence of *S. mutans* to tooth surfaces, which could ultimately increase dental plaque production and caries [6–8].

Due to the high cost of dental treatments, caries preventative measures are recommended to protect teeth. However, routine methods using mechanical/chemical approaches have drawbacks, such as the increased risk of fluorosis and the destruction of healthy bacteria by antimicrobial mouthwashes, which leads to the colonization of pathogens [9–11]. The potential to treat the oral environment and its various reservoirs using molecules of diverse densities and concentrations for distinct purposes offers a promising approach to modulating the host's inflammatory response in the oral environment [12]. This could lead to significant improvements without the need for invasive therapies. Because of the rising resistance to synthetic antimicrobials and their side effects, research on alternative natural products like probiotic microorganisms and their



metabolites has been recommended.

Probiotics are living microorganisms that promote host health and immunological homeostasis when administered appropriately [13]. They have been shown to decrease caries by suppressing oral pathogens and altering the microbial composition of dental plaque [14–16]. Also, among their attributes are inhibition of plaque induction by free radical scavenging and controlling mucosal permeability, in addition to destroying pathogens through secretion of lactic acid, hydrogen peroxide, and bacteriocins [17,18]. Probiotics are generally well-tolerated, but people with compromised immune systems should not take them due to the possibility of infections [19]. Some of them, like *Lactobacillus* and *Bifidobacterium*, are acidogenic, making them unsuitable for treating dental caries [20,21]. Postbiotics are a mixture of deactivated probiotic cells and their metabolites, which deliver some of the benefits of probiotics without the drawbacks [22]. They are suggested to suppress *S. mutans* biofilms [23] and oral multispecies biofilms [24] and might be used to create potent anticaries agents [25]. “Postbiotic mediators” (PMs) is a relatively new term that is gaining popularity and refers to byproducts of the metabolic activity of probiotics. PMs extracted from various microorganisms have been reported to prevent infection through attenuating pathogen growth and biofilm development [25].

Various *Lactobacillus* strains have shown promising effects against *S. mutans* in the oral cavity. But the number of studies on their PMs is relatively limited [25]. *Lactobacillus rhamnosus* GG (LGG) and *Lactobacillus reuteri* (LR) belong to the *Lactobacillus* genus. They have beneficial effects such as good growth capacity, strong adhesion ability, efficacy against pathogens, and production of antimicrobial and anti-colonization substances that make them attractive as probiotic strains [26,27]. The exact effect of various lactobacilli and their byproducts and the mechanisms involved in their effectiveness against *S. mutans* is unclear. On the other hand, the beneficial effects of postbiotics on *S. mutans* have been suggested to depend upon the specific strains of the probiotics [22,23]. The gene regulation of *gtfs* is crucial in *S. mutans* adhesion and biofilm formation and has been the focus of many investigations [28–31]. Therefore, the current study aims to examine the anticaries effect of PMs derived from LGG and LR by investigating their antibiofilm effect and metabolic activity on *S. mutans* and assessing the expression of the *gtfB* gene.

2. Materials and Methods

2.1 Bacterial Strains, Culture Media, and Growth Conditions

Both *L. rhamnosus* GG (ATCC 53103) and *L. reuteri* (ATCC 23272) kept in de Man, Rogosa, and Sharpe (MRS) agar (Merck, Darmstadt, Germany). Before each test, *Lactobacillus* strains were cultured in MRS broth (Merck, Darmstadt, Germany) and placed in a CO₂ incubator at 37 ± 1 °C for 48 hours. *S. mutans* ATCC 35668 was obtained

from the Iranian Biological Resource Centre (Tehran, Iran). Bacteria were incubated at 37 ± 1 °C in a brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) under capneic conditions (4–5% of CO₂) for 18 hours. Approximately 1.5 × 10⁸ colony forming units (CFUs)/mL of test bacteria were prepared by adding 0.5 mL of the culture to 9.5 mL of new BHI broth and incubating the tube for 4–5 hours until *S. mutans* cells reached the mid-log phase of growth. The final concentration of *S. mutans* was determined by spectrophotometry to be 1.5 × 10⁸ CFU/mL (optical density at 600 nm: 0.08–0.13).

2.2 Postbiotic Mediators (PMs)

After the incubation time of the *Lactobacillus* species was completed, centrifugation (10,000 × g, 8000 rpm) was performed for 10 minutes at 4 °C, and the supernatant was separated from the sediment and transferred to a new tube. All supernatants were filtered through a 0.22 μm polyethersulfone membrane syringe filter (Millipore, Burlington, MA, USA), and their pH was adjusted to physiological conditions (pH 7.2–7.4) using 5 M sodium hydroxide prior to use.

2.3 Evaluation of Antibacterial and Antibiofilm Activity

2.3.1 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of PMs

The Clinical and Laboratory Standards Institute (CLSI) guideline [32] was used to calculate MIC and MBC. Briefly, 100 μL of BHI broth was poured into round-bottom 96-well microplates, and 100 μL of each PMs was added to the first well of each row at 1.56–100% [v/v] concentration. Starting from the first well, 100 μL of the contents of each well was transferred to the next well, and the contents of the last well (10th) were discarded. *S. mutans* culture was diluted (1:10) in Mueller Hinton broth to give a 10⁷ CFU/mL suspension. In a microplate, 5 μL of this suspension was added to 100 μL of the PM solutions at 1:2 dilutions to give a final suspension of bacteria around 5 × 10⁵ CFU/mL in two-fold dilutions of PMs. The plate was then incubated for 24 hours at 37 °C in the presence of 5% CO₂, and the MIC was defined as the lowest concentration of PMs at which no growth was observed. A positive control (wells containing bacterial suspension and nutrient media, devoid of PMs) and a negative control (wells containing PMs and nutrient media, devoid of bacterial suspension) were also used.

Sub-culturing broth dilutions determined the MBC of PMs at or above the MIC that inhibits the growth of *S. mutans* on BHI agar (Merck, Darmstadt, Germany). It was defined as the lowest concentration of an antimicrobial that caused at least 99.999% (3-log reduction) killing of the initial inoculum. The tests were repeated at least three times.

2.3.2 Determination of the Biofilm Destruction Effect of PMs

Two hundred μL of *S. mutans* suspension at a concentration of 1.5×10^8 CFU/mL was added to each well of a flat-bottom 96-well microplate and was incubated at 37°C with 5% CO_2 for 48 hours to form a microbial biofilm. After aspirating the suspension and washing the wells three times with sterile phosphate-buffered saline (pH 7.4), the $2 \times$ MIC of each PM (100 μL) was added into each well, and the microplate was incubated at 37°C . After 24 hours, the bacterial biofilm was scraped from the bottom of the microplate, followed by dilution plating to determine CFU/mL [33]. All tests were repeated at least three times.

2.3.3 Microscopic Visualization of Biofilm

Scanning electron microscopy (SEM, Zeiss, EVO 40, Jena, Germany) was used for the structural analysis of the PM-treated biofilms. *S. mutans* biofilm was allowed to develop on the surface of the sectioned teeth. Three intact permanent human premolar that was previously extracted due to orthodontics were selected. Informed consent was obtained from each donors. The teeth were preserved in distilled water at 37°C until the experiment. Before the operation, the teeth were scaled by hand and cleaned using a fine-grain pumice-water slurry (Dental AG Ltda, São Paulo, SP, Brazil) and Robinson bristle brushes (Labor Dental Ltda, São Paulo, SP, Brazil) in the low-speed handpiece for the 30 s. Using a micromotor handpiece, each tooth was cut in longitudinal sections with a diamond disc. Before treatment, sectioned teeth were autoclaving at 121°C , 15 lbs psi for 30 minutes, followed by treatment with $2 \times$ MIC of the PMs. After the incubation period, the biofilm on the teeth was fixed with 2.5% glutaraldehyde, dehydrated through a series of 50%, 60%, 70%, 80%, 90%, and $2 \times 100\%$ ethanol solutions, and coated with a thin film of palladium-gold (Gatan 682PECS, Gatan, Pleasanton, CA, USA) for visualization [34].

2.3.4 Evaluation of Metabolic Activity Using XTT Reduction Assay

The XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) reduction assay (XTT Kit; Roche Applied Science, Indianapolis, IN, USA) was used to measure the metabolic activity of *S. mutans*, according to the manufacturer's instructions. Briefly, 100 μL of *S. mutans* suspension (1.5×10^8 CFU/mL) was added to the wells of a flat-bottom 96-well microplate and incubated at 37°C for 24 hours, followed by treatment with $1/2 \times$ MIC doses of each PM. This concentration was to specifically study the effects of the PMs on metabolic activity and to avoid cell death-related effects brought about by MIC and high concentrations. After 24 hours, 50 μL of the XTT solution was added to each well, and the microplate was incubated in the dark at 37°C for two hours. Absorbance was detected spectrophotometri-

cally by a microplate reader at 490 nm [35].

2.4 Quantification of *gtfB* Gene Using Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Immediately after treating *S. mutans* with $1/2 \times$ MIC doses of PMs, total RNAs were extracted using the super RNA extraction kit (AnaCell, Tehran, Iran). After removing any remaining genomic DNA using RNase-free DNase I treatment, total RNA (150 ng) was reverse transcribed in a 10 μL cDNA reaction volume using the RevertAid First Strand cDNA Synthesis Kit following the manufacturer's instructions. The specific primers related to *gtfB* and *16S rRNA* internal control genes are listed in Table 1 [36]. Distilled water served as the negative control. A LightCycler® 96 System (Roche Diagnostics, Indianapolis, IN, USA) was used to perform amplification. The obtained Ct (Cycle threshold) values of the target genes were normalized to the *16S rRNA* housekeeping gene, and the $2^{-\Delta\Delta\text{CT}}$ method was used to calculate fold changes (Livak and Schmittgen) method [37].

Table 1. Primers of target and housekeeping genes used in this study.

Gene		Sequence (5'-3')	Size
<i>gtfB</i>	Forward	TGTTGTTACTGCTAATGAAGAA	103 bp
	Reverse	GCTACTGATTGTCGTTACTG	
<i>16S rRNA</i>	Forward	GCAGAAGGGGAGAGTGGAAAT	182 bp
	Reverse	GGCCTAACACCTAGCACTCA	

2.5 Statistical Analysis

All tests were repeated at least thrice, and data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD (honestly significant difference) tests. All results were presented as mean \pm standard deviation, and *p*-values less than 0.05 were considered significant.

3. Results

3.1 MIC and MBC of the PMs

To obtain the lowest concentration of PMs that inhibited the growth of *S. mutans*, serial dilutions of PMs were utilized. No turbidity at concentrations of more than 25% (v/v) in both PMs from LGG and LR was seen, which showed regarded as the MIC value. Also, in both PMs, there was no growth of *S. mutans* on BHI agar at concentrations above 25% (v/v), which confirms as MBC value ($\text{MIC}_{\text{PMs}} = \text{MBC}_{\text{PMs}}$, concentrations of more than 25% (v/v) in both PMs from LGG and LR).

3.2 Biofilm Destruction of *S. mutans* by PMs

CFU/mL count was used to assess the potential inhibitory effect of the PMs on *S. mutans* biofilm (Fig. 1) and

showed values of $3.2 \pm 0.38 \times 10^7$, $2.04 \pm 0.29 \times 10^7$, and $2.77 \pm 0.41 \times 10^7$ in controls, LGG-, and LR- PMs, respectively. Based on these findings, both PMs caused biofilm destruction compared to controls; however, destruction was significant in the LGG group ($p = 0.02$) but not in the LR group ($p = 0.39$).

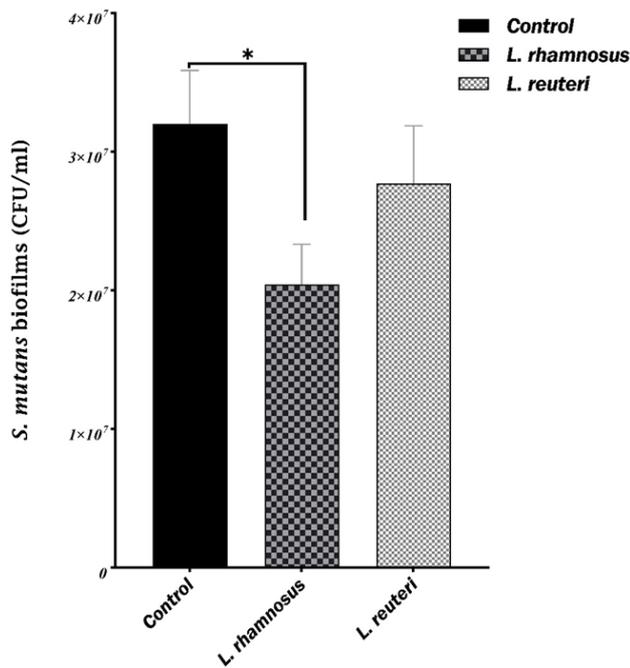


Fig. 1. Changes in *S. mutans* colony forming unit treated with postbiotic metabolites. *significant difference at $p < 0.05$.

3.3 SEM Analysis

SEM images (Fig. 2) demonstrated fewer microorganisms and smaller microcolonies on the surface when PMs interacted with *S. mutans*, suggesting the destruction of *S. mutans* biofilm. In line with our CFU/mL counts, LGG PMs had a greater effect on the biofilm compared to the LR PMs.

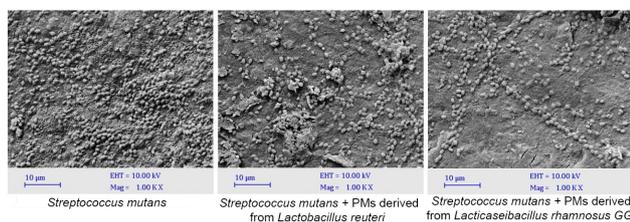


Fig. 2. Scanning electron microscopy images of *S. mutans* biofilms treated with postbiotic metabolites (Scale bar = 10 μm ; Mag = 1.00 KX).

3.4 Inhibitory Effect of PMs on *S. mutans* Metabolic Activity

The XTT reduction assay was employed to measure the metabolic activity of *S. mutans* biofilms (Fig. 3). The absorbance values of the control, LGG PMs and LR PMs were 1.56, 0.72, and 0.93, respectively. The PMs from LGG and LR groups showed significant reductions in the metabolic activity of *S. mutans* compared to the control ($p = 0.0001$ and $p = 0.002$, respectively). These results indicate that PMs have the capacity to inhibit *S. mutans* metabolic activity.

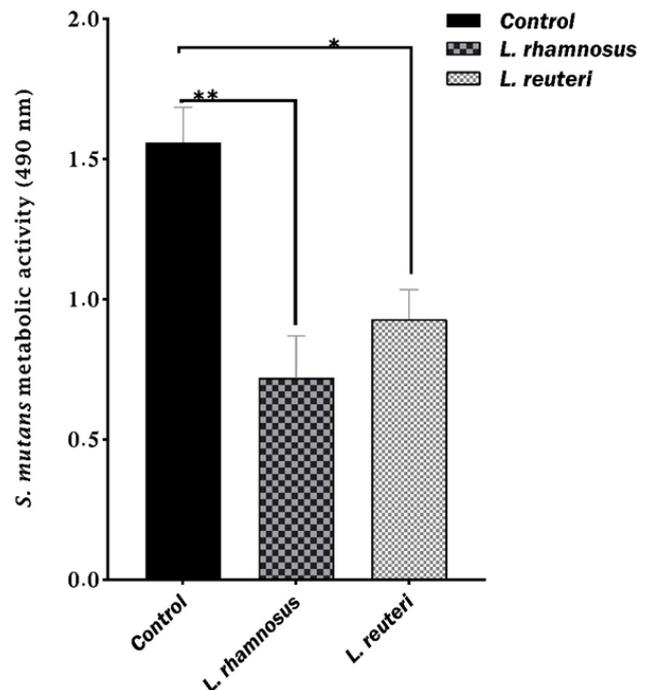


Fig. 3. The mean absorbance values of *S. mutans* metabolic activity based on the XTT reduction assay. *significant difference at $p = 0.002$; **significant difference at $p = 0.0001$. Error bars display standard deviations of the means.

3.5 Effect PMs on *S. mutans* *gtfB* Gene Expression

The effect of PMs derived from LGG and LR on the expression of the *S. mutans* *gtfB* gene was examined using qRT-PCR and presented as fold change using the $2^{-\Delta\Delta C_t}$ method. According to our findings (Fig. 4), treatment with the LGG and LR PMs resulted in 92% and 90% decrease in *gtfB* gene expression, respectively ($p = 0.01$ for both comparisons).

4. Discussion

Lactobacillus spp. probiotics inhibit dental caries by generating hydrogen peroxide, organic acids, or antimicrobial peptides [25,38–40]. However, low culture viability and susceptibility to environmental conditions limit their

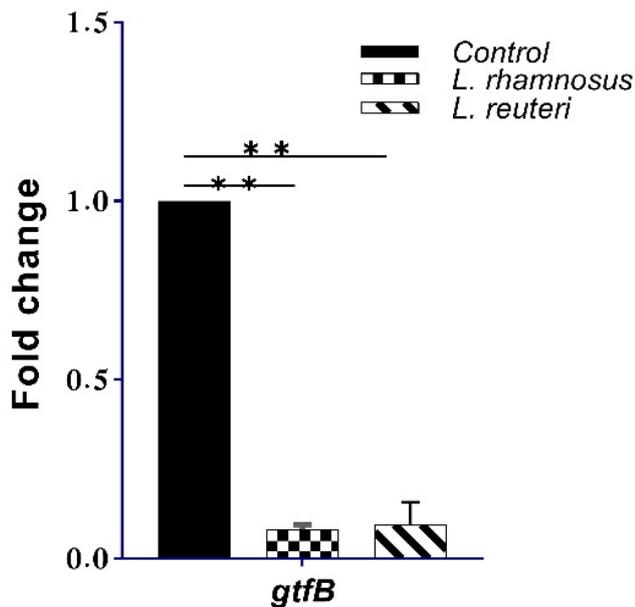


Fig. 4. The expression of *gtfB* gene in the control and PMs derived from LGG and LR. **significant difference at $p < 0.01$.

application in functional products, hence, the increasing interest in postbiotics. PMs are substances produced or released during the metabolic activity of microorganisms and are superior to probiotics because of their clear chemical structures, safety dose parameters, and longer shelf life. They have exhibited the potential to prevent caries by inhibiting pathogens' growth and biofilm formation [25,41]. Applying purified byproducts or cellular components of probiotics for therapeutic investigations focusing on a certain disease would help understand the primary molecular mechanisms related to each molecule [42]. To further introduce viable options for possible use in caries control, we investigated the inhibitory activity of PMs derived from LGG and LR against *S. mutans*.

Our MIC and MBC results showed that the effective bactericidal concentration of PMs derived from both bacteria against *S. mutans* was 25% (v/v), which confirms their antibacterial potential against *S. mutans*. Based on the biofilm assay (Fig. 1), the CFU value in both PMs was lower than the controls, and the LGG PMs exhibited lower counts than those extracted from LR. These data indicate that PMs produced by LGG were more effective in reducing the number of *S. mutans* (CFU/mL) and had higher antimicrobial activity. SEM micrographs also showed a significant reduction of *S. mutans* biofilm in the PMs of both bacteria. Consistent with the results of the CFU counts, the LGG PMs showed a better inhibitory effect compared to the LR PMs. The biofilm-reducing activity of postbiotics is crucial for dental caries prevention.

Different factors are responsible for the various antimicrobial, antibiofilm, and anti-adherence activities of pro/postbiotics, including multiple strains of *Lactobacilli* and/or their PBs [25]. These factors can be byproducts

or cellular components of live microorganisms or their dead/inactivated forms. Examples include biosurfactants, lipoteichoic acid, cell-free supernatant *etc.* [25]. According to former studies, biosurfactants significantly suppressed *S. mutans* biofilm [31,43], lipoteichoic acids showed anticaries properties [23,24], metabolites of *L. fermentum* TcUESC01 had anti-adherence and bactericidal activities against *S. mutans* planktonic cells [44], and organic acids demonstrated growth-inhibiting effects on *S. mutans* [25]. In agreement with our results, many studies have shown that the cell-free supernatant obtained from numerous *Lactobacillus* strains, including *L. reuteri* ATCC 23272, *L. rhamnosus* [30], *L. rhamnosus* HN001 [45], and *L. reuteri* AN417 [46] exhibited antibacterial activities and caused a significant decrease in the levels of *S. mutans* biofilms. In contrast to our findings, Chen *et al.* [47] reported that viable *L. reuteri* suppressed the cariogenic effects of multispecies biofilms, but the cell-free supernatant was not as effective. This conflict may be explained by the different biofilms used in the studies: we employed a *S. mutans* monospecies biofilm, while they applied *S. mutans*, *L. rhamnosus* and *Actinomyces naeslundii* multispecies biofilms in their research.

The XTT results obtained in the present study demonstrated that PMs from LGG and LR significantly suppressed the metabolic activity of *S. mutans* which decreased its ability to form biofilms (Fig. 3). The *S. mutans* samples treated with LGG PMs showed a higher metabolic activity reduction than LR. Our XTT findings were strongly confirmed by the CFU quantitative test results, which assess biofilms. A previous study suggested that decreases in cell metabolism may also reduce the synthesis of extracellular matrix material [48]. In line with our observations and using the XTT assay, Srivastava *et al.* [49] observed an 89% reduction in the activity of *S. mutans* following initial incubation with *L. plantarum* supernatant. They also reported 33% inhibition of *S. mutans* biofilm after 12 h treatment [49]. Similarly, *L. reuteri* and *S. oligofermentans* PMs have been suggested to decrease the survival and metabolic activity of *S. mutans* [50].

The glucosyltransferase *gtfB* gene encodes the GtfB enzymes involved in forming insoluble extracellular glucans in *S. mutans*, which is the major polymeric matrix of biofilms [38,51]. This gene also regulates bacterial adhesion to the tooth surface, aggregation and coaggregation of the bacterial cells, and the integrity and stability of the biofilm structure. Therefore, its suppression may be associated with reduced biofilm/plaque formation and, ultimately, caries control [4,5]. We employed qRT-PCR to examine the expression of *gtfB* in *S. mutans*. It showed that the coculture of this bacteria with the PMs from both LGG and LR significantly down-regulated the expression level of *gtfB* (Fig. 4). This was in agreement with several other coculture studies using *S. mutans* and multiple *Lactobacilli* strains, including LGG [6,38,52], and their PMs,

including LR PMs [3,8,25,29–31,49,53], who showed significant suppression of glucosyltransferase-encoding genes *gtfB*, *gtfC*, and/or *gtfD*.

To investigate the antimicrobial effects of PMs derived from two reference strains, we evaluated *S. mutans*, the main component of dental plaque responsible for causing tooth decay. However, the dynamic oral cavity contains many more species which could be involved in forming different microbial communities. Furthermore, there is great variability in biofilm formation and content among individuals with tooth decay. Therefore, it is suggested that future studies address this limitation and, in addition to testing other strains, conduct clinical studies to confirm the results reported in the current investigation. Also, evaluating the PMs of *Lactobacilli* isolated from dairy and digestive samples can offer further information on the role of postbiotics in the control of dental caries.

5. Conclusions

In conclusion, this study demonstrated the antimicrobial and antibiofilm properties of probiotic-derived PMs from LGG and LR strains against *S. mutans*. Our findings showed that MIC and MBC values were achieved at concentrations above 25% (v/v) for both PMs, suggesting their potential as effective antimicrobial agents. Biofilm destruction was observed in both PMs. However, destruction was significant in the LGG group. SEM analysis supported these findings, revealing fewer microorganisms and smaller microcolonies in the presence of PMs. Both PMs also significantly reduced the metabolic activity of *S. mutans*, as indicated by the XTT reduction assay. Moreover, treatment with LGG and LR PMs resulted in a substantial decrease in *gtfB* gene expression, which plays a crucial role in *S. mutans* biofilm formation. Overall, our results suggest that PMs from LGG and LR strains have promising potential as a natural alternative for preventing dental caries caused by *S. mutans*. However, further *in vivo* studies and clinical trials are needed to evaluate the efficacy and safety of PMs in oral environments.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

MB, MA, SE, RH, MM and MHY designed the research study. MB, MA, SE, RH, MM and MHY performed the research. RF and MP provided the methodology for the research and contributed to the writing of the draft and deep revision of the manuscript. MA analyzed the data. MB, MA, SE, RH, MP, RF, MM. and MHY wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final

manuscript.

Ethics Approval and Consent to Participate

This study was conducted with the approval and supervision of the National Institute for Medical Research Ethics Committee (IR.NIMAD.REC.1400.148) and the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.DENTISTRY.REC.1400.191). All procedures were carried out following the relevant guidelines and regulations.

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Conflict of Interest

The authors declare no conflict of interest.

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