Effect of *Lactobacillus* species on *Streptococcus mutans* Biofilm formation

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Abstract: *Streptococcus mutans* is the primary pathogen responsible for initiating dental caries and decay. The presence of sucrose, stimulates *S. mutans* to produce insoluble glucans to form oral biofilm also known as dental plaque to initiate caries lesion. The *GtfB* and *LuxS* genes of *S. mutans* are responsible for formation and maturation of biofilm. *Lactobacillus* species as probiotic can reduces the count of *S. mutans.* In this study effect of different *Lactobacillus* species against the formation of *S. mutans biofilm* was observed. Growing biofilm in the presence of sucrose was detected using 96 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. All strains of *Lactobacillus* potently reduced the formation of *S. mutans* biofilm whereas *Lactobacillus acidophilus* reduced the genetic expression by 60-80%. Therefore, probiotic *Lactobacillus* species can be used as an alternative instead of antibiotics to decrease the chance of dental caries by reducing the count of *S. mutans* and their gene expression to maintain good oral health.

Keywords: *Streptococcus mutans*, *Lactobacillus*, Probiotic, *GtfB*, *LuxS*, Biofilm.

INTRODUCTION

Streptococcus mutans is one of the primary pathogen that responsible for tooth decay and cavity formation (Meiers *et al*., 1982; Loesche 1996; Tanzer *et al*., 2001; Becker *et al*., 2002). In the presence of sugar especially sucrose, it produces water insoluble glucans which accumulates on the tooth surfaces as dental plaque or oral biofilm that provides a base for the development of polymicrobial communities. These untreated biofilms may result in oral diseases (Mukasa 1973; Kolenbrander *et al*., 2002; Marsh 2003; Jenkinson *et al*., 2005; Kuramitsu, He *et al*., 2007). The accumulating microorganisms and their metabolites also may result in dental caries and periodontitis (Chung, Ha *et al*., 2004). Dental plaque is well organized bacterial community (Wood *et al*., 2000) that provides bacterial adhesion (Busscher *et al.,* 1997; Bos *et al*., 1999) shelter and antibiotic resistance (Zaura-Arite *et al*., 2001). Bacterial adherence is the key step in the formation and development of oral biofilm.

Streptococcus mutans possesses Glucosyltransferases enzyme encoded by *GtfB, -C & -D* (Banas and Vickerman 2003; Banas 2004). These enzymes are responsible for producing α (1-3) or (1-6) linked glucan polymer, which are responsible for dental plaque formation (Burne 1998; Wen and Burne 2004). Cell to cell communication, either inter or intra species, is necessary for the development of mature dental plaque which contains polymicrobial communities. *S. mutans* also possesses a *LuxS*-mediated signaling pathway that affects the biofilm formation, maturation and bacteriocin production (Merritt *et al*. 2003; Wen and Burne 2004; Huang *et al*., 2009). It catalyzes the reaction for the formation of autoinducer 2 (AI-2) which is a universally recognized signal and may be used to mediate interspecies interactions in a multispecies biofilm such as the dental plaque (Kolenbrander *et al*., 2002). *LuxS* gene of *S. mutans* is required for numerous cellular processes ranging from the formation of biofilm to acid tolerance (Wen and Burne 2004; Merritt *et al*., 2005).

Probiotics are living microorganisms that may confer a health benefit on the host (Food and Agriculture Organization, FAO). *Lactobacillus* species as probiotic bacteria are being used in many dairy products to gain health benefits. Recently, several evidence suggest that probiotic therapy might be applied for the maintenance of the oral health as a part of oral multispecies biofilm (Caglar *et al*., 2005; Meurman 2005; Hatakka *et al*., 2007). *Lactobacillus* species have been tested to confer probiotic effect in the oral cavity. *Lactobacilli* comprise of ≤ 1% of the total cultivable microbiota in the oral cavity. It is also considered to be cariogenic (Beighton 2005; Haukioja *et al*., 2008) which makes it controversial in the oral cavity. However different studies support the idea of beneficial rather than harmful effects on oral health (Nase *et al*., 2001; Hatakka *et al*., 2008; Stecksen-Blicks *et al*., 2009).

Streptococcus mutans are among the bacteria that are early colonizer of oral cavity, responsible for formation of the biofilm in the oral cavity. Any therapeutic procedure

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toward the reduction of *S. mutans* in the oral cavity is useful to control and maintain good oral health. The objective of this study is to detect the effect of *Lactobacillus acidophilus, Lactobacillus salivarius* and *Lactobacillus rhamnosus* on formation of biofilm of *Streptococcus mutans* and also to detect the effect of *L. acidophilus* on *GtfB* and *LuxS* genes expression that are responsible for formation of biofilm. *Lactobacillus acidophilus* is selected for detail study or their effect of gene expression as they are commonly used as probiotic strain in different products, which are available commercially.

MATERIALS AND METHOD

Bacterial culture and growth conditions

Streptococcus mutans was maintained in Brain Heart Infusion (BHI) broth. *Lactobacillus acidophilus, Lactobacillus bulgaricus Lactobacillus rhamnosus* and *Lactobacillus salivarius* were maintained in deMan, Rogosa and Sharpe (MRS) Broth. For Biofilm assays *S. mutans* was grown in BHI containing 2% sucrose while *Lactobacillus* species were grown in MRS broth with or without sucrose. All microorganisms were incubated at 37^{o}C in an anaerobic jar for 24-48hrs.

The effect of Lactobacillus species on formation of biofilm

To evaluate the effect of *Lactobacillus spp.* on formation of *Streptococcus mutans* biofilm, an overnight culture of each isolate was grown in their respective media as stated above. The suspensions were adjusted with their respected broth to 0.5 McFarland turbidity standards. Formation of *S. mutans* biofilm was assayed in the presence or absence *Lactobacillus* strains. *Lactobacillus* strains and *S. mutans* were mixed at equal ratio (1:1). Blank wells contained PBS instead of probiotic strains. Plates were incubated at 37^{o}C for 24 and 48 hrs. Quantitation of biofilms was performed using crystal violet based microtitre plate assay (Stepanovic *et al*., 2000).

Growth of dual-species biofilms

Sterile glass slides were used as substratum and biofilms were grown in 50ml falcon tube by same procedure as mentioned above. The biofilms, grown on the glass slides, were deposited in 50ml falcon tubes and were transferred aseptically, daily to keep medium fresh. After 48 hours, the biofilms were scratched off with a sterile spatula and suspended in 7.5ml of 10mM potassium phosphate buffer adjusted to pH 7.0. To de chain and separate the cells, the biofilms were sonicated using a sonicator at energy level 3 for 25 seconds, twice, with 2 minutes on ice between treatments. After sonication cells were stored at -80^{o}C freezer in RNase free tube until RNA extraction.

RNA extraction

RNA extraction was performed by using Trizol (invitrogen) according to manufacturer protocol. The samples were resuspended in 1mL Trizol reagent and vortexed to mix completely and put in ice for 10mins. 200µl chloroform was added and mixed gently by inverting tubes 10-15 times and keep in ice for 15mins. Samples were centrifuged at 12000xg for 15mins at 4^{o}C. Aqueous phase was transferred 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 4^{o}C. After discarding supernatant 1ml of 75% ethanol was added and vortexed then centrifuged at 7500xg for 5mins at 4^{o}C. After discarding the supernatant, the RNA pallet was air dried to remove traces of ethanol and resuspend in 30µl RNase free water. Quality of RNA was evaluated on agarose gel and quantified using Biophotometer plus (NanoVue, USA). cDNA was synthesized 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 acidophilus on Streptococcus mutans GtfB* and *LuxS* gene expression was evaluated 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 detection 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 performed at denaturation at 95^{o}C for 2mins followed by 40 cycles of 95^{o}C for 30secs, 55^{o}C for 1min and 72^{o}C for 1 min. All samples were run in triplicate and relative quantification was done by using $2^{-\Delta\Delta C}{}_{t}$ method.

Table 1: Primer for the detection of genes responsible for *S. mutans* biofilm formation (Wen, Yates *et al.,* 2010)

Primer	Sequence (5' – 3')	Product
Ldh-F	TTGGCGACGCTCTTGATC TTAG	92bp
Ldh-R	GTCAGCATCCGCACAGTC TTC	
GtfB-F	AGCAATGCAGCCATCTAC AAAT	98bp
GtfB-R	ACGAACTTTGCCGTTATT GTCA	
LuxS-F	ACTGTTCCCCTTTTGGCT GTC	93bp
LuxS-R	AACTTGCTTTGATGACTG TGGC	

STATISTICAL ANALYSIS

Independent T test was used to compare groups for significance using SPSS version 11.5.

RESULTS

Bacterial culture

Streptococcus mutans was obtained from Department of Stomatology, Dalian Medical University which was further confirmed by Gram staining and biochemical characterization using API strips for *Streptococcus* species identification and 16s rRNA sequencing. Three *Lactobacillus* strains of *Lactobacillus* species (*Lactobacillus acidophilus, Lactobacillus salivarius* and *Lactobacillus rhamnosus*) were purchased from China General Microbiological Culture Collection Center.

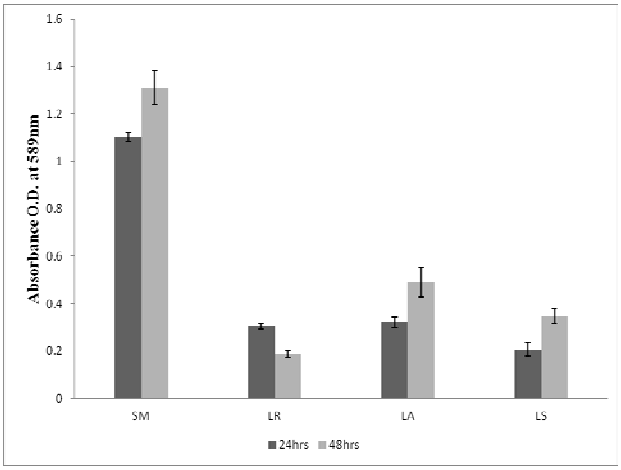

Fig. 1: Biofilm formation by microorganism used at 24 and 48 hrs. SM= *Streptococcus mutans*, LR= *Lactobacillus rhamnosus*, LA= *Lactobacillus acidophilus* and LS= *Lactobacillus salivarius*

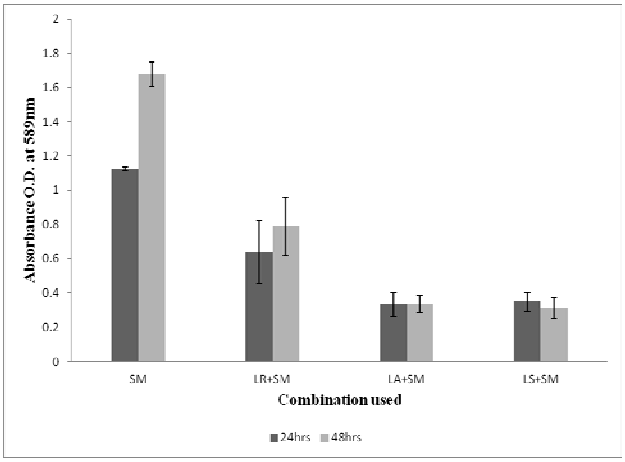

Fig. 2: Biofilm formation by *Streptococcus mutans* (SM) in presence of *Lactoacillus* species used at 24 and 48 hrs. LR= *Lactobacillus rhamnosus*, LA= *Lactobacillus acidophilus* and LS= *Lactobacillus salivarius*

Biofilm formation

Streptococcus mutans produced significant biofilm as compared to *Lactobacillus* species (fig. 1). All *Lactobacillus* species used in this experiment significantly reduced the biofilm formation by *Streptococcus mutans. Lactobacillus acidophilus* and *Lactobacillus salivarius* reduced the biofilm of *S. mutans* to a great extent ($p \leq 0.005$) as compare to *Lactobacillus rhamnosus* ($p \leq 0.005$) (fig. 2). These *Lactobacillus* strains also reduced the viable count of *S. mutans* (Data was not shown).

Effect of L. acidophilus on GtfB and LuxS expression of S. mutans

Expression of *GtfB* and *LuxS* genes were quantified using real time RT-PCR. *Ldh* gene was used as a reference control. In the presence of *L. acidophilus* the 80% expression of *GtfB* was significantly reduced as compared to internal control *Ldh* gene ($p=0.001$). *L. acidophilus* also reduced 56% of the *LuxS* gene expression ($p=0.006$) as compared to internal control *Ldh* gene (fig. 3).

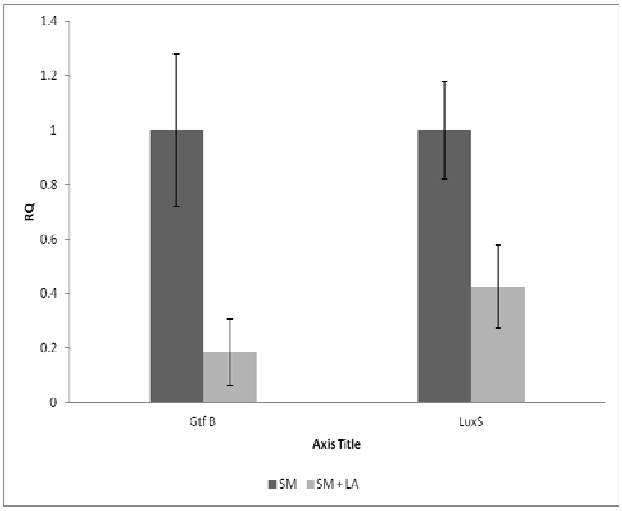

Fig. 3: Effect of *Lactobacillus acidophilus* on *GtfB* and *LuxS* gene expression responsible for biofilm formation in *Streptococcus mutans*

DISCUSSION

Streptococcus mutans is one of the early colonizer and primary pathogen of the oral cavity. It is responsible for dental caries and tooth decay by producing acid. In the presence of sugar, it produces water insoluble glucans which deposited to form dental plaque and provides a platform to build multispecies microbial community. Any strategy that directs toward the reduction of *S. mutans* count is beneficial to maintain healthy oral cavity. Probiotic therapy was first introduced by Elli Metchinikoff (1908) and used to achieve beneficial effect on the digestive tract. Recent years, researchers are taking more interest in the use of probiotics to maintain the oral health (Tahmourespour *et al*., 2011a; Nase *et al*., 2001; Chung, Ha *et al*., 2004; Stamatova 2007; Strahinic *et al*., 2007; Koll *et al*., 2008) *Lactobacillus* is one of the most studied species in oral cavity which comprises of ≤ 1% of the total cultivable microbiota in the oral cavity. These

species are cariogenic and can lead to tooth decay but in different studies it has also been mentioned that *Lactobacillus* has beneficial effect of in the oral cavity.

In this experiment three strains of *Lactobacilli* decreased the biofilm formation of clinically isolated *Streptococcus mutans Lactobacillus acidophilus* and *Lactobacillus salivarius* reduced the biofilm formation more potently as compared to *Lactobacillus rhamnosus*. Appropriate mechanism of the inhibition of biofilm was not determined but it might be due to interference of adherence, fighting for nutrients and antibacterial peptide production by *Lactobacillus* species. *L. rhamnosus* and *L. acidophilus* also reduced the viable count of *S. mutans* (results are not shown). Several studies using different strain of *Lactobacillus* reduced the salivary mutans count. In the presence of *Lactobacillus acidophilus* DSM 20079, the adherence of *Streptococcus mutans* was reduced (Tahmourespour *et al.,* 2011a) the adherence of non mutans streptococci more than mutan streptococci was also reduced in presence of *L. fermentum (*Tahmourespour *et al*. 2011b). *S. mutans* was also reduced or inhibited in the presence of *L. rhamnosus GG, L. reutri and L plantarum* (Soderling *et al*., 2011). Still the exact mechanism of inhibitory effect of biofilm by *Lactobacillus* species is not clear.

In this study the effect of *Lactobacillus acidophilus* on *GtfB* and *LuxS* gene expression of *S. mutans* were also determined. *GtfB* is responsible for the production of insoluble glucans which in turn form dental plaque and provide shelter and food for other bacterium which can cause further damage to oral cavity (Schilling and Bowen 1992). Expression of *GtfB* level in saliva is directly proportional to the presence of caries (Vacca Smith *et al*., 2007). *GtfB* is considered to be the virulence factor, which needs to be reduced to prevent dental caries. Our result showed that *L. acidophilus* reduced 80% expression of the *GtfB* gene. Reduction of *GtfB* directly related to less accumulation of insoluble glucans, which in turn reduces the chance of dental caries. *L. acidophilus* also reduced the gene expression of *LuxS* that code for an enzyme that catalyzed the production of autoinducer 2 molecule (Wen and Burne, 2004) and is responsible for inter and intra species microbial communication. In *S. mutans LuxS* based quorum sensing facilitates the ability to withstand environmental stress conditions, competence and biofilm formation (Lemos *et al*., 2004; Wen and Burne 2004). *L. casei* was reduced *LuxS* genes expression to 7 folds (Wen and Burne 2004). Other studies also showed that *S. mutans* with Knockout *LuxS* gene had impaired biofilm growth and stress tolerance of bacteria (Merritt *et al*., 2005; Huang *et al*., 2009). *LuxS* mutant in the presence of sucrose failed to form a biofilm.

In conclusion all the *Lactobacillus* species reduce the biofilm formation of *S. mutans* as well as *Lactobacillus acidophilus* reduces the gene expression of *GtfB* gene and *LuxS* gene responsible for biofilm formation and maturation. We can use the fighting among bacterial species as an alternative to antibiotic therapy and lower the chances of antibiotic resistance among microbial world. Still further research is needed to target other genes responsible for biofilm formation like *GtfC, GtfD, GpbA, GpbB, GpbC, BrpA, ftf* and *Spa P* to find out the complete mechanism involved in reduction of *S. mutan* biofilm formation.

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