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The effect of *Lactobacillus plantarum* on expression of biofilm genes of *Streptococcus mutans* isolated from dental caries

Zahra K. Saeed[1], Basil A. Abbas[2], & Rasha M. Othman[2]

ABSTRACT

Background: **Dental caries and decay caused by *Streptococcus mutans*. Sucrose, stimulates *S. mutans* to yield insoluble glucans to form oral biofilm also called dental plaque to start process of caries. The *GtfB* and *LuxS* genes of *S. mutans* are responsible for development and evolution of biofilm. *Lactobacillus plantarum* as probiotic can affect the biofilm formation of *S. mutans.***

Aim of the study: **To evaluate the effect of *Lactobacillus plantarum* against *S. mutans* biofilm formation.**

Results: **Growing biofilm in the presence of sucrose was detected using 100 well microtiter plate crystal violet assay and biofilm formation by *S. mutans* in the presence of *Lactobacillus* was detected. Gene expression of biofilm forming genes (*GtfB* and *LuxS*) was quantified through Real- time PCR. The transcriptional levels of *GtfB* and *LuxS* genes were remarkably down regulated 9.43 -and 4.24- fold change, respectively, when treated with *lactobacillus plantarum* in comparison with the control group.**

Conclusions: Lactobacillus plantarum **as probiotics can be used effectively to reduce the chance of dental caries by decreasing the effects of *S. mutans* and their gene expression to conserve good oral health.**

Key words: Lactobacillus plantarum, **goat, raw milk, biofilm, *Streptococcus mutans*.**

تأثير بكتريا Lactobacillus plantarum على التعبير عن جينات الأغشية الحيوية للبكتريا Streptococcus mutans المعزولة من تسوس الأسنان.

الخلفية: تسوس الأسنان والتسوس الناجم عن العقدية الطافرة. السكروز، يحفز S. mutans لإعطاء glucans غير قابلة للذوبان لتشكيل بايو فيلم عن طريق الفم وتسمى أيضا لوحة الأسنان لبدء عملية تسوس الأسنان. إن جينات GtfB و LuxS في S. mutans مسؤولة عن تطوير وتطور الأغشية الحيوية Lactobacillus plantarum .كما بروبيوتيك يمكن أن تؤثر على تشكيل بايو فيلم .S الطفرات.

الهدف من الدراسة: لتقييم تأثير Lactobacillus plantarum ضد S. mutans تشكيل بيوفيلم.

النتائج: تم اكتشاف نمو بيوفيلم في وجود السكروز باستخدام ١٠٠ فحص دقيق للألواح البنفسجية البلورية الدقيقة وتشكيل بيوفيلم بواسطة S. mutans في وجود Lactobacillus. تم تقدير كمية التعبير الجيني لجينات تشكيل الغشاء الحيوي (GtfB و LuxS) من خلال PCRفي الوقت الحقيقي. كانت مستويات النسخ من جينات GtfB و LuxS منظمة بشكل ملحوظ أسفل ٩,٤٣ – و ٤,٢٤ أضعاف التغيير، على التوالي، عندما تعامل مع plantarum اللبنيك بالمقارنة مع المجموعة الضابطة.

الاستنتاجات: Lactobacillus plantarum كما البروبيوتيك يمكن استخدامها بشكل فعال للحد من فرصة تسوس الأسنان عن طريق تقليل آثار S. mutans وتعبير الجينات الخاصة بهم للحفاظ على صحة الفم جيدة.

الكلمات المفتاحية: Lactobacillus plantarum،الماعز، الحليب الخام، البيوفيلم ، الطفرات العقدية.

INTRODUCTION

Tooth decay, caries and periodontal disease are very common health problem that cost a lot in the world.[1] Developed countries have addressed this health problem and controlled it with fluoride enriched water and personal hygiene products since early in the 1960s.[1] Dental disease remains a "soundless epidemic" in the world that threatens children and adults.[1,2] The oral streptococci principally mutans streptococci are related with the development of caries in humans.[3] The adhesion of oral streptococci such as *Streptococcus mutans* to tooth surfaces has the chief role in their pathogenicity. Because of

[1]Dentistry college, University of Basrah; [2]College of Veterinary Medicine, University of Basrah, Iraq
Corresponding Author; basilabbas63@yahoo.com

increasing antibiotic resistance of bacteria, new methods like whole bacteria replacement therapy for decreasing of oral cavity pathogens must be investigated. [4,5] In general, a probiotic, is a living microorganism which play role in the health of the host positively. It's valued effects may be through direct antagonist effect against particular groups of organisms, leading to decrease its numbers or by an effect on metabolic pathways or by stimulation of immunity. [1,6,7] *L. plantarum* is a Gram positive, short-rod, micro-aerophilic, acid-tolerant, non-spore forming, non-respiring, low G + C content, hetero-fermentative group of lactobacilli with a range of applications in the food industry as a starter culture and preservatives.[8] It is a non-spore forming bacterium which produces organic acids such as acetic acid, succinic acid and lactic acid as major metabolites. The antibacterial, antifungal and probiotic properties of LAB strains have been widely known. [9] *L. plantarum* grow under low buffering capacity in the stomach and other complex bile salt secretions in humans and other mammals. Besides applications in the food industry, *L. plantarum* has wide applications in the pharma industry by contributing significantly to human medicine without contributing to any side effects. Recently, *L. plantarum* has been applied in medical fields for the treatment of various chronic and cardiovascular diseases such as Alzheimer's, Parkinson's, diabetes, obesity, cancer, hypertension, urinogenital complications, liver disorders, etc.[10] The present study aimed to investigate the in vitro importance of *L. plantarum* related to the medical field. *Lactobacilli plantarum* is reported to interfere with infection by pathogens. For example, *L. plantarum* inhibits production of pro-inflammatory cytokines, chemokines, and endotoxin shock induced by *Shigella flexneri* and *Vibrio anguillarum.*[11-14] *L. plantarum* induces production of anti-inflammatory cytokines without inducing inflammatory responses. [13] However, the regulatory effect of lactobacillus plantarum on *S. mutans* biofilm formation that is closely associated with dental caries is rarely studied. In this study we tried to evaluate the effects of *Lactobacillus plantarum* on S. mutans dental biofilm formation.

MATERIAL AND METHODS

Bacteria were previously isolated from dental carries in microbiology Lab at college of veterinary university of Basrah. Bacterial culture and growth conditions *Streptococcus mutans* was maintained in Brain Heart Infusion (BHI) broth. *Lactobacillus plantarum* are maintained in deMan, Rogosa and Sharpe (MRS) Broth. For Biofilm assays *S.mutans* was grown in BHI containing 2% sucrose while *Lactobacillus* were grown in MRS broth with or without sucrose. All microorganisms were incubated at 37°C in an anaerobic jar for 24-48hrs. To evaluate the effect of *Lactobacillus plantarum.* on formation of *Streptococcus mutans* biofilm, an overnight culture of each isolate was grown in their corresponding media as stated above. The suspensions were adjusted with their respected broth to 0.5 McFarland turbidity standards. Formation of *S. mutans* biofilm was evaluated in the presence or absence *Lactobacillus plantarum*. *Lactobacillus plantarum* and *S. mutans* were mixed at equal ratio (1:1). Blank wells contained PBS instead of probiotic strains. Plates were incubated at 37°C for 24 and 48 hrs. Quantitation of biofilms was performed using Stepanovic [15] crystal violet based microtitre plate assay. RNA extraction was achieved by using Trizol (invitrogen) according to manufacturer procedure. The samples were resuspended in 1mL Trizol reagent and vortexed to mix entirely and placed in ice for 10 mins. 200µl chloroform was added and mixed smoothly by inverting tubes 10-15 times and keep in ice for 15mins. Samples were centrifuged at 12000xg for 15mins at 40C. Aqueous phase was transported to a new microfuge tube.500µl isopropanol was added to aqueous phase and kept at room temperature for 10mins followed by

centrifugation at 12000xg for 10mins at 4o C. After discarding supernatant 1ml of 75% ethanol was added and vortexed then centrifuged at 7500xg for 5mins at 4oC. The RNA pallet was air dried to eliminate traces of ethanol and re-suspend in 30µl RNase free water. Quality of RNA was assessed on agarose gel and quantified using Biophotometer plus (NanoVue, USA). cDNA was created from extracted RNA using TaKaRa RNA PCR kit (AMV) Ver 3.0 (TaKaRa, Japan) by using random nanomers according to manufacturer protocol.

Real-time PCR

The effect of *Lactobacillus plantarum* on *Streptococcus mutans GtfB* and *LuxS* gene expression was assessed by Real-time PCR. *S. mutans Ldh* gene was used as an internal control to normalize the target genes. *S. mutans* specific *Ldh, GtfB* and *LuxS* primers as shown in (Table-1), were used. The reactions were performed in Real-time PCR recognition system (Agilent, USA). Each 25µl reaction mixture contained 12.5µl of 2x SYBR Green PCR Mix (TaKaRa, Japan), 1µl of each primer (20µM) and 0.4µl ROX reference dye, 1µl of sample cDNA and 9.1µl sterile deionized water. Amplification was achieved at denaturation at 95C for 2mins followed by 40 cycles of 95C for 30secs, 55o C for 1min and 72C for 1 min. All samples were run in triplicate and relative quantification was done by using 2-ΔΔC t method.

Table 1. Primer for the detection of genes responsible for *S. mutans* biofilm formation.

Genes	References
Target *LuxS* and *gtfB* genes: ***LuxS*-F:ACTGTTCCCCTTTTGGCTGTC** ***LuxS*-R: AACTTGCTTTGATGACTGTGGC**	Wen, Yates *et al.,* 2010 [16]
***gtfB*-F: AGCAATGCAGCCATCTACAAAT** ***gtfB*-R:ACGAACTTTGCCGTTATTGTCA**	Wen, Yates *et al.,* 2010 [16]
Reference *16SrRNA* gene: **F:TATTGTTGCTGTGGGACCTGAG** **R:CCTGAGAATCTGAGTAAATCCACT**	***In the present study***

RESULTS

Biofilm Formation: The results of biofilm formation of *S. mutans* indicates that most strains (80%) produce strong biofilm (Table-2).

Table 2. Biofilm formation ability of *S. mutans* on microtiter plate

No. of *S. mutans* Isolate (%)	Biofilm producer			
	None No. (%)	Weak No. (%)	moderate No. (%)	Strong No. (%)
10	0	0	2(20)	8(80)

Ant biofilm activity of *Lactobacillus plantarum*:

In presence of *L. plantarum* the biofilm activity of *S. mutans* reduced up to 20% of original activity (Table-3).

Table 3. Antibiofilm activity of *Lactobacillus plantarum* against *S. mutans* isolates.

Sample No.	C+3*CD	O.D 630 *S. mutans*	Biofilm formation	O.D 630 of *S. mutans* in the presence of *L. plantarum*	Percentage of biofilm formation in the presence of *plantarum*
1	0.072	0.413	Strong	0.103	25
2	0.072	0.450	Strong	0.091	20
3	0.072	0.502	Strong	0.121	24
4	0.072	0.390	Strong	0.118	30
5	0.072	0.460	Strong	0.159	35
6	0.072	0.422	Strong	0.170	41
7	0.072	0.355	Moderate	0.089	25
8	0.072	0.433	Strong	0.087	20
9	0.072	0.399	Strong	0.120	30
10	0.072	0.350	Moderate	0.101	29

Effects of *lactobacillus* on the *GTF* and *LUX* genes of *S. mutans*:

Relative expressions (fold-change or fold-difference of expression levels) of the representative genes were compared to those within the control untreated isolates and the results showed that, both tested genes were found to be down regulated after exposure to 0.5x MIC of cinnamon aldehyde (Table-4,5).

Table 4. *GTF* gene expression after treated with sub MIC of *lactobacillus plantarum*.

GTF gene expression						Gene expression	
Exp. Group (t1)	CT (GTF)	CT (16SrRNA)	ΔCT (Test)	ΔCT (control)	ΔΔCT	Fold change (2^-ΔΔCT)	Mean
T	30.70	30.10	0.60	-1.09	1.69	0.311	
T	29.40	28.10	1.30	-1.09	2.39	0.191	
T	30.10	29.10	1.00	-1.09	2.09	0.235	
T	30.10	28.40	1.70	-1.09	2.79	0.145	
T	32.50	29.60	2.90	-1.09	3.99	0.063	0.141
T	32.30	29.80	2.50	-1.09	3.59	0.083	
T	32.50	31.20	1.30	-1.09	2.39	0.191	
T	32.00	29.70	2.30	-1.09	3.39	0.096	
T	31.80	29.30	2.50	-1.09	3.59	0.083	
T	32.30	27.30	5.00	-1.09	6.09	0.015	
C	25.40	26.10	-0.70	-1.09	0.39	0.76	
C	26.00	28.80	-2.80	-1.09	-1.71	3.28	
C	25.50	28.50	-3.00	-1.09	-1.91	3.77	
C	28.00	26.80	1.20	-1.09	2.29	0.20	
C	25.00	26.60	-1.60	-1.09	-0.513	1.43	1.33
C	26.00	28.10	-2.10	-1.09	-1.013	2.02	
C	27.00	26.90	0.10	-1.09	1.187	0.44	
C	26.00	27.10	-1.10	-1.09	-0.013	1.01	
C	29.00	28.10	0.90	-1.09	1.987	0.25	
C	28.10	26.10	2.00	-1.09	3.087	0.12	
Mean C	26.60	27.31	-0.71				

In this method one of the experimental samples is the calibrator such as (Control samples) each of the normalized target values (CT values) is divided by the calibrator normalized target value to generate relative expression level.

Expert.	Fold
T	0.141
C	1.328
Expert.	st. error
T	0.000
C	0.000

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:

ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)

Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:

ΔCT (Test) = CT (target, test) – CT (ref, test)

ΔΔCT= ΔCT (test) – ΔCT (calibrator)

Fold change = 2-ΔΔCT (Normalized expression ratio)

Table 5. *LUX* gene expression after treated with sub MIC (minimum inhibitory concentration) of *lactobacillus plantarum*.

***Lux* gene expression**						**Gene expression**	
Exp. Group (t1)	CT (LUX)	CT (16SrRNA)	ΔCT (Test)	ΔCT (control)	ΔΔCT	Fold change (2^-ΔΔCT)	
T	30.50	30.10	0.40	-1.09	1.49	0.357	
T	30.00	28.10	1.90	-1.09	2.99	0.126	
T	30.70	29.10	1.60	-1.09	2.69	0.155	
T	29.90	28.40	1.50	-1.09	2.59	0.166	
T	29.50	29.60	-0.10	-1.09	0.99	0.505	0.316
T	31.00	29.80	1.20	-1.09	2.29	0.205	
T	30.30	31.20	-0.90	-1.09	0.19	0.878	
T	30.00	29.70	0.30	-1.09	1.39	0.382	
T	29.90	29.30	0.60	-1.09	1.69	0.311	
T	30.00	27.30	2.70	-1.09	3.79	0.072	
C	25.00	26.10	-1.10	-1.09	-0.01	1.01	
C	23.00	25.80	-2.80	-1.09	-1.71	3.28	
C	24.10	26.50	-2.40	-1.09	-1.31	2.48	
C	26.00	26.80	-0.80	-1.09	0.29	0.82	
C	25.00	26.60	-1.60	-1.09	-0.513	1.43	1.34
C	26.00	28.10	-2.10	-1.09	-1.013	2.02	
C	30.00	26.90	3.10	-1.09	4.187	0.05	
C	26.00	27.10	-1.10	-1.09	-0.013	1.01	
C	26.90	28.10	-1.20	-1.09	-0.113	1.08	
C	27.40	26.10	1.30	-1.09	2.387	0.19	
Mean C	25.94	26.81	-0.87				

Expert.	Fold
T	0.316
C	1.34
Expert.	st. error
T	0.000
C	0.000

First, normalize the CT of the reference (ref) gene to that of the target gene, for calibrator sample:
ΔCT (calibrator) = CT (target, calibrator) – CT (ref, calibrator)
Second, normalize the CT of the reference (ref) gene to that of the target gene, for the test sample:
ΔCT (Test) = CT (target, test) – CT (ref, test)
ΔΔCT= ΔCT (test) – ΔCT (calibrator)
Fold change = 2-ΔΔCT (Normalized expression ratio)

The effects of sub inhibitory concentrations of *lactobacillus* supernatant on the transcriptional modulation of biofilm genes (*GTF and LUX)* of *S. mutans* were assessed by quantitative RT-PCR (Fig-1).

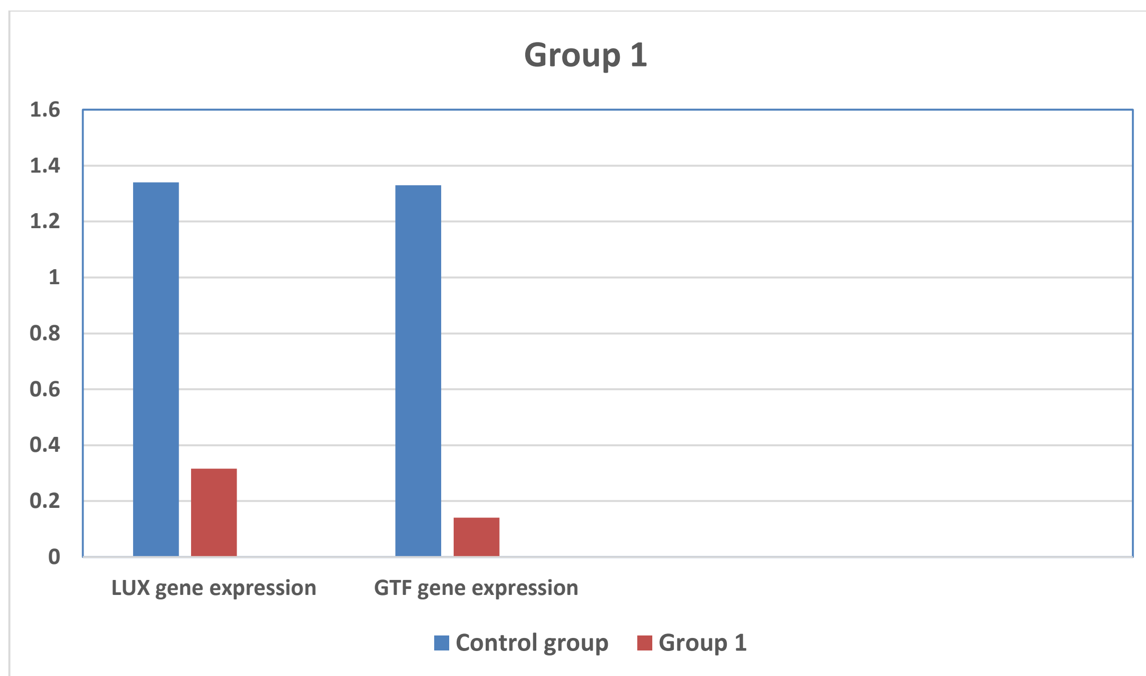

Fig 1. Relative expression of *LUX* and *GTF* genes of *S. mutans* in the presence of lactobacillus 1.Transcript levels were monitored by real-time PCR as described in the text. Using the 2−ΔΔCt method, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (16 rRNA) and relative to the untreated control. Blue bars express control group while green bars express test group. Values represent the mean ± SD for three independent experiments. P < 0.05.

DISCUSSION

S. mutans gives its name to a group of seven closely related species, together referred to as the mutans streptococci. The primary homes of *S. mutans* are mouth, pharynx, and intestine.[17] Numerous factors, such as adhesion to enamel surfaces, creation of acidic metabolites, ability to form glycogen reserves and the capacity to manufacture extracellular polysaccharides are present in dental caries.[17,18] *S. mutans* have a fundamental role in the pathogenesis of dental caries, [19] because these can attached to the enamel salivary pellicle and to other plaque bacteria.[6] Mutans streptococci are strong acid makers and therefore cause an acidic environment forming the possibility for cavity formation. [20] Usually, we need 6-24 months for the appearance of *S. mutans* in the tooth cavities following caries. [12] The acidogenic *S. mutans* are able to make extra-cellular polysaccharides (EPS) provided that sucrose must be present, [22,23] but also from fructose and glucose. The extra-cellular poly-saccharides are long-chained and high molecular mass polymers. [24] The energy rich glycosidic bond between the glucose and fructose moieties supplies. energy needed for the synthesis of EPS. Glucose homo-polysaccharides are named glucans while fructose homopolysaccharides are termed fructans.[15,25] Glucans are created by glucosyltransferases (GTF) while fructans are formed by fructosyl transferases (FTF). [26] The manufacture of large quantities of EPSs from

sucrose is significant factor of *S. mutans* cariogenicity.[27] Antibacterial substances created by probiotic lactobacilli include lactic acid, which can stop microbial growth by depressing the pH; hydrogen peroxide, which can stop bacterial DNA synthesis;[28] and bacteriocins, which can distracts bacterial cell membranes to kill gram-positive bacteria. As MS(mutans species) is acid-tolerant and hydrogen peroxide making by lactobacilli is low, it is possible that the antibacterial substances in probiotics may be primarily bacteriocins or bacteriocin-like proteins. Studies have presented that Lactobacilli showed significant inhibitory effects on MS due to bacteriocin making. [29] However, at present, the antimicrobial mechanism of probiotic lactobacilli against MS is still not fully assumed and requires further research. Dental plaque is a biofilm structure which progressively sediments on the tooth surface. This structure composed of a variety of bacteria that make a complex ecological environment in which streptococci and other caries-related microorganisms create acid, which is the direct reason of tooth decay [30] To be effective at stopping caries, probiotics need to have an inhibitory effect on Streptococcus species. Researches on intestinal microbes have proved that some lactic acid bacteria such as *L. rhamnosus* GG are competent to have significant inhibition effects on pathogens.[31] In the present study, *Lactobacilli plantarum* reduced the biofilm formation of isolated *Streptococcus mutans*. Suitable mechanism of the inhibition of biofilm was not firm but it might be due to impediments of adherence, struggling for nutrients and antibacterial peptide production by *Lactobacillus plantarum,* also may decrease the viable count of *S. mutans*. Numerous studies using different strain of Lactobacillus have shown reduction in salivary mutans count. [31] In the presence of Lactobacillus, the adherence of *Streptococcus mutans* was reduced.[32] *S. mutans* was also decreased or stopped in the presence of *L. rhamnosus GG, L. reutri and L plantarum*. [33] Till now the exact mechanism of inhibitory effect of biofilm by Lactobacillus species is not clear. In the present study, the effect of Lactobacillus *plantarum* on GtfB and LuxS gene expression of S. mutans was evaluated. GtfB is blamed for the production of insoluble glucans which in turn create dental plaque and afford shelter and food for other bacterium which can cause more destruction to oral cavity. [34] GtfB is considered to be the virulence factor, which needs to be decreased to prevent dental caries. Our result showed that the transcriptional levels of *GTF* and *LUX* genes were remarkably down regulated 9.43 -and 4.24- fold change, respectively, when trated with lactobacillus 1 in comparison with the control group (Fig-1). while The transcriptional levels of *GTF* and *LUX* genes were down regulated 8.05 -and 5.83- fold change, respectively, when treated with lactobacillus 2 in comparison with the control group. Decrement of GtfB directly related to less gathering of insoluble glucans, which cause reduction of the chance of dental caries. Lacobacilli also diminish the gene expression of LuxS that code for an enzyme that catalyzed the manufacture of autoinducer 2 molecule [35] and is blamable for inter and intra species microbial communication. In *S. mutans* LuxS based quorum sensing enables the capability to tolerate environmental stress situations, competence and biofilm formation. [15,36] *L. casei* was decreased LuxS genes expression to 7 folds.[36] Other studies also revealed that *S. mutans* with Knockout LuxS gene had impaired biofilm growth and stress tolerance of bacteria.[37,38]

CONCLUSIONS

Lactobacillus plantarum as probiotics can be used effectively to reduce the chance of dental caries by decreasing the effects of *S. mutans* and their gene expression to conserve good oral health.

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