Master's Thesis of Science in Agriculture

Screening and Evaluation of *Lactobacillus paracasei* M9-1 against Biofilm Formation by *Streptococcus mutans*

*Streptococcus mutans*의 biofilm 형성을 억제하는 *Lactobacillus paracasei* M9-1의 선발 및 평가

February 2019

Heesung Shin

Department of International Agricultural Technology

Graduate School of International Agricultural Technology

Seoul National University

Screening and Evaluation of *Lactobacillus paracasei* M9-1 against Biofilm Formation by *Streptococcus mutans*

A thesis
submitted in partial fulfillment of the requirements to the faculty
of Graduate School of International Agricultural Technology
for the Degree of Master of Science in Agriculture

By
Heesung Shin

Supervised by
Prof. Chul Sung Huh

Major of International Agricultural Technology
Department of International Agricultural Technology
Graduate School of International Agricultural Technology
Seoul National University

December 2018

Approved as a qualified thesis
for the Degree of Master of Science in Agriculture
by the committee members

Chairman	Byung-Chul Park, Ph.D.	
Member	Chul Sung Huh, Ph.D.	
Member	Tae Sub Park, Ph.D.	

Abstract

Screening and Evaluation of *Lactobacillus paracasei* M9-1 against Biofilm Formation by *Streptococcus mutans*

Heesung Shin

Major of International Agricultural Technology

Department of International Agricultural Technology

The Graduate School

Seoul National University

Dental caries is a disease caused by demineralization of enamel layer in tooth. Acidic condition in oral cavity results in destruction of hydroxyapatite, which is a major component of enamel layer. Cariogenic microorganisms in oral microbiota are one of the reasons that causes acidic condition in oral cavity. They utilize carbohydrates as their energy source and produce organic acids and glucans by using glucosyltransferases to form biofilm. In normal oral cavity, acidic condition is usually neutralized by human saliva. However, when biofilm is formed, it blocks human saliva and keeps

organic acids around the enamel layer. As a result, acidic condition is maintained within biofilm. *Streptococcus mutans* is well known for acceleration of biofilm formation by providing glucosyltransferases (Gtfs) to other microbiota which can't produce Gtfs, thereby accelerating biofilm formation. The aim of this study is to screen and evaluate lactic acid bacteria (LAB) which has inhibitory effects against biofilm formation of *S. mutans*. 21 LAB, which have been proven for their antimicrobial activity against pathogenic bacteria in previous study were used in this study, The results of safety assessment showed all of tested LAB satisfied criteria from EFSA, which suggests that all of tested LAB are safe when consumed by mouth. *Lactobacillus paracasei* M9-1 showed highest antimicrobial activity (> 14mm), higher co-aggregation ability than that of reference strain, *Lactobacillus rhamnosus GG* and highest inhibition ability in biofilm formation against *S. mutans* among tested LAB. Therefore, *L. paracasei* M9-1 was selected as a promising probiotic strain which has inhibitory effects against biofilm formation of *S. mutans*. The effects of *L. paracasei* M9-1 on the caries-inducing related gene expression in *S. mutans* caused the reduction of adhesion related gene expression. In conclusion, *L. paracasei* M9-1 is the promising probiotic strain which can reduce dental caries by their antimicrobial ability to reduce cell concentration of *S. mutans*, co-aggregation ability to prevent *S. mutans* from binding to tooth

surface, and inhibition ability to reduce adhesion related gene expression of *S. mutans,* thereby reducing biofilm formation of *S. mutans.*

Keyword : Dental caries, *Streptococcus mutans*, biofilm, lactic acid bacteria, probiotics

Student Number : 2017-28580

Contents

List of Tables

List of Figures

List of Abbreviations

ATP: Adenosine triphosphate

BHI: Brain Heart Infusion

EFSA: European Food Safety Authority

EPS: Exopolysaccharide

FDI: *Federation Dentaire Internationale* World Dental Federation

Ftf: Fructosyltransferase

Gtf: Glucosyltransferase

HAP: Hydroxyapatite

LAB: Lactic acid bacteria

L. fermentum: *Lactobacillus fermentum*

L. paracasei: *Lactobacillus paracasei*

L. plantarum: *Lactobacillus plantatum*

L. reuteri: *Lactobacillus reuteri*

L. rhamnosus: *Lactobacillus rhamnosus*

L. lactis: *Lactococcus lactis* subsp. *lactis*

P. acidilactici: *Pediococcus acidilactici*

W. cibaria: *Weissella cibaria*

MIC: Minimum Inhibitory Concentration

MRS: de Man, Rogosa and Sharpe

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

S. mutans: *Streptococcus mutans*

SpaP: Streptococcal protein antigen P

SrtA: Sortase A

Subsp.: Subspecies

WapA: Wall-associated protein A

Chapter 1. Introduction

Dental caries is the most prevalent multifactorial disease in the world, affecting almost 44 % of the world population (FDI, 2015). Among the multifactorial factors which cause dental caries, oral microbiota take a significant role in the process of dental caries and formation of dental plaque. There are more than 700 bacteria in the oral cavity, and some of bacteria which cause dental caries are called cariogenic bacteria. These bacteria form biofilm on surface of tooth under sucrose present condition, which is also known as dental plaque, and keep acidification of oral cavity by producing and trapping organic acids inside the biofilm as their end product of fermentation. This process results in degradation of hydroxyapatite, which is a main constituent of enamel layer in tooth surfaces. *Streptococcus mutans*, the most significant cariogenic bacteria, accelerates the tooth decay by secreting enzymes responsible for formation of biofilm to others that can't synthesize those enzymes. As a result, other bacteria use glucosyltransferases (Gtfs) to form biofilm, thereby enhancing accumulation of biofilm (Forssten *et al.*, 2010).

Probiotics are defined as live organisms which give beneficial

effects on the host when administered in adequate amounts (Hill *et al.*, 2014). Most of studies have reported beneficial role of probiotics in prevention or treatment of dental caries caused by *S. mutans,* although a few studies have reported negative effects of probiotics in dental caries due to their high acidogenic and aciduric properties (Soderling, 2012). According to those studies, probiotics inhibit the growth of *S. mutans* or reduce the virulence factors in *S. mutans* which cause dental caries.

In this study, we screened the lactic acid bacteria (LAB) which has biofilm inhibition ability against *S. mutans* to reduce dental caries by measuring the antimicrobial, co-aggregation, and biofilm formation inhibition ability. Then we examined the potential cariogenicity of our selected probiotic strains and the effects of the selected probiotic strain supernatant on the caries-inducing related gene expression of the *S. mutans*.

Chapter 2. Review of Literature

2.1. Dental caries in oral cavity

2.1.1. Dental caries

Dental caries is a disease, caused by with continuous, phase shift of demineralization and remineralization in tooth, thereby resulting tooth decay. Sugars and other fermentable carbohydrates can be used as energy source for oral bacteria which cause dental caries. Therefore, dental caries is significantly affected by the sugar consumption. It has been considered as one of the important global oral disease with periodontal disease and the major problem in most of developed countries. In most of developing countries, the prevalence rates of dental caries were low when compared to developed countries. However, since 2005, dental caries is tending to increase due to large consumption of sugars in developing countries (Petersen *et al.*, 2005). Moreover, dental caries is also the most prevalent disease occurring in children (Figure 1). It is more likely to develop into the inflammation because deciduous tooth is smaller than permanent tooth.

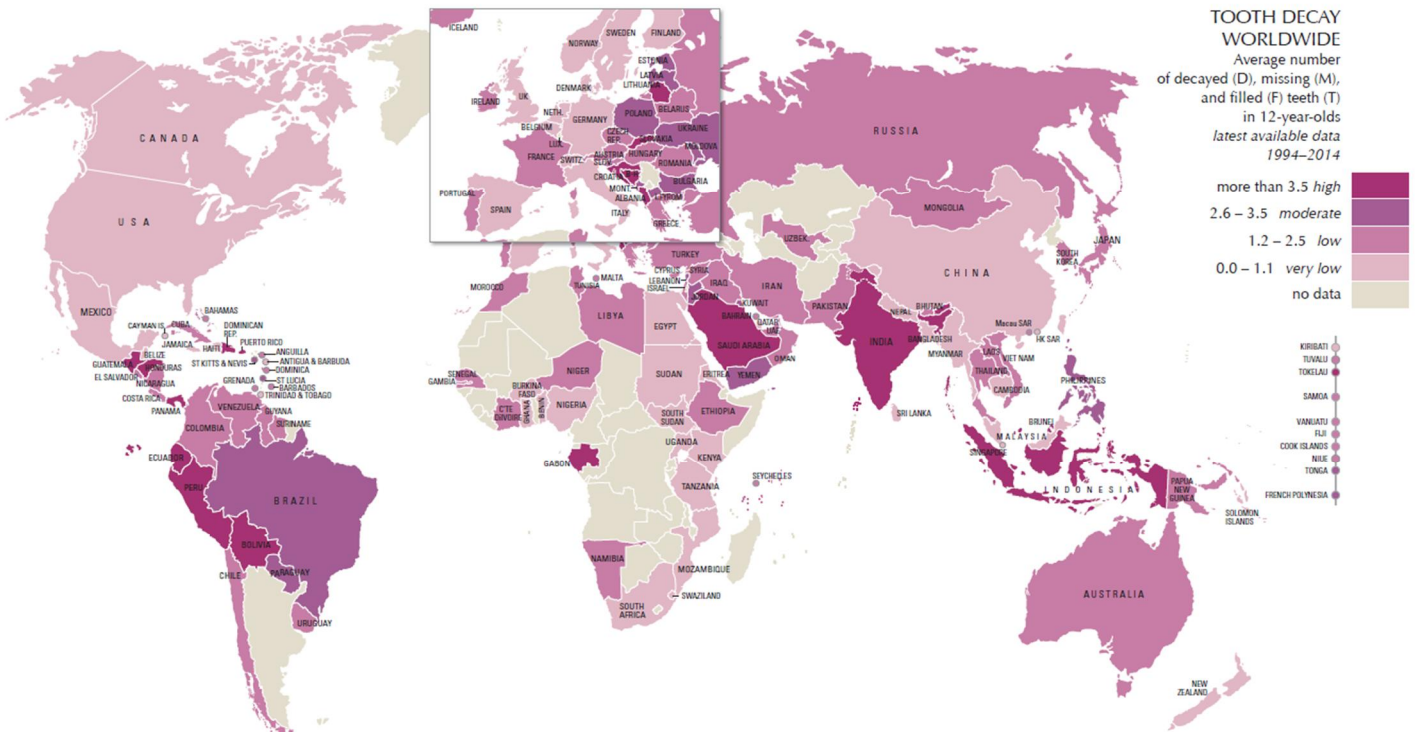

Figure 1. The global prevalence of dental caries in 12 years-old children (The challenge of oral disease, World Dental Federation, 2015).

A tooth consists of enamel, dentin, and pulp layers (Figure 2). The outer structure exposed to oral cavity is dental crown while inner one is known as tooth root. More than 95 % of enamel layer is composed of mineral complex such as carbonated hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). The unit cell of carbonated hydroxyapatite (HAP) is in hexagonal shape and with repetition of cells, they form crystal structure (Abou Neel *et al.*, 2016). The crystal formation of carbonated HAP makes the tooth hard and gives physical force to tooth (Sun *et al.*, 2017). In progress of dental caries, organic acids are produced by oral microbiota attached on enamel layer and dissolves carbonated HAP (Featherstone, 2000).

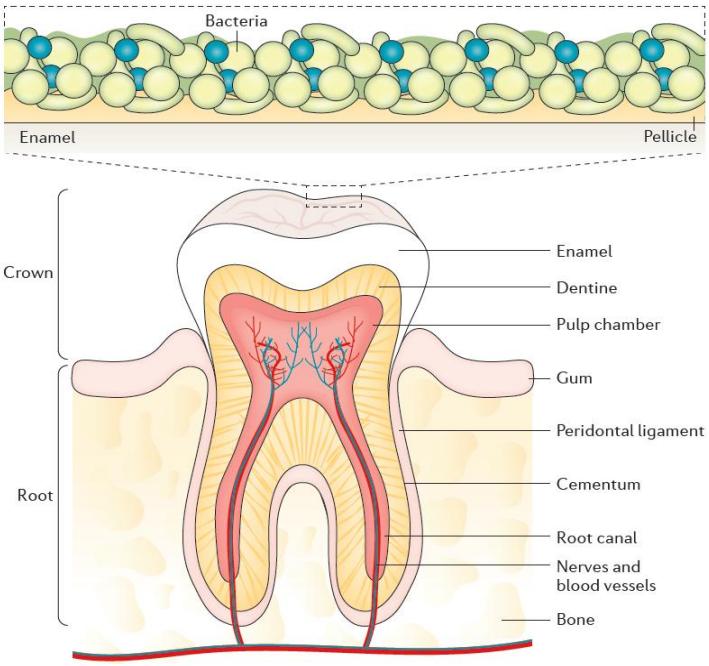

Figure 2. Structure of normal tooth (Pitts *et al.*, 2017).

2.1.2. Demineralization of hydroxyapatite

When HAP is exposed to water, a small amount of HAP dissolves, releasing its component such as calcium, phosphate, and hydroxyl ions. This process stops when water reaches the equilibrium state. At this equilibrium state, the rate of mineral dissolution is equal to rate of mineral precipitation (Fejerskov, 2009).

If pH decreases by one unit, the solubility of HAP increases up to about 10−fold. Under low pH condition, hydroxyl ions from HAP are removed to form water with hydrogen ions. As a result, HAP can't maintain its crystal form and release calcium and phosphate ions (Dawes, 2003). This process is called demineralization of HAP (Figure 3).

In healthy oral cavity, tooth is always covered by saliva fluid. The role of saliva fluid is to remove remaining food debris and bacteria by swallowing. Also, saliva fluid contains calcium and phosphate, which are component of HAP, and thereby decreases solubility of HAP. HCO_3^-, known as saliva bicarbonate, increases pH and buffer capacity in saliva fluid (Bardow *et al.*, 2000; Bardow *et al.*, 2001). The buffering capacity of saliva prevents degradation of HAP. The pH of saliva is reported as approximately 7.0.

The penetration of saliva can be blocked by biofilms and organic acids produced by bacteria are trapped inside biofilm. Therefore,

environment in biofilm causes the tooth to maintain acidic condition by losing its neutralizing capacity. The demineralization of HAP starts when pH decreases to its critical pH, 5.5 (Barron *et al.*, 2003).

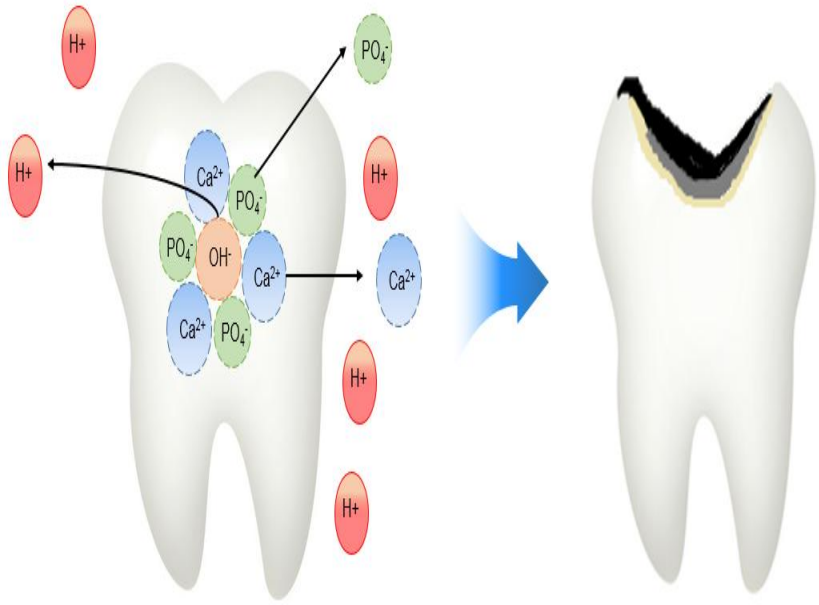

Figure 3. The demineralization of tooth under low pH condition.

2.1.3. Acidic condition is maintained by cariogenic oral microbiota

Even though the mouth is not ideally homogenous for its resident microbiota, it provides several different habitats for oral microbiota (Dewhirst *et al.*, 2010). More than 700 species have been reported as oral microbiota, but less than half of them are cultivatable (Paster *et al.*, 2001). The oral microbiota in healthy oral cavity has high diversity and is subject specific (Aas *et al.*, 2005). *Streptococcus* has been reported as the most dominant genus in healthy oral microbiota (Costalonga *et al.*, 2014; Zaura *et al.*, 2009). It is also the most dominant genus in dental plaque, which is a cause of dental caries (Peterson *et al.*, 2014). Due to highly personal and structural difference variability, species enumeration is generally very difficult to achieve (Griffen *et al.*, 2011). The early colonizers on the enamel layer include *Streptococcus*, *Actinomyces*, *Haemophilus*, *Neisseria* and *Veillonella* (Marsh, 1994).

Dental caries is in close relationship with oral microbiota. The oral microbiota which causes dental caries is classified as cariogenic oral microbiota. The cariogenic oral microbiota utilizes the remaining ingredients such as sugars in saliva to produce organic acid, which results in decrease of pH in the oral environment and degradation of enamel layer (Takahashi *et al.*, 2011). Another feature of cariogenic oral microbiota is to produce the biofilm. Biofilm is a 3 dimensional

complex of exopolysaccharides (EPS) which enables microorganisms to be attached on tooth surface. At initial stage, few early colonizers are attached on tooth surface, especially on acquired enamel pellicle, and produce biofilm to make the attachment irreversible (Whittaker, 1996). As first colonizers are multiplied, their metabolites change the surrounding environment, such as use of O_2 to produce CO_2, making anaerobic condition. The consequence of this process is favorable for secondary colonizers such as facultative anaerobes and they bind to receptors of already colonized bacteria. The diversity of microbial community in biofilm increases and the attached bacteria also begin to produce EPS. When biofilm gets matured and highly densified, it blocks penetration of extra cellular compounds such as antimicrobial factors or antibiotic factors and traps metabolites from microorganism inside biofilm (Figure 4). As a result, the organic acid is produced during carbohydrate metabolism of microbiota inside the biofilm and gets trapped inside, lowering pH condition in biofilm (Kolenbrander *et al.*, 2000; Vu *et al.*, 2009; Marsh, 2010). Another function of biofilm is the communication between microbiota called 'quorum-sensing'. The quorum sensing is the regulation of specific gene expression which is triggered by signal peptides from different bacteria, depending on concentration of cells. In low cell density, signal peptides are lower

than threshold level. As bacteria are multiplied, signal peptides are accumulated and when it reach its threshold level, gene expression is activated for adaption to environment. A good example of quorum-sensing is an increase in antibiotic resistance of embedded microbiota in biofilm. Specific bacteria in biofilm may transfer its antibiotic resistance gene to one another (Socransky *et al.*, 2002).

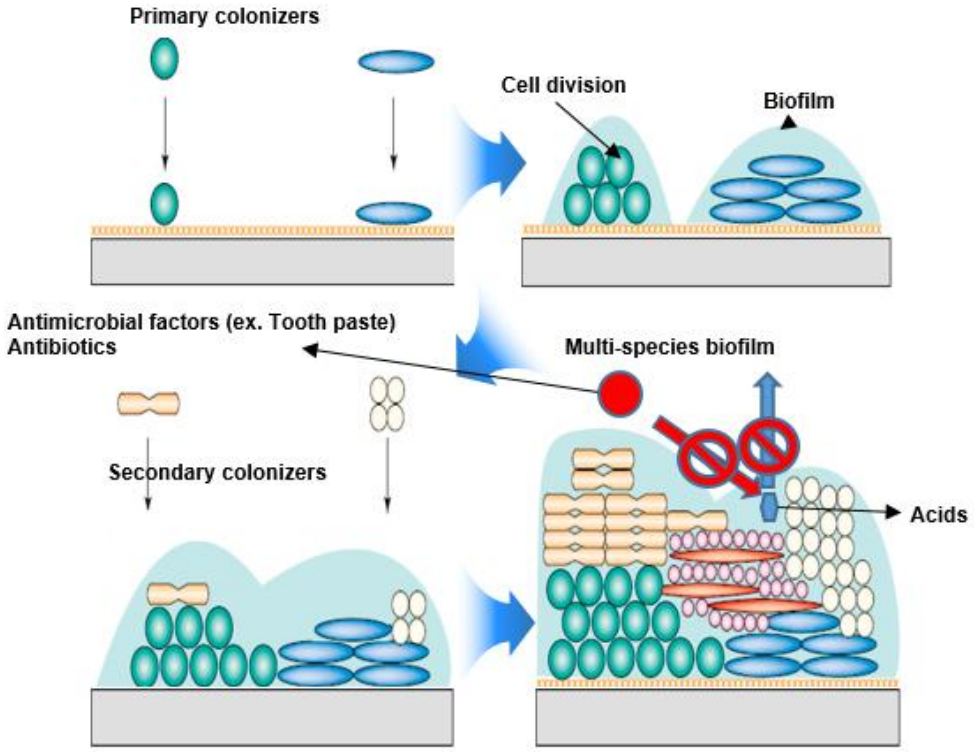

Figure 4. The biofilm formation by cariogenic oral microbiota (Modified from Lof *et al.*, 2017). The primary colonizers attach to acquired enamel pellicle. The growth of first colonizers begins to produce biofilms and makes the surrounding environment favorable for secondary colonizers. When secondary colonizers co-aggregate with first colonizers, they also produce biofilm. The matured biofilm blocks extracellular compounds penetrating into biofilm and traps intracellular compounds such as organic acids inside the biofilm.

2.1.4. *Streptococcus mutans*

Streptococcus mutans is a facultative anaerobes, gram−positive in cocci form. It has been reported as the most common species found in dental caries and a key contributor in the formation of biofilm (Gross *et al.*, 2012; Koo *et al.*, 2010; Beighton, 2005). *S. mutans* utilizes sucrose to produce EPS, organic acid, adheres to enamel pellicles, and has acid tolerance ability.

Regarding to its genome data, carbohydrate metabolism of *S. mutans* has been reported that it can utilize various carbohydrates than any other gram−positive bacteria. *S. mutans* can utilize glucose, fructose, sucrose, lactose, galactose, mannose, cellobiose, β−glucosides, trehalose, maltose/maltodextrin, raffinose, ribulose, melibiose starch and other carbohydrates (Ajdic *et al.*, 2002). The uptake of sugars from the environment is processed by its phosphoenolpyruvate: sugar phosphotransferase, which can be commonly used in sugar transport system of gram−positive bacteria. In addition, *S. mutans* also has at least five sugar ABC transport system (Russell *et al.*, 1992).

2.2. The caries-inducing factors of *S. mutans*

2.2.1. Biofilm formation

Several groups of *Streptococcus spp.*, including *S. mutans*, secrete enzyme such as Gtfs and fructosyltransferase (Ftf). The function of these enzymes is to synthesize EPS from sucrose, which is only substrate that glucan can be made from. Among Gtfs, GtfB catalyzes the cleavage of the glycosidic bond in sucrose thereby forming covalent glucosyl-enzyme intermediate and fructose. Then growing α-glucan chains are synthesized from covalent glucosyl-enzyme intermediate. When α-1.3-glucan is formed, it is water-insoluble (Figure 5). This prevents the constructed biofilm from dissolving or washing out by saliva (Krzysciak *et al.*, 2014: Raj *et al.*, 2017).

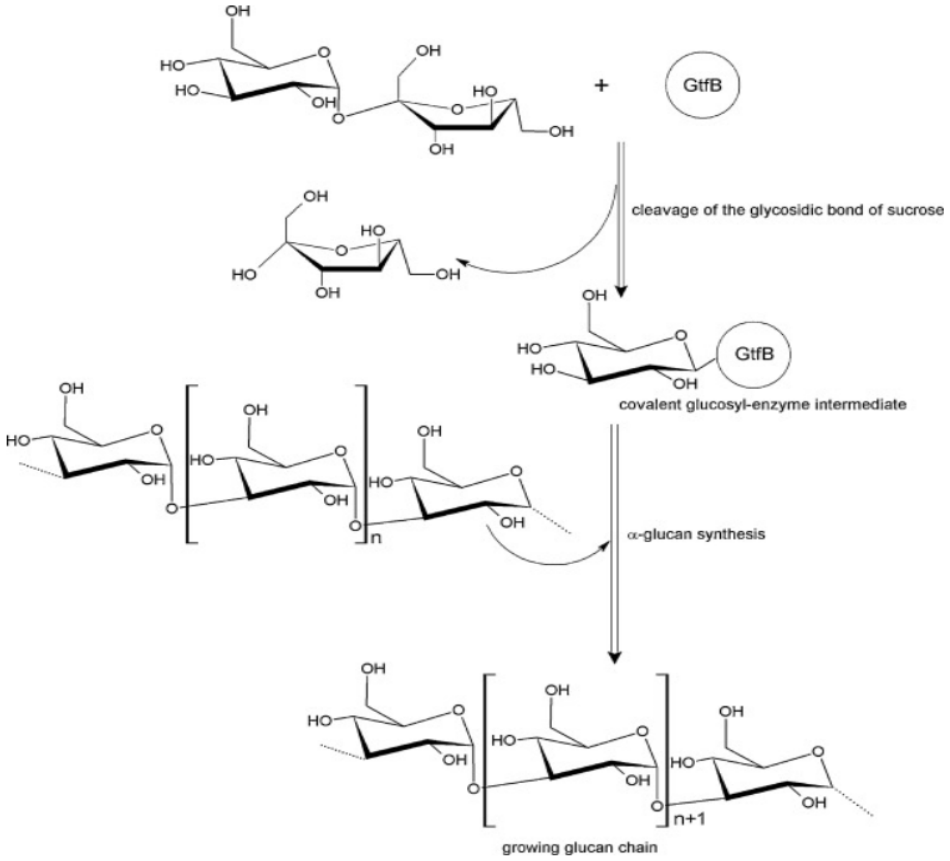

Figure 5. Synthesis of water-insoluble glucan by glucosyltransferase B in *S. mutans* (Pleszczynska *et al.*, 2015)

In addition, due to its structure, biofilm can also bind to metal ions, which makes biofilm stronger attachment (Sutherland, 2001). *S. mutans* produces various kinds of Gtfs, but well known for its functions are GtfB, GtfC, GtfD and Ftf. GtfD synthesizes water soluble, α-1,6-glucan. Determining structure of glucans synthesized by Gtfs has not been fully understood, but there is a study that decrease of carboxyl-terminal repeats increases water solubility of glucan product (Monchois *et al.*, 1999). GtfC has similar homology to that of AgI/II family of proteins although it's binding domain has not been fully identified. This indicates that GtfC is a cell wall bound protein and may acts as a receptor in cell surface for adhesion of *S. mutans* to enamel pellicle (Banas *et al.*, 2003). GtfB may has this function too (Hanada *et al.*, 1988). Ftf produces β-2,1-fructan by hydrolyzing fructose (Bergeron *et al.*, 2001).

Another significant feature of *S. mutans* is that it excretes Gtfs in active form to other bacteria which can't synthesize Gtfs (Figure 6). As a result, other bacteria can utilize sucrose to make water-insoluble glucan and accelerates accumulation of glucan (Vacca-Smith *et al*, 1998).

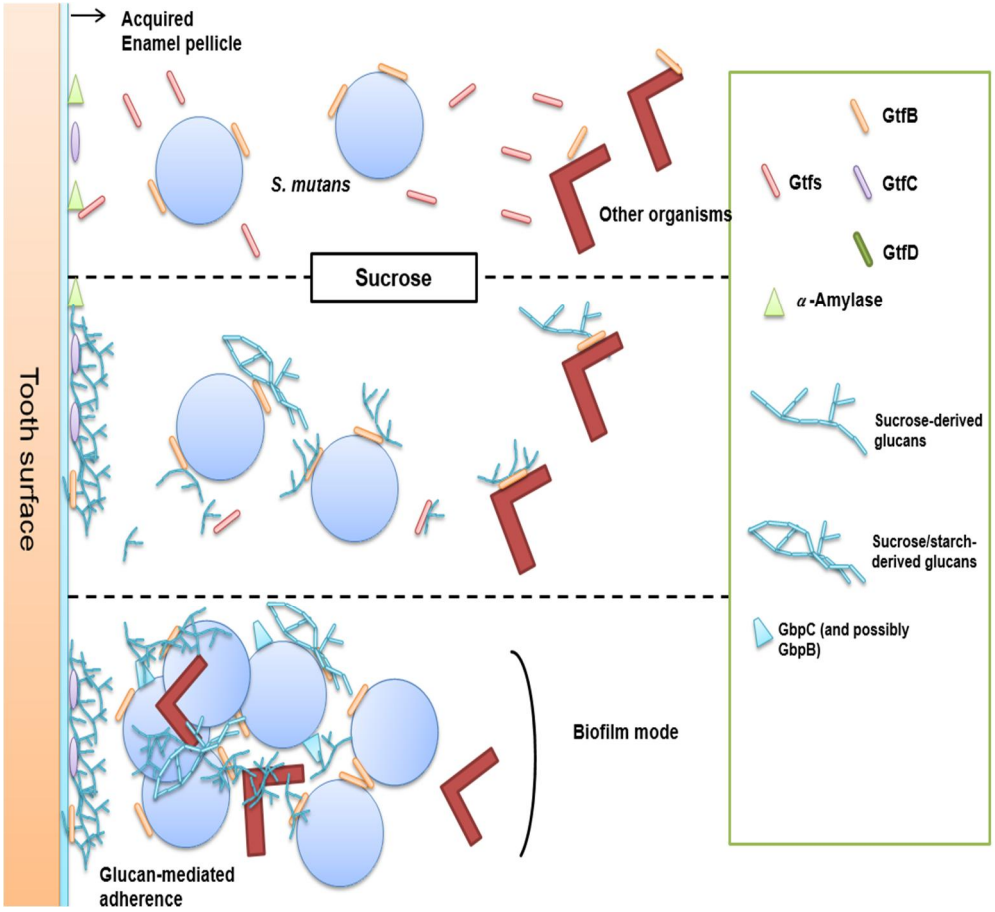

Figure 6. Model of glucan-mediated bacterial adherence (Modified from Bowen *et al.*, 2011).

GtfB, GtfC, and GtfD are encoded by *gtfB*, *gtfC*, and *gtfD*. The *gtf*B (4.4 kbp) and *gtf*C (4.3 kbp) are in an arrangement, however, *gtf*D (5.3 kbp) is not linked to the *gtf*BC locus (Bowen *et al.*, 2011). GtfB and GtfC share about 75 % of homologous amino acid sequence, but GtfD only shares about less than 50 % of homologous amino acid sequence. The homologous sequence region is related in signal sequence. The closeness of *gtf*B and *gtf*C locus suggests that two gtf genes could be regulated by same signal (Ueda *et al.*, 1988).

Two component signal transduction systems are control systems which regulate gene expression for bacterial adaptation, survival and virulence in harsh condition. The function of these system is to regulate gene expression in response to changes in surrounding environment. When *S. mutans* is exposed to environmental changes, signal transduction is processed through two regulatory elements, which are histidine kinase and response regulator (Gao *et al.*, 2009; Stock *et al.*, 2000). In the beginning, the signal gets autophosphorylation by using ATP at histidine residue in histidine kinase, transferring phosphate group to an aspartate residue in response regulator, resulting in conformational changes. Then response regulator regulates gene expression by binding to their promoter region, thereby initiating transcription. The VicKR system is one of the two component signal transduction system in *S. mutans* (Figure 7). A study has reported that only *vicR* gene is essential for

survival of *S. mutans* and *vicK* knockout mutant of *S. mutans* resulted in abnormal formation of biofilm or reduction in biofilm. (Senadheera *et al.*, 2005). This study indicates that VicKR system regulates expression of *gtf*s and *ftf*. Another study has reported that VicKR system induce cell division, production of EPS under sucrose present condition (Stipp *et al.*, 2013).

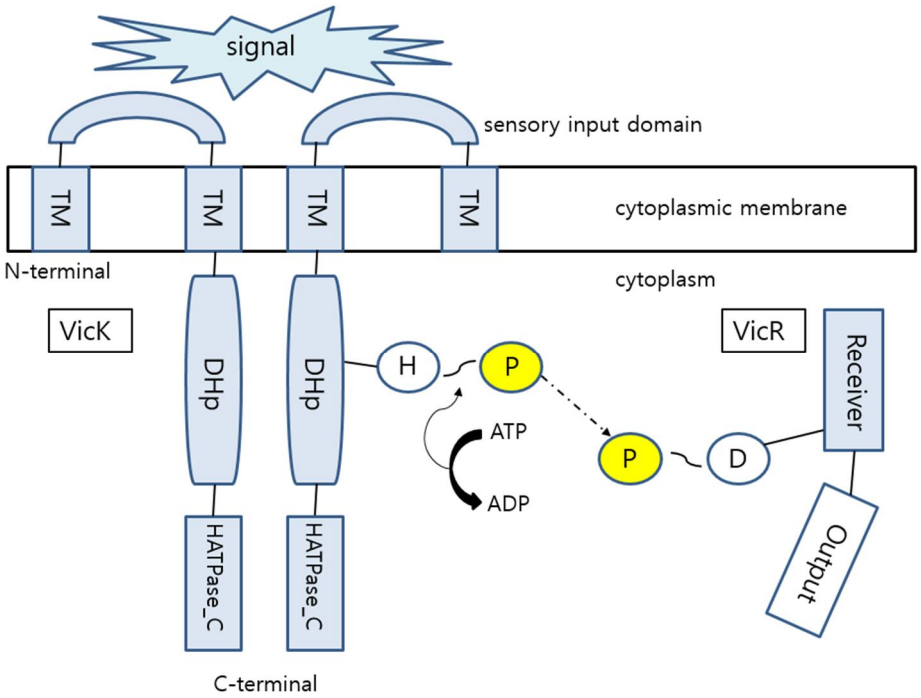

Figure 7. Schematic representation of two component signal transduction system VicKR (Modified from Mattos-Graner *et al.*, 2017).

2.2.2. Acid tolerance response

com*CDE* system is another two component signaling transduction system in *S. mutans*. The *comC* encodes a competence-stimulating peptide and *comDE* does histidine kinase and regulatory receptor. When competence-stimulating peptide reaches its threshold level, it is detected by histidine kinase ComD which is encoded by *comD*, and promotes autophosphorylation. Then ComD transfers phosphate group to regulatory receptor, ComE which initiates transcription of genes necessary for genetic competence and cell separation (Figure 8). The *comCD* defected mutants shows reduction in acid tolerance response (Li *et al.*, 2001; Li *et al.*, 2002; Matsui *et al.*, 2010).

The *aguD* in *S. mutans* is acid tolerance related gene which directly regulates acid tolerance response (Figure 9). It encodes an agmatine-putrescine antiporter which functions as a transporter for agmatine uptake. In acidic condition, acid-sensitive bacteria in the biofilm produce agmatine. When *S. mutans* uptakes agmatine, it converts agmatine into putrescine, ammonia and ATP. The production of ammonia neutralizes pH in acidic condition (Griswold *et al.*, 2006).

Another study has reported that F1-Fo-ATPase, a proton pump encoded by *atpD* gene, removes intracellular protons to maintain intracellular pH in *S. mutans* (Nguyen *et al.*, 2014).

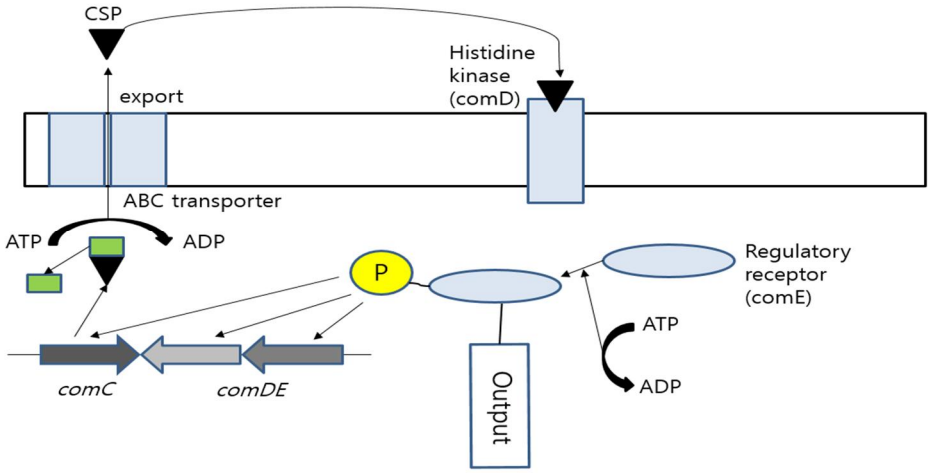

Figure 8. Competence stimulating peptide and role of comCDE system (Modified from Li *et al.*, 2002).

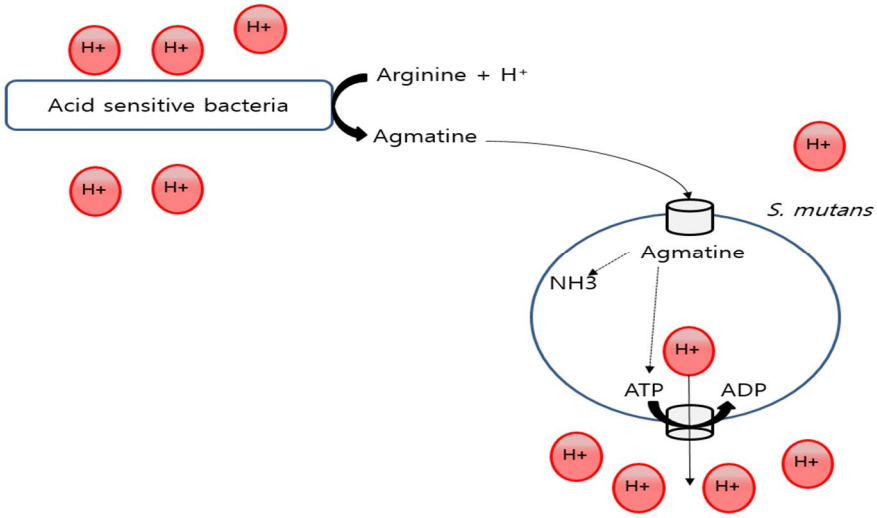

Figure 9. Proposed role of acid tolerance system in *S. mutans* (Modified from Grisworld *et al.*, 2006).

2.2.3. Adhesion factors

One of the caries-inducing factors in *S. mutans* is its ability to attach on tooth surface and form a biofilm. The mechanism of adhesion by *S. mutans* has two pathways. One is sucrose dependent pathway, usually catalyzed by Gtfs, and other is sucrose independent pathway. Wall-associated protein A has been reported as its function for attachment although the exact mechanism is not clear. Several studies have reported that deletion of *wapA* gene resulted in reduction of bacterial attachment (Zhu *et al.*, 2006; Russell *et al.*. 1995).

S. mutans also expresses several adhesion molecules, such as streptococcal protein antigen P (SpaP) encoded by *spaP*. This protein has ability in binding to a salivary agglutinin glycoproteins with association of sortase A (SrtA) enzyme. When SpaP is detected by SrtA, it functions as a signal. The surface proteins are synthesized as precursors which have signal peptide in N-terminal, and LPXTG motif in C-terminal. Then SrtA cleaves the site between threonine and the glycine in LPXTG motif. Then the intermediate is formed between cysteine residue in SrtA and carboxyl-group of threonine at cleaved LPXTG motif (Figure 10). After the formation of intermediate, the amino acid group of cell wall performs a nucleophilic attack at intermediate. The breakdown of intermediate frees remaining LPXTG motif and forms amide bond between the

threonine site in LPXTG and cell wall lipid II (Schneewind and Missiakas, 2012, Mitchell, 2003).

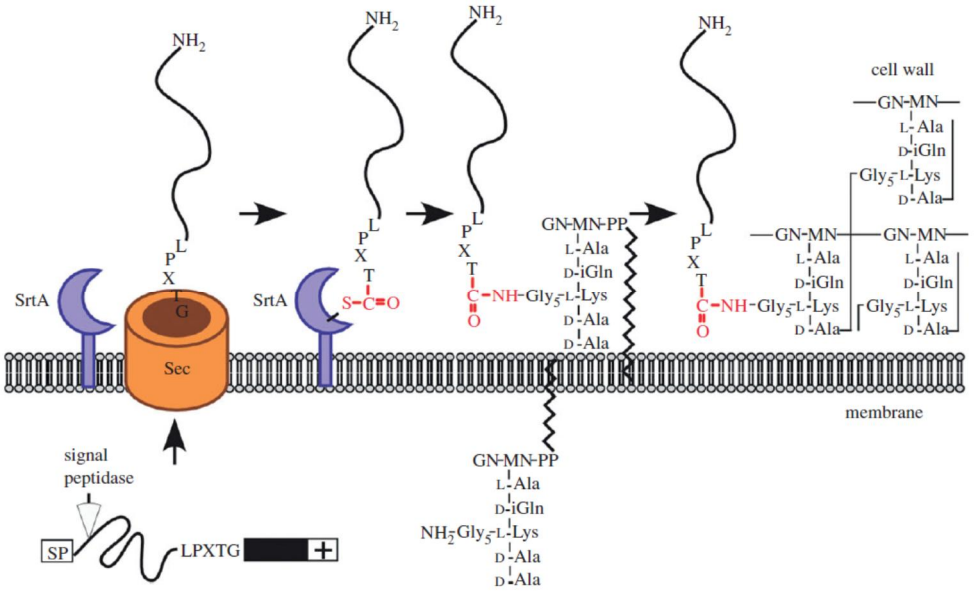

Figure 10. The role of srtA in Gram-positive bacteria (Schneewind and Missiakas, 2012).

2.3 Probiotics and their anti-cariogenic role

2.3.1 Probiotics

Probiotics are defined as living microorganisms which give host beneficial health effects when consumed in adequate concentration by WHO at 2012. The most well-known probiotics are LAB. In particular, *lactobacilli* and *bifidobacteria* are generally used genus in probiotics (Ouwehand *et al.*, 2002). However, the exact answer to how probiotics work on their beneficial effects to host is not a single answer. Their beneficial actions vary through each strains.

There are several reports that propose mechanisms in action of probiotics. Probiotics may compete the adhesion site with pathogens for their receptor site and thereby block pathogen from entering into intracellular space (Ingrassia *et al.*, 2005; Wu *et al.*, 2008), or they compete nutrition with pathogens (Markowiak *et al.*, 2017). Also they induce secretion of mucin to enhance epithelial barrier for colonization resistance of pathogens (Schroeder *et al.*, 2018) or enhance expression of tight junction proteins (Ukena *et al*, 2007; Ashida *et al.*, 2011). Furthermore, probiotics produce antimicrobial factors such as lactic acid or bacteriocin (Dasari *et al.*, 2014; Avonts *et al.*, 2004). In particular, bacteriocin can be categorized into three groups. Class I prevents normal cell wall synthesis or creates pore to membrane of pathogens. Class II is inserted into the membrane and enhances e-polarization. Class III induces the membrane lysis (Jozefiak and Sip, 2013). Another function of probiotics is enhancing

immunological defense of the host by regulating inflammation. Several studies have reported that probiotics reduce inflammation by decreasing expression level of pro-inflammatory cytokines (Plaza-Diaz *et al.*, 2017). Also, probiotics regulate inflammation by increasing expression of regulatory cytokine, IL-6 (Lescheid, 2014). Lastly they produce short-chain fatty acid for energy source for intestinal cells (LeBlanc *et al.*, 2017).

Traditionally, researches on probiotics have been usually focused on health of gut and most of clinical studies proved that probiotics can prevent or alleviate gastrointestinal diseases. However, in recent years, several researches have reported that probiotics also can be used for maintaining oral health as well (Haukioja, 2010).

2.3.2 Beneficial effects of probiotics in oral cavity

Due to increasing use of antibiotics and emergence of antibiotic resistant pathogens, the attention to living therapeutics is rising quickly in nowadays. Probiotic strains including *lactobaciili* have the ability of colonizing not only in gastrointestinal tract, but also in other part of body such as vagina and oral cavity (Selle and Klaenhammer, 2013). The proposed role of probiotics in oral cavity is to restore microbial homeostasis balance between healthy microbiota and pathogens. The mechanism of probiotic action as a living therapeutics is in direct interactions with pathogenic bacteria, or modulation of immune system, In direct interactions with

pathogenic bacteria which cause oral disease, probiotics compete for nutrition or receptor binding sties, produce antimicrobial factors such as bacteriocin to inhibit growth of oral pathogens, and reduce biofilm formation (Meurman and Stamatova, 2007; Mahasneh and Mahasneh, 2017; Allaker and Stephen; 2017). Modulation of immune system by probiotics will cause alteration in production of inflammatory cytokines, or enhance epithelial barrier function. In addition, probiotics are reported to have ability in maintaining healthy state of oral cavity without significant alteration of microbial community in salivary ecosystem (Toiviainen, 2015). The summary of probiotics and their actions in oral cavity are listed on Table 1.

Table 1. The summary of probiotics and their actions in oral cavity.

Probiotics	Mode of action	Reference
L. rhamnosus	Inhibits growth of *S. mutans*	Allaker and Stephen, 2017
L. reuteri	Co-aggregates with pathogenic bacteria	Jorgensen *et al.*, 2017
*L. salivarius*i	Reduces biofilm formation of *S. mutans*	Wu *et al.*, 2015
L. acidophilus	Inhibits growth of *S. mutans*	Schwendicke *et al.*, 2017
L. casei	Reduces biofilm formation of *S. mutans*	Schwendicke *et al.*, 2017
L. paracasei	Inhibits growth of oral pathogens	Chuang *et al.*, 2011
L. plantarum	Reduces inflammation in gingivitis	Montero *et al.*, 2017
L. reuteri	Reduces pregnancy gigivitis	Schlagenhauf *et al.*, 2016
L. salivarius	Reduces pocket depth in chronic periodontitis	Penala *et al.*, 2016
L. rhamnosus	Improvements in chronic periodontitis	Morales *et al.*, 2016
L. brevis	Delays gingivitis development	Lee *et al.*, 2015
W. cibaria	Co-aggregates with pathogenic bacteria	Kang *et al.*, 2005
W. cibaria	Reduces volatile Sulphur compounds in halitosis	Kang *et al.*, 2006
L. plantarum	Reduces pocket depth in chronic periodontitis	Iwasaki *et al.*, 2016
Bifidobacteria	Manages gingivitis or periodontitis	Gruner *et al.*, 2016
S. salivarius	Antimicrobial activity in halitosis	Masdea *et al.*, 2012
S. salivarius	Blocks pneumococcal binding sites	Manning *et al.*, 2016
S. salivarius	Reduces volatile Sulphur compounds in halitosis	Burton *et al.*, 2006

2.3.3 Potential cariogenicity of probiotics

Although there are many reports that proved beneficial role of probiotics in dental caries, the efficacy of probiotics is limited and the potential cariogenicity of probiotics is still need to be concerned (Cagetti *et al.*, 2013). *Lactoabacilli* was the first microoraganism to be indicated as a cariogenic agent in dental caries due to their high acid tolerance and acidogenecity in 1990's (Houte, 1994). A study has reported that some LAB have ability to form biofilm and high resistance to environmental stress (Kubota *et al.*, 2008). In particular, few studies have reported genes responsible in adhesion and formation of biofilm in LAB (Lebeer *et al.*, 2007). However it is not still clear whether LAB in raw ingredients from foods can form biofilm or not. Several studies have reported the cariogenicity of LAB in early childhood (Houte *et al.*, 1982, Becker *et al.*, 2002). These findings suggest that *lactobacilli* may play a role as a cariogenic agent even though it is less significant factor than *S. mutans*. Also, a recent study reported that *L. rhamnosus* didn't have ability to reduce cariogenicity of *S. mutans* in *in vitro* biofilm model (Fernandez *et al.*, 2015).

Chapter 3. Materials and methods

3.1. Screening of potential probiotics with inhibition ability against biofilm formation by *S. mutans*

3.1.1. Bacterial strains and growth condition

Two *S. mutans* strains namely ATCC25175 and ATCC700610 were selected in this study. *S. mutans* were cultured in Brain–Heart Infusion (BHI) media (Oxoid, USA) at 37 ℃ under aerobic condition. The LAB listed in Table 2, were cultured in deMan, Rogosa and Shape (MRS) media (Difco, USA) and M17 media (Difco, USA) supplemented with 10 % lactose (Difco, USA) at 37 ℃ and 30 ℃ in aerobic condition, respectively. For biofilm inhibition assay, RNA extraction, degradation of HAP, all of tested LAB were cultured in BHI broth.

Table 2. List of bacterial strains used in this study.

Species	Strains	Growth media	Temperature (℃)	Origin
S. mutans	ATCC25175	BHI broth	37	KCTC
S. mutans	ATCC700610	BHI broth	37	ATCC
L. rhamnosus	GG	MRS broth	37	Human intestine
L. fermentum	KM6-5	MRS broth	37	Human intestine
L. fermentum	LM15-1	MRS broth	37	Human intestine
L. fermentum	LM16-10	MRS broth	37	Human intestine
L. gasseri	LM8-5	MRS broth	37	Human intestine
L. paracasei	LM1-1	MRS broth	37	Human intestine
L. paracasei	LM1-3	MRS broth	37	Human intestine
L. paracasei	LM3-5	MRS broth	37	Human intestine
L. paracasei	M9-1	MRS broth	37	Raw milk
L. plantarum	KM14-5	MRS broth	37	Human intestine
L. reuteri	LDTM7503	MRS broth	37	Pig intestine
L. reuteri	LDTM7504	MRS broth	37	Pig intestine
L. reuteri	LDTM7505	MRS broth	37	Pig intestine
L. rhamnosus	HM15-5	MRS broth	37	Human intestine
L. rhamnosus	HM26-1	MRS broth	37	Human intestine
L. rhamnosus	LM11-1	MRS broth	37	Human intestine
L. rhamnosus	LM14-3	MRS broth	37	Human intestine
L. lactis	LDTM6801	M17 broth	30	Cheese
L. lactis	LDTM6804	M17 broth	30	Raw milk
P. acidilactici	LDTM5201	MRS broth	37	Korean traditional food
W. cibaria	LDTM8901	MRS broth	37	Korean traditional food

3.1.2. Preparation of culture supernatant

The preparation of culture supernatant was followed by Lin *et al.*, 2015 with slight modification. One ml (1.5 x 10^6 CFU/ml) of LAB was incubated aerobically for 24 hours in 4 ml of BHI broth at 37 ℃ or MRS broth at 37 ℃ or M17 at 30 ℃. The overnight cultured bacteria were centrifuged at 4,000 rpm, 10 minutes, 4 ℃ to remove all bacterial cells. After removing bacterial cells, supernatant was filtered through a 0.22−um filter and stored at 4 ℃ until further use.

3.1.3. Safety assessment

Hemolytic activity The overnight cultured LAB were streaked on sheep blood agar plates (7 % v/v sheep blood). Plates were incubated at 37 ℃ and *L. lactis* at 30 ℃ for 48 hours in aerobic condition (Yadav *et al.*, 2016). The zone around colonies was observed.

Biogenic amine production assay The medium was prepared, based on Bover−Cid *et al.*, 1999. Its composition is presented in Table 3. The overnight cultured strains were streaked in decarboxylase medium plates and zone around colonies was observed.

Table 3. Composition (%) of decarboxylase medium.

Component	Modified medium (%)
Trpytone	0.5
Yeast extract	0.5
Meat extract	0.5
Sodium chloride	0.25
Glucose	0.05
Tween 80	0.1
$MgSO_4$	0.02
$MnSO_4$	0.005
$FeSO_4$	0.004
Ammonium citrate	0.2
Thiamine	0.001
K_2PO_4	0.2
$CaCO_3$	0.01
Pyridoxal-5-phosphate	0.005
Histidine monohydrochloride	0.25
Tyrosine free base	0.25
Ornithine monohydrochloride	0.25
Lysine monohydrochloride	0.25
Bromocresol purple	0.006
Agar	2
pH	5.3

Antibiotic susceptibility Antibiotic susceptibility of LAB was measured by minimum inhibitory concentration (MIC). Microbiological cut-off values (mg/L) were measured based on European Food Safety Authority guidelines (EFSA, 2012). The antibiotics used in this study were ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol. A sterilized cotton swab was dipped into the overnight cultured LAB and then streaked over whole LSM agar surface. LSM agar is composed of 90 % Isosensitext broth (Oxoid, USA), 10 % MRS broth (Difco, USA), and 1.5 % Bacto agar (Difco, USA). After streaking, each antibiotic's E-test strips was loaded on the center of plates and incubated at each strain's optimum temperature for 24 hours, aerobically. MIC was calculated as at the start of clear zone.

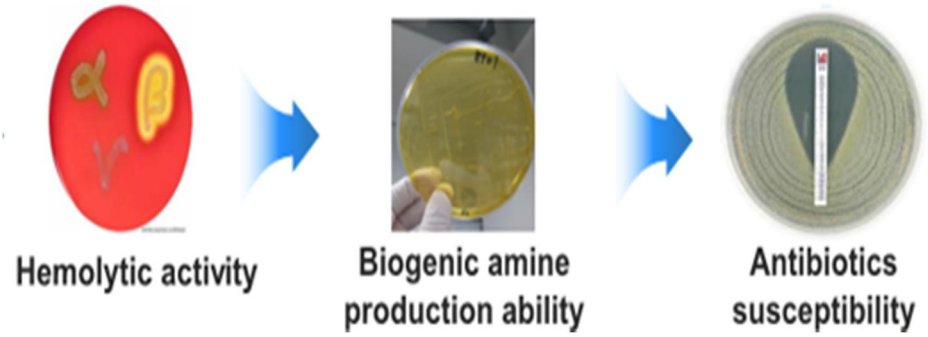

Figure 11. The schematic diagram of safety assessment.

3.1.4. Antimicrobial activity test

Agar well diffusion assay The overnight cultured *S. mutans* were centrifuged at 4,000 rpm, 10 minutes, 4 ℃. After centrifuge, the supernatant was discarded and pellets were washed twice with 1X PBS. *S. mutans* were adjusted to optical density 1.0 ($1.5x10^8$ CFU/ml) at 600 nm with 1X PBS. Adjusted *S. mutans* suspension were inoculated at melted BHI agar held at 45 ℃. After pouring melted BHI agar onto top of BHI agar, 7 mm diameter well were made by the end of sterile pipet tip. The 100 ul overnight cultured LAB and supernatant of LAB were inoculated into each wells. The plates were incubated for 48 hours at their optimum growth temperature in aerobic condition. The inhibition activity was measured by measuring the diameters of inhibition zones (Balouiri *et al.*, 2016)

3.1.5. Co-aggregation assay

The interactions among cells were determined by modifying assay from Xu *et al.*, 2009. Overnight cultured bacteria were harvested at 4,000 rpm for 10 minutes at 4 ℃, washed twice with 1X PBS and adjusted to $1.0x10^8$ CFU/ml. For auto aggregation assay, 3 ml of each bacterial cells was vortexed for 5 seconds and incubated at room temperature for 2 hours. The absorbance of upper supernatant was measured at 600 nm using SpectraMax3

spectrophotometer (Molecular Devices, USA). The auto-aggregation assay was calculated with the following equation.

$$\text{Auto-aggregation (\%)} = (1-A_{2h}) \text{ X } 100$$

For co-aggregation assay, 2 ml of *S. mutans* ATCC25175 and ATCC700610 and same volume of LAB were mixed, then vortexed for 5 seconds and incubated at room temperature for 2 hours. The absorbance of upper supernatant was measured at 600 nm using SpectraMax3 spectrophotometer. The co-aggregation value was calculated as following

$$\text{Co-aggregation (\%)} = [1-A_{mix}/(A_{\text{LAB auto-aggregation}}+A_{\text{S.mutans auto-aggregation}})/2] \text{ X } 100$$

3.1.6. Biofilm inhibition assay by LAB against *S. mutans*

Reduction of biofilm was measured by modifying method from Wasfi *et al.*, 2018. The overnight cultured *S. mutans* were centrifuged at 4,000 rpm, 10 minutes, at 4 ℃. The cell supernatant was discarded and pellets were resuspended in BHI broth with addition of 0.2 % sucrose and adjusted to O. D 1.0 ($1.5x10^8$ CFU/ml). Then adjusted *S. mutans* 100 ul and LAB filtered supernatant were added into each well of 96-well microplate. The plates were incubated at 37 ℃, aerobically for 24 hours. After incubation, the supernatant of each well was removed and reduction in biofilm was measured by crystal violet staining, which was modified a method

from O'Toole *et al.*, 1999. In brief, each well was washed with 1X PBS three times after removal of supernatant. Then, 0.1 % (w/v) crystal violet solution was added to each well and incubated for 30 minutes at room temperature. The crystal violet was removed and each well was washed with deionized water until remaining crystal violet was completely removed. The plates were air-dried in 70 ℃ dry-oven for 10 minutes. Then, 95 % ethanol 200 ul was added to each well for dissolving biofilm and incubated for 15 minutes at room temperature. The 150 ul of dissolved biofilm in 95 % ethanol was moved to new 96-well microplate. The optical density at 545 nm was measured by using SpectraMax3.

For effects of supernatant on preformed biofilm, *S. mutans* were added into 96-well microplate and incubated for 24 hours. After incubation, the supernatant was removed and LAB filtered supernatant was added to each well and incubated for 24 hours (Figure 12). Reduction of biofilm was measured as previously described.

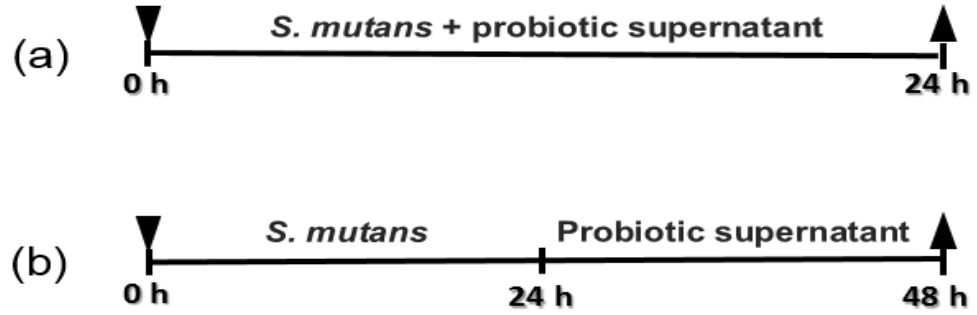

Figure 12. Time schedule for the (a) prevention, and (b) inhibition assays of biofilm formation by *S. mutans*.

3.1.7. Carbohydrate fermentative pattern and enzymatic profiling

Carbohydrate fermentative pattern and profiling enzymes were tested by using API 50 CHL and API zym kit (Bio-Merieux, France). The overnight cultured probiotic strains were harvested and washed twice with sterile PBS and adjusted to optical density 1.0 at 600 nm. Each protocol of the test was performed as manufacturer's instructions.

3.2. Potential cariogenic ability of selected probiotic strains

3.2.1. Biofilm formation of selected probiotic strains

To estimate the cariogenic potential of selected probiotic strains, the following assay was performed. The overnight cultured probiotic strains were centrifuged at 4,000 rpm, 10 minutes at 4 ℃ to remove residual media. The supernatant was discarded and pellets were adjusted to optical density 1.0 at 600 nm with 0.2 % sucrose added BHI broth. Each of adjusted probiotic strains were added into each well of 96-well microplate and incubated for 24 hours. After incubation, supernatant was removed and crystal violet assay was performed as previously described.

3.2.2. The hydroxyapatite degradation

To estimate the cariogenic properties of selected probiotic strains and *S. mutans* in degradation of HAP, we modified a method from Nikawa *et al.*, 2004. The 25 mg of HAP powder (Sigma, USA) was added to each well of 24-multiwell cell culture plates (Falcon, USA) and 1.5 ml of 0.2 % sucrose added BHI broth was also added to each well. The overnight cultured bacteria were centrifuged at 4,000 rpm, 10 minutes, at 4 ℃. The cell pellets were harvested and washed twice with 1X PBS and adjusted to optical density 1.0 at 600 nm in BHI broth with 0.2 % sucrose. 50 ul of adjusted bacteria were added to each well. For co-inoculation, 50 ul of adjusted *S. mutans* and 50 ul of adjusted probiotic strains were added to each well. The amount of calcium ion release was measured by using calcium colorimetric assay kit (Biovision, USA) as following manufacturer's instruction.

3.3. Inhibitory effects of selected probiotic strains on the gene expression in *S. mutans*

3.3.1. Extraction of total RNA

We studied the effects of LAB filtered supernatant on *S. mutans*

gene expression in planktonic form, and biofilm formation state by modifying method from Wasfi *et al.*, 2018. The overnight cultured *S. mutans* were centrifuged at 4,000 rpm, 10 minutes, at 4 ℃. The supernatant of cell suspension was discarded and pellets were harvested in 0.2 % sucrose added BHI broth. Optical density of *S. mutans* were adjusted to 1.0 at 600 nm and 250 ul of adjusted *S. mutans* and same volume of LAB filtered supernatant were added to each well of 24-multiwell cell culture plates. Then 1.5 ml of 0.2 % sucrose added BHI broth was also added to each well and incubated for 24 hours at 37 ℃. After incubation, cells in suspension were collected as planktonic group and cells in biofilm were washed twice with 1X PBS and 1 ml of Accuzol (Invitrogen, USA) was added to each well. Cell scrapper was used to collect cells in biofilm group and suspensions were moved to new 2.0 ml e-tube. The total RNA ware extracted by slightly modifying manufacturer's instruction. In brief, 0.2 ml of chloroform was added to each sample and incubated for 3 minutes at room temperature. Then cell suspensions were centrifuged at 15,000 rpm, 10 minutes at 4 ℃. After centrifuge, aqueous phase of each sample was collected into new 1.7 ml e-tube. Same volume of isopropanol was added to each sample and inverted 15 times and incubated at room temperature for 10 minutes. Then, samples were centrifuged at 15,000 rpm, 10 minutes, at 4 ℃. The

supernatant was discarded as carefully as possible, and 1 ml of 70 % ethanol was added to each sample and vortexed for 5 minutes. Then samples were centrifuged at 8,000 rpm, 5 minutes at 4 ℃ again and supernatant was discarded. The pellets were air-dried in 70 ℃ dry oven for removal of remaining ethanol. Pellets were suspended with 30 ul of RNase-free water, and RNA concentration and purity were determined by Nanodrop (SPECTROSTAR nano, BGM Labtech, Germany). Finally, the ReverTra AceR qPCR RT Master Mix with gDNA remover kit (Toyobo, Japan) was used to synthesize cDNA from 600 ng of extracted RNA, according to manufacturer's instruction.

3.3.2. Quantitative real-time polymerase chain reaction (qRT-PCR) and data analysis

By using qRT-PCR, we measured the alterationd in gene expressions of *S. mutans*, under association with selected probiotic strains. We used *gtfB*, *gtfC*, *gtfD,* which are involved in glucan synthesis, and *sacB*, *vicK*, *vicR*, which are involved in fructan synthesis and regulation of *gtf*s. Secondary, we used *aguD*, *atpD*, *comC*, and *comD*, which are involved in acid tolerance response. Lastly, we used *srtA*, *spaP*, and *wapA*, which are involved in cell adhesion to enamel layer and other bacteria under sucrose absent

condition. The primers used in this study were designed according to Wasfi *et al.*, 2018; Levesque *et al.*, 2005; Li *et al.*, 2013 and synthesized by Bioneer, South Korea (Table 4). qRT-PCR was performed by Biorad CFX 96 (Biorad, USA), using TOPreal^{TM} qPCR SYBR Hi-ROX master kit (Biorad, USA). All reactions (25 ul) were performed using five technical replicates. Each reaction mixture contained 100 ng of cDNA and 10 pmole of primers. The RT-PCR cycling condition were as follows: one cycle with 95 ℃ for 10 minutes, then 55 cycles of denaturation at 95 ℃ for 10 seconds, annealing at 52-62 ℃ (depending on primers used), and elongation and fluorescent data was collect at 72 ℃ for 30 seconds according to a method from Wasfi *et al.*, 2018. A melting curve was generated at the end of each reaction at 65-95 ℃. The 16s rRNA gene was used as a housekeeping gene. The relative mRNA expression levels were normalized to the expression of the housekeeping gene using ΔΔCt value analysis. The qRT-PCR data were expressed as in fold change of their levels to control group (BHI broth treated).

Table 4. List of primers used in this study.

Related function	Target gene	Primer name	Oligonucleotide sequence 5' -3'	TM (℃)	Product size (bp)	References
Biofilm formation	*gtfB*	Gtfb-F	ACGAACTTTGCCGTTATTGTCA	58.2	96	Lee and Kim, *et al.*, 2014
		Gtfb-R	AGCAATGCAGCCAATCTACAA	57.6		
	gtfC	Gtfc-F	CTCAACCAACCGCCACTGTT	60.1	91	Lee and Kim, *et al.*, 2014
		Gtfc-R	GTTTAACGTCAAAATTAGCTGTATTAG	56.9		
	gtfD	Gtfd-F	TGTCTTGGTGGCCAGATAAAC	57.2	132	Lee and Kim *et al.*, 2014
		Gtfd-R	GAACGGTTTGTGCAGCAAGG	59.7		
	sacB	Sacb-F	CCTGCGACTTCATTACGATTGGTC	61	103	Wasfi *et al.*, 2018
		Sacb-R	ATTGGCGAACGGCGACTTACTC	62.5		
	vicK	Vick-F	CACTTTACGCATTCGTTTTGCC	58.7	102	Senadheera *et al.*, 2005
		Vick-R	CGTTCTTCTTTTTCCTGTTCGGTC	59.6		
	vicR	Vicr-F	CGCAGTGGCTGAGGAAAATG	58.9	157	Senadheera *et al.*, 2005
		Vicr-R	ACCTGTGTGTGTCGCTAAGTGATG	62.1		
Acid tolerance	*aguD*	Agud-F	ATCCCGTGAGTGATAGTATTTG	54.5	80	Jeon *et al.*, 2009
		Augd-R	CAAGCCACCAACAAGTAAGG	56		
	atpD	Atpd-F	CGTGCTCTCTCGCCTGAAATAG	60.2	85	Jeon *et al.*, 2009
		Atpd-R	ACTCACGATAACGCTGCAAGAC	60.3		
	comC	Comc-F	TATCATTGGCGGAAGCGGAA	58.8	71	Wasfi *et al.*, 2018
		Comc-R	TCCCCAAAGCTTGTGTAAAACT	57.4		
	omD	Comd-F	CGCGATTGGAGCCTTTAG	55.1	133	Wasfi *et al.*, 2018
		Comd-R	CCTGAAATTCAGTTAGCCTTT	53.1		

Adhesion	*srtA*	Srta−F	GAAGCTTCCTGTAATTGGCG	56.9	108	Levesque *et al.*, 2005
		Srta−R	TATGGTGCTGGAACGATGAA	58		
	spaP	Spap−F	GGATCTGGCTGGGATAGTTCAG	61.6	70	Li *et al.*, 2013
		Spap−R	GACCAGACATGCGGATAGCA	62.3		
	wapA	Wapa−F	TCAAACGAATGTTCCGACAA	55.1	109	Zhu *et al.*, 2006
		Wapa−R	GACCAGACATGCGGATAGCA	57.3		
Housekeeping	*16s rRNA*	16s−F	CCTACGGGAGGCAGCAGTAG	60.8	101	Salehi *et al.*, 2014
		16s−R	CAACAGAGCTTTACGATCCGAAA	58.4		

Chapter 4. Results

4.1 Screening of potential probiotics with inhibition ability against biofilm formation by *S. mutans*

4.1.1. Safety assessment

Antibiotic susceptibility of the tested LAB was determined by Minimum Inhibitory Concentration (MIC) of each type of antibiotics recommended by microbiological breakpoints of EFSA, 2012. We confirmed that all of tested LAB satisfied cut off values from EFSA (Table 5).

Production of hemolytic substances and biogenic amines by LAB was measured by observing zone around colonies using streaking. None of tested LAB showed hemolytic and biogenic amine production activity.

Table 5. Minimum inhibitory concentrations (ug/ml) of antibiotics to LAB.

Species	Strain	Antibiotics susceptibility								
		AMP	VAN	GEN	KAN	STR	ERY	CLI	TET	CHL
L. rhamnosus	GG	0.5	>256	2	24	6	<0.015	0.06	0.12	2
L. fermentum	KM6-5	0.12	>256	1	16	8	0.12	<0.015	2	4
L. fermentum	LM15-1	0.12	32	0.25	16	8	<0.015	<0.015	2	1.5
L. fermentum	LM16-10	0.12	>256	0.5	16	4	0.015	<0.015	2	2
L. gasseri	LM8-5	0.25	2	8	2	8	0.25	<0.015	2	2
L. paracasei	LM1-1	0.5	>256	4	16	32	0.015	0.12	1	0.12
L. paracasei	LM1-3	1	>256	2	24	16	0.06	0.12	1	0.12
L. paracasei	LM3-5	1	>256	2	24	64	0.015	0.12	2	4
L. paracasei	M9-1	0.25	>256	8	8	64	<0.015	<0.015	0.5	2
L. plantarum	KM14-5	1	>256	2	24	16	0.5	0.12	8	4
L. reuteri	LDTM 7503	4	64	4	8	32	0.25	0.5	1	2
L. reuteri	LDTM 7504	2	>256	8	8	32	0.25	0.25	4	2

L. reuteri	LDTM 7505	1	>256	2	24	16	0.015	0.5	2	1
L. rhamnosus	HM15-5	2	>256	8	24	8	0.015	1	4	1
L. rhamnosus	HM26-1	1	>256	2	12	8	0.015	1	2	4
L. rhamnosus	LM11-1	1	>256	2	24	8	0.03	0.06	1	4
L. rhamnosus	LM14-3	1	>256	2	12	16	0.03	0.06	2	4
L. lactis	LDTM 6801	0.12	1	4	4	12	0.06	0.12	1	2
L. lactis	LDTM 6804	0.12	0.5	4	4	24	0.06	0.12	0.06	1
P. acidilactici	LDTM 5201	1	>256	4	64	32	0.25	<0.015	8	0.06
W. cibaria	LDTM 8901	0.25	>256	4	4	24	0.06	0.12	0.06	1
Suggested breakpoint in accordance to the European Food Safety Authority (EFSA)										
L. obligate homofermentative		1	2	16	16	16	1	1	4	4

L. obligate heterofermentative	2	n.r[a].	16	32	64	1	1	8	4
L. paracasei	4	n.r.	32	64	64	1	1	4	4
L. plantarum	2	n.r.	16	64	n.r	1	2	32	8
L. reuteri	2	n.r.	8	64	64	1	1	16	4
L. rhamnosus	4	n.r.	16	64	32	1	1	8	4
L. lactis	2	4	32	64	32	1	1	4	8
Pediococcus	4	n.r.	16	64	64	1	1	8	4
Other gram positive	1	2	4	16	8	0.5	0.25	2	2

Susceptibility of *Lactobacillus*, *Pediococcus*, *Weissela* and *Lactococcus* subsp. *lactis* was determined according to European Food Safety Authority (EFSA 2012). AMP, VAN, GEN, KAN, STR, ERY, CLI, TET, CHL refer to ampicillin, vancomycin, gentamycin, kanamycin, streptomycin, erythromycin, clindamycin, tetracyclin, and chloramphenicol.

[a]n.r: not required.

4.1.2. Antimicrobial activity

To measure antimicrobial activity of LAB, we used agar well diffusion assay. It showed that inhibition zone of LAB by cell suspension varied among different species. *L. fermentum, L. reuteri*, and *W. cibaria* didn't have antimicrobial activity against *S. mutans* ATCC 25175. *L. paracasei*, *L. gasseri*, *L rhamnosus*, *L. lactis*, and *P. acidilactici* showed inhibition zone against *S. mutans* ATCC25175. Among those, *L. paracasei* M9-1 showed highest antimicrobial activity (14.45333 mm) followed by *L. paracasei* LM1-3 (12.884 mm), *L. plantarum* KM14-5 (12.78267 mm), and *L. rhamnosus* LM11-1 (12.70667 mm) which were higher than *L. rhamnosus* GG (12.61667 mm), a reference strain (Table 6). However, antimicrobial activity of LAB supernatant was not detected against *S. mutans* ATCC25175. This result indicates that tested LAB can only inhibit growth of *S. mutans* when inoculated as in cell suspension. Agar well diffusion assay was not able to be used on *S. mutans* ATCC700610 because it was un-cultivatable on agar plates.

Table 6. The antimicrobial activity of LAB against *S. mutans* ATCC25175.

Species	Strain	Diameter of the inhibition zone (mm)[a]	
		Cell suspension	Cell supernatant
L. rhamnosus	GG	++	−
L. fermentum	KM6-5	−	−
L. fermentum	LM15-1	−	−
L. fermentum	LM16-10	−	−
L. gasseri	LM8-5	++	−
L. paracasei	LM1-1	+	−
L. paracasei	LM1-3	++	−
L. paracasei	LM3-5	+	−
L. paracasei	M9-1	+++	−
L. plantarum	KM14-5	++	−
L. reuteri	LDTM7503	−	−
L. reuteri	LDTM7504	−	−
L. reuteri	LDTM7505	−	−
L. rhamnosus	HM15-5	+	−
L. rhamnosus	HM26-1	+	−
L. rhamnosus	LM11-1	++	−
L. rhamnosus	LM14-3	+	−
L. lactis	LDTM6801	+	−
L. lactis	LDTM6804	+	−
P. acidilactici	LDTM5201	+	−
W. cibaria	LDTM8901	−	−

[a]+++, >14 mm; ++, >12 mm; −, no clear zone.

4.1.3. Co-aggregation ability with *S. mutans*

The LAB were co-inoculated with *S. mutans* ATCC25175 and 700610 to evaluate co-aggregation ability. Most of tested LAB showed higher co-aggregation ability than *L. rhamnosus* GG. *L. plantarum* KM14-5 and *L. rhamnosus* HM15-5 showed lower co-aggregation ability than *L. rhamnosus* GG with *S. mutans* strains (Figure 13a,b). The co-aggregation ability of LAB with *S. mutans* ATCC700610 was lower than those on *S. mutans* ATCC25175. *L. gasseri* LM8-5 and *L. reuteri* LDTM7503, 7504 and 7505 were highly aggregated with both of *S. mutans* strains. These result suggested that co-aggregation ability of LAB was species specific.

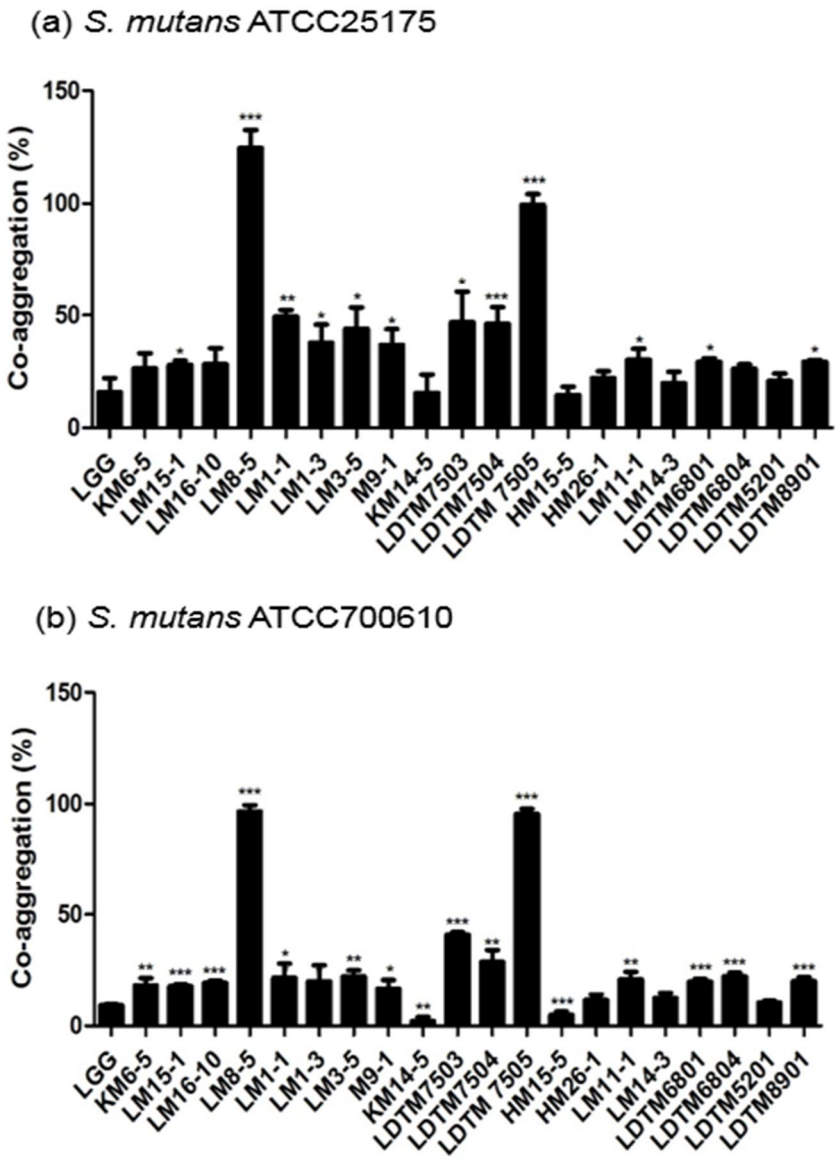

Figure 13. Co-aggregation ability of LAB with *S. mutans* (a) ATCC25175, and (b) ATCC700610. Data are mean values ± standard deviation (S.D.). Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with *L. rhamnosus* GG group.

4.1.4. Biofilm inhibition ability against *S. mutans*

Biofilm inhibition ability of LAB filtered supernatant was measured by using crystal violet assay. To evaluate the preventive effect of the LAB filtered supernatant on biofilm formation of *S. mutans*, *S. mutans* and LAB filtered supernatant were inoculated to BHI broth with addition of 0.2 % sucrose at same time. Also, to evaluate the inhibitory effect of LAB filtered supernatant on the biofilm, *S. mutans* were inoculated to BHI broth with 0.2 % sucrose and incubated for 24 hours at 37 ℃. After incubation, LAB filtered supernatant was treated to the preformed biofilm and incubated another 24 hours at 37 ℃. In biofilm prevention assay, *L. fermentum* KM6-5, *L. paracasei* LM1-1, LM1-3, LM3-5, and M9-1, *L. plantarum* KM14-5, *L. rhamnosus* HM15-5, LM11-1, and LM14-3 showed higher prevention ability against *S. mutans* ATCC25175 compared to that of *L. rhamnosus* GG (Figure 14a). *L. fermentum* KM6-5, LM15-1, and LM16-10, *L. paracasei* M9-1, *L. rhamnosus* HM26-1 and LM11-1 showed higher inhibition ability against biofilm formation of *S. mutans* ATCC700610 than *L. rhamnosus* GG (Figure 14b). When inoculated after preformed biofilm, *L. paracasei* M9-1 showed higher inhibition ability than *L. rhamnosus* GG against *S. mutans* ATCC 25175 among tested LAB (Figure 15a). *L. fermentum* LM15-1, *L. gasseri* LM8-5, *L. paracasei* M9-1, *L. rhamnosus* LM14-3 showed higher inhibition ability against *S. mutans* ATCC700610 when inoculated after preformed biofilm

(Figure 15b).

Among tested LAB, *L. paracasei* M9-1 showed the highest reduction in prevention and inhibition of biofilm formation against *S. mutans* ATCC25175 and ATCC700610.

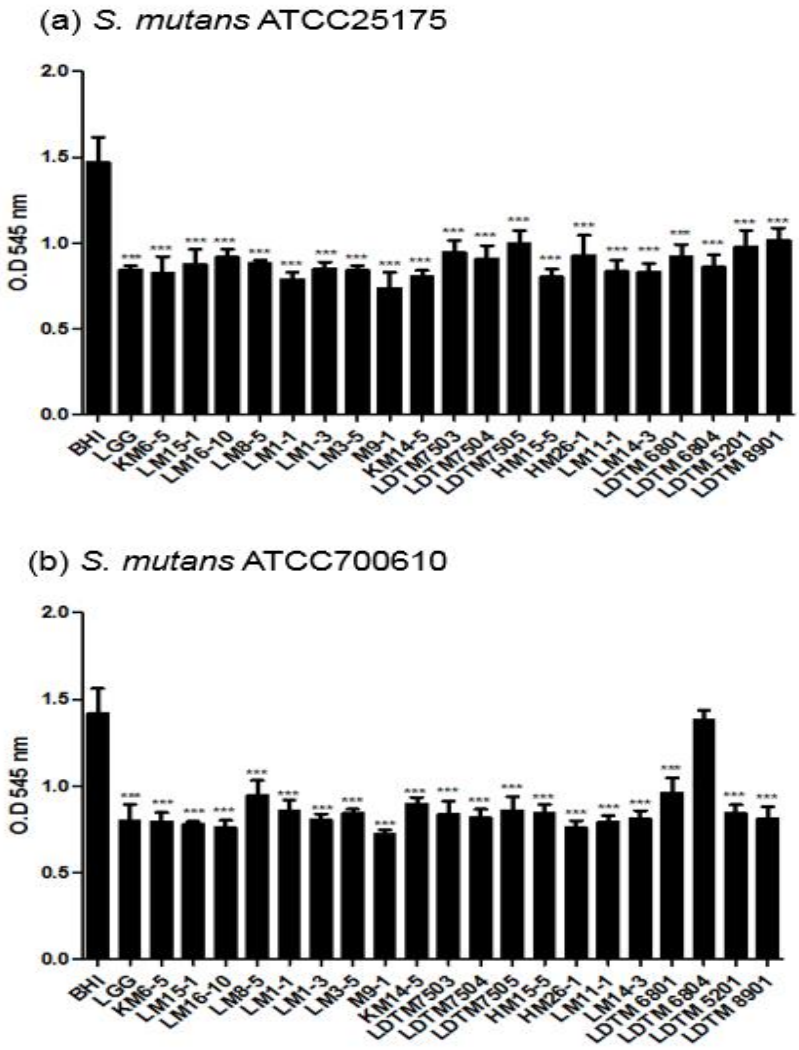

Figure 14. Inhibitory effects of LAB on biofilm-forming *S. mutans* (a) ATCC25175, and (b) ATCC700610. Data are mean values ± standard deviation (S.D.). Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

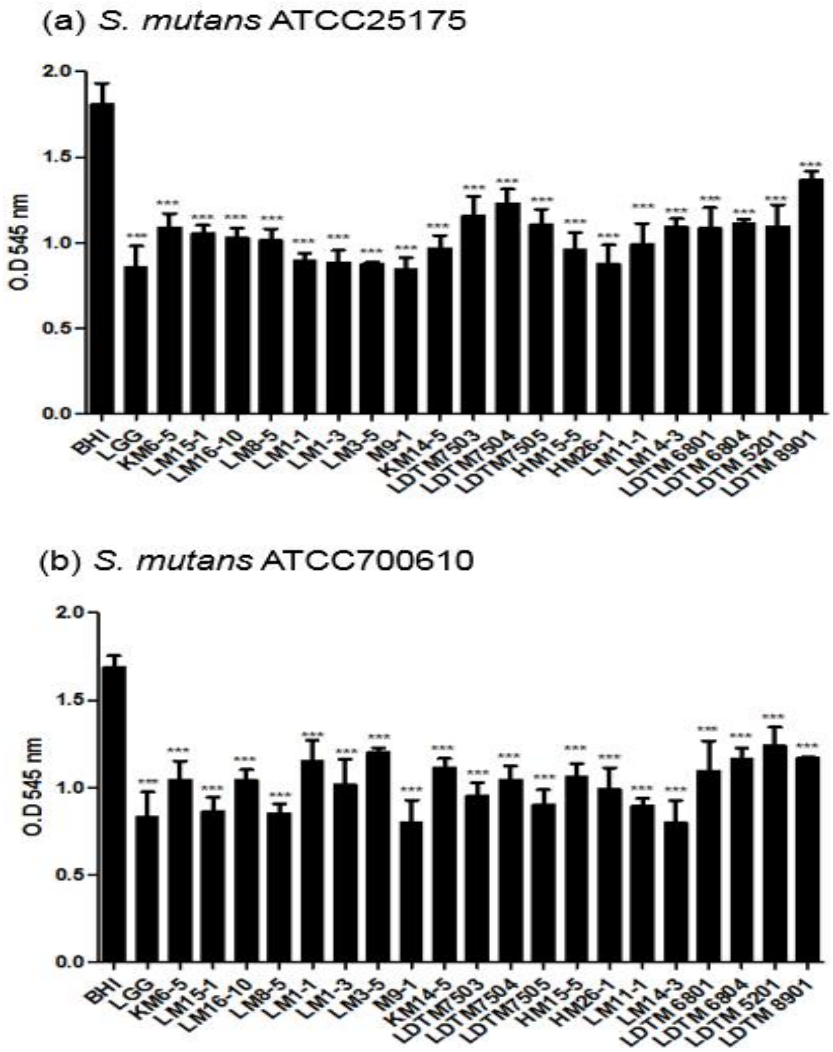

Figure 15. Inhibitory effects of LAB on preformed biofilm of *S. mutans* (a) ATCC25175, and (b) ATCC700610. Data are mean values ± standard deviation (S.D.). Significant differences were determined by using Student's *t*−test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

4.1.5. Selection of potential probiotics

Our data concluded that *L. paracasei* M9-1 was the most promising anti-cariogenic LAB, which has higher abilities than *L. rhamnosus* GG and satisfied criteria from EFSA to be classified as probiotics. Therefore, *L. paracasei* M9-1 was selected as our probiotic strains and applied to next step. In addition, *P. acidilactici* LDTM5201, *W. cibaria* LDTM 8901, *L.* subsp. *lactis* LDTM6804 were also used for genus diversity and *L. rhamnosus* GG as a reference strain.

4.1.6. Carbohydrate fermentative pattern and enzymatic profiling

We analyzed carbohydrate fermentative pattern and enzymatic profiling for analyzing biochemical characteristic of the selected probiotic strains. These results showed that *S. mutans* strains can utilize more kinds of carbohydrates than our selected probiotic strains. –*L. paracasei* M9-1 showed similar carbohydrate pattern to *S. mutans* strains while others showed differences (Figure 16a). The clear differences were observed in enzymatic profiling between each bacteria (Figure 16b).

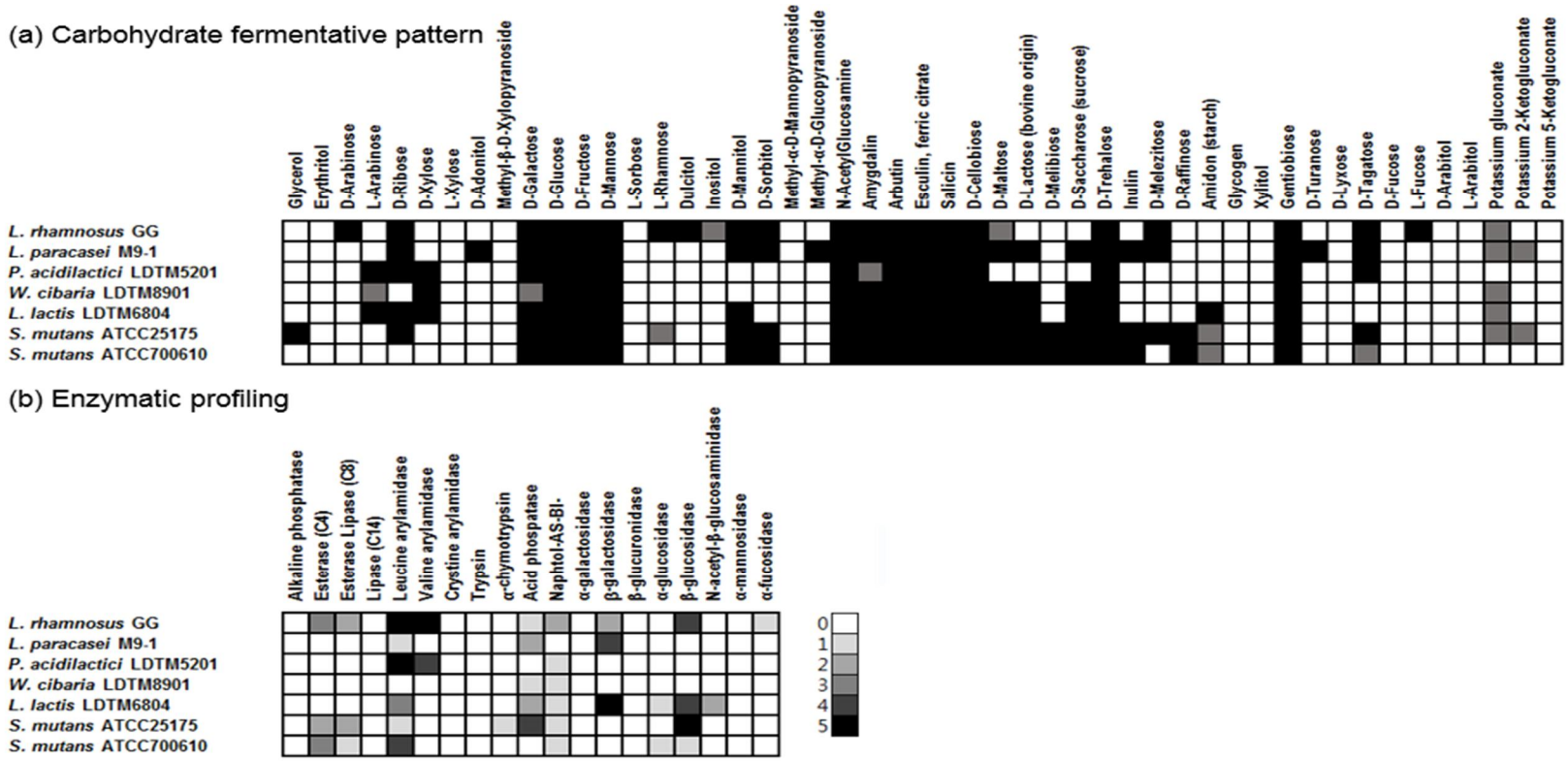

Figure 16. Heatmap of carbohydrates fermentative pattern and enzymatic profiling of selected probiotic strains and *S. mutans* strains. (a) Carbohydrate fermentative pattern, and (b) enzymatic profiling. The colorimetric intensity is indicated by color gradients: black represents high activity, while white represents no reaction.

4.2 Potential cariogenic ability of selected probiotic strains

4.2.1. Biofilm formation of selected probiotic strains

Some studies reported that probiotics may be the potential cariogenic agent due to production of organic acids and high acid tolerance. Therefore, we examined potential cariogenic ability of selected probiotic strains by inducing biofilm formation without *S. mutans*. All of tested strains except *L. lactis* LDTM6804 exhibited no biofilm formation (Figure 17).

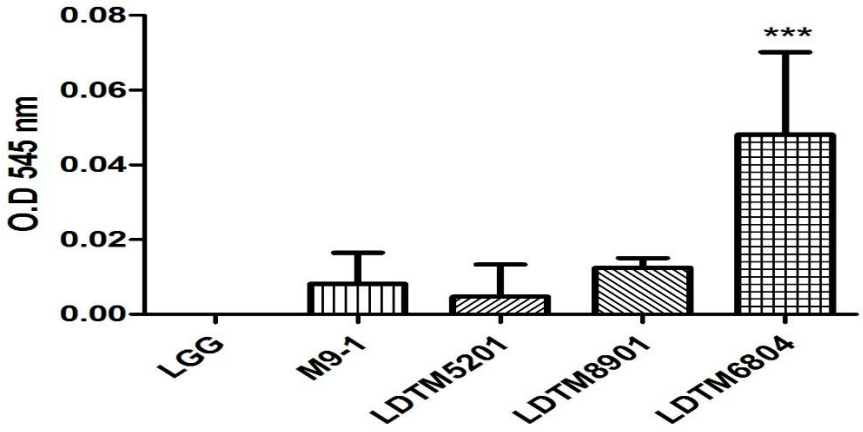

Figure 17. Biofilm formation ability of selected probiotic strains. Each strains were cultured in BHI broth with 0.2 % sucrose to induce biofilm formation. Data are mean values ± standard deviation (S.D.). Significant differences were determined by using Student's t-test, $***P \leq 0.001$ compared with *L. rhamnosus* GG group.

4.2.2. Degradation of hydroxyapatite

Dental caries is directly related to degradation of hydroxyapatite (HAP) due to low pH condition. We examined HAP degradation ability of the selected probiotic strains and *S. mutans* by measuring the amount of the calcium release. The selected probiotic strains except *L. lasti*s LDTM6804 showed lower calcium release than that of *S. mutans* ATCC25175 and ATCC700610 although the difference between ATCC700610 and selected probiotic strains were relatively small. After 24 hours incubation, all of the selected probiotic strains showed similar calcium release compared to *S. mutans* strains. *L. lactis* LDTM6804 showed higher calcium release than *S. mutans* strains until 12 hours of incubation (Figure 18a). In addition, we also assessed the HAP degradation when the selected probiotic strains were co-cultured with *S. mutans*. The selected probiotic strains treated group showed similar amount of the calcium release compared to untreated group in *S. mutans* ATCC25175. After 24 hours incubation, *L. paracasei* M9-1 treated group showed lower amount of the calcium release compared to untreated group (Figure 18b). In *S. mutans* ATCC700610, all of the selected probiotic strains treated group showed higher calcium release until 12 hours of incubation except *L. paracasei* M9-1 showed similar to *S. mutans* ATCC700610. However, after 24 hours of incubation, all of the selected probiotic strains treated groups showed lower calcium

release compared to untreated group. These data indicated that *L. paracasei* M9-1 didn't aggravate calcium release when co-cultured with *S. mutans* (Figure 18c).

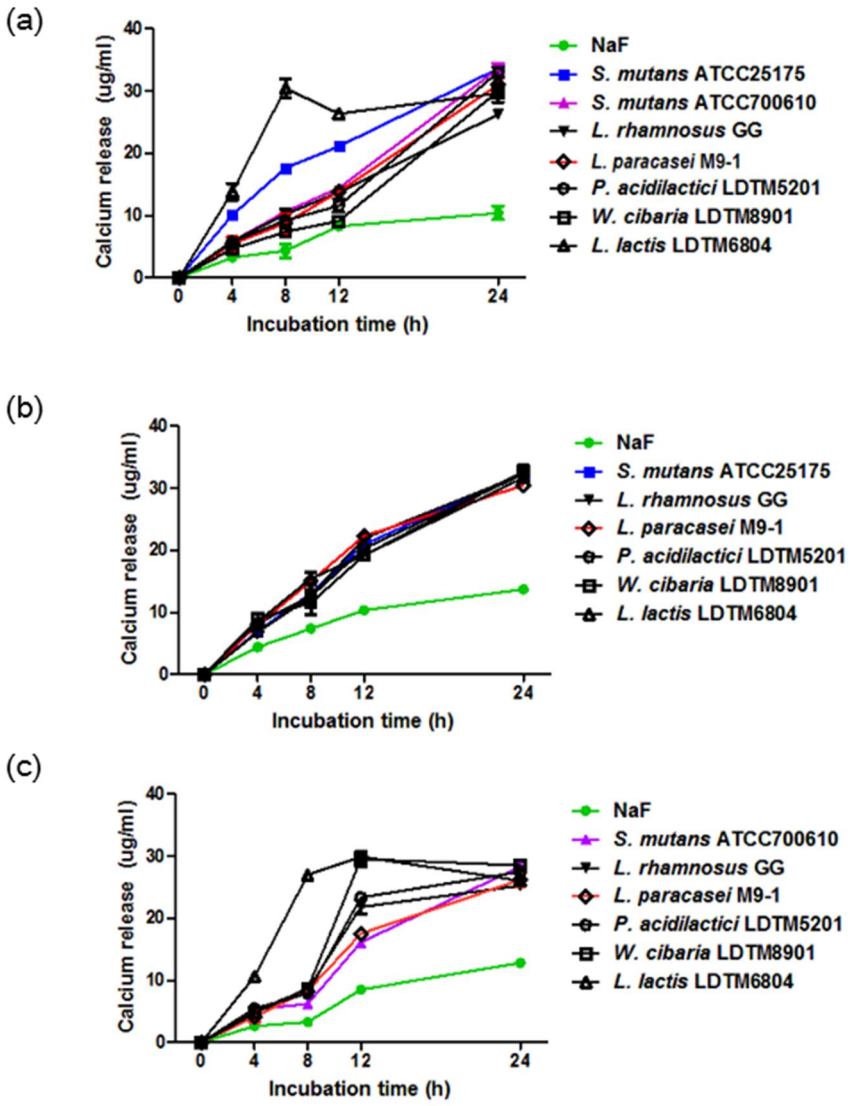

Figure 18. The calcium releasing ability of *S. mutans* and selected probiotic strains. (a) Calcium release by individual strains from HAP, Selected probiotic strains with *S. mutans* (b) ATCC 25175, and (c) ATCC700610.

4.3 Inhibitory effects of probiotic strains on the gene expression in *S. mutans*

4.3.1. Alteration in biofilm formation associated gene expression

To determine the effects of *L. paracasei* M9-1 supernatant on caries-inducing related gene expression in *S. mutans*, we measured biofilm-formation, acid tolerance, and adhesion associated mRNA expression by qRT-PCR.

*gtf*s are known to be major contributor genes in biofilm formation of *S. mutans*. *vic*KR, two component signal system encoding genes, have been reported for their function in regulating *gtf*s. *vicKR* defected mutants showed abnormal or reduced biofilm formation (Senadheera *et al.*, 2005).

In biofilm-forming *S. mutans*, *L. rhamnosus* GG and *L. paracasei* M9-1 significantly reduced *gtfC*, *vicK* and *vicR* expression level in both *of S. mutans* strains. However, *L. paracasei* M9-1 didn't significantly reduced *gtfB* in *S. mutans* ATCC25175 while *L. rhamnosus* GG significantly reduced *gtfB* in both of *S. mutans* strains. Both of selected probiotic strains significantly increased expression level of *gtfD* in *S. mutans* ATCC25175 but *L. paracasei* M9-1 significantly increased *gtfD in S. mutans* ATCC700610 while *L. rhamnosus* GG significantly decreased *gtfD* expression level in *S.*

mutans ATCC700610 (Figure 19a,b). *L. paracasei* M9-1 significantly reduced *sacB* expression level in *S. mutans* ATCC25175 and *L. rhamnosus* GG and *L. paracasei* M9-1 significantly increased *sacB* expression level in *S. mutans* ATCC700610 (Figure 20a,b).

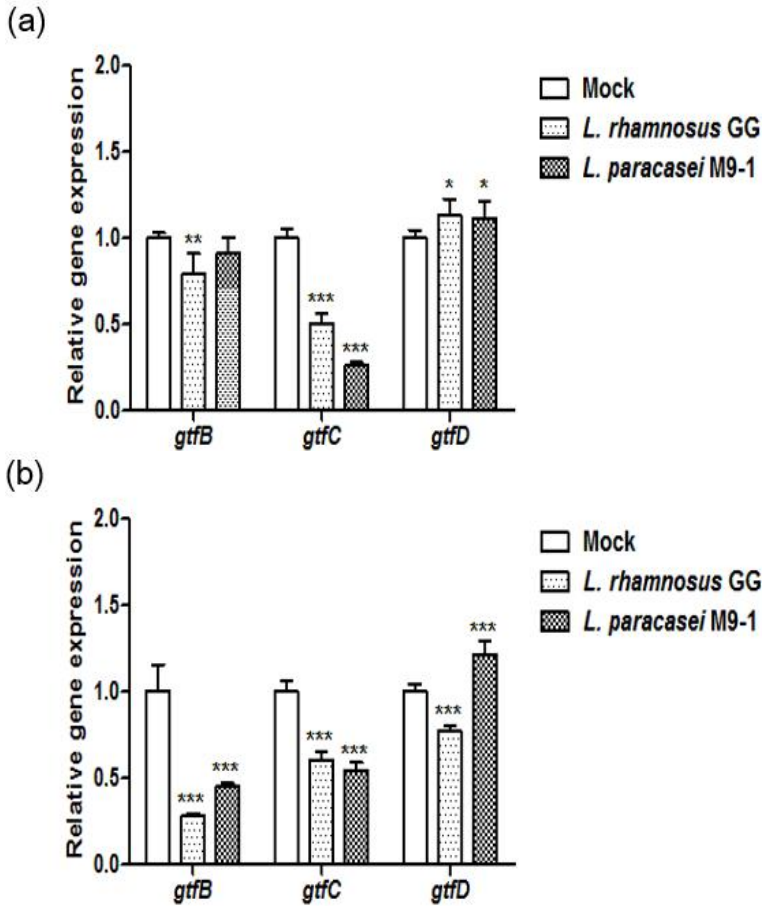

Figure 19. Effects of probiotics on expression of biofilm-associated genes in biofilm-forming *S. mutans*. *gtf*s in biofilm-forming *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control. Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

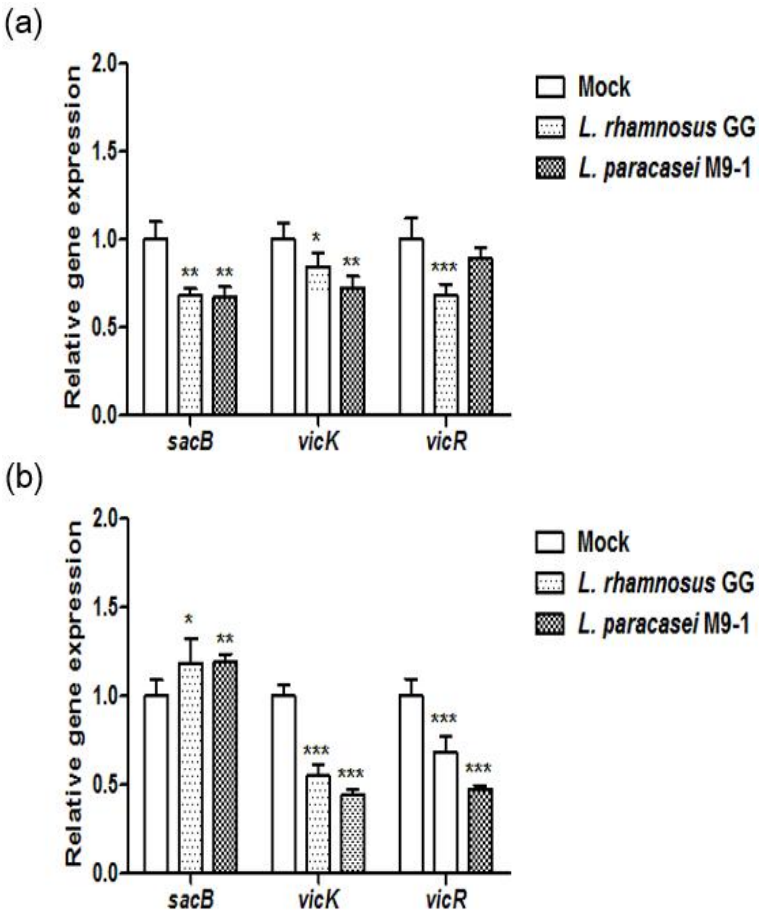

Figure 20. Effects of probiotics on expression of *sacB*, *vicK*, and *vicR* genes in biofilm-forming *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control. Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

In planktonic *S. mutans*. *L. rhamnosus* GG significantly decreased *gtfB* expression level and *L. paracasei* M9-1 increased *gtfB* expression level significantly in *S. mutans* ATCC25175. On the contrary, *L. rhamnosus* GG increased *gtfB* expression level and *L. paracasei* M9-1 significantly decreased expression level of *gtfB* and *gtfC* in *S. mutans* ATCC700610. In *gtfD* expression level, *L. paracasei* M9-1 significantly decreased (Figure 21a,b). *L. rhamnosus* GG and *L. paracasei* M9-1 significantly decreased *sacB* expression level in *S. mutans* ATCC25175 and *sacB* expression level in *S. mutans* ATCC700610 (Figure 22a,b). *L. rhamnosus* GG decreased *vicK* and *vicR* expression level in both of *S. mutans* strains significantly.

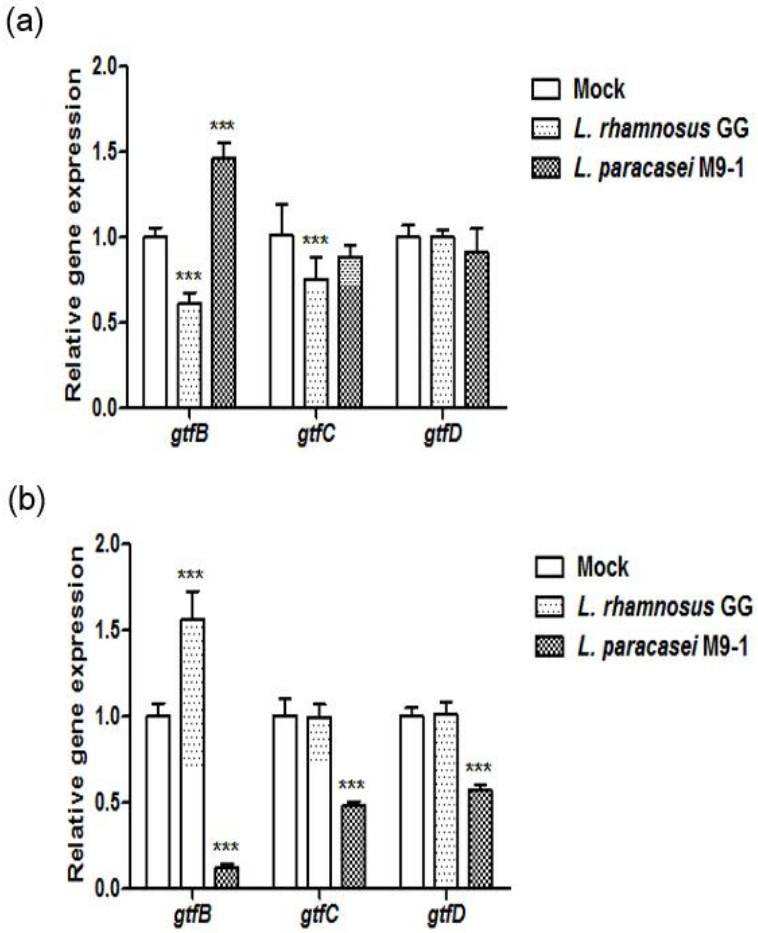

Figure 21. Effects of probiotics on expression of biofilm-associated genes in planktonic *S. mutans*. *gtf*s in planktonic *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control. Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

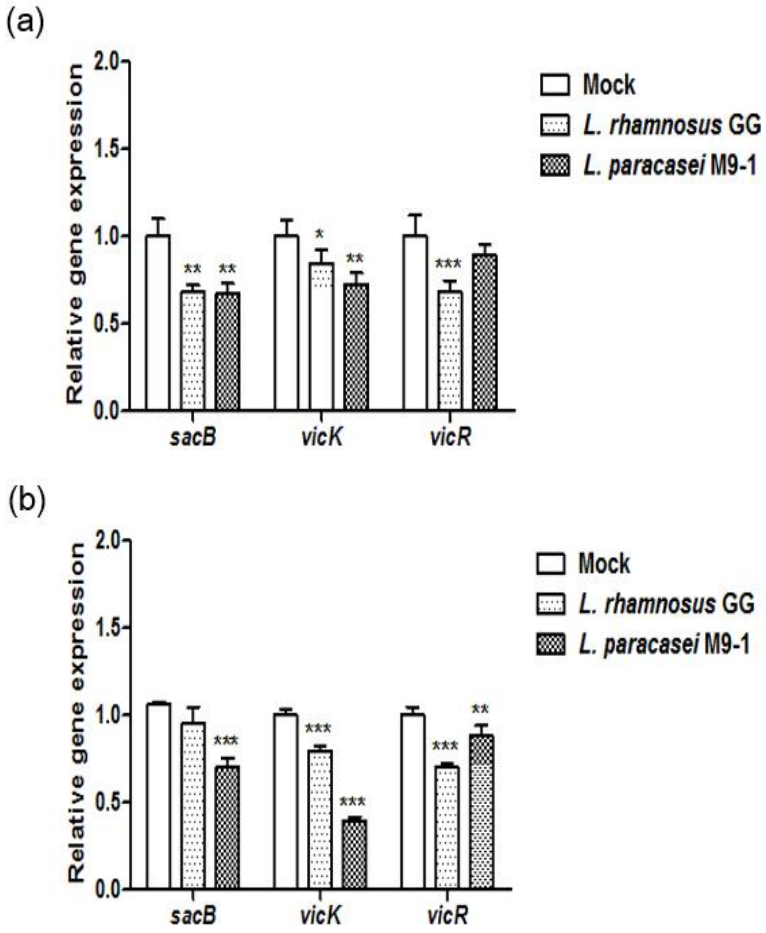

Figure 22. Effects of probiotics on expression of *sacB*, *vicK*, and vic*R* genes in planktonic *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control. Significant differences were determined by using Student's $t-$test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

4.3.2. Alteration in acid tolerance associated gene expression

According to Li *et al.*, 2001, *comCDE*, which is one of two component signal system, is involved in acid tolerance of *S. mutans*. When *comC*, *comD*, or *comE* genes was defective in constructed mutants, they all showed decreased in the log phase acid tolerance response. Also, Griswold *et al.*, 2006, reported that *S. mutans* converts arginine into agmatine to produce ammonia, thereby neutralizing acidic condition and use F-ATpase to pump out intracellular hydrogen ion.

In biofilm formation *S. mutans*, *L. rhamnosus* GG significantly increased *comC* expression level. Both of *Lactobacillus* strains significantly decreased *comD* expression level in *S. mutans* ATCC 25175. However, in *S. mutans* ATCC700610, both strains showed the exact opposite results from ATCC25175. They significantly decreased *comC* expression level. *L. rhmanosus* GG and significantly decreased *aguD* expression level in *S. mutans* ATCC 25175 and both of the selected probiotic strains significantly decreased *aguD* expression level in *S. mutans* ATCC700610. In *atpD* expression level, *L. rhamnosus* GG significantly increased *atpD* expression in *S. mutans* ATCC25175 and decreased *atpD* expression in *S. mutans* ATCC700610, significantly. *L. paracasei* M9-1 significantly increased *atpD* expression level only in *S. mutans* ATCC700610 (Figure 23a,b).

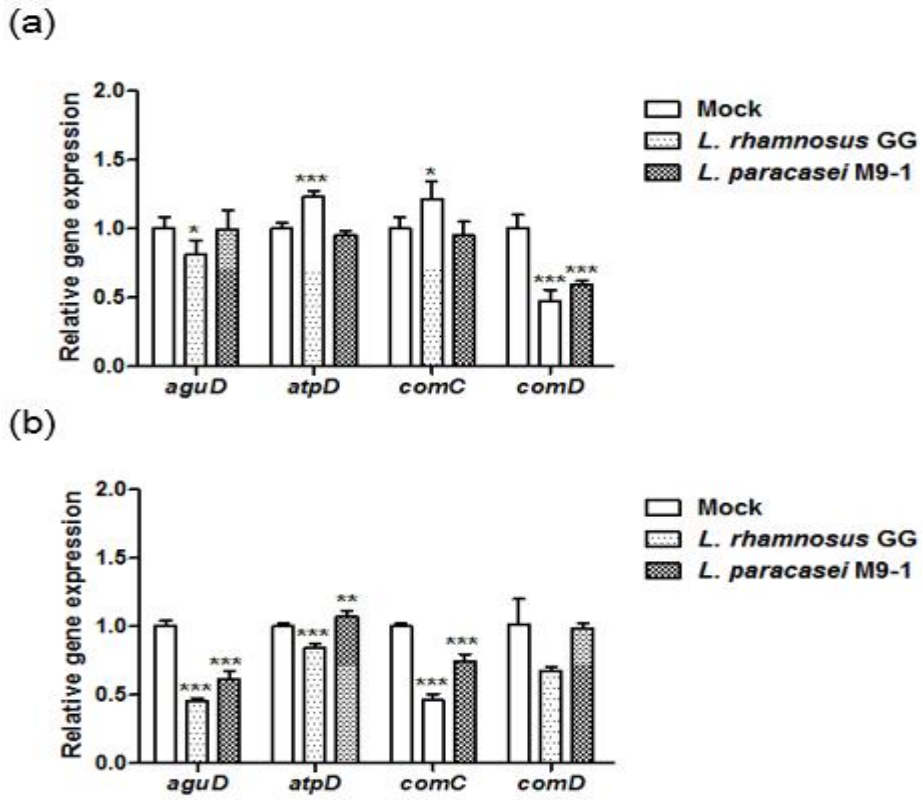

Figure 23. Effects of probiotics on expression of acid tolerance genes in biofilm-forming *S. mutans* (a) ATCC 25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control, BHI group. Error bars indicates standard deviation. Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

In planktonic *S. mutans*, *L. rhamnosus* GG decreased *comC* and *comD* expression level significantly and *L. paracasei* M9-1 increased *comC* and *comD* expression level significantly in *S. mutans* ATCC 25175. In *S. mutans* ATCC700610, *L. rhmanosus* GG significantly increased *comD* expression level and *L. paracasei* M9-1 significantly decreased *comD* expression level. In *aguD* expression level, both strains increased *aguD* expression in *S. mutans* ATCC25175 and decreased *aguD* expression in *S. mutans* ATCC700610, significantly. *L. rhamnosus* GG and *L. paracasei* M9-1 both significantly decreased *atpD* expression level in *S. mutans* ATCC25175, but *L. rhamnosus* GG increased *atpD* expression while *L. paracasei* M9-1 decreased *atpD* expression level significantly (Figure 24a,b).

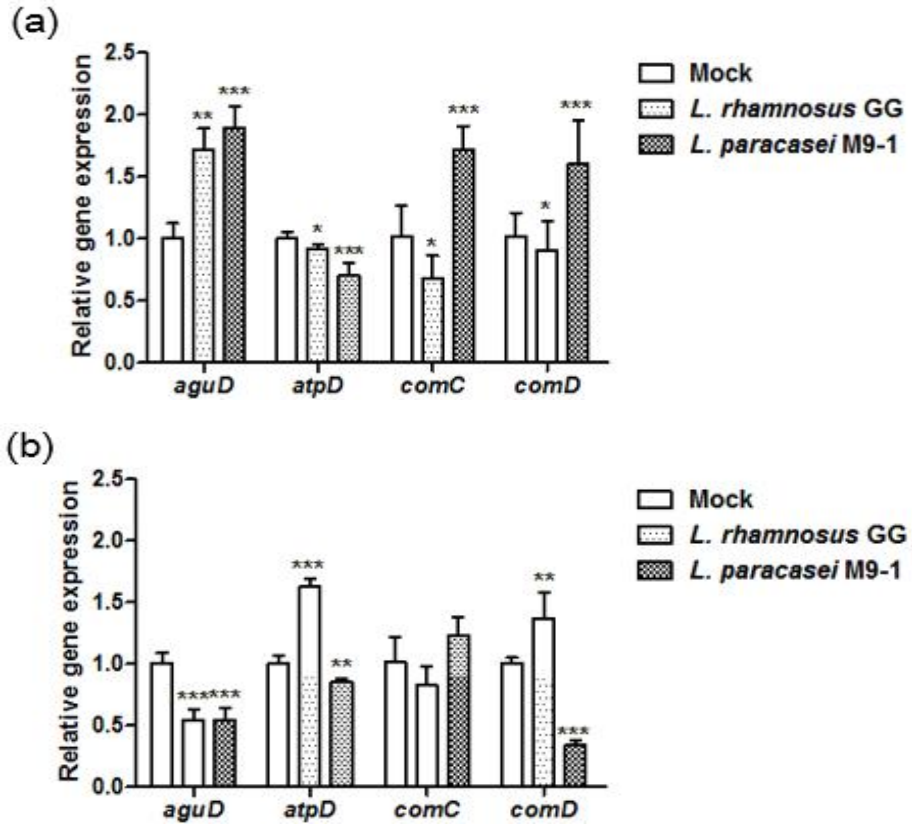

Figure 24. Effects of probiotics on expression of acid tolerance genes in planktonic *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control, BHI group. Error bars indicates standard deviation. Significant differences were determined by using Student's t−test, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ compared with control group.

4.3.3. Alteration in adhesion associated gene expression

Lastly, we examined the alteration of adhesion associated gene in *S. mutans* when treated with the selected probiotic strains filtered supernatant. In biofilm formation *S. mutans*, both selected probiotic strains significantly decreased *srtA* expression level in both of *S. mutans* strains. Also, selected probiotic strains significantly increased *spaP* and *wapA* expression level in *S. mutans* ATCC25175 and decreased *wapA* expression level in *S. mutans* ATCC700610 (Figure 25a,b).

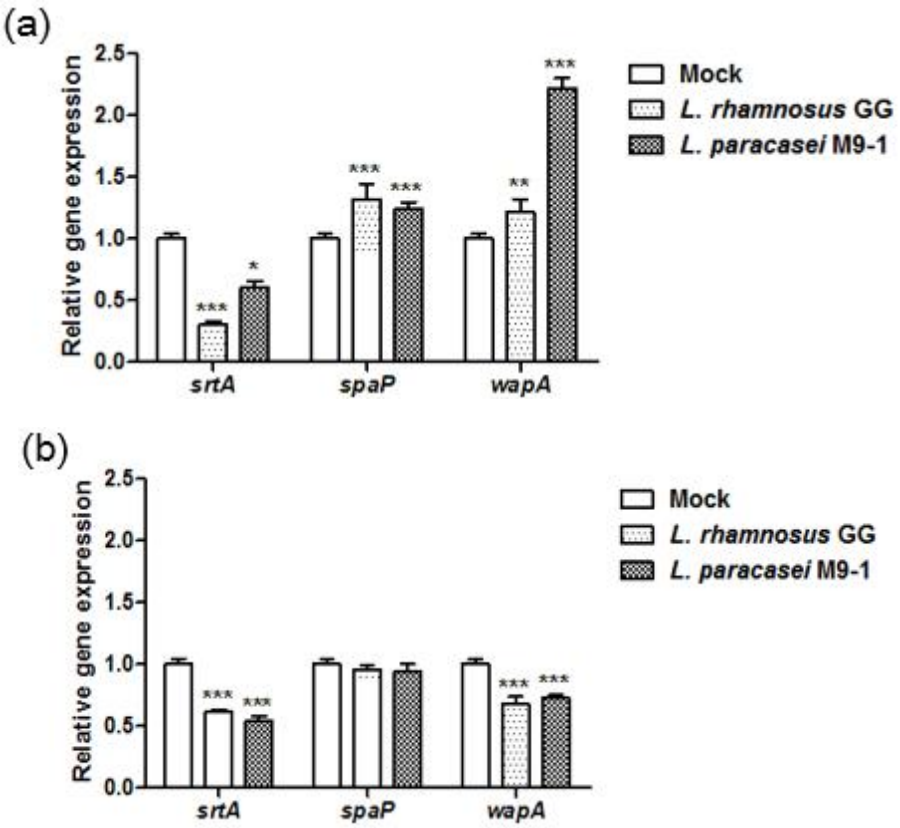

Figure 25. Effects of probiotics on expression of adhesion genes in biofilm-forming *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control, BHI group. Error bars indicates standard deviation. Significant differences were determined by using Student's *t*-test, $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$ compared with control group.

In planktonic *S. mutans*, *L. rhamnosus* GG and *L. paracasei* M9-1 significantly decreased *srtA* expression level in both of *S. mutans* strains. Both of lactobacillus strains significantly increased *spaP* expression level in *S. mutans* strains. *L. rhamnosus* GG decreased *wapA* expression level in both of *S. mutans* ATCC 25175 while *L. paracasei* M9-1 increased *wapA* expression level in *S. mutans* ATCC 25175 and decreased *wapA* expression level in *S. mutans* ATCC 700610 significantly (Figure 26a,b).

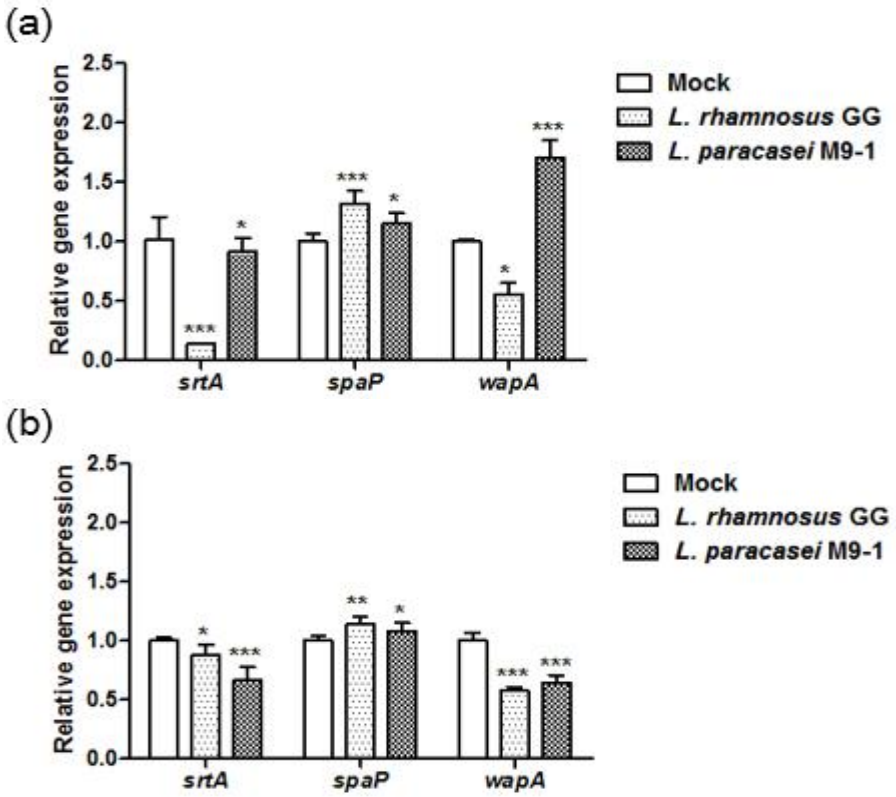

Figure 26. Effects of probiotics on expression of adhesion genes in planktonic *S. mutans* (a) ATCC25175, and (b) ATCC700610. In each panel, data are expressed as fold change, calculated by using $2^{-\triangle\triangle Ct}$ method relative to untreated probiotics as a control, BHI group. Error bars indicates standard deviation. Significant differences were determined by using Student's $t-$test, $*P \leq 0.05$, $** P \leq 0.01$, $***P \leq 0.001$ compared with control group.

Chapter 5. Discussion

The cariogenic microbiota in oral cavity produces organic acids derived from fermentation using dietary carbohydrates. In addition, it produces EPS such as glucans to form biofilm which is known as dental plaque. The function of biofilm is not only to block harmful materials entering into biofilm from outside, but also to trap nutrients and end products such as organic acids produced by bacteria (Huang *et al.*, 2011). These results in degradation of enamel layer in tooth surface, which is composed of HAP. In past, treatment of dental caries almost exclusively relied on physical cleansing of tooth surface by tooth brushing. However, it was unsuccessful in reducing dental caries. The interesting fact is that dental caries was the major reason of rejection from military service during 1940s (Loesche, 1996).

Among the oral microbiota, *S. mutans* is one of the normal microbiota in oral cavity and recognized as a major cariogenic agent (Shukla *et al.*, 2016). A study reported that it can persist without detection of hydroxyapatite degradation, which means that *S. mutans* is one of the normal microbiota in healthy oral cavity and can survive without biofilm formation (Marsh, 2003). The major reason why *S. mutans* is known as the most significant contributor in development

of the dental caries comes along with their specific enzyme, called glucosyltransferase. The major function of glucosyltransferase is to produce extracellular polysaccharide named glucan via degrading sucrose (Balakrishnan *et al.*, 2000).

Phenotypic variations such as serotype polysaccharide, carbohydrate fermentation, binding to tooth surface, and formation of biofilm are strain dependent on *S. mutans* (Waterhouse *et al.*, 2007). These phenotypic variations may result in different cariogenicity in each strain of *S. mutans*. Therefore, we used *S. mutans* ATCC25175 and ATCC700610 strains as indicator strains for screening and evaluation of anti-cariogenic LAB. *S. mutans* ATCC700610 was the first *S. mutans* strain to have their genome sequence identified. These findings provided not only uncharacterized genes involved in pathogenesis of *S. mutans* but also a future insight to identification of different strains in *S. mutans* (Ajdic *et al.*, 2002).

A study reported that genetic difference in *comC* gene among different *S. mutans* strains and others revealed ATCC25175 has unique *comC* residue of "LGKIR" at end of its C-terminal while ATCC 700610 has "LGK" residue at end of its C-terminal (Petersen and Scheie, 2000; Song *et al.*, 2013).

For treatment of the dental caries, tooth extraction method was widely used when there was little information about caries

prevention. However, as dental caries forms quickly, and progression rate is high, there were several limitations in tooth extraction. In clinical practices, caries management are focused on restoration or retention of teeth. Still, the restoration without a prevention are such a short-term effects and if the causes of disease were not removed, the recurrence of dental caries are another limitation in restoration. In nowadays, many clinical practices are focusing on prevention of dental caries by removal of the biofilm, or using fluoride (Selwitz *et al.*, 2007). However sometimes fluoride alone is not enough for prevention of dental caries. Even with fluoride, the carious lesion still develops when there are more than 6 dietary sugar uptake (Philip *et al.*, 2018). Probiotics have been emerged as an alternative therapy for the prevention or treatment of dental caries. The role of probiotics in dental caries reduction is to reduce biofilm formation or to reduce the concentration of *S. mutans*.

Therefore, the aim of this study is to screen LAB which has biofilm formation reducing ability against different *S. mutans* strains and to evaluate effects of selected probiotic strain on alteration of caries-inducing related mRNA expressions in *S. mutans*.

We used 21 LAB, whose antimicrobial activity against several pathogens including psychrotrophic bacteria, IBD-related pathogens, and food-spoilage pathogens were already qualified in previous

study, (data not published) because a numerous kinds of LAB are proposed as probiotics or used as probiotics (Ljungh and Wadstrom, 2006). To be classified as probiotics, the safety assessment of probiotics must be evaluated under criteria of reliable organization such as EFSA. Although each strains of LAB which are not pathogenic, does not need to be treated with antibiotics, however as they are living organism when consumed in high number, they must not possess antibiotic resistance gene that can be transferred to other microorganism, especially to pathogens (Broaders *et al.*, 2013). Biogenic amines are formed through undesirable decarboxylation of amino acids. When accumulated in high concentration, Biogenic amines may have toxicological effects on human. Most of LAB do not produce biogenic amine, but some species of LAB have been reported for its production of biogenic amine (Spano *et al.*, 2010). The hemolysis is considered as one of the major virulence factors in pathogens and hemolytic activity in *lactobacilli* is evaluated in many of current probiotic studies (Halder *et al.*, 2017). We evaluated each of selected 21 LAB on their antibiotics susceptibility, biogenic amine production, and hemolytic ability. None of those LAB showed such harmful effects and all of the LAB satisfied criteria from EFSA. This result indicates that our 21 LAB can be considered as safe when ingested and accepted as probiotics,

The agar diffusion assay is widely used antimicrobial activity assay because it is a simple, inexpensive assay which has advantages in easy to reproduce (Magaldi *et al.*, 2004). Due to variation of probiotic capacity among different strains and species (Guantario *et al.*, 2018), there is no exact reference strain in functional study of probiotics. However, even though *L. rhamnosus* GG does not always show the highest functionality, it is the most widely used and studied commercial probiotics, Thus, *L. rhamnosus* GG, is used as reference strain in many studies (Segers *et al.*, 2014). In this study, we also used *L. rhamnosus* GG as a reference strain to give convenience in re-verification and for commercial values. The antimicrobial activity of 21 LAB was determined by measuring diameter of clear zone. Our results differed among strains, however it showed similar tendency among species. In *S. mutans* ATCC25175, the highest antimicrobial activity was detected in *L. paracasei* M9-1 cell suspension among tested LAB. In the meanwhile, the antimicrobial activity from filtered supernatant of each LAB were not detected. This result indicates that the antimicrobial activity of our tested LAB were available only in the presence of living cell which may produce antimicrobial substances response to presence of other bacteria in same environment. This result was correspondent to result reported by Oldak *et al.*, 2017. In *S. mutans* ATCC700610, we couldn't use the agar well diffusion assay due to uncultivatable

feature of *S. mutans* ATCC700610 in solid culture.

Co-aggregation takes a significant role in formation of dental plaque. One of proposed mechanism in probiotic action against dental caries is their competition for binding site with pathogens or aggregation to binding receptors of oral pathogens, thereby blocking the adhesion of oral pathogens, otherwise which will bind to salivary pellicle (Haukioja *et al.*, 2008). To compare co-aggregation ability between LAB, we first examined their auto-aggregation ability and then examined co-aggregation ability by the calculation described in Materials and Methods. Interestingly *L. rhamnosus* GG showed lower co-aggregation ability than most of other strains. This result was correspond to findings from Keller *et al.*, 2011 which reports that *L. rhamnosus* strains have low co-aggregation abilities. In the contrast, *L. reuteri* and *L. gasseri* showed remarkable co-aggregation ability in our result. *L. reuteri* are reported to have higher auto-aggregation rate than *L. gasseri* and *L. rhamnosus* (Leccese *et al.,* 2014), but in this study, *L. gasseri* showed higher co-aggregation ability than *L. reuteri*. This result suggests that *L. gasseri* LM8-5 may expresses highest *apf* gene among tested LAB. Also, several studies reported about positive correlation between auto-aggregation and co-aggregation ratio (Xu *et al.*, 2009; Collado *et al.*, 2007). On the contrast, with regard to Twetman *et al.*, 2009, co-aggregation abilities vary through different strains of LAB. However,

our result showed similar tendency between each species, which indicates that auto-aggregation and co-aggregation depends on each species rather than strains.

Biofilm formation is recognized as the most significant factor in virulence of *S. mutans* (Pitts *et al.*, 2017). Therefore, measuring quantification of biofilm is necessary for screening probiotics which has anti-cariogenic function against *S. mutans*. Crystal violet staining assay was first designed by Christensen *et al.*, 1985 and Stepanovic *et al.*, 2000 modified the crystal violet assay to decrease technical errors in quantifying biofilm production. Crystal violet primarily binds to compounds in bacterial cell such as RNA or DNA or proteins. When dissolved in ethanol, it shows blue violet colors and thereby the absorbance is directly related to bacteria with crystal violet binding. In nowadays, it is used as a simple, inexpensive, and effective assay in many biofilm quantification assays. However, according to Monteiro *et al.*, 2015, there are clear disadvantages in using crystal violet assay. The crystal violet assay can't distinguish alive or dead cells because crystal violet stains whole bacterial cell, despite alive or dead, and even the matrix. This result also indicates that when LAB are used as in their cell suspension form, the crystal violet assay can't distinguish LAB from *S. mutans* cell thereby lowering accuracy of quantification in

reduction of biofilm production by *S. mutans*. To overcome this disadvantages, many studies used cell-free supernatant of LAB to measure inhibitory effects against biofilm formation of *S. mutans*. In this study, for prevention assay in inhibiting initial colonization of *S. mutans*, we inoculated *S. mutans* with LAB filtered supernatant at same time. For therapeutic assay, the biofilm of *S. mutans* were induced for 24 hour and subsequently the filtered LAB supernatant were treated to those preformed biofilm. All of LAB filtered supernatant showed inhibition in biofilm formation of both *S. mutans* strains regardless of treatment time and inhibition ability of LAB filtered supernatant varied among with different strains. This result is quite different from Wasfi *et al.*, 2018. In their result, all of the tested LAB supernatant showed antimicrobial activity against *S. mutans* ATCC25175 which indicates that reduction in biofilm formation may have been caused by inhibition of growth in *S. mutans* ATCC25175. However in our data, even if LAB supernatant has no antimicrobial activity against *S. mutans* strains, all of tested LAB supernatant still showed reduction in biofilm formation. Therefore, specific metabolites in LAB supernatant seems to have inhibitory effects against biofilm formation of *S. mutans*. For example, Ahn *et al.*, 2018 reported lipoteichoic acid from *L. plantarum*, which is a major component of cell wall in Gram-positive bacteria, showed reduction of biofilm formation without influencing growth of *S.*

mutans. Among tested LAB, *L. paracasei* M9-1 showed highest biofilm inhibition ability in both of *S. mutan*s strains for their biofilm colonization, and preformed biofilm, which was higher than reference strain, *L. rhamnosus* GG.

Considering our results from antimicrobial activity, co-aggregation ability, biofilm inhibition activity, *L. paracasei* M9-1 was selected as a promising probiotics which has inhibition abilities against biofilm formation by *S. mutans*. To give genus diversity, we also selected *P. acidilactici* LDTM5201, *W. cibaria* LDTM8901, *L. lactis* LDTM6804, and *L. rhamnosus* GG as reference strain for further characterizing and evaluation.

To analyze selected probiotic's carbohydrate fermentative pattern and enzymatic profiling, we used API assay. Two different strains of *S. mutans* showed clear difference in carbohydrate pattern and enzymatic profiling. This indicates the ability to utilize carbon source varies among strains and it may explain why cariogenic ability varies among different strains of *S. mutans*. Furthermore, *L. paracasei* M9-1 had most similar carbohydrate fermentation pattern to *S. mutans* ATCC25175. This result supports the possibility that *L. paracasei* M9-1 may compete for nutrition with *S. mutans* in oral cavity, thereby inhibiting growth of *S. mutans*.

Probiotics have been also reported for their ability in biofilm

formation. By forming biofilm, probiotics can enhance resistance to environmental conditions, and colonization to intestinal epithelial cells (Salas-Jara *et al.*, 2016). In gastrointestinal tract, the ability to produce biofilm may benefit the host for enhancing colonization rate and survival of probiotics. This function can be harmful to host when biofilm formation process takes place in oral cavity due to probiotic's high acid production and high acid tolerance. To test cariogenicity of selected probiotic strains, we induced biofilm formation of selected probiotic strains with same methods used in inducing biofilm formation of *S. mutans*. Our result showed that selected probiotics can't produce biofilm under sucrose added condition except *L. lactis* LDTM6804 showed possibility of biofilm formation compared to that of *L. rhamnosus* GG.

As mentioned in literature review, about 95 % of enamel is HAP. HAP is made up of hydroxyl ions, phosphate ions, and calcium ions. Due to positive charges from calcium ions, HAP also contains positive charge, and this provides bondage to *S. mutans*. The biofilms of *S. mutans* with calcium ions trapped in their covalent bond, increase the degradation of HAP (Lin and Pan, 2014). Therefore, the formation of biofilm induces the demineralization of HAP and release the calcium ions from HAP (Venegas *et al.*, 2006). By examining the amount of released calcium ions from HAP, we can

also measure cariogenecity of selected probiotic strains and *S. mutans*. Furthermore, we evaluated degradation of HAP when *S. mutans* co-cultured with selected probiotics. Our results showed that when cultured individually, all of selected probiotics showed the degradation of HAP, although which were lower than those of *S. mutans* strains and even *S. mutans* strains showed difference in calcium release. This result can also support that cariogenicity of *S. mutans* strains are different among strains. After 24 hours of incubation, all of selected probiotic strains showed lower calcium release than that of *S. mutans* strains, although the difference was relatively small. This result is clearly different from Nikawa *et al.*, 2004. In their study, they reported calcium release by *S. mutans* begins after 24 hours of incubation, and *L. reuteri* showed no release of *S. mutans*. The modification of HAP degradation assay from Nikawa *et al.*, 2004 might explain this difference. They used highly compressed HAP beads but in this study, we used HAP as in powder form. In powder form, the binding force might be lower than compressed bead form, thereby the degradation rate in powder form may be faster than the compressed bead form. Furthermore, we added sucrose to induce biofilm. The addition of sugar may have affected the degradation rate of HAP due to faster growth of *S. mutans* and the selected probiotic strains. A study from Lin and Pan, 2014, also reports that *L. paracasei* showed the release of calcium

from HAP, which were lower than that of *S. mutans*. This result corresponds with our result. However, we must consider that in this method, we couldn't check the influence of selected probiotics when trapped within the *S. mutans* biofilm. Therefore, we co-cultured selected probiotics with *S. mutans* and examined release of calcium amount in the same way. Our result showed clear difference between each strain of *S. mutans*. In *S. mutan*s ATCC25175, all of the selected probiotic strains showed similar calcium release compared to control group. Among those, *L. paracasei* M9-1 showed lower calcium release compared to control group. In *S. mutans* ATCC700610, all of selected probiotic strains showed higher calcium release compared to control group at initial stage except *L. paracasei* M9-1. *L. paracasei* M9-1showed similar calcium release compared to *S. mutans* ATCC700610. However after 24 hours of incubation, all of the selected probiotic strains showed lower calcium release compared to control group. Therefore, *P. acidilactici* LDTM 5201, *W. cibaria* LDTM8901, and *L. lactis* LDTM6804 were disqualified for further assays due to enhancing the degradation of HAP in *S. mutans* ATCC25175 and ATCC700610.

In line with previous results in this study, we selected *L. paracasei* M9-1 as promising probiotics which has inhibitory effects against biofilm formation by *S. mutans* ability. To evaluate the

effects of *L. paracasei* M9-1 supernatant on *S. mutans* caries-inducing factors, we examined alteration of caries-inducing related gene expression in *S. mutans* by qRT-PCR. The genes examined in this study were biofilm associated gene, such as *gtfB*, *gtfC*, *gtfD*, and *sacB*, and the two component signal system which can regulate biofilm-associated gene, *vicK*, and *vicR*. Our study also included the acid tolerance related gene *aguD*, and *atpD*, and the two component signal system *comC* and *comD*. In addition, the adhesion related gene *srtA*, *spaP*, and *wapA* were included. In biofilm-forming group, *L. paracasei* M9-1 decreased *gtfC*, *vicK*, and *vicR* expression level in both of *S. mutans* strains, while other biofilm associated genes showed different tendency. According to Wasfi *et al.*, 2018, the decrease in *vicK* and *vicR* level resulted in decrease of *gtf*s and *ftf* because biofilm formation are regulated by VicKR system. However, in our study, *L. paracasei* M9-1 didn't significantly decreased *gtfB* in *S. mutans* ATCC25175 and the expression of *gtfD* and *sacB* varied along with different strains of *S. mutans*. In planktonic group, *L. paracasei* M9-1 increased *gtfB* expression level in *S. mutans* ATCC 25175 despite reduction in VicKR system. These data suggest that *gtfc* may be directly regulated by VicKR system, and other biofilm associated genes are not. *S. mutans* are reported to have at least 13 kinds of two component signal system, and other

two component signal system rather than VicKR system may have caused the difference between expression levels of each biofilm associated genes. Also, increases in *gtfD* expression level would increase the production of water-soluble glucans. As a result, the solubility of biofilm by saliva increases and cause the biofilm to be easily removed. We assume that the high level of *gtfD* and low level of *gtfB* might cause the conversion of water-insoluble glucan into water-soluble glucan although more evidence are needed to prove the theory. In acid tolerance gene expression, our result showed that *L. paracasei* M9-1 didn't have much influence on the acid tolerance response as in correlation with our agar well diffusion assay. *L. paracasei* M9-1 showed negligible effect on expression of acid tolerance gene in *S. mutans* ATCC25175 or increased *atpD* gene expression level in *S. mutans* ATCC700610 despite the reduction of *comC* and *comD* expression level. This result indicates that ComCD signal transduction system regulates the acid tolerance related gene rather than *aguD* and *atpD*. Instead, a study reported that ComCD also regulates the *gtf*s and glucan binding sites such as *gbpB* (Li *et al*., 2002). Therefore, different alteration in expression of *gtf*s might be caused by different alteration in expression of *comCD* system. In biofilm-forming *S. mutans,* reduction in *comC* expression level might have caused the reduction in *gtfB* expression

level. However, in planktonic *S. mutans*, the reduction in *comD* expression level seemed to have caused the reduction in all of tested biofilm associated gene expression level, which suggest that ComCD system regulation depends on presence of biofilm. Also ComCD system is regulated by cell density in biofilms (Jarosz *et al.*, 2009). In our result, *comC* and *comD* was expressed higher in planktonic group, which indicates that the cell density became lower in biofilm group due to the decreased expression level of *gtfC*. In adhesion related gene, *L. paracasei* M9-1 decreased *srtA* expression level in biofilm group in both of *S. mutans* strains. This suggest that *L. paracasei* M9-1 might inhibits biofilm formation in the absence of sucrose. In total, *L. paracasei* M9-1 inhibits attachment of *S. mutans* to tooth surface rather than inhibiting production of the biofilm even without presence of sucrose.

Although our result showed biofilm formation reducing effects of *L. paracasei* M9-1 against *S. mutans*, there are limitations before applying *L. paracasei* M9-1 to clinical study. We showed *L. paracasei* M9-1 as a new probiotics for its biofilm forming reducing ability against *S. mutans*, but we couldn't confirm that *L. paracasei M9-1* would show its anti-caries activity when treated as live cell due to limitation in crystal violet assay. Indeed, when probiotics are used as live cell form to examine the reduction in biofilm formation,

the crystal violet may also bind to probiotic strains and result in the increase of biofilm formation which will cause misleading in analysis. Moreover, if probiotics is trapped inside biofilm without inhibitory activity, it can aggravate the dental caries. However, according to our results, *L. paracasei* M9-1 couldn't produce biofilm and showed anti-microbial activity against *S. mutans* when treated as live cell form. Therefore, we assume that when *L. paracasei* M9-1 are treated as live cell form, the reduction ability against biofilm formation of *S. mutans* would be enhanced rather than aggravated.

A weakness in our study is that we used HAP degradation without presence of human saliva. Human saliva is one of the critical components in oral ecological environment. It plays a significant role in maintaining neutral pH in oral cavity by possessing several buffering peptides such as phosphate or bicarbonate (Kumar *et al.*, 2017). We believe that the increase in the calcium release of selected probiotic strains treated group is related to the absence of human saliva because selected probiotic strains can't produce biofilm and thereby human saliva might wash out the organic acid produced from the selected probiotic strains. Also, the temporal persistence of probiotics may be another concern in evaluating the efficacy of probiotics against dental caries. A study has reported that the effect of probiotics on dental caries disappeared after stop consuming

probiotics (Petti *et al.*, 2001). Therefore, more precise study such as *in vivo* evaluation of *L. paracasei* M9-1 in caries induced rats or extraction of specific metabolite which can reduce biofilm formation of *S. mutans* by using chromatography are needed before applying to clinical studies.

In conclusion, our study found a noble probiotics which has preventive effects on reducing dental caries caused by *S. mutans* through biofilm inhibition against two different *S. mutans* strains even though there are some limitations. Our result indicates that *L. paracasei* M9-1 can prevent and inhibit biofilm formation of *S. mutans* by antimicrobial, co-aggregation and the reduction of adhesion related gene expression in *S. mutans* despite the presence of sucrose. *In vivo* study is needed to confirm safety in alive cell form and efficacy in long term use. However, our study also provides possibility of using *L. paracasei* M9-1 as in supernatant form. According to Tanzer *et al.*, 2010, even heat-killed *L. paracasei* can reduce the number of *S. mutans* and caries lesion score in caries induced rat model by co-aggregation. In addition, Holz *et al.*, 2013 used sugar-free candies as a delivery substance. Therefore, if we can extract the metabolites which can reduce biofilm formation, we may add those to gum or gargling solution. Also, it will help us to understand exact mechanism of biofilm reducing ability of *L. paracasei* M9-1.

References

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., and Dewhirst, F. E. (2005) Defining the Normal Bacterial Flora of the Oral Cavity. *J Clin Microbiol* 43:5721-5732.

Abou Neel, E. A., Aljabo, A., Strange, A., Ibrahim, S., Coathup, M., Young, A. M., Bozec, L., and Mudera, V. (2016) Demineralization-remineralization Dynamics in Teeth and Bone. *Int J Nanomedicine* 11:4743-4763.

Ahn, K. B., Balk, J. E., Park, O., Yun, C., and Han, S. H. (2018) *Lactobacillus plantarum* Lipoteichoic Acid Inhibits Biofilm Formation of *Streptococcus mutans*. *PLOS* 13:e0192694

Ajdic, D., McShan, W. M., McLauhglin, R. E., Savic, G., Chang, J., Crason, M. B., Preimeaux, C., Tian, R., Kenton, S., Jia, H., Lin, S., Qian, Y., Li, S., Zhu, H., Najra, F. Lai, H., White, J., Roe, B. A., and Ferretti, J. J. (2002) Genome Sequence of *Strptococcus mutans* UA159, A Cariogenic Dental Pathogen. *PNAS* 99:1-6.

Allaker, R. P., and Stephen, A. S. (2017) Use of Probiotics and Oral Health. *Curr Oral Health Rep* 4:309-318.

Ashida, H., Ogawa, M., Kim, M., Mimuro, H., and Sasakawa, C. (2011) Bacteria and Host Interactions in the Gut Epithelial Barrier. *Nat Chem Biol* 8:36-45.

Avonts, L., Uytven, E. V., and Vuyst, L. D. (2004) Cell Growth and Bacteriocin Production of Probiotic *Lactobacillus* Strains in Different Media. *Int Dairy J* 14:947-955.

Balakrishnan, M., Simmonds, R. S., and Tagg, J. R. (2000) Dental Caries is a Preventable Infectious Disease. *Aust Dent J* 45:235-245.

Balouiri, M., Sadiki, M., and Ibnsouda, S. K. (2016) Methods for in vitro Evaluating Antimicrobial Activity: A Review. *J Pharm Anal* 6:71-79.

Banas, J. A., and Vickerman, M. M. (2003) Glucan-Binding Proteins of the Oral *Streptococci*. *Crit Rev Oral Biol Med* 14:89-99.

Bardow, A., Moe, D., Nyvad, B., and Nauntofte, B. (2000) The Buffer Capacity and Buffer Systems of Human Whole Saliva Measured Without Loss of CO_2. *Arch Oral Biol* 45:1-12.

Bardow, A., Nyvad, B., and Nauntofte, B. (2001) Relationships Between Medication Intake, Compalints of Dry Mouth, Salivary Flow Rate and Composition, and the Rate of Tooth Demineralization in situ. *Arch Oral Biol* 46:413-423.

Barron, R. P., Carmichael, R. P., Marcon, M. A. and Sandor, G. K. B. (2003) Dental Erosion in Gastroesophageal Reflux Disease. *J Can Dent Assco* 69:84-89.

Becker, M. R., Paster, B. J., Leys, E. J., Moeschberger, M. L., Kenyon, S. G., Galvin, J. L., Boches, S. K., Dewhirst, F. E., and Griffen, A. L. (2002) Molecular Analysis of Bacterial Species

Associated with Childhood Caries. *J Clin Microbiol* 40:1001-1009.

Beighton, D. (2005) The Complex Oral Microflora of High-risk Individuals and Groups and Its Role in the Caries Process. *Community Dent Oral Epidemiol* 33:248-255.

Bergeron, L. J., and Burne, R. A. (2001) Roles of Fructosyltransferase and Levanase-Sucrase of *Actinomyces naeslundii* in Fructan and Sucrose Metabolism. *Infect Immun* 69:5395-5402.

Bover-Cid, S., and Holzapfel, W. H. (1999) Improved Screening Procedure for Biogenic Amine Production by Lactic Acid Bacteria. *Int J Food Microbiol* 53:33-41.

Bowen, W. H., and Koo, H. (2011) Biology of *Streptococcus mutans*-derived Glucosyltransferases: Role in Extracellular Matrix Formation of Cariogenic Biofilms. *Caries Res* 45:69-86.

Broaders, E., Gahan, C. G., and Marchesi, J. R. (2013) Mobile Genetic Elements of the Human Gastrointestinal Tract: Potential for Spread of Antibiotic Resistance Genes. *Gut Microbes* 4:271-280.

Burton, J. P., Wescombe, P. A., Moore, C. J., Chilcott, C. N. and Tagg, J. R. (2006) Safety Assessment of the Oral Cavity Probiotic *Streptococcus salivarius* K12. *Appl Environ Microbiol* 72:3050-3053.

Cagetti, M. G., Mastroberardino, S., Milia, E., Cocco, F., Lingstrom, P., and Campus, G. (2013) The Use of Probiotic Strains in Caries

Prevention: A Systematic Review. *Nutrients* 5:2530-2550.

Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M., and Beachey, E. H. (1985) Adherence of Coagulase-Negative *Staphylococci* to Plastic Tissue Culture Plates: A Quantitative Model for the Adherence of *Staphylococci* to Medical Devices. *J Clin Microbiol* 22:996-1006.

Chuang, L. C., Huang, C. S., Ou-Yang, L. W., and Lin, S. Y. (2011) Probiotic *Lactobacillus paracasei* Effect on Cariogenic Bacterial Flora. *Clin Oral Investig* 15:471-476.

Collado, M. C., Meriluoto, J., and Salminen, S. (2007) Measurement of Aggregation Properties Between Probiotics and Pathogens: *in vitro* Evaluation of Different Methods. *J Microbiol Methods* 71:71-74.

Costalonga, M., and Herzberg, M. C. (2014) The Oral Microbiome and the Immunobiology of Periodontal Disease and Caries. *Immunol Lett* 162:22-38.

Dasari, S., Shouri, R. N. D., Wudayagiri, R., and Valluru, L. (2014) Antimicrobial Activity of *Lactobacillus* Against Microbial Flora of Cervicovaginal Infections. *Asian Pac J Trop Dis* 4:18-24.

Dawes, C. (2003) What Is the Critical ph and Why Does a Tooth Dissovle in Acid? *J Can Dent Assoc* 69:722-724.

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmanan, A., and Wade, W. G. (2010) The Human Oral

Microbiome. *J Bacteriol* 192:5002-5017.

European Food Safety Authority. (2012) Guidance on the Assessment of Bacterial Susceptibility to Antimicrobials of Human and Veterinary Importance: EFSA Panel on Additives and Products or Substances Used in Animal Feed (FEEDAP). *The EFSA J* 2740:1-10.

Featherstone, J. D. B. (2000) The Science and Practice of Caries Prevention. *J Am Dent Assoc* 131:887-899.

World Dental Federation. (2015) The Challenge of Oral Disease - A Call for Global Action. The Oral Health Atlas, 2nd edition, Geneva: FDI World Dental Federation ,

Fejerskov, O., and Kidd, E. A. M. (2009) Dental Caries: The Disease and Its Clinical Management. Oxford: Blackwell Munksgaard.

Fernandez, C. E., Giacaman, R. A., Tenuta, L. M., and Cury, J. A. (2015) Effect of the Probiotic *Lactobacillus rhamnosus* LB21 on the Cariogenicity of *Streptococcus mutans* UA159 in a Dual-Species Biofilm Model. *Caries Res* 49:583-590.

Forssten, S. D., Bjorklund, M., Ouwehand, A. C. (2010) Streptococcus mutans, Caries and Simulation Models. *Nutrients* 2:290-298.

Gao, R., and Stock, A. M. (2009) Biological Insights from Structures of Two-component Proteins. *Annu Rev Microbiol* 63:133-154.

Griffen, A. L., Beall, C. J., Firestone, N. D., Gross, E. L., Difranco, J.

M., Hardman, J. H., Vriesendorp, B., Faust, R. A., Janies, D. A., and Leys, E. J. (2011) CORE: a Phylogenetically-curated 16S rDNA Database of the Core Oral Microbiome. *PLoS One* 6:1-10.

Griswold, A. R., Jameson-Lee, M., and Burne, R. A. (2006) Regulation and Physiologic Significance of the Agmatine Deiminase System of *Streptococcus mutans* UA159. *J Bacteriol* 188: 834-841.

Gross, E. L., Beall, C. J., Kutsch, S. R., Firestone, N. D., Leys, E. J., and Griffen, A. L. (2012) Beyond *Streptococcus mutans*: Dental Caries Onset Linked To Multiple Species By 16S rRNA Community Analysis. *PLoS One* 7:1-11.

Gruner, D., Paris, S., and Schwendicke, F. (2016) Probiotics for Managing Caries and Periodontitis: Systematic Review and Meta-analysis. *J Dent* 48:16-25.

Guantario, B., Zinno, P., Schifano, E., Roselli, M., Perozzi, G., Palleschi, C., Uccelletti, D., and Devirgiliis, C. (2018) *In vitro* and *in vivo* Selection of Potentially Probiotic *Lactobacilli* From Nocellara del Belice Table Olives. *Front Microbiol* 9:595-610.

Halder, D., Mandal, M., Chatterjee, S. S., Pal, N. K., and Mandal, S. (2017) Indigenous Probiotic *Lactobacillus* Isolates Presenting Antibiotic like Activity against Human Pathogenic Bacteria. *Biomed* 5:1-11.

Hanada, N., and Kuramitsu, H. K. (1988) Isolation and Characterization of the *Streptococcus mutans gtfC* Gene, Coding for

Synthesis of Both Soluble and Insoluble Glucans. *Infect Immun* 56:1999-2005.

Haukioja, A. (2010) Probiotics and Oral Health. *Eur J Dentistry* 4:348-355.

Haukioja, A., Loimarant, V., and Tenovuo, J. (2008) Probiotic Bacteria Affect the Composition of Salivary Pellicle and *Streptococcal* Adhesion *in vitro*. *Oral Microbiol Immuno* 23:336-343.

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, F. S., Calder, P. C., and Sanders, M. E. (2014) The International Scientific Association for Probiotics and Prebiotics Consensus Statement on the Scope and Appropriate Use of Term Probiotic. *Nat. Rev. Gastroenterol. Hepatol* 11:506-514.

Holz, C., Alexander, C., Balcke, C., More, M., Auinger, A., Bauer, M., Junker, L., Grunwald, J., Lang, C., and Pompejus, M. (2013) *Lactobacillus paracasei* DSMZ16671 Reduces Mutans Streptococci: A Short-term Pilot Study. *Probiotics Antimicrob Proteins* 5:259-263.

Houte, J. V. (1994) Role of Micro-organisms in Caries Etiology. *J Dent Res* 73:672-681.

Houte, J. V., Gibbs, G., and Butera, C. (1982) Oral Flora of Children with "Nursing Bottle Caries". *J Dent Res* 61:382-385.

Huang, R., Li, M., and Gregory, R. L. (2011) Bacterial Interactions in

Dental Biofilm. *Virulence* 2:435-444.

Ingrassia, I., Leplingard, A., and Darfeuille-Michaud, A. (2005) *Lactobacillus casei* DN-114 001 Inhibits the Ability of Adherent-invasive *Escherichia coli* Isolated From Crohn's Disease Patients to Adhere to and to Invade Intestinal Epithelial Cells. *Appl Environ Microbiol* 71:2880-2887.

Iwasaki, K., Maeda, K., Hidaka, K., Nemoto, K., Hirose, Y., and Deguchi, S. (2016) Daily Intake of Heat-Killed *Lactobacillus plantarum* L-137 Decreases the Probing Depth in Patients Undergoing Supportive Periodontal Therapy. *Oral Health Prev Dent* 14:207-214.

Jarosz, L. M., Deng, D. M., van der Mei, H. C., Crielaard, W., and Krom, B. P. (2009) *Streptococcus mutans* Competence-Stimulating Peptide Inhibits *Candida albicans* Hypha Formation. *Eukaryot Cell* 8:1658-1664.

Jeon, JG., Klein, M. I., Xiao, J., Gregoire, S., Rosalen, P. D., and Koo, H. (2009) Influences of Naturally Occurring Agents in Combination with Fluoride on Gene Expression and Structural Organization of Streptococcus mutans in Biofilms. *BMC Microbiol* 9:228-238.

Jorgensen, M. R., Kragelund, C., Jensen, P. O., Keller, M. K., and Twetman, S. (2017) Probiotic *Lactobacillus reuteri* Has Antifungal Effects on Oral *Candida* Species *in vitro*. *J Oral Microbiol* 9:1-8.

Jozefiak, D., and Sip, A. Bacteriocins in Poultry Nutrition – a Review.

Ann Anim Sci 13:449-462.

Kang, M. S., Kim, B. G., Chung, J., Lee, H. C., and Oh, J. S. (2006) Inhibitory Effect of *Weissella cibaria* Isolates on the Production of Volatile Sulphur Compounds. *J Clin Periodontol* 33:226-232.

Kang, M. S., Na, H. S., and Oh, J. S. (2005) Coaggregation Ability of *Weissella cibaria* Isolates with *Fusobacterium nucleatum* and Their Adhesiveness to Epithelial Cells. *FEMS Microbiol Lett* 253: 323-329.

Keller, M. K., Hasslof, P., Stecksen-Blicks, C., and Twetman, S. (2011) Co-aggregation and Growth Inhibition of Probiotic *Lactobacilli* and Clinical Isolates of *Mutans Streptococci*: An *in vitro* Study. *Acta Odontol Scand* 69:263-268.

Kolenbrander, P. E., Palmer, R. J., Rickard, A. H., Jakubovics, N. S., Chalmers, N. I., and Diaz, P. I. (2000) Bacterial Interactions and Successions During Plaque Development. *Periodontol* 42: 47-79.

Koo, H., Xiao, J., Klein, M. I., and Jeon, J. G. (2010) Exopolysaccharides Produced by *Streptococcus mutans* Glucosyltransferases Modulate the Establishment of Microcolonies Within Multispecies Biofilms. *J Bacteriol* 192:3024-3032.

Krzysciak, W., Jurczak, A., Koscielniak, D., Bystrowska, B., and Skalniak, A. (2014) The Virulence of *Streptococcus mutans* and the Ability to Form Biofilms. *Eur J Clin Microbiol Infect Dis* 33:499-515.

Kubota, H., Senda, S., Nomura, N., Tokuda, H., and Uchiyama, H.

(2008) Biofilm Formation by Lactic Acid Bacteria and Resistance to Environmental Stress. *J Biosci Bioeng* 106: 381-386.

Kumar, B., Kashyap, N., Avinash, A., Chevvuri, R., Sagar, M. K., and Shrikant, K. (2017) The Composition, Function, and Role of Saliva in Maintaining Oral Health: A Review. *Int J Contemporary Dent Medi Rev* 2017:1-6.

Lebeer, S., De Keersmaecker, S. C. J., Verhoeven, T. L. A., Fadda, A. A., Marchal, K., and Vanderleyden, J. (2007) Functional Analysis of *luxS* in the Probiotic Strain *Lactobacillus rhamnosus* GG Reveals a Central Metabolic Role Important for Growth and Biofilm Formation. *J Bacteriol* 189:860-871.

LeBlanc, J. G., Chain, F., Martin, R., Bermudenz-Humaran, L. G., Courau, S., and Langella, P. (2017) Beneficial Effects on Host Energy Metabolism of Short-chain fatty Acids and Vitamins Produced by Commensal and Probiotic Bacteria. *Microb Cell Fact* 16:79-90.

Leccese Terraf, M. C., Mendoza, L. M., Juarez Tomas, M. S., Silva, C., and Nader-Macias, M. E. F. (2014) Phenotypic Surface Properties (Aggregation, Adhesion and Biofilm Formation) and Presence of Related Genes in Beneficial Vaginal *Lactobacilli*. *J Appl Microbiol* 117:1761-1772.

Lee, J. K., Kim, S. J., Ko, S. H., Ouwehand, A. C., and Ma, D. S. (2015) Modulation of the Host Response by Probiotic *Lactobacillus*

brevis CD2 in Experimental Gingivitis. *Oral Dis* 21:705-712.

Lee, SH., and Kim, YJ. (2014) A Comparative Study of the Effect of Probiotics on Cariogenic Biofilm Model for Preventing Dental Caries. *Arch Microbiol* 196:601-609.

Lescheid, D. W. (2014) Probiotics as Regulators of Inflammation: A Review. *Func Food Health Dis* 4:299-311.

Levesque, C. M., Voronejskaia, E., Huang, Y. C., Mair, R. W., Ellen, R. P., and Cvitkovitch, D. G. (2005) Involvement of Sortase Anchoring of Cell Wall Proteins in Biofilm Formation by *Streptococcus mutans*. *Infect Immun* 73:3773-3777.

Li, M. Y., Huang, R. J., Zhou, X. D., and Gregory, R. L. (2013) Role of Sortase in *Streptococcus mutans* Under the Effect of Nicotine. *Int J Oral Sci* 5:206-211.

Li, Y. H., Hanna, M. N., Svensater, G., Ellen, R. P., and Cvitkovitch, D. G. (2001) Cell Density Modulates Acid Adaptation in Streptococcus mutans: Implications for Survival in Biofilms. *J Bacteriol* 183:6875-6884.

Li, Y. H., Tang, N., Aspiras, M. B., Lau, P. C. Y., Lee, J. H., Ellen, R. P., and Cvitkovitch, D. G. (2002) A Quorum-Sensing Signaling System Essential for Genetic Competence in *Streptococcus mutans* is Involved in Biofilm Formation. *J Bacteriol* 184:2699-2708.

Lin, T. H., and Pan, T. M. (2014) Inhibitory Effect of *Lactobacillus paracasei* subsp. *paracasei* NTU 101 on Rat Dental Caries. *J Funct*

Foods 10:223-231.

Lin, X., Chen, X., Chen, Y., Jiang, W., and Chen, H. (2015) The Effect of Five Probiotic *Lactobacilli* Strains on the Growth and Biofilm Formation of *Streptococcus mutans*. *Oral Dis* 21:e128-e134.

Ljungh, A., and Wadstrom, T. (2006) Lactic Acid Bacteria As Probiotics. Curr. *Issues Intestinal Microbiol* 7:73-90.

Loesche, W. J. (1996) Microbiology of Dental Decay and Periodontal Disease. Medical Microbiology 4th edition, Galveston: Texas.

Lof, M., Janus, M. M., and Korm, B. P. (2017) Metabolic Interactions between Bacteria and Fungi in Commensal Oral Biofilms. *J Fungi* 3: 1-13

Magaldi, S., Mata-Essayang, S., Hartung de Capriles, C., Perez, C., Colella, M. T., Olaizola, C., and Ontiveros, Y. (2004) Well Diffusion for Antifungal Susceptibility Testing. *Int J Infect Disease* 8:39-45.

Mahasneh, S. A., and Mahasneh, A. M. Probiotics: A Promising Role in Dental Health. *Dent J* 5:1-10.

Manning, J., Dunne, E. M., Wescombe, P. A., Hale, J. D., Mulholland, E. K., Tagg, J. R., Robins-Browne, R. M., and Satzke, C. (2016) Investigation of *Streptococcus salivarius*-mediated Inhibition of *Pneumococcal* Adherence to Pharyngeal Epithelial Cells. *BMC Microbiol* 16:1-9.

Markowiak, P., and Slizewska, K. (2017) Effects of Probiotics, Prebiotics, and Synbiotics on Human Health. *Nutrients* 9:1021.

Marsh, P. D. (1994) Microbial Ecology of Dental Plaque and its Significance in Health and Disease. *Adv Dent Res* 8:263-271.

Marsh, P. D. (2003) Are Dental Diseases Examples of Ecological Catastrophes? *Microbiol* 149:279-294.

Marsh, P. D. (2010) Microbiology of Dental Plaque Biofilms and Their Role in Oral Health and Caries. *Dent Clin North Am* 54:441-454.

Masdea, L., Kulik, E. M., Hauser-Gerspach, I., Ramseier, A. M., Filippi, A., and Waltimo, T. (2012) Antimicrobial Activity of *Streptococcus salivarius* K12 on Bacteria Involved in Oral Malodour. *Arch Oral Biol* 57:1041-1047.

Matsui, R. and Cvitkovitch, D. (2010) Acid Tolerance Mechanisms Utilized by *Streptococcus mutans*. *Future Microbiol* 5:403-417.

Mattos-Graner, R. P., and Duncan, M. J. (2017) Two-component Signal Transduction Systems in Oral bacteria. J Oral Microbiol 9:1400858.

Meurman, J. H., and Stamatova, I. (2007) Probiotics: Contributions to Oral Health. *Oral Dis* 13:443-451.

Mitchell, T. J. (2003) The Pathogenesis of *Streptococcal* Infections: From Tooth Decay to Meningitis. *Nat Rev Microbiol* 1:219-230.

Monchois, V., Lakey, J. H., and Russell, R. R. B. (1999) Secondary Structure of *Streptococcus downei* GTF-I Glucansucrase. *FEMS Microbiol Lett* 177:243-248.

Monteiro, D. R., Feresin, L. P., Arias, L. S., Barao, V. A., Barbosa, D. B., and Delbern, A. C. (2015) Effect of Tyrosol on Adhesion of *Candida albicans* and *Candida glabrata* to Acrylic Surfaces. *Med Mycol* 53:656-665.

Montero, E., Iniesta, M., Rodrigo, M., Marin, M. J., Figuero, E., Herrera, D., and Sanz, M. (2017) Clinical and Microbiological Effects of the Adjunctive Use of Probiotics in the Treatment of Gingivitis: A Randomized Controlled Clinical Trial. *J Clin Periodontol* 44:708-716.

Morales, A., Carvajal, P., Silva, N., Hernandez, M., Godoy, C., Rodriguez, G., Cabello, R., Garcia-Sesnich, J., Hoare, A., Diza, P. I., and Gamonal, J. (2016) Clinical Effects of *Lactobacillus rhamnosus* in Non-Surgical Treatment of Chronic Periodontitis: A Randomized Placebo-Controlled Trial With 1-Year Follow-Up. *J Periodontol* 87:944-952.

Nguyen, P. T., Falsetta, M. L., Hwang, G., Gonzalez-Begne, M.. and Koo, H. (2014) alpha-Mangostin Disrupts the Development of *Streptococcus mutans* Biofilms and Facilitates Its Mechanical Removal. *PLoS One* 9:e111312.

Nikawa, H., Makihira, S., Fukushima, H., Nishimura, H., Ozaki, Y., Ishida, K., Darmawan, S., Hamada, T., Matsumoto, A., Takemoto, T., and Aimi, R. (2004) *Lactobacillus reuteri* in Bovine Milk Fermented Decreases the Oral Carriage of *Mutans Streptococci*. *Int J Food Microbiol* 95:219-223.

Oldak, A., Zielinska, D., Rzepkowska, A., and Kolozyn-Krajewska, D. (2017) Comparison of Antibacterial Activity of *Lactobacillus plantarum* Strains Isolated from Two Different Kinds of Regional Cheeses from Poland: Oscypek and Korycinski Cheese. *Biomed Res Int* 2017: 1-11.

O'Toole, G. A., Pratt, L. A., Watnic, P. I., Newman, D. K., Weaver, V. B>, and Kolter, R. (1999) Genetic Approaches to Study of Biofilms. *Methods Enzymol* 310:91-109.

Ouwehand, A. C., Salminen, S., and Isolauri, E. (2002) Probiotics: An Overview of Beneficial Effects. *Anton Leeuw* 82: 279-289.

Paster, B. J., Boches, S. K., Galvin, J. L., Ericson, R. E., Lau, C. N., Levanos, V. A., Sahasrabudhe, A., and Dewhirst, F. E. (2001) Bacterial Diversity in Human Subgingival Plaque. *J Bacteriol* 183:3770-3783.

Penala, S., Kalakonda, B., Pathakota, K. R., Jayakumar, A., Koppolu, P., Lakshmi, B. V., Pandey, R., and Mishra, A. (2016) Efficacy of Local Use of Probiotics as an Adjunct to Scaling and Root Planing in Chronic Periodontitis and Halitosis: A Randomized Controlled Trial. *J Res Pharm Pract* 5:86-93.

Petersen, F. C., and Scheie, A. Aa. (2000) Genetic Transformation in Streptococcus mutans Requires a Peptide Secretion-like Appartus. *Oral Microbiol Immuno* 15:329-334.

Petersen, P. E., Bourgeois, D., Ogawa, H., Estupinan-Day, S., and

Ndiaye, C. (2005) The Global Burden of Oral Diseases and Risks to Oral Health. *Bulletin of the WHO* 83:661-669.

Peterson, S. N., Meissner, T., Su, A. I., Snesrud, E., Ong, A. C., Schork, N. J., and Bretz, W. A. (2014) Functional Expression of Dental Plaque Microbiota. *Front Cell Infect Microbiol* 4:1-13.

Petti, S., Tarsitani, G., and D'Arca, A. S. (2001) A Randomized Clinical Trial of the Effect of Yoghurt on the Human Salivary Microflora. *Arch Oral Biol* 46:705-712.

Philip, N., Suneja, B., and Walsh, L. J. (2018) Ecological Approaches to Dental Caries Prevention: Paradigm Shift or Shibboleth? *Caries Res* 52:153-165.

Pitts, N. B., Zero, D. T., Marsh, P. D., Ekstrand, K., Weintraub, J. A., Ramos-Gomez, F., Tagami, J., Twetman, S., Tsakos, G., and Ismail, A. (2017) Dental caries. *Nat Rev Dis Primers* 3:1-16.

Plaza-Diaz, J., Ruiz-Ojeda, F. J., Vilchez-Padial, L. M., and Gil, A. (2017) Evidence of the Anti-Inflammatory Effects of Probiotics and Synbiotics in Intestinal Chronic Diseases. *Nutrients* 9:1-19.

Pleszczynska, M., Wiater, A., Janczarek, M., and Szczodrak, J. (2015) (1→3)-α-D-Glucan Hydrolases in Dental Biofilm Prevention and Control: A Review. *Int J Biol Macromol* 79:761-778

Raj, A., Bhati, P., and Bhadekar, R. (2017) Effect of Lactic Acid bacteria on Biofilm Formation by Streptococcus mutans: An in vitro Study, Int J Pharmaceutical Sci Research 8:2533-2538.

Russell, M. W., Harrington, D. J., and Russell, R. R. B. (1995) Identity of *Streptococcus mutans* Surface Protein Antigen III and Wall-Assocaited Protein Antigen A. *Infect Immun* 63:733-735.

Russell, R. R. B., Opoku, J. A., Sutcliffe, I. C., Tao, L., and Ferretti, J. J. (1992) A Binding Protein-Dependent Transport System in *Streptococcus Mutans* Responsible For Multiple Sugar Metabolism. *J Biol Chem* 267:4631-4637.

Salas-Jara, M. J., Ilabaca, A., Vega, M., and Garcia, A. (2016) Biofilm Forming *Lactobacillus*: New Challenges for the Development of Probiotics. *Microorganisms* 4:35.

Salehi, R., Savabi, O., Kazemi, M., Kamali, S., Salehi, A. R., Eslami, G., and Tahmourespour, A. (2014) Effects of Lactobacillus reuteri-derived Biosurfactant on the Gene Expression Profile of Essential Adhesion Genes (gtfB, gtfC and ftf) of *Streptococcus mutans*. Adv Biomed Res 3:169.

Schlagenhauf, U., Jakob, L., Eigenthaler, M., Segerer, S., Jockel-Schneider, Y., and Rehn, M., (2016) Regular Consumption of *Lactobacillus reuteri*-containing Lozenges Reduces Pregnancy Gingivitis: An RCT. *J Clin Periodontol* 43:948-954.

Schneewind, O., and Missiakas, D. M. (2012) Protein Secretion and Surface Display in Gram-positive Bacteria. *Philos Trans R Soc Lond B Biol Sci* 367:1123-1139.

Schroeder, B. O., Birchenough, G. M. H., Stahlman, M., Arike, L.,

Johansson, M. E. V., Hansson, G. C., and Backhed, F. (2018) *Bifidobacteria* or Fiber Protects against Diet-Induced Microbiota-Mediated Colonic Mucus Deterioration. *Cell Host Microbe* 23:27-40.

Schwendicke, F. Korte, F., Dorfer, C. E., Kneist, S., Fawzy El-Sayed, K., and Paris, S. (2017) Inhibition of *Streptococcus mutans* Growth and Biofilm Formation by Probiotics *in vitro*. *Careis Res* 51:87-95.

Segers, M. E., and Lebeer, S. (2014) Toward a Better Understanding of *Lactobacillus rhamnosus* GG - Host Interactions. *Microb Cell Fact* 13:1-16.

Selle, K., and Klaenhammer, T. R. (2013) Genomic and Phenotypic Evidence for Probiotic Influences of *Lactobacillus gasseri* on Human Health. *FEMS Microbiol Rev* 37:915-935.

Selwitz, R. H., Ismail, A., and Pitts, N. B. (2007) Dental Caries. *Lancet* 369:51-59.

Senadheera, M. D., Guggenheim, B., Spatafora, G. A., Huang, Y. C., Choi, J., Hung, D. C., Treglown, J. S., Goodman, S. D., Ellen, R. P. and Cvitkovitch, D. G. (2005) A *vicRK* Signal Transduction System in *Streptococcus mutans* Affects *gtfBCD*, *gbpB*, and *ftf* Expression, Biofilm Formation, and Genetic Competence Development. *J Bacteriol* 187:4064-4076.

Shukla, C., Maurya, R. K., Singh, V., and Tijare, M. (2016) Evaluation of Chagnes in *Streptococcus mutans* Colonies in Microflora of the Indian Population with Fixed Orthodontics

Appliances. *Dent Research J* 13:309-314.

Socransky, S. S., and Haffajee, A. D. (2002) Dental Biofilms: Difficult Threapeutic Targets. *Periodontol* 28:12-55.

Soderling, E. (2012) Probiotics and Dental caries. *Microb Ecol Health Dis* 23:55-56.

Song, L., Wang, W., Conrads, G., Rheinberg, A., Sztajer, H., Reck, M., Wagner-Doble, I., and Zeng, A. (2013) Genetic Variability of *Mutans Streptococci* Revealed by Wide Whole-genome Sequencing. *BMC genomics* 14:2-24.

Spano, G. Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernandez, M., Lopez, P., de Palencia, P. F., Corbi, A., Trip, H., and Lolkema, J. S. (2010) Biogenic Amines in Fermented Foods. *Eur J Clin Nutr* 64:S95-S100.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., and Svabic-Vlahovic, M. (2000) A Modified Microtiter-plate Test for Quantification of *Staphylococcal* Biofilm Formation. *J. Microbiol Methods* 40:175-179.

Stipp, R. N., Boisvert, H., Smith, D. J., Hofling, J. F., Duncan, M. J., and Mattos-Graner, R. O. (2013) *CovR* and *VicRK* Regulate Cell Surface Biogenesis Genes Required for Biofilm Formation in *Streptococcus mutans*. *PLoS One* 8:1-13.

Stock, A. M., Robinson, V. L., and Goudreau, P. N. (2000) Two-

Component Signal Transduction. *Annu Rev Microbiol* 69:183-215.

Sun, M., Wu, N., and Chen, H. (2017) Laser-assisted Rapid Mineralization of Human Tooth Enamel. *Sci Rep* 7:9611.

Sutherland, I. W. (2001) Biofilm Exopolysaccharides : A Strong and Sticky Framework. *Microb* 147:3-9.

Takahashi, N., and Nyvad, B. (2011) The Role of Bacteria in the Caries Process: Ecological Perspectives. *J Dent Res* 90: 294-303.

Tanzer, J. M., Thompson, A., Lang, C., Cooper, B., Hareng, L., Gamer, A., Reindl, A., and Pompejus, M. (2010) Caries Inhibition by and Safety of Lactobacillus paracasei DSMZ16671. *J Dent Res* 89:921-926.

Toiviainen, A., Jalasvuori, H., Lahti, E., Gursoy, U., Salminen, S., Fontana, M., Flannagan, S., Eckert, G., Kokaras, A., Paster, B. J., and Soderling, E. (2015) Impact of Orally Administered Lozenges with *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on the Number of Salivary *Mutans streptococci*, Amount of Plaque, Gingival Inflammation and the Oral Microbiome in Healthy Adults. *Clin Oral Invest* 19:77-83.

Twetman, L., Larsen, U., Fiehn, N. E., Stecksen-Blicks, C., and Twetman, S. (2009) Coaggregation between Probiotic Bacteria and Caries-Associated Strains: An *in vitro* Study. *Acta Odontol Scand* 67:284-288.

Ueda, S., Shiroza, T., and Kuramitsu, H. K. (1988) Sequence

Analysis of the *gtfC* Gene from *Streptococcus mutans* GS-5. *Gene* 69: 101-109.

Ukena, S. N., Singh, A., Dringenberg, U., Engelhardt, R., Seidler, U., Hansen, W., Bleich, A., Bruder, D., Franzke, A., Rogler, G., Suerbaum, S., Buer, J., Gunzer, F., and Westendorf, A. M. (2007) Probiotic *Escherichia coli* Nissle 1917 Inhibits Leaky Gut by Enhancing Mucosal Integrity. *PLoS One* 2:1-9.

Vacca-Smit, A. M., and Bowen, W. H. (1998) Binding Properties of *Streptococcal* Glucosyltransferases for Hydroxyapatite, Saliva-coated Hydroxyapatite, and Bacterial Surfaces. *Arch Oral Biol* 43:103-110.

Venegas, S. C., Palacios, J. M., Apella, M. C., Morando, P. J., and Blesa, M. A. (2006) Calcium Modulates Interactions betwwen Bacteria and Hydroxyapatite. *J Dent Res* 85:1124-1128.

Vu, B., Chen, M., Crawford, R. J., and Ivanova, E. P. (2009) Bacterial Extracellular Polysaccharides Involved in Biofilm Formation. *Molecules* 14:2535-2554.

Waterhouse, J. C., Swan, D. C., and Russell, R. R. B. (2007) Comparative Genome Hybridization of *Streptococcus mutans* Strains. *Oral Microbiol Immuno* 22:103-110.

Wasfi, R., Abd El-Rahman, O. A., Zafer, M. M., and Ashour, H. M. (2018) Probiotic *Lactobacillus* sp. Inhibit Growth, Biofilm Formation, and Gene Expression of Caries-inducing *Streptococcus mutans*. *J*

Cell Mol Med 22:1972-1983.

Whittaker, C. J., Klier, C. M., and Kolenbrander, P. E. (1996) Mechanisms of Adhesion by Oral Bacteria. *Annu Rev Microbiol* 50:513-552.

Wu, C. C., Lin, C. T., Wu, C. Y., Peng, W. S., Lee, M. J., and Tsai, Y. C. (2015) Inhibitory Effect of *Lactobacillus salivarius* on *Streptococcus mutans* Biofilm Formation. *Mol Oral Microbiol* 30:16-26.

Wu, X., Vallance, B. A., Boyer, L., Bergstrom, K. S., Walker, J., Madsen, K., O'Kusky, J. R., Buchan, A. M., and Jacobson, K. (2008) Saccharomyces boulardii Ameliorates Citrobacter rodentium-induced Colitis Through Actions on Bacterial Virulence Factors. *Am J Physiol Gastrointest Liver Physiol* 294:G295-306.

Xu, H., Jeong, H. S., Lee, H. Y., and Ahn, J. (2009) Assessment of Cell Surface Properties and Adhesion Potential of Selected Probiotic Strains. *Lett Appl Microbiol* 49:434-442.

Yadav, R., Puniya, A. K., and Shukla, P. (2016) Probiotic Properties of *Lactobacillus plantarum* RYPR1 from an Indigenous Fermented Beverage Raabadi. *Front Microbiol* 7:1683.

Zaura, E., Keijser, B. J., Huse, S. M., and Crielaard, W. (2009) Defining the Healthy "Core Microbiome" of Oral Microbial Communities. *BMC Microbiol* 9:259.

Zhu, L., Kreth, J., Cross, S. E., Gimzewski, J. K., Shi, W., and Qi, F. (2006). Functional Characterization of Cell-Wall-Associated Protein WapA in *Streptococcus mutans*. *Microbiology* 152:2395-2404.

Abstract in Korean

충치는 치아 표면에 존재하는 enamel의 demineralization에 의하여 발생한다. 구강 내의 높은 산성도는 enamel 층의 주요 구성 성분인 수산화인회석의 분해를 일으키게 된다. 구강 미생물 중에서도 충치를 유발하는 미생물 들은 구강 내에서 산성도를 높이는 여러 요인 중 하나이다. 충치 유발 미생물들은 구강 내 잔여 탄수화물을 에너지원으로 사용하고, glucosyltransferase 라는 효소를 이용하여 바이오필름을 형성하며, 유기산들을 생성한다. 건강한 구강 내에서는 일반적으로 인간의 침에 의해 낮은 pH 환경이 중화된다. 그러나 바이오필름이 형성될 시, 바이오필름은 인간의 침이 바이오필름 내부로 들어오는 것을 방지하고, 내부의 유기산들이 밖으로 방출되는 것을 억제한다. 결과적으로 바이오필름 내부에서는 유기산들이 갇혀 높은 산성 환경이 유지되게 된다. *Streptococcus mutans*는 glucosyltransferase를 생성하지 못하는 타 미생물에게 glucosyltransferase를 전달하여 해당 미생물 또한 glucan 형성이 가능하게 되어 바이오필름 형성을 가속화시키기 때문에 충치 유발 주요 원인 균으로 잘 알려져 있다. 따라서 본 연구의 목적은 충치의 주요 원인 균인 *S. mutans*의 바이오필름 형성을 감소시켜 충치 발생을 억제 할 수 있는 유산균 주의 스크리닝 및 평가이다. 본 연구에서는 기존에 타 미생물에 대하여 항균활성 능력이 검증 된 유산균 21 종류를 사용하였다. 안전성 검사를 실시한 결과 EFSA가 제시하는 기준에 21가지 유산균이 모두 부합한

것을 확인하였고, 이는 구강으로 섭취하였을 때, 건강에 이상이 없음을 의미한다. *Lactobacillus paracaei* M9-1은 실험에 사용된 유산균 중에서 가장 높은 항균 활성 능력을 보였으며 (> 14mm), reference strain인 *Lactobacillus rhamnosus* GG보다 더 높은 co-aggregation 능력과 가장 높은 biofilm 형성을 억제 능력을 보였다. 따라서 *L. paracasei* M9-1이 *S. mutans*의 biofilm 형성을 억제할 수 있는 잠재적인 프로바이오틱스 후보로 선발 되었다. *L. paracasei* M9-1이 *S. mutans*의 충치 유발 관련 유전자들에 미치는 영향을 평가해 본 결과, 부착 기능과 관련된 유전자들의 발현을 감소시키는 것을 확인하였다. 그러므로 본 논문은 *L. paracasei* M9-1이 항균활성 능력, co-aggregation 능력, *S. mutans*의 부착 단백질들을 발현하는 유전자들의 발현을 억제하여 *S. mutans*의 바이오필름 생성을 억제할 수 있기 때문에 충치를 감소시킬 수 있는 프로바이오틱스임을 확인하였다.

주요어 : 충치, *Streptococcus mutans*, 바이오필름, 유산균, 프로바이오틱스

학 번 : 2017-28580

Acknowledgement

2017년 2월 1일에 평창으로 와서 실험실 생활을 시작한지가 엊그제 같은데 어느 새 벌써 2년의 시간이 흘러갔습니다. 저의 31년 생활 중, 가장 빠르게 지나갔던 2년인 것 같습니다. 인생을 살면서 한번쯤은 후회하지 않게 살아보자 라는 생각을 가지고 입학하였었는데, 2년이 지나서 졸업할 시기가 다가오니 여러모로 감회가 새롭습니다.

가장 먼저 2년 동안 저를 지도해주셨던 허철성 교수님께 감사의 인사를 드리고 싶습니다. 대학원 진학을 생각하기 전, 실험실 생활을 경험해보기 위하여 수 차례 타 실험실들에 인턴 지원서를 제출하였을 때, 수락 해주는 곳은 아무 곳도 없었습니다. 아무런 연락조차 없었고, 심지어 학부 전공도 다른 상태로 지원하였던 저를 받아주셨습니다. 교수님께서 지도해주신 덕분에 2년이라는 시간 동안 유산균이란 미생물을 재미있게 배워갈 수 있었으며, 능력이 많이 부족한 저를 걱정해주시고, 졸업 이후 제 미래에 대하여 조언을 해주셔서 감사합니다. 앞으로도 유산균은 정말 저에게 큰 비중을 차지할 것 같습니다.

다음으로는 저희 졸업 논문 심사를 위하여 심사위원으로 참석해주신 박병철 교수님과 박태섭 교수님께 감사의 인사를 드리고 싶습니다. 바쁘신 와중에도 제가 작성한 논문을 세세히 읽어주시면서 완성도를 높여주시기 위해 첨삭까지 해주셔서 감사 드리며, 운이 좋게 두 교수님의 수업을 모두 수강할 수 있어서 견문을 조금 더 넓힐 수 있었습니다.

또한, 제가 처음 평창캠퍼스에 입학한 시점부터 본 논문을 작성하는데

있어서 처음 구상 단계부터 완성하는 순간까지 함께 토론해주시고 질문이 생길 때마다 매번 자세히 조언해주시고, 건강에는 이상이 없는지 걱정해주시며, 실험이 잘 진행되지 않을 때는 직접 실험을 지도해주셨던 김인선 박사님, 입학한지 얼마 안되고 기초 지식과 실험 능력이 부족한 저를 지도해주시고 가르쳐 주셨던 여소영 연구원님, 비록 주제는 바뀌었지만 바뀌기 전 주제에서 실험이 막혀있을 때, 본인 과제가 아님에도 불구하고 지도해주셨던 두은희 박사님, 논문 리뷰 시 발생한 의문점들을 질문할 때마다 밤늦게 까지 함께 남아서 조언해주신 류리 박사님, 질문 할 때마다 항상 웃으면서 직접 찾아서 답변해주시고 실험 또한 알려주셨던 김지희 연구원님께도 대단히 감사의 말씀을 드리고 싶습니다. 처음 왔을 때, 졸업 학기 임에도 불구하고 첫 오리엔테이션 대상자인 저에게 다양한 실험을 알려주었던 도선이 형, 승일 선배, 혜원 선배, 저에게 실험을 알려주고 유산균에 대하여 소개해 준 유경 선배, 졸업을 한 지금까지도 모르는 부분에 대하여 알려주는 은지 선배와 실험실의 맏형으로서 저희 생활에 어려움이나 불편함이 없는지 세세히 체크해주시고 어려울 때 기댈 수 있는 범주 형, 실험 기법이나 논문 작성법에 대하여 질문하였을 때 귀찮아하지 않고 조언해주었던 손상모 박사과정 학생, 밤늦게까지 실험을 하고 있을 때 도와줄 건 없는지, 도와줄 게 있다면 함께 남아 도와주었던 상현이 형, 입학 학기는 다르지만 거의 비슷한 시기에 와서 항상 챙겨주고 도와주었던 원준이, 옆자리에서 있으면서 걱정해주고 도와주었던 은솔이, 본인도 바쁘지만 도와달라고 했을 때 거리낌 없이 도와준 예림이, 첫 학기라 바쁜 와중에도 두 팔 걷고 도와준 정욱이와 입학 학기보다 먼저 와서

대부분의 실험을 함께하였던 규민이에게도 큰 은혜를 받았습니다. 제가 이곳에서 아쉬운 점이 있다면 여러분들께서 도와주신 만큼, 저 또한 여러분들께 도움을 드렸어야 했는데 저 바쁘다는 핑계로 제대로 도와드리지 못한 것 같아 많이 후회가 됩니다. 함께 해준 여러분들 덕분에 2년이란 시간, 힘들 때도 있었지만 웃으면서 살아 갈 수 있었습니다. 정말 감사합니다.

이제 졸업을 하여 사회로 나가게 된다면, 서울대학교 국제농업기술대학원 유가공 및 장내미생물학 실험실 졸업생으로서, 저희 연구실에 부끄럽지 않도록 훌륭한 연구자가 되겠습니다. 여러분과 함께 할 수 있어서 정말 즐겁고 행복하였으며 또한 영광이었습니다. 평창에서의 2년은 잊지 못할 추억이 될 것이고, 이곳에서의 인연이 계속해서 유지되었으면 좋겠습니다.

마지막으로, 돌이켜 보면 방황이 참 많았는데, 그 때마다 저의 선택을 존중해주시고 지원해주셨던 저희 가족, 2년 동안 룸메이트로 같이 살았던 범석이, 같은 층에서 사무실을 쓰면서 지냈던 국제협력개발 트랙 식구들, 실험에 관련하여 질문하였을 때 도움을 많이 주신 가금방 식구들에게도 감사의 인사를 전하며 이만 마치겠습니다. 감사합니다.