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Short Communication

Effect of *Lactobacillus* Species on the Expression of Gene Related to Biofilm Formation by *Streptococcus mutans*

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ABSTRACT

Streptococcus mutans, in relation to the expression of genes encoding the enzymes glucosyltransferases, leading to formation of biofilm has been associated with tooth decay. Several reports have suggested that species of *Lactobacillus* provide probiotic effect against oral cavity. The current research was therefore conducted to investigate the effect of *Lactobacillus* species on the expression of gene associated with biofilm formation by *Streptococcus mutans*. Eighty-six saliva swab samples were obtained from patients suffering from tooth decay. *Streptococcus* species was isolated from saliva samples and characterized using cultural and biochemical methods. Species identity of *Streptococcus* and *Lactobacillus* was confirmed by sequencing of the 16S rRNA gene. Biofilm formation assay was set up using culture of *S. mutans* (control) or co-cultures with either of the two species of *Lactobacillus*, respectively. RT-qPCR was used to examine the expressions of *gtfB* gene in all the incubated cell cultures. The results revealed that out of the 86 test saliva samples, 34.8% was positive for *Streptococcus mutans.* The molecular identification revealed the identities of the species of *Lactobacillus* to be *L. salivarius and L. acidophilus*. It was observed that both *L. acidophilus* and *L. salivarius* lowered biofilm formation by *S. mutans* to a significant level. Furthermore, there was an increase in the level of *gtfB* gene expression in *S. mutans* when co-cultured with *L. salivarius* (2.9-fold) or *L. acidophilus* (1.9-fold), compared with a lone culture of *S. mutans.* Our finding indicates the role of *Lactobacillus* species in the control of dental caries.

***Keywords*:** Biofilm, *GtfB* gene, *Streptococcus mutans*, *Lactobacillus* species, RT-qPCR

Introduction

Dental caries is a chronic oral condition that is disabling and can affect adults, as well as a child's health.[1] The disease is infectious and it emerges from the homoeostatic host-microbiota imbalance.[4] A number of studies have shown connections between poor oral hygiene and cardiac illness.[2,3] A change from commensalism to parasitism of non-pathogenic micro-organisms leads to changes in oral condition as a result of poor sanitation, smoking, decrease in saliva flow and infectious diseases.[5] *Streptococcus mutans* has been described as a major contributor to tooth decay.[6] The enzyme glucosyltransferases, encoded by *gtfB*, *-C* and *-D*,[7,8] have been linked to *Streptococcus mutans*. These enzymes are responsible for the production of α (1-3) or (1-6) glucan-linked polymer, which contributes to the production of dental plaque.[9]

In some dairy products, *Lactobacillus* strain is used to gain health benefits as a probiotic bacterium. Recent evidence suggests that probiotic therapy can be administered to enhance oral safety, as *Lactobacillus* strain is a member of oral multi-species biofilm.[10] Species of *Lactobacillus* have been evaluated to provide probiotic effect against oral cavity. In oral cavity, lactobacilli constitute 1 % of the total microbiota that can be formed, often referred to as cariogenic,[11] that make the oral cavity questionable. However, multiple studies support the concept of lactobacilli being beneficial, rather than having adverse effects on oral health.[12-13] The aim of the present study was to investigate the effect of *Lactobacillus* species on gene expression associated with biofilm formation by *Streptococcus mutans*.

Materials and Methods

Sample Collection

Between October 2015 and February 2016, eighty-six (86) saliva swab samples were obtained from patients suffering from tooth decay in Al-Esraa University College, Baghdad, Iraq. The samples were collected into approved cryovials, held on ice immediately and then stored at -20°C until required for analysis. *Lactobacillus* species used for this study were obtained from the bacterial bank of Al-Nahrain University, Baghdad, Iraq. All the samples were collected after ethical approval (No3912) was obtained from Al-Esraa University College ethical committee.

Identification of Streptococcus and Lactobacillus species

The saliva samples were inoculated into Brain Heart Infusion (BHI) agar and incubated in an aerobic jar at 37°C. Colonies were sub cultured into a fresh medium until single colonies were obtained. The isolates were identified by Gram staining and biochemical characterization. The *Streptococcus* and *Lactobacillus* species were confirmed by molecular biological approach. PCR-amplification products of the 16S rRNA gene were sequenced and the nucleotide sequences were subjected to bioinformatics analysis.

Growth Conditions of Bacterial Isolates

For assay involving biofilm formation, *S. mutans* were grown in BHI with 2% sucrose, while *Lactobacillus* species were grown with or without sucrose in MRS broth. The bacteria were all incubated in an aerobic jar for a period of 48 h at 37°C.

Investigating the Effect of Lactobacillus species on Biofilm Formation

An aliquot of 100 µl of *L. salivarius* or *L. acidophilus* culture supernatant was added to 100 µl of BHI broth containing 10^5–10^6

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CFU of *S. mutans* (co-culture). The control sample was set up by introducing 100 µL MRS medium to *S. mutans* (lone culture), instead of the culture supernatant. Each sample (200 µL) was placed into a 96-well polystyrene culture plate, following incubation at 37°C for 24 hr. In order to evaluate the degree of biofilm formation in each microplate, the culture medium was removed and the microplate was washed two times with 200 µL of phosphate-buffered saline (PBS). Peritoneal biofilm cells were stained with 200 µL of 0.1% (w/v) purple crystal for 15 min and washed two times with PBS. The bound dye was removed from the stained cells with 200 µL of 99% ethanol. Using a spectrophotometer, the quantity of biofilm formed was determined by measuring absorbance of the solution at 595 nm. For each sample, three replicates were prepared.[14]

RNA Extraction and Gene Expression Analysis

RNA was extracted from all the incubated cell cultures by utilizing Zymo Quick-RNA Micro-prep Kit, according to the manufacturer's protocol. The isolated RNAs were quantified, reverse-transcribed into cDNA and used to perform a reverse transcription real-time polymerase chain reaction (RT-qPCR). A set of primers was used to amplify specific region within the prolactin receptor gene, *gtfB*; forward primer sequence is 5'-ACGAACTTTGCCGTTATTGTCA-3' and reverse primer sequence is 5'-AGCAATGCAGCCAATCTACAA-3'.[15] Another set of primers was used to amplify the reference gene, 16S rRNA; forward primer sequence is 5'-CCCCGGAAAGGGTCTAACAC-3' and reverse primer sequence is 5'-TGAGTGCAAGAGGGGAGAGT-3'. The thermal cycling program used for the amplification involved: enzyme activation at 95 ^{o}C for 7 min, followed by 40 cycles of denaturation at 95 ^{o}C for 20 sec, annealing and florescence screening at 55 ^{o}C for 20 sec and extension at 72 ^{o}C for 20 sec. At the end of the amplification procedure, the products were expressed as Ct value for each sample. Gene folding expression was calculated according to Livack method after abstracting the Ct values of reference gene.

Statistical Analysis

All experiments were conducted in three replicates. Data were presented as mean ± standard deviation (SD) using SPSS (version 24.0). Student's t-test and ANOVA were employed to compare means. The level of statistical significance was $P<0.05$.

Results and Discussion

Of the 86 test saliva swab samples collected from patients, 30 (34.8 %) were positive for *Streptococcus mutans*. Cultural method using Gram staining and biochemical characterization were used to identify *Streptococcus* species. More so, sequencing of PCR-amplified 16S rRNA gene, followed by bioinformatic analysis were employed to verify species of the *Lactobacillus*. The outcome of the molecular analysis revealed that two species of *Lactobacillus* were involved; *L. salivarius and L. acidophilus.*

S. mutans produced a significantly higher biofilm compared to *Lactobacillus* species. The Lactobacillus species used in this study significantly lowered the formation of biofilm by *S. mutans.* It was found that both L. acidophilus and *L. salivarius* lowered biofilm of *S. mutans* to a significant degree ($P \leq 0.005$) as shown in Figure 1. The results obtained for the RT-qPCR are presented as curves (Figure 2), which represent amplification of target gene, gtfB. There was an increase in the level of gtfB gene expression in S. mutans when co-cultured with L. salivarius (2.9-fold), compared to a lone culture of S. mutans (control). A lower gene expression level was observed (1.9-fold) in *S. mutans* in a co-culture containing *S. mutans* and L. acidophilus, although higher than that observed in the control (Figure 3). Formation of oral cariogenic biofilm involved different phases. It starts by the colonization of pellicle by non-mutans Streptococci, and this phase provides the conditions for the next phase in which the growth of *S. mutans* initiates biofilm formation.16 These bacteria cause caries formation by using virulence factors such as the production of acid, which is required for destruction of dental hard tissues and its ability to use sucrose in order to form exopolysaccharides (EPS) by the action of several glucosyltransferases (Gtfs) encoded by the genes, gtfB, gtfC and gtfD.17 In this study, the expression of an essential gene, gtfB was detected in the presence of probiotic bacteria, which can influence oral health of the host when present in large amount. In general, most of these probiotic bacteria are Gram-positive, which belong to the genera Lactobacillus.18 Gtf gene products can be considered as virulence factors linked with dental caries etiology and a high degree of unsolvable caries in dental plaque connected with an increased danger of biofilm cariogenicity in humans.

Our study showed that there was a down-regulation of *gtfB* gene in *S. mutans* isolate that was grown in the presence of *Lactobacillus* species, in relation to the control isolate which was grown in the absence of *Lactobacillus* species (Figure 3). This observation agrees with a previous study carried out by Salehi *et al.*, in which they applied bio-surfactant extracted from *Lactobacillus* species and studied their effect on the expression of different *gft* genes. They found out that *L. casei* biosurfactant was highly effective in down-regulating these genes in *S. mutans*, hence it was proposed as an effective therapy for dental caries.[19] The results obtained in our study do not correlate with the finding of Wasfi *et al.*, who demonstrated that *L. salivarius* exhibited the highest level of antibiofilm and peroxide-dependent antimicrobial activity.[4] Both biofilm-forming cells treated with *Lactobacillus* species supernatants showed a decreased expression level of the genes involved in the synthesis of exopolysaccharides.

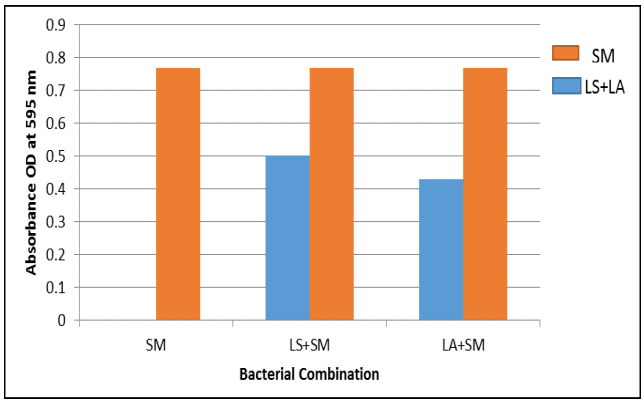

Figure1: Biofilm formation by *Streptococcus mutans*
SM: *Streptococcus mutans*; LA: *Lactobacillus acidophilus*; LS: *Lactobacillus salivarius*

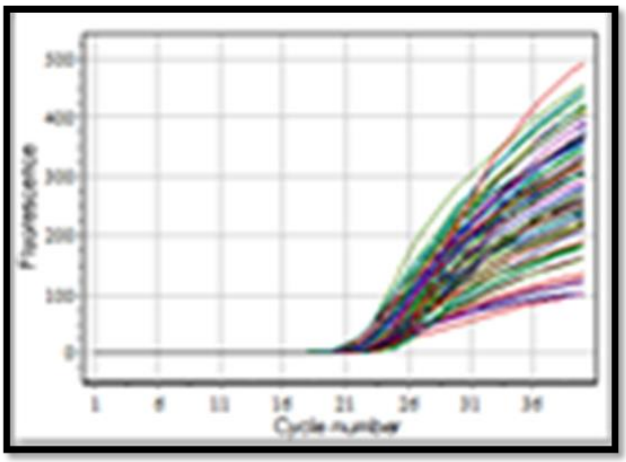

Figure 2: RT -qPCR curves of *gtfB* gene amplification

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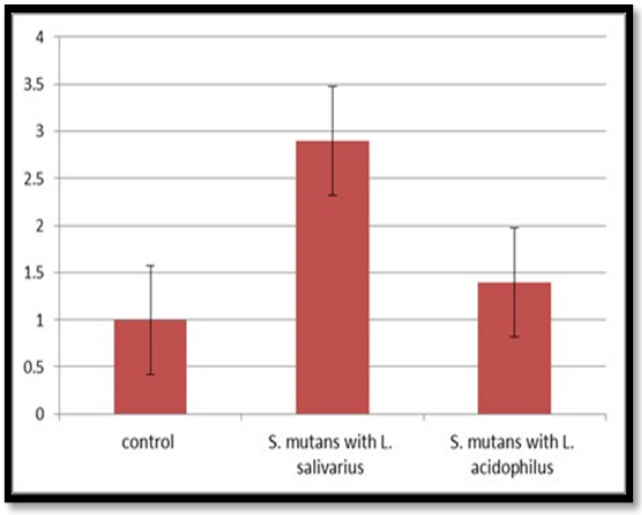

Figure 3: Expression of *gtfB* gene in *S. mutans* in single and co-cultures with *Lactobacillus* species

Conclusion

Our results indicated that both species of *Lactobacillus* used in the study reduced biofilm formation by *S. mutans*, as well as lowered the expression level of *gtfB* gene. Therefore, the findings from this research suggest that *Lactobacillus* species can be employed in the management of oral caries

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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