Eastern Journal of Agricultural and Biological Sciences (EJABS)
ISSN: 2753-3247
Website: https://qabasjournals.com/index.php/ejabs

Impact of *Lactobacillus plantarum* on expression of biofilm-related genes in *Streptococcus mutans* isolated from patients of dental caries

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ARTICLE INFO

Received: March 25, 2024
Accepted: April 29, 2024
Volume: *4*
Issue: 2

KEYWORDS

Probiotic, Molecular qPCR, *lux S* gene, *gtfBCD* genes, Iraq

ABSTRACT

Streptococcus mutans causes caries and decay. During congestion sucrose, *S. mutans* digests insoluble glucans leading to tooth plaque or oral biofilm which starts the caries process. *GtfB* and *LuxS* genes affect growth and development of biofilm in *S.mutans*. *Lactobacillus plantarum* probiotic can influence the formation of *Streptococcus mutans*biofilm. The main aim of this investigation is to discover what effect does *Lactobacillus plantarum* supernatant have on virulence genes expression and *Streptococcus mutans* growth.PCR (quantitative real-time)and microtiter plate assay were used for evaluating antibacterial properties of *Lactobacillus plantarum"*spent culture supernatant (SCS). According to data, Lactobacillus plantarum supernatant significantly suppressed the growth of *S. mutans* ($P < 0.01$). Compared with control group, treatment with Lactobacillus plantarum SCS caused a marked reduction ($P < 0.01$) in both planktonic and biofilm forms expression levels of *luxS*, *gtfB*, *gtfC* and *gtfD* genes in *Streptococcus mutans*. This research suggests that *Lactobacillus plantarum* may inhibit the growth and virulence factors of streptococcus mutans strains and Probiotics containing *Lactobacillus plantarum* may be useful in lowering dental caries risk and maybe promoting better oral health.

1. Introduction

Probiotics are living microorganisms that provide the host with health advantages when consumed in sufficient quantities [1]. Probiotic studies conducted during the last ten years have had a substantial influence on food technology, improving consumer health outcomes. Extensive research supports the various advantages of probiotics, including lowering cholesterol levels, boosting the immune system, promoting gut health, and potentially reducing the risk of cancer [2]. Additionally, there is substantial evidence supporting the use of probiotics in managing acute diarrhea, preventing antibiotic-induced diarrhea, and enhancing lactose metabolism [3]. With the escalating concern over bacterial resistance to antibiotics on a global scale [4, 5], there is a growing interest in exploring alternative therapies for oral health. It is imperative to identify safer and more effective alternatives to pharmaceutical antibiotics. Recent assessments have indicated that probiotic strains can be utilized to prevent dental conditions such as caries [6].

Dental caries is a prevalent and persistent dental condition that can impact an individual's health at any stage of life. Numerous research studies have associated cardiovascular diseases with inadequate dental hygiene. Dental caries is a condition that arises from an imbalance resulting from internal interactions between the microbiota and the host [7]. Alterations to the oral environment due to factors such as smoking, poor hygiene, systemic diseases, and reduced saliva production may cause non-pathogenic bacteria to transition from a commensal relationship to parasitism. *Streptococcus mutans* has been identified as the primary cause of dental caries [8, 9]. There are many processes involved in the production of cariogenic biofilm in the oral cavity. The first stage is the attachment of non-mutans streptococci to the pellicle, which facilitates the growth of *Streptococcus mutans* and the beginning of biofilm formation.Caries are partially formed by the virulence factors of *Streptococcus mutans,* including the production of acid that damages the hard tissues of the teeth. Other contributing factors include the presence of an agmatine deiminase system and F-ATPase, which are encoded by the *aguBDAC* operon and *atpD*

gene, respectively [10, 11]. These components are important in the acid-adaptive response and contribute to the acid-resistant properties of the bacteria. (iii) The capacity to produce exopolysaccharides (EPS) from sucrose through the activity of several glucosyltransferases (Gtfs) encoded by the genes gtfb, gtfc, and gtfd, as well as fructosyltransferase encoded by the *sacB* (ftf) gene [12-14]. The enzymes glucosyltransferase and fructosyltransferase facilitate the production of extracellular glucan and fructan polymers from sucrose, respectively [15]. The EPS formed is believed to have two functions: facilitating microbial adherence to surfaces and providing protection to embedded bacteria [16]. These pathogenic traits operate under the regulation of quorum sensing mechanisms. The two-components signal transduction systems (TCSTS), such as comCDE and vicRKX, are regulatory linkages that control gene expression in reply to stimuli from the environment. These systems are crucial for the survival and modulation of virulence in bacteria [17, 18]. Probiotics have the potential to influence oral pathogens through general mechanisms. While the specific ways in which probiotics exert their effects are not fully understood, it is widely believed that they compete with pathogens for nutrients and space. Additionally, probiotics may have immunomodulatory effects and impact the production of lactic acid, peroxide, or bacteriocin [19]. Our hypothesis is that Lactobacillus species can inhibit the growth, biofilm formation, and gene expression of *Streptococcus mutans*. The aim of this study is to examine how the supernatant of *Lactobacillus plantarum* affects the growth and expression of virulence genes in *Streptococcus mutans*.

2. Methodology

Bacterial strains

Streptococcus mutans and *Lactobacillus plantarum*, initially isolated from cow milk and dental caries patients [20, 21], were cultured and preserved in Brain Heart Infusion (BHI) broth under optimal growth conditions at the microbiology laboratory of the University of Wasit, College of Medicine.

Process of SCS production

To produce SCS from *Lactobacillus plantarum*, the method described by Lin et al. [22] was followed. After being prepared, the supernatant was filtered using 0.45-µm filters from "Millipore, Bedford, MA, USA". At this point, it was divided into four parts. The first part went without any treatment; whereas the other three parts were treated with organic acids, H_2O_2 and bacteriocin for neutralization purposes respectively. To counterbalance the influence of organic acid on SCS, its pH value was adjusted to 6.5 with 1 N NaOH. Then, two remaining portions were subjected to 1 mg/ml trypsin from "SigmaAldrich, USA" and 0.5 mg/ml catalase (Sigma-Aldrich, C1345 , USA) for eliminating effects of bacteriocin and H2O2 respectively [23]. Treated or non-treated supernatants were preserved in freezing condition.

Antibacterial testing of SCS

Streptococcus mutans was cultured for 16–18 hours at 37°C in brain heart infusion broth in order to evaluate the antibacterial efficacy of the SCS. *Streptococcus mutans* was grown and diluted to a turbidity comparable to McFarland 0.5 (1.5×10^8 cells/ml) in brain heart infusion broth; and subsequently, eight copies of each Lactobacillus SCS (Greiner Bio-One, Kremsm Unster, Austria) were added to wells of a 96-well microtiter plate containing 100 µl of the *S. mutans* suspension and 100 µl of supernatants. The plates were then anaerobically incubated for 24 hours at 37°C. Control wells utilized sterile MRS broth instead of SCS. Following incubation, the optical density 600 nm was measured using a microplate reader (Stat Fax2100) [23-25]. The same procedures were conducted with treated supernatants to assess changes in antimicrobial activity after neutralizing the effects of acidic pH, peroxides, and bacteriocin.

Effect of SCS on virulence genes of S. mutans

Quantitative real-time PCR was used to examine the effect of SCS on expressions of biofilm-related genes (*luxS, gtfB, gtfC, and gtfD*) in *S. mutans*. The impact of this filtered supernatant from Lactobacillus plantarum on both the planktonic and biofilm forms of *Streptococcus mutans* was investigated. *Streptococcus mutans* was diluted to McFarland 0.5 after overnight culture at 37°C in BHI broth. A mixture of 1.5 ml of BHI broth, 250 µl of the SCS, and 250 µl of the *Streptococcus mutans* suspension was incubated anaerobically at 37°C for a full day. Control wells utilized MRS broth instead of the *Lactobacillus planetarium*supernatant [26]. To extract planktonic bacterial RNA, the culture suspension was removed from the wells post-incubation. Following two sterile saline washes, cells adhered to the plate wells were scraped into a centrifuge tube, suspended in saline, and released. *Streptococcus mutans* RNA was isolated entirely. Total RNA extraction from *S. mutans* adherent and planktonic cells was carried out according to the manufacturer's instructions utilising the TRIzol® reagent kit. The purity of the extracted total RNA was assessed using a Nanodrop spectrophotometer (THERMO, USA). DNase I enzyme treatment of the extracted RNA samples was performed following the Promega company's instructions in the USA to eliminate any residual genomic DNA. The mixture was incubated at 37°C for 30 minutes with the addition of EDTA to deactivate the DNase enzyme. The DNase-treated RNA samples the used for cDNA synthesis by using the "AccuPower® RocktScript RT PreMix kit".

The inner control for tolerating the target gene expression was the "*Streptococcus mutans*16SrRNA gene". Specific primers for the *luxS*, *gtfB*, *gtfC*, and *gtfD* genes of *S. mutans* (Table 1) were utilized. Real-time PCR amplification was carried out using a

Real-time PCR thermocycler from Agilent, USA. Each reaction mixture of 25 µl comprised 12.5 µl of 2x SYBR Green PCR Mix from TaKaRa, Japan, 1 µl of each primer (20 µM), 0.4 µl of ROX reference dye, 1 µl of sample cDNA, and 9.1 µl of sterile deionized water. The amplification process included a first denaturation step at 95°C for 2 minutes, proceeded by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. All samples were run in triplicate, and the relative quantification was conducted using the 2-ΔΔCt method. Each experiment was performed with triplicate samples and the results were based on three independent experiments.

Table 1. This study's primers and the corresponding nucleotide sequences

Gene	Primer sequence (5'-3')	Size (bp)	References
16S rRNA	"F: CCTACGGGAGGCAGCAG"	196	27
	"R:ATTACCGCGGCTGCTGG"		
luxS	"F: ACTTGCTTTGATGACTGTGGC"	115	27
	"R:TCAGCGTATTGACGGGATG"		
gtfB	"F: AGCAATGCAGCCAATCTACAAAT"	59	28
	"R: ACGAACTTTGCCGTTATTGTCA"		
gtfC	"F: GTGCGCTACACCAATGACAGAG"	107	28
	"R: GCCTACTGGAACCCAAACACCTA"		
gtfD	"F: TGGCACCGCAATATGTCTCTTC"	103	28
	"R: CAATCCGCAATAACCTGAATACCG"		

Statistical analysis

The GraphPad Prism Software was served to identify significant differences between values of G1 and G2 groups at $P<0.05$ (*) [27].

3. Results and Discussion

Antimicrobial effect L. plantarum supernatant against S. mutans

Streptococcus mutans growth was strongly and significantly inhibited ($P < 0.01$) by the Lactobacillus plantarum's untreated supernatant (Fig. 1). The antibacterial impact was strongly ($P < 0.01$) reduced when the acidity of the supernatant was neutralized, although the growth of Streptococcus mutans was still significantly ($P < 0.05$) decreased. When trypsin or catalase were added, Lactobacillus plantarum's antimicrobial efficacy against *Streptococcus mutans* significantly decreased ($P <0.05$) (Fig. 1), suggesting that peroxides are a factor in this action.

The mutans streptococci consist of seven faithfully related species collectively known as *S. mutans. S. mutans* primarily resides in the oral cavity, throat, and gastrointestinal tract. Dental caries is caused by multiple factors, such as binding to enamel surfaces, generating acidic byproducts, storing glycogen, and synthesizing extracellular polysaccharides. Because of their capacity to attach to other plaque-forming bacteria and the enamel salivary film, *S. mutans* plays a significant part in the improvement of dental caries [28].

Mutans streptococci are known for their ability to produce high levels of acid, creating an acidic environment that can lead to the formation of cavities. After the onset of caries, *S. mutans* typically colonizes tooth cavities within 6 to 24 months. Acidogenic *S. mutans* can produce extracellular polysaccharides (EPS) from fructose and glucose as well as sucrose when available, which serve as energy-dense molecules due to their complex structure involving long chains with high molecular weight. The production process involves fructosyl transferases creating fructans (FTF) and glucosyltransferases manufacturing glucans (GTF): two different types of polysaccharides via glycosidic bond synthesis between fructose and glucose monomers [29]. Among various factors contributing to its cariogenicity, *S. mutans'* high synthesis rate of extracellular polymer (EPS) from sucrose significantly exacerbates the situation thus sustaining the tooth decay dynamics initiated by these microbial actions.Probiotic lactobacilli produce antibacterial compounds like lactic acid, hydrogen peroxide, and bacteriocins that are effective in killing Gram-positive bacteria and hampering bacterial growth. The low production of hydrogen peroxide by lactobacillus planetarium and the acid tolerance of mutans species suggest that bacteriocins or similar proteins may be responsible for the antibacterial effects of probiotics [30].

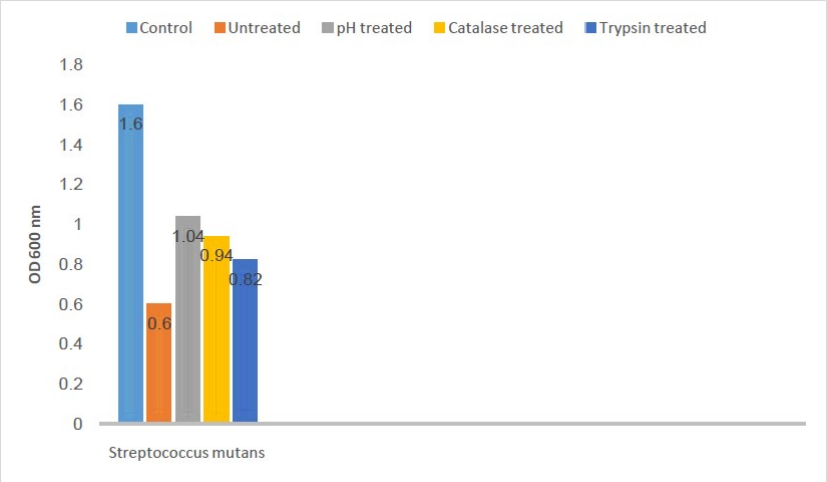


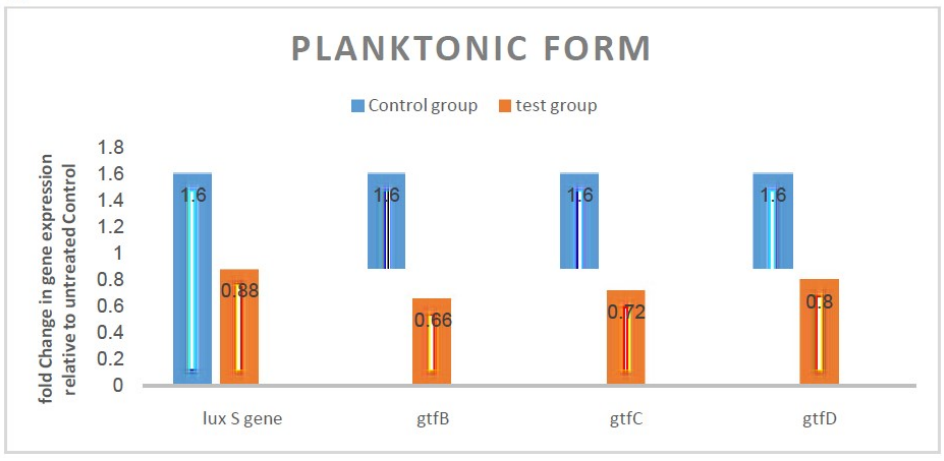
Figure 1 Streptococcus mutans growth in the presence of treated and untreated L. plantarum supernatant. Optical density (OD) of S. mutans growth in the presence of treated and untreated L. plantarum supernatants. Control: Streptococcus mutans grown in BHI broth. Untreated: Spent Culture Supernatant (SCS) of each strain supernatant, pH treated: supernatant with adjusted pH 6.5, catalase treated: supernatant after addition of 0.5 mg/ml catalase enzyme and trypsin treated: supernatant after addition of 1 mg/ml trypsin enzyme. Data are expressed as the mean S.D., P < 0.01 compared with Streptococcus mutans grown in BHI broth as control

Effects of L. plantarum filtered supernatants on the luxS, gtfB, gtfC and gtfD genes expression of S. aureus

We performed qPCR analysis to evaluate the impact of *Lactobacillus plantarum* SCS (diluted at a 1:8 ratio in BHI) on *Streptococcus mutans* cells. Specifically, we examined the expression levels of four genes associated with *S. mutans* virulence in both planktonic and biofilm cells following exposure to the SCS. The control group comprised untreated cells prepared under identical conditions but without the SCS being tested. The four genes analyzed were *gtfB, gtfC, and gtfD* (related to EPS formation) along with *LuxS* (involved in interspecies quorum sensing and other behaviors). In both planktonic and biofilm states, the levels of expression of *luxS, gtfB, gtfC*, and *gtfD* genes in the *S. mutans* were assessed, with the results depicted in figure (5). A significant decrease in the expression of these genes was observed post-treatment with Lactobacillus plantarum SCS compared to the control group.

In the planktonic state, the expression of the *luxS* gene was clearly reduced by 1.81-fold, while the levels of *gtfB, gtfC, and gtfD* genes related to EPS formation were decreased by 2.42-fold, 22.22-fold, and 2.0-fold respectively. Conversely, in the biofilm state, the expression level of *luxS* gene was reduced by 2.66-fold, and the levels of expression of *gtfB, gtfC, and gtfD* genes were reduced by 4.0-fold, 3.63-fold, and 3.07-fold respectively.

A



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B

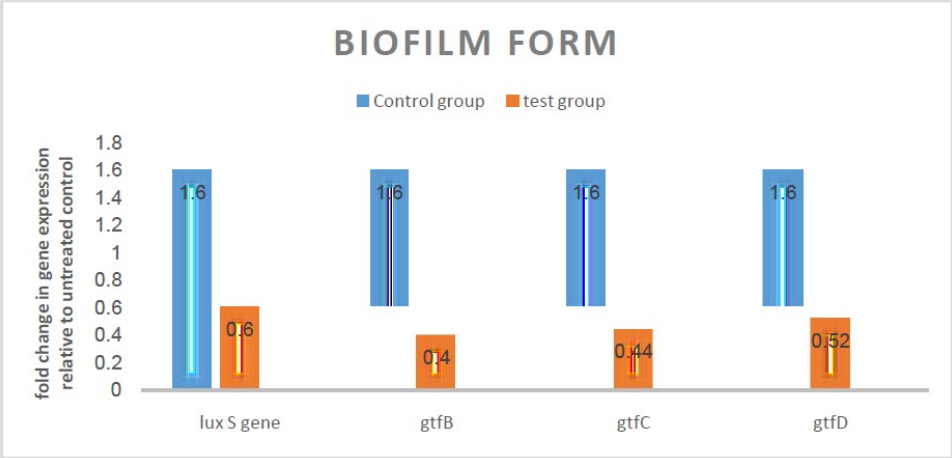

Figure 2 Changes in gene expression patterns linked to the exposure of Streptococcus mutans to the tested spent culture supernatant of Lactobacillus plantarum were assessed using qPCR, both in planktonic and biofilm-forming states. The fold change in gene expression levels, calculated through the ΔΔCt method relative to the untreated control, is depicted in each panel. Statistical analysis indicated significant variances (P < 0.01) between the experimental and control groups

Studies show that by producing bacteriocin, lactobacilli may efficiently suppress mutans streptococci. However, further studies is needed to fully understand the antibacterial mechanisms of probiotic lactobacilli against Mutans streptococci.In the current investigation, the influence of the SCS of *L. plantarum* on the growth and expression levels of *LuxS , GtfB , GtfC and GtfC* gene s of *S. mutans* was evaluated [31]. Our result presented thatthe examined *L. planetarium* strains caused important decline in the bacterial growth of *Streptococcus mutans* in Brain heart infusionmedia, as determined by the adjustment in 0btical density 600. *Streptococcus mutans* is a bacterium that produces organic acid through sugar fermentation and can withstand acidic conditions in the plaque environment, making it acid-tolerant. To assess the impact of organic acids, H_2O_2, and bacteriocin produced by tested *Lactobacillus planetarium*, their effects were neutralized using methods such as neutralization, catalase, and trypsin addition, respectively. The antimicrobial effect of the tested substances was significantly reduced when the supernatant was neutralized to pH 6.5. Despite this, the neutralized supernatant still exhibited a notable growth reduction observed in*Streptococcus* mutans compared to the control, indicating that neutralized substances were less effective in inhibiting microbial growth than untreated ones. This indicates the presence of other antimicrobial agents working alongside acid to suppress bacterial development, such as H_2O_2, bacteriocin, and biosurfactant. *Streptococcus mutans*, a key contributor to dental caries, typically resides in biofilm form within the oral cavity. Apart from forming a protective barrier against chemicals, *Streptococcus mutans* EPS (extracellular polysaccharides) aids in biofilm formation, which contributes to tooth decay. Targeting biofilms with treatment medications may be the most effective approach to preventing dental caries.

The cariogenic goods of *Streptococcus mutans* biofilms are controlled by diverse crucial genes. Therefore, the expression of typical biofilmrelated genes was examined. The genes investigation involvedgenes for synthesis of insoluble glucan (*gtfB, gtfC*), soluble glucan (*gtfD*) and quorum sensing gene (lux S) [32].

In the present study, the levels of expression of"*luxS, gtfB, gtfC*, and *gtfD* genes" in S. mutans were). Immensely down-regulated after treatment with *L. plantarum* SCS in comparison to the control group. The advocation of the reduced production of extracellular polysaccharides and oral bacterial aggregation thereby inhibiting biofilm formation is due to the downregulation of the expression of genes in outr study. Additionally, cinnamaldehyde demonstrated a decrease in *LuxS* gene expression, which codes for an enzyme that helps to catalyze the production of autoinducer 2 (AI-2). AI-2 is a vital component of "inter- and intra-species microbial communication" in oral bacteria, along with *S. mutans.* Consequently, the formation of biofilms in *S. mutans,* which relies on communication between different species, was disrupted by reducing the expression of luxS, thereby inhibiting AI-2 synthesis [33, 34]. Current studies have shown these *S. mutans luxS* mutants exhibit a more granule appearance in biofilms and are not capable of generating the AI-2 signal. Furthermore, it has been brought to our attention that disabling the luxS results in a lower expression of various genes that encode for proteins association with the

membrane which are responsible for maintaining envelope structure and integrity and the tolerance of the acid. Hence, the observed lowerence in *luxS gene* expression in our research may have greatly participated to the reduced acid tolerance and biofilm development [35, 36].

4. Conclusion

The conclusions of the study advise that it is possible to employ *Lactobacillus plantarum* as probiotics with effectiveness.to demonstrate a decrease in the Probability of tooth decay by (i) formation of organic acids and peroxide which originally promoted the deactivation of *Streptococcus mutans* growth; (ii) several *Streptococcus mutans* virulence genes were down-regulated, such as those that produce EPS (*gtfB, gtfC, and gtfD*), and quorum-sensing genes (lux S).

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