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Research Article

Lactobacillus rhamnosus and *Lactobacillus salivarius* Modulate Planktonic, and Biofilm State of Cariogenic *Streptococcus mutans*

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Abstract

Streptococcus mutans is one of the early colonizers of the oral cavity and contributes to dental caries and decay. Therefore, any intervention that reduces its number in the oral cavity is considered helpful in preventing dental infections. Probiotics confer health benefits, and numerous studies have highlighted their curative effects on the oral cavity. In this study, the antimicrobial and biofilm-inhibiting potential of *Lactobacillus rhamnosus* and *Lactobacillus salivarius* in co-culture with cariogenic bacterium *S. mutans* was evaluated. *S. mutans* formed a significant biofilm both in the presence and absence of sucrose with optical density > 3-4 at 48h and 72h. On the contrary, *L. rhamnosus,* and *L. salivarius* formed weaker biofilm compared to *S. mutans*. Both *Lactobacillus* strains significantly reduced the bacterial load by up to 4-5 folds with 70 – 80% biofilm formation of *S. mutans* in co-culture experiments. The cell-free supernatant of both *Lactobacillus* strains also demonstrated a reduction in biofilm formation and bacterial load. Propidium monoazide (PMA) assays further revealed a significant decrease in *S. mutans* count when co-cultured with *L. rhamnosus* and *L. salivarius*. Gene expression analysis indicated the down-regulation of the quorum sensing (*LuxS*) gene when co-cultured with the *Lactobacillus* strains. In conclusion, our findings suggest that *L. rhamnosus* and *L. salivarius* can be used to control cariogenic bacteria, *S. mutans*.

Keywords: *Streptococcus mutans*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, probiotics, biofilm formation

1. Introduction

Streptococcus mutans is a facultative anaerobic bacterium, usually present in the oral cavity. It is recognized as the main etiological agent of dental caries. Dental caries is a multifactorial disease influenced by dietary intake, oral hygiene, human genetics, and the presence of cariogenic bacteria such as *S. mutans* (Weber et al. 2018). Virulence factors such as acid and insoluble glucan production in the presence of sucrose are among the key contributors to tooth decay(Moynihan 2016). The production of insoluble glucans initiates biofilm formation and provides a binding site for other free-floating bacteria. Under acidic conditions, it creates a cycle that provides a favorable breeding ground for *S. mutans* in the oral ecology (Baker and Edlund 2019).

Furthermore, dental plaque represents a dynamic, intricate, and multidimensional bacterial community enmeshed in exopolymeric insoluble substances, forming a biofilm that can adhere to either enamel or implants (Valm 2019). Biofilms provide a dynamic environment that, in addition to facilitating adherence, enables the horizontal transmission of antimicrobial genes. This creates an atmosphere of bacterial synergism or antagonism within a multispecies community. Factors affecting oral biofilm include interbacterial co-adhesion, pH, oxygen, and nutrients (Sharma et al. 2023). This microbial and resulting

Table 1: Primer Used to Detect *Streptococcus mutans*.

Primer	Sequence (5′ – 3′)	Product Size (bp)
16S rRNA - Forward	TCGCGTTGCTTCGAATTAAA	115
16S rRNA – Reverse	GGGAGTACGACCGCAAGGTT	
***LuxS* - Forward**	ACTGTTCCCCTTTTGGCTGTC	93
***LuxS* - Reverse**	AACTTGCTTTGATGACTGTGGC	

metabolite- accumulation leads to dental diseases such as dental caries, and periodontitis. *S. mutans* is typically present in the mouth as a component of normal flora. However, due to ecological imbalance, its titer increases, leading to the onset of disease. Hence reducing the population of *S. mutans* in the oral cavity is a prime objective of most of the therapies.

Recently, the use of probiotics in maintaining oral health has gained significant attention (Seminario-Amez et al. 2017). Probiotics are live microorganisms that confer health benefits upon administration by modulating the oral microbiota via competitive or non-competitive inhibition. This leads to the production of antimicrobial substances such as bacteriocins, enhancing the immune system and reducing the *S. mutans* count in the oral cavity (Homayouni Rad, Pourjafar, and Mirzakhani 2023). Many studies have reported the use of probiotic bacteria, such as *Lactobacillus* and *Bifidobacterium*, in maintaining oral health. *Lactobacilli* comprise of ≤ 1% of the total cultivable microbiota in the oral cavity and produce acid; thus, further investigations are needed to assess their suitability as therapeutic agents (Zaura and Twetman 2019). However, literature and many clinical studies support the idea of beneficial effects rather than harmful ones on oral health (Navidifar et al. 2023, Homayouni Rad, Pourjafar, and Mirzakhani 2023). Therefore, this study was designed to evaluate the modulating effect of *Lactobacillus salivarius* and *Lactobacillus rhamnosus* on the planktonic and biofilm formation of *S. mutans.*

2. Material and Method

2.1. Bacterial Culture and Growth Condition

S. mutans (ATCC 25175) were maintained in Heart Infusion (HI) broth. Whereas *L. rhamnosus* (ATCC 53103) and *L. salivarius* (ATCC 11741) were maintained in deMan, Rogosa, and Sharpe (MRS) broth. For Biofilm assay, *S. mutans* were grown in BHI containing 2% sucrose, while *Lactobacillus* species were in MRS broth with or without sucrose. Incubation was done at 37°C in an anaerobic jar for 24-72 hours.

2.2. Biofilm Production Assay by Microtiter Plate Test

To assess the biofilm formation, bacterial culture was refreshed overnight in their respective medium with and without 0 – 1.5% sucrose and incubated anaerobically for 18-20 hours at 37°C. The following day, the respective bacteria (5×10^6) were inoculated in 200µl of HI broth in a 96-well plate and incubated anaerobically for 24 – 72 hours at 37°C. Only media was also added as a negative control. At respective time points, non-adhered cells were washed thrice with phosphate buffer saline (PBS). Plates were dried and heat-fixed at 60°C for 30 minutes. The biofilm was stained with 200µL of 0.1% crystal violet for 20 minutes. Followed by washing to remove excess stain, and the absorbed crystal violet was dissolved in 200µl of 30% (v/v) glacial acetic. For the quantitative analysis of biofilms, the absorbance was read at 589nm on an ELISA reader (Usmani et al. 2021).

2.3. The Effect of *Lactobacillus* Species on Biofilm Formation

Co-culturing *S. mutans* with *L. rhamnosus* and *L. salivarius* was done to evaluate the antimicrobial and biofilm inhibitory potential of *S. mutans*. The bacterial inoculum was adjusted to 5×10^6 cells/ml. *S. mutans* (100 µl) were mixed with *L. rhamnosus* (100 µl) and *L. salivarius* (100 µl), respectively. On the other hand, *S. mutans* without *Lactobacillus* strain served as a positive control, whereas only media served as a negative control. Plates were incubated at 37°C for 24 hours, 48 hours, and 72 hours. After respective time points, quantitation of biofilms and *S. mutans* growth were determined by crystal violet assay and colony-forming unit (CFU).

2.4. Antimicrobial Properties of *Lactobacillus salivarius* Cell-Free Extract

The cell-free spent medium of the *Lactobacillus* strains was also analyzed for their inhibitory activity against *S. mutans* biofilm. The overnight cultures of *L. rhamnosus*

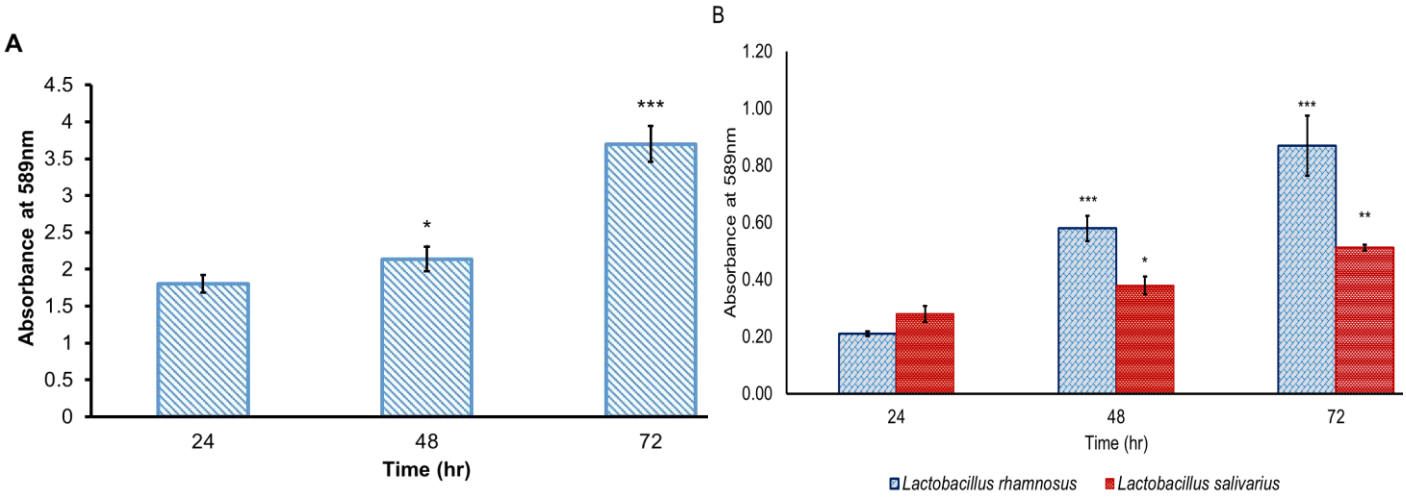

Figure 1: Biofilm Formation S. mutans (A) and L. rhamnosus and L. salivarius (B). The statistical significance was calculated by evaluating 24-hour biofilm data.

and *L. salivarius* were centrifuged at 5000 rpm to pallet down bacterial cells. The cell-free supernatant was further passed through a 0.45µm syringe filter to remove any bacteria present. The cell-free supernatant of each *Lactobacillus* strain was mixed with HI broth in a 1:1 ratio i.e. 100µl supernatant and 100µl media in a 96-well plate. After mixing, *S. mutans* (5 x 10^6 cfu/ml) were inoculated in the well, and plates were incubated at 37°C for 24 hours, 48 hours, and 72 hours. After incubation, the biofilm and growth inhibition were estimated by a crystal violet assay and CFU.

2.5. Depth Analysis Using Confocal Laser Scanning Microscopy

The biofilm depth analysis of the co-culture experiment was performed using a fluorescent microscope. *S. mutans* (5 x 10^6 cfu/ml) alone, and with *L. rhamnosus* (5 x 10^6 cfu/ml) and *L. salivarius* (5 x 10^6 cfu/ml) were co-cultured respectively in a 6-well plate containing coverslip. The plate was incubated for 48 hours at 37°C in an anaerobic jar. Following incubation, the coverslips were removed from the well, washed thrice with PBS, and stained with 5µL of 0.5% (w/v) fluorescein isothiocyanate (FITC) stock solution in 1mL of PBS in the dark. Coverslips were washed again to remove excess stain and then fixed with 4% paraformaldehyde for 1 hour at room temperature. The biofilm was visualized, and depth was determined using A Leica CLSM TCSSP2 confocal scanning microscope with the excitation and emission wavelength set to 488nm by an adjustable spectrum slit.

2.6. Propidium Monoazide (PMA) Linked Real-Time PCR Analysis of *S. mutans* After Co-Culturing

S. mutans was grown in co-culture with *Lactobacillus* species, as mentioned above, but now in a 50ml Falcon tube containing a glass slide as a substratum. The glass slide was aseptically transferred daily to a fresh medium until 48 hours. The biofilms were scratched off with a sterile spatula and suspended in 7.5 ml of 10 mM potassium phosphate buffer (pH 7.0). To de-chain and separate the cells, the biofilms were subjected to sonication by using a sonicator, at energy level 3 for 25 seconds, twice, with 2 minutes on ice between treatments. The bacterium was palleted down after centrifugation at 5000 rpm. To determine the total number of viable bacterial cells, PMA coupled with real-time PCR was used(Lee et al. 2022).

Briefly, the bacterial pellet was re-suspended in 500µl culture aliquot, and 20µl of 0.5 mg/mL PMA was added to 500µl culture aliquot to get 20µg/mL as the final concentration. Samples were incubated for 10 minutes in the dark, followed by exposure to 650W halogen lamp light for 10 minutes. Samples were kept at 20cm from the light source and were laid horizontally on ice to avoid excessive heating, with shaking after 30 sec to 1 minute for homogenous light exposure. Following light exposure, samples were centrifuged at 10,000 x g for 5 min to pallet out the cells and washed twice with distilled water to remove residual PMA. The DNA was extracted using a bacterial DNA isolation kit (Thermo Scientific, USA) as per manufacturer protocol.

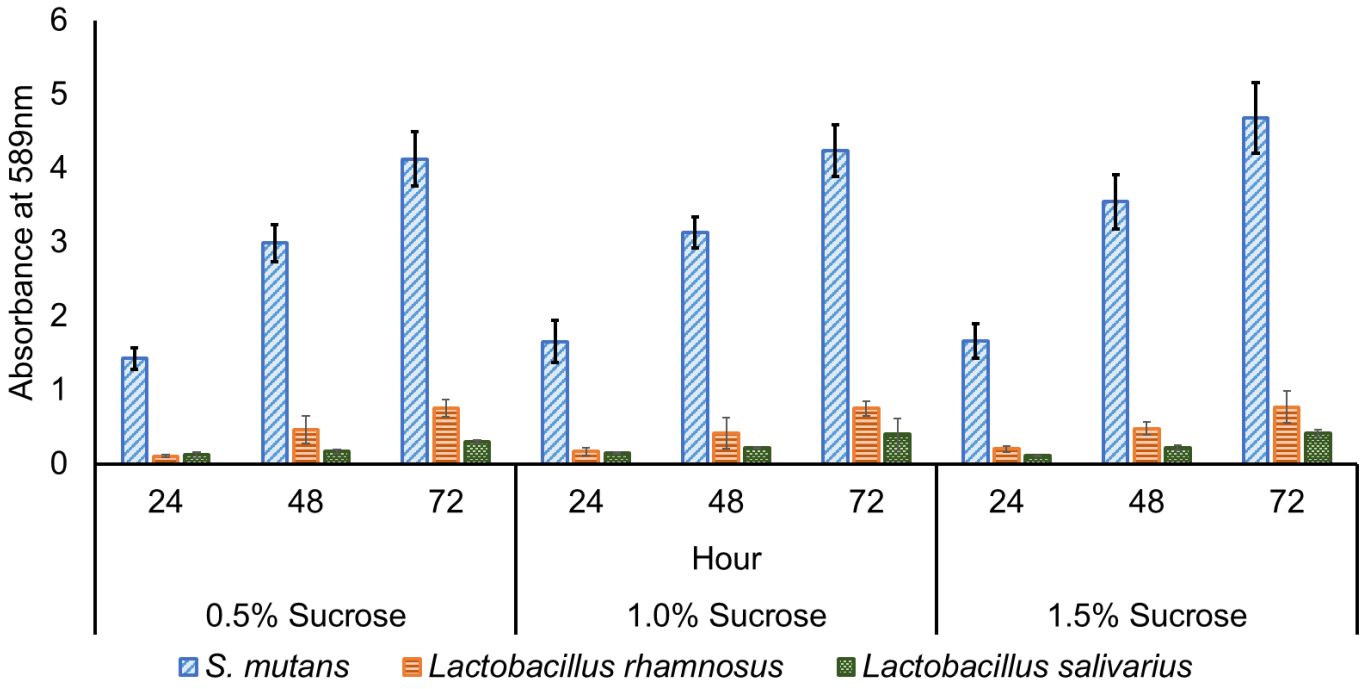

Figure 2: Biofilm Formation of *S. mutans*, *L. rhamnosus*, and *L. salivarius* in the presence of 0.5%, 1.0%, and 1.5% Sucrose, respectively.

2.7. Real-Time PCR

Real-time PCR was used to detect the copy number of *S. mutans* and quorum-sensing (*LuxS*) gene expression from PMA-linked DNA and isolated RNA, respectively. PMA linkage of DNA was done, as previously mentioned, and RNA was extracted from probiotic-treated cells using TRIzol reagent (Thermo Scientific, USA). RNA was reverse transcribed to cDNA, using a reverse transcription revert aid cDNA synthesis kit (Thermo Scientific, USA). Sybergreen real-time kit was used for real-time PCR. Each 25µl reaction mixture contained 12.5µl of 2x SYBR Green PCR Mix, 1µl of each primer (20 µM) (table 1), 0.4µl ROX reference dye, 1µl of sample DNA and 9.1µl sterile deionized water. Amplification was performed, starting with denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. A tenfold dilution series of PCR products was used to generate standard curves for the quantitation of target DNA. The correlation coefficient value of the standard curves was 0.95. Samples were run in triplicate. For gene expression of *LuxS*, relative quantification was done by using the $2^{-\Delta\Delta Ct}$ method.

2.8. Statistical Analysis

The results are presented as mean ± standard deviation. All the experiment was conducted in triplicate. The statistical significance of the data was analyzed using one two-tailed t-test, and one-way ANOVA using SPSS software v 20 (IBM, USA). The p-value of <0.05 was considered significant. The statistical significance was presented as $*p = < 0.05$; $**p = < 0.01$, and $***p = < 0.001$, respectively.

3. Results and Discussion

S. mutans is a cariogenic bacterium, that usually resides in the oral cavity and induces infection by virtue of its biofilm-forming and acid-producing ability. So, any strategy that reduces their count and biofilm formation will be considered. With the increase in antimicrobial resistance, the use of probiotics is emerging as an alternative therapy to control various infections, including dental caries. Probiotics, by competitive and non-competitive inhibition, such as antimicrobial substance release, promoting the immune system, can potentially become a good option to cure infectious diseases. Thus, the probiotic strains *L. salivarius* and *L.*

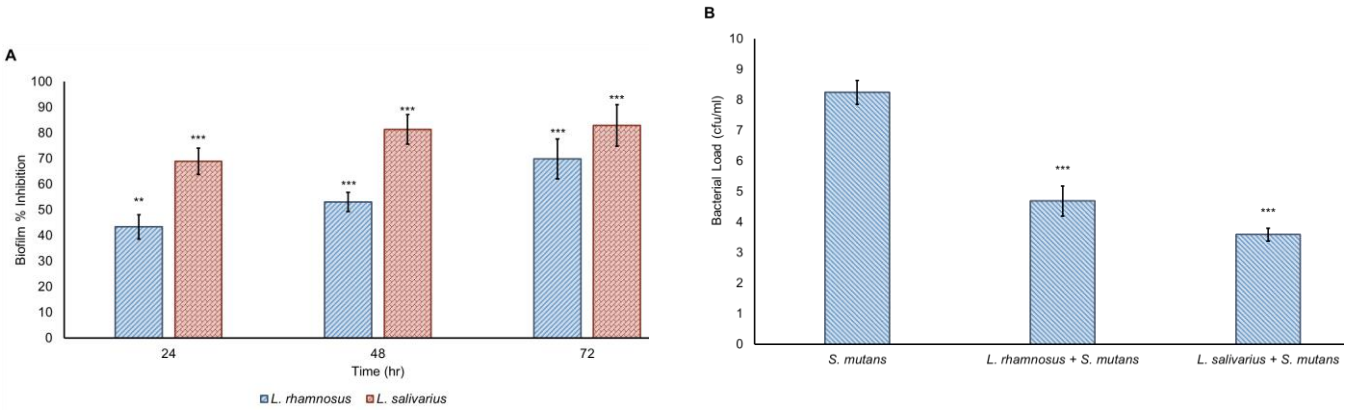

Figure 3: Co-culturing of *S. mutans* with *L. rhamnosus* and *L. salivarius* (A) Biofilm inhibition at 24h, 48h, and 72h (B) Bacterial load at 48h.

rhamnosus were tested for their ability to reduce cariogenic *S. mutans* and their biofilm.

The result exhibited that the used strains of *S. mutans* formed significantly strong biofilm as compared to *L. rhamnosus* and *L. salivarius* (*Figure 1 A and B*). The results corroborate with other studies that showed that isolated *S. mutans* from cariogenic teeth were all strong biofilm producers, with all the genes, required for biofilm formation, intact for initiating dental decay (Zayed et al. 2021, Pisarska et al. 2022). When the biofilm formation of all the tested strains was checked in the presence of 0-1.5% sucrose, again *S. mutans* showed significant biofilm formation compared to the *Lactobacillus* strains used (*Figure 2*). This indicates the competence of *S. mutans* biofilm formation as compared to the *Lactobacillus* species used. The growth and biofilm formation of *Lactobacillus* species were found to be similar either in the presence or absence of sucrose, which demonstrated that increasing concentration of sucrose, has no impact on their growth. However, there is a slight increase of biofilm in the presence of 1-1.5% sucrose (*Figure 2*). The obtained results are in agreement with other studies which showed that cariogenicity of *S. mutans* significantly increases in the presence of sucrose or dietary sugars (Leme et al. 2006, Waldman et al. 2023).

Probiotics are microbes, which are generally regarded as safe (GRAS) that upon administration provide health benefits by altering the conditions required for infectious and metabolic diseases (Milner et al. 2021). Probiotics are known for their ability to restore normal flora within the gut, as well as oral microflora by reducing cariogenic bacteria such as *S. mutans* (Zeng et al. 2023). Numerous studies highlight the importance of probiotics such as *L. acidophilus, L. casei, L. bulgaricus,* and *L. plantarum* in reducing cariogenic pathogens after their consumption (Zeng et al. 2023). *L. casei*, a probiotic strain found in the commercial dairy drink Yakult, was reported to reduce cariogenic bacteria and displayed cariostatic effects by reducing acid production and biofilm formation (Lin, Chou, and Hsu 2017). Biofilm formation is one of the key contributors to the pathogenicity of *S. mutans*. In addition, *S. mutans* biofilm allows other cariogenic bacteria to adhere to produce cariogenic plaque to destroy dentine. Both the strains used in this study, *L. rhamnosus* and *L salivarius*, not only significantly reduced the biofilm formation but also inhibited *S. mutans* growth in vitro. However, *L. salivarius* was found to be more active in reducing *S. mutans* count and biofilm as compared to *L. rhamnosus* (*Figure 3A and B*). *L. salivarius* inhibited 70% biofilm of *S. mutans* at 24h, the inhibition further increased to >80% after 48h, and 96h (*Figure 3A*). Furthermore, it reduced the *S. mutans* bacterial load by 5 folds at the 48-hour mark (*Figure 3B*). On the other hand, *L. rhamnosus* inhibited >40% biofilm at 24h and further inhibited >50% and >70% biofilm respectively at 48h, and 72h (*Figure 3A*). *L. rhamnosus* also reduced the bacterial load by 3.5-fold (*Figure 3B*). Notably, probiotic strains are reported to produce antimicrobial substances; therefore, the cell-free extract of both probiotic strains was analyzed for biofilm and bacterial inhibitory potential. The cell-free extract of *L. salivarius* significantly inhibited 40% biofilm at 24h and the inhibition further increased to >50% and >60% at 48h and 72h, respectively (*Figure 4A*). In contrast, the

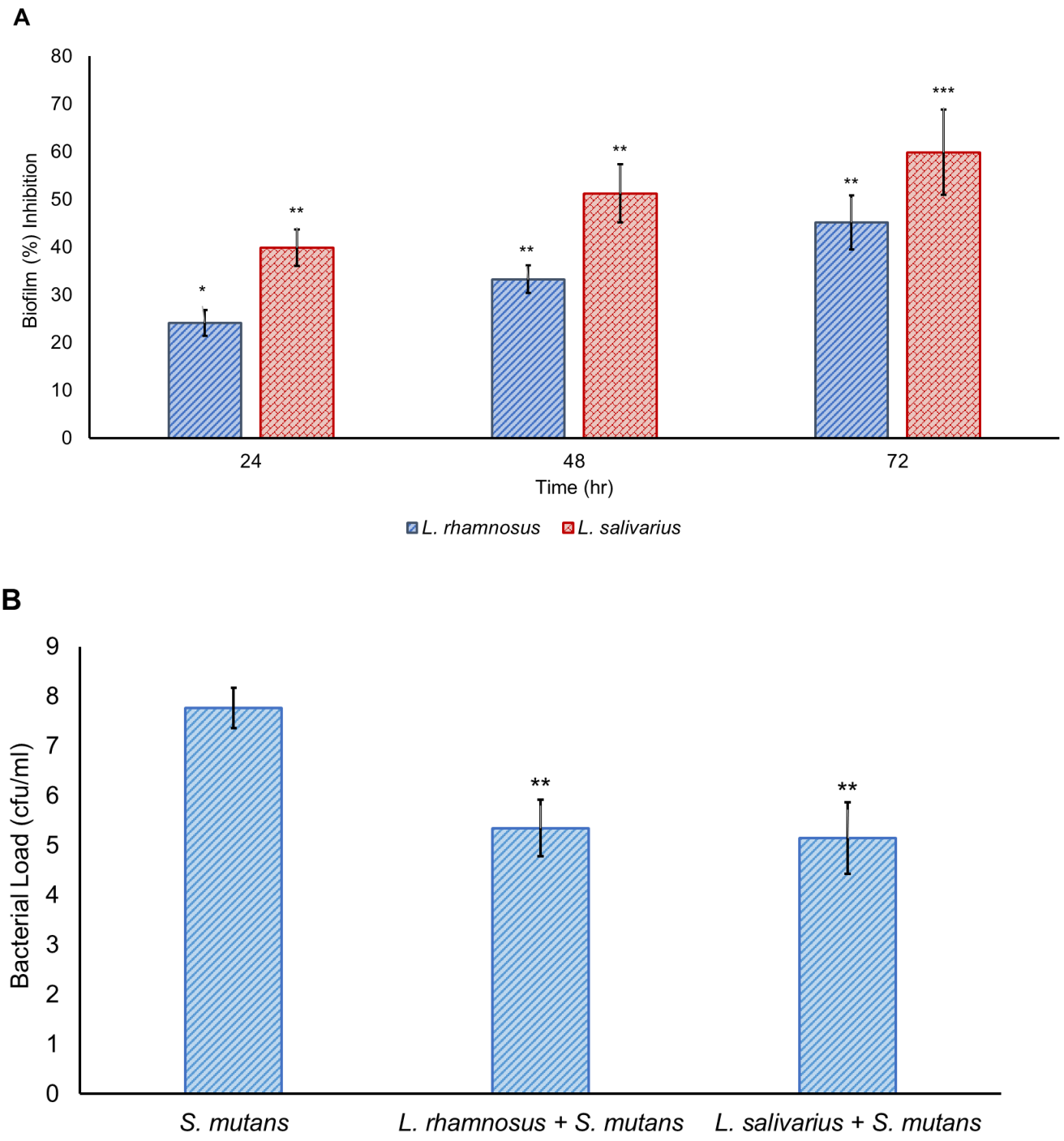

Figure 4: Effect of cell-free extract of *L. rhamnosus* and *L. salivarius* on *S. mutans* (A) Biofilm inhibition at 24h, 48h, and 72h (B) Bacterial load at 48h

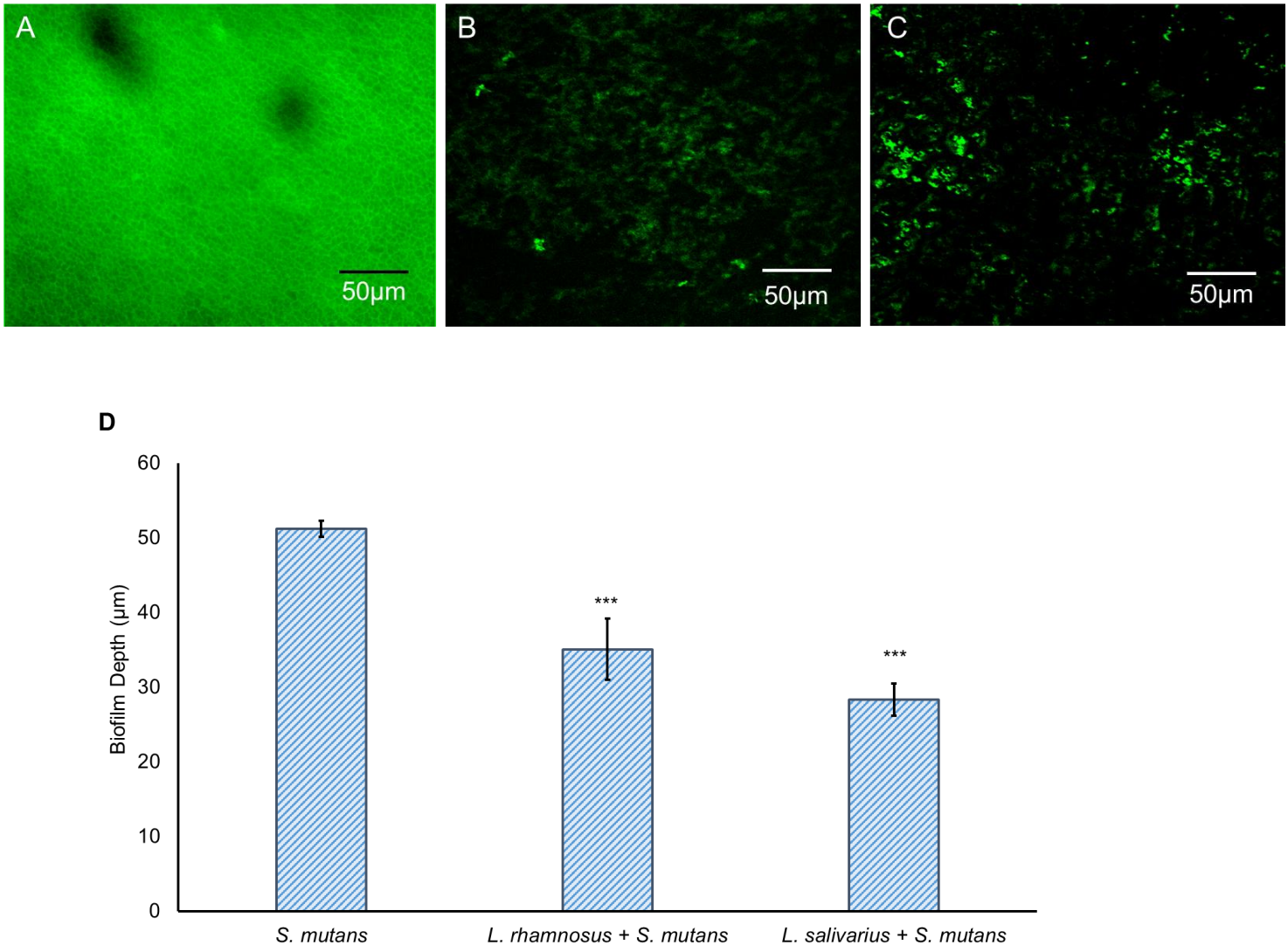

Figure 5: ***S. mutans*** **biofilm thickness when grown with** ***L. rhamnosus*** **and** ***L. salivarius*** **at 48h (A, B, C) Confocal microscope analysis (D) Quantitative thickness.**

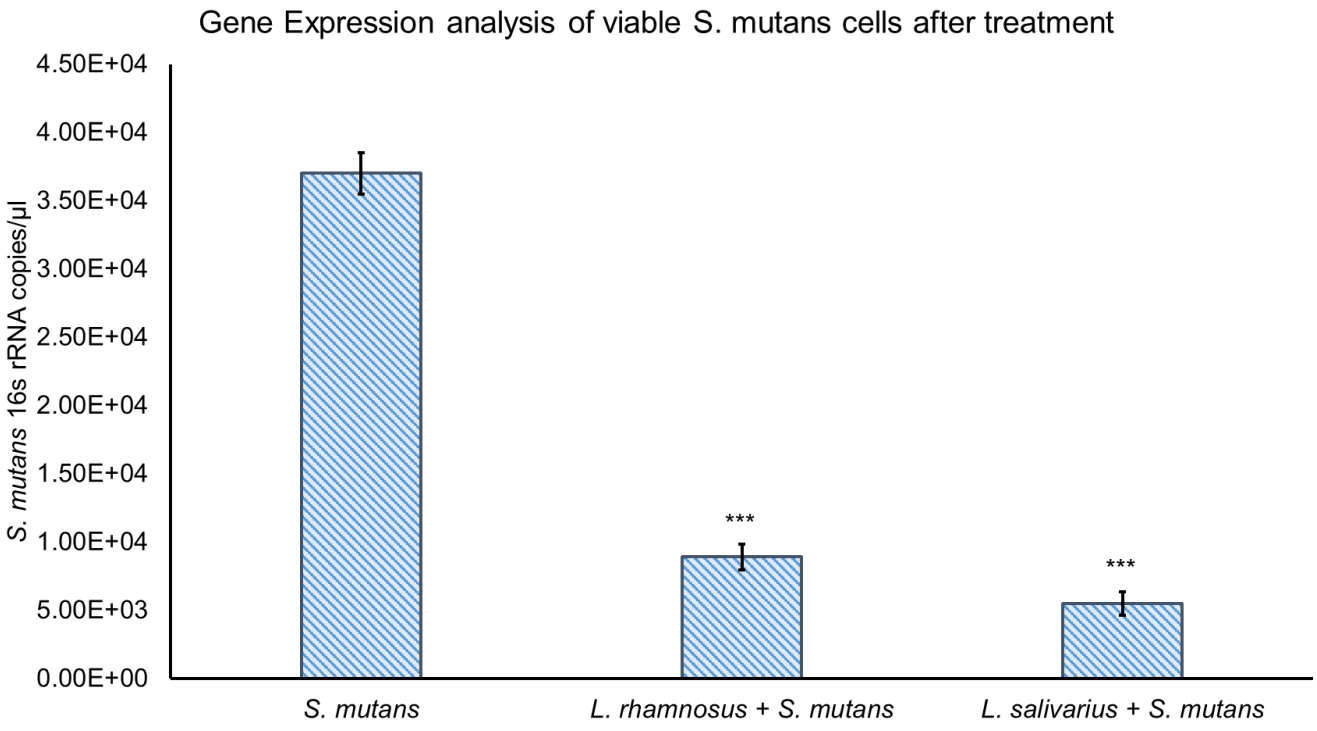

Figure 6: Propidium monoazide (PMA) cross-linked PCR assay to analyze S. mutans count after treatment with *L. rhamnosus* and *L. salivarius*

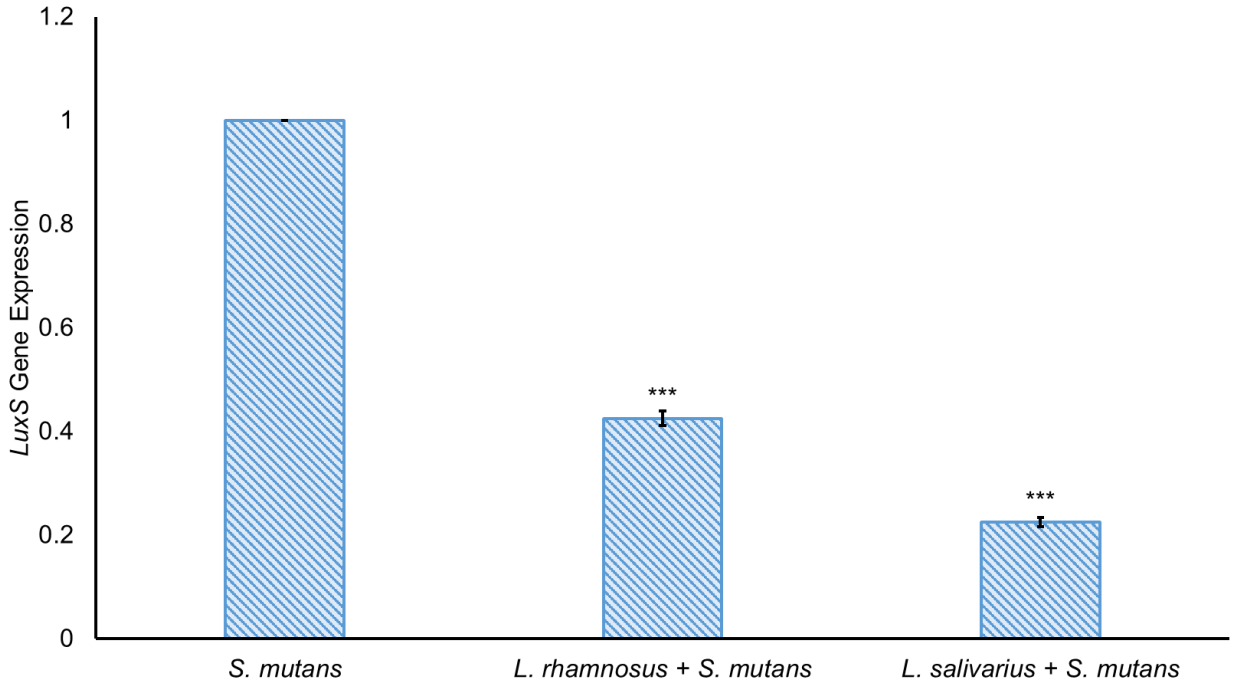

Figure 7: Quorum sensing (*LuxS*) gene expression analysis in S. mutans after treatment with L. *rhamnosus* and *L. salivarius.*

biofilm reduction was significantly less in the cell-free extract of *L. rhamnosus*. It reduced only 20% biofilm at 24h with a maximum reduction of 50% at 72h (*Figure 4A*). Interestingly, the cell-free extract of both strains reduced the bacterial load by 2.5 folds (*Figure 4B*). The biofilm depth analysis was also performed, and it was observed that co-culture of *L. rhamnosus* and *L. salivarius* with *S. mutans* significantly reduced the biofilm depth to 35µm and 29µm, respectively, as compared to *S. mutans* alone, which form a biofilm with depth 50µm (*Figure 5A and B*). The result showed the maximum reduction of *S. mutans* and their biofilm in co-culture experiments, unlike those in cell-free extract. This further solidifies the theory that the probiotic strains can compete for nutrients and space with the production of antimicrobial peptides, a phenomenon that significantly impacted *S. mutans'* viability and pathogenicity. Studies also showed that isolated *L. rhamnosus* and *L. salivarius* reduce *S. mutans* count (Wu et al. 2015, Tahmourespour, Kasra-Kermanshahi, and Salehi 2019).

The advent of molecular methods facilitated the detection of viable and non-viable cells in the co-culture experiments with species-specific primers. PMA-linked real-time assay has been reported to identify live and dead cells within the bacterial community (Lee et al. 2022, Trigueros et al. 2023). PMA reportedly crosslinks dead cells within the bacterial community, thus inhibiting their amplification in PCR (Deshmukh, Bhand, and Roy 2020). However, the use of this assay was not reported earlier in the co-culturing experiment of *Lactobacillus* species with *S. mutans*. So, it was used in this study with *S. mutans*-specific 16s rRNA primers. The PCR results exhibited a significant reduction in *S. mutans* copy number when co-culture with *L. rhamnosus* and *L. salivarius* (*Figure 6*). Quorum sensing is a bacterial phenomenon, with its aid bacteria communicate with each other in dense populations. In addition to quorum sensing mechanisms, *LuxS*-dependent quorum sensing is primarily responsible for *S. mutans* biofilm maturation and cariogenic attributes (Merritt et al. 2003). The quorum sensing inhibition might be a target to reduce the pathogenicity of *S. mutans* to avoid cariogenicity and biofilm formation (Wasfi et al. 2018). In co-culturing experiments, the gene expression of *LuxS* was significantly down-regulated by 60% and 70% in *L. rhamnosus* and *L. salivarius* treated groups, respectively. This further lends credence to the claim that biofilm inhibition might be due to the quorum-quenching capability of *Lactobacillus* strains used.

In conclusion, *Lactobacillus salivarius* and *Lactobacillus rhamnosus* significantly inhibited *S. mutans* growth and biofilm formation. They significantly reduced the biofilm depth, as evidenced by microscopy, as well as reduced the bacterial load, analyzed via CFU and PMA crosslink PCR assay. Probiotic strains significantly reduced the quorum sensing gene (*LuxS*) expression in co-culture experiments, potentially inhibiting biofilm activities. Therefore, reported probiotic strains can be used as a control strategy for dental caries after further pre-clinical experimentation.

Conflict of Interest

The authors declare that they have no competing interests.

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Study Approval

This study was approved by the Panjwani Center for Molecular Medicine & Drug Research, University of Karachi, Pakistan.

Consent Forms

NA.

Authors Contribution

AA and LJ carried out all the data collection, bench work, and manuscript writing. XY helped in data collection and statistical analysis. AA and XY conceptualized and supervised the study.

Data Availability

Data is available upon reasonable request from the corresponding author.

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