

**ORAL PROBIOTIC EFFECT OF LACTOBACILLUS
CASEI SHIROTA ON STREPTOCOCCUS MUTANS**

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.


Chen Huizhen

March 16, 2014

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Summary

Being one of the most common infectious diseases, dental caries is a transmissible bacterial infection caused by acids from bacterial metabolism which accumulate on enamel surface and dissolve the enamel hydroxyapatite crystals. *Streptococcus mutans* is one of the important cariogenic bacteria with high acidogenicity and acid tolerance. The traditional preventive therapies, like fluoride and chlorhexidine, have limitations and potential side effects. Probiotic treatments have demonstrated some promising effects in caries prevention. However, the exact mechanisms remain unclear.

Yakult[®] is a popular sweet probiotic drink containing live *Lactobacillus casei* Shirota strain. Its beneficial effects on gastrointestinal diseases, allergy, and immune system have been reported. However, there is no study reporting the effect of Yakult[®] or the probiotic bacteria *L. casei* Shirota on *S. mutans*.

In this study, the inhibitory effects of Yakult[®] and *L. casei* Shirota on *S. mutans* biofilm formation and acidogenicity were evaluated, as well as the potential mechanisms. The major findings are summarized as follows:

(1) Yakult[®] and *L. casei* Shirota decreased *S. mutans* biofilm formation on the tooth surface with the reduced biofilm acidogenicity and lesion depth in enamel

demineralization.

(2) Although there is no bactericidal effects, *L. casei* Shirota significantly decreased *S. mutans* extracellular polysaccharide (EPS) production, biofilm formation and acidogenicity by suppressing *gtfB*, *gtfC* and *ldh* expression in *S. mutans*.

(3) *L. casei* Shirota cell free culture supernatant (CFCS) inhibited the growth of *S. mutans*. The inhibitory compounds were heat stable and active in an acidic environment.

This study has deepened the understanding of cariostatic/probiotic effect of Yakult[®] and *L. casei* Shirota, and may facilitate the clinical application of this non-fluoride “sweet” therapy to prevent caries formation, particularly in children.

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Abbreviations

AEP: acquired enamel pellicle
AMCA: aminomethylcoumarin
ATR: acid tolerance response
AUC: area under the curve
BHI: Brain heart infusion
BHIS: BHI supplemented with 1% sucrose
BOT: biofilm-on-tooth
CFCS: cell free culture supernatant
cfu: colony forming unit
CHX: chlorhexidine
CLSM: confocal laser scanning microscope
Cy: cyanine
d: day
DexA: dextranase
DMFT: decayed, missing, filled teeth
EPS: Extracellular polysaccharides
FAO: Food and Agriculture Organization of the United Nations
FISH: fluorescence *in situ* hybridization
FITC: fluorescein isothiocyanate
FruA: fructanase
Ftf: fructosyltransferase
GA: glutaraldehyde
Gtf: glucosyltransferase
h: hour
HA: hydroxyapatite
IFN- γ : Interferon- γ
IL: induce interleukin
IPS: intracellular polysaccharides
LAB: lactic acid bacteria
LcS: *Lactobacillus casei* Shirota
LGG: *Lactobacillus rhamnosus* GG
LPS: lipopolysaccharide
min: minute
MRS: De Man, Rogosa, Sharpe
MS: mutans streptococci
MS agar: Mitis salivarius agar
PBS: phosphate buffer saline
PFA: *Paraformaldehyde*
PLM: polarized light microscope
RCT: randomized controlled trial

RTE-VPMP: ready-to-eat vacuum-packaged meat products

sd: standard deviation

SEM: Scanning electronic microscope

sHA: saliva-coated HA

SM: *Streptococcus mutans*

SS: *Streptococcus sanguinis*

TNF- α : tumour necrosis factor- α

TRITC: tetramethylrhodamine isothiocyanate

WapA: wall-associated protein A

WHO: world health organization

Publications

International Conferences:

(1) Hui-Zhen Chen, Chin-Ying Stephen Hsu, Lian Hui Zhang, Yuan Kun Lee. Antibacterial effect of probiotic *Lactobacillus casei* Shirota towards *Streptococcus mutans*. The 25th IADR-SEA Division Annual Scientific Meeting. 2011, Singapore.

(2) Hui-Zhen Chen, Chin-ying Stephen Hsu, Yuan Kun Lee, Lian Hui Zhang. Potential Cariostatic Effect of *Lactobacillus casei* Shirota. The sixth International Association for Dental Research Pan-European Region Meeting (IADR/PER). 2012, Helsinki, Finland.

(3) Hui-Zhen Chen, Chin-ying Stephen Hsu, Yuan Kun Lee, Lian Hui Zhang. Interaction of *Streptococcus mutans* and *Lactobacillus casei* Shirota in Biofilm. The 26th IADR-SEA Division Annual Scientific Meeting. 2012, Hong Kong.

(4) Hui-Zhen Chen, Chin-ying Stephen Hsu, Yuan Kun Lee, Lian Hui Zhang. Inhibitory Effect of Probiotics on *Streptococcus mutans* Biofilm. 2nd Meeting of the International Association of Dental Research-Asia Pacific Region (IADR-APR). 2013, Bangkok, Thailand.

CHAPTER 1

LITERATURE REVIEW

Chapter 1:

Literature Review

1.1 Dental caries

1.1.1 Caries and its prevalence

Dental caries is one of the most common and costly diseases in the world. It is a chronic diet-related infection, with bacterial metabolic by-products inflicting damage to the dental structures. Although caries is not a life-threatening condition, it has an important role in the manifestation of tooth pain and loss, leading to a decrease in the quality of life (Petersen, 2003). In many developing countries, access to oral health services is limited and teeth are often left untreated or are extracted because of pain or discomfort. Moreover, oral health presents a close association with the individual's general health, and may be a risk factor for systemic diseases, such as cardiovascular disease and diabetes (Petersen, 2003).

According to the World Health Organization (WHO), dental caries affected 60% to 90% of school going children as well as the majority of adults (Petersen, 2003). Caries is the most prevalent oral disease in most industrialized countries. In 2010, it was reported that oral diseases affected 3.9

billion people globally, and caries in permanent teeth was the most prevalent (average prevalence of 35% for all ages combined) (Marcenes et al., 2013). The prevalence of dental caries in USA during 1999-2002 was 41% among children aged 2-11 years, and 86.8% for 20-39 years old adults (Beltrán-Aguilar ED, 2005). The prevalence in Singapore was 48.9% for 5-6 years old children as reported in 2008 (Gao XL, 2009) and 96.6% for 20-65 years old adults in 1996 (Loh T, 1996). Dental caries has historically been considered the most important global oral health burden (Petersen, 2003). These epidemiologic data indicate the importance and urgent need for effective caries prevention and control.

1.1.2 Tooth structure and hydroxyapatite dissolution

1.1.2.1 Composition and structure of dental hard tissues

The dental hard tissues consist of enamel, dentin and cementum (Fig. 1.1). Enamel is the outer layer of the tooth, covering dentin and pulp cavity. It is composed of 96% wt% of mineral and 4% of water and organic material. The enamel mineral consists mainly of calcium, phosphate and hydroxyl ions in a stoichiometric ratio $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ which actually is the formula of the mineral “hydroxyapatite (HA)” (Weatherell, 1975). The underlying dentine consists of more organic components than enamel, including type I collagen

and non-collagenous proteins (Smith et al., 2012). Cementum is a calcified substance covering the root of a tooth. The organic matrix of human cementum consists mainly of type I collagen and type III collagen (Bosshardt and Selvig, 1997).

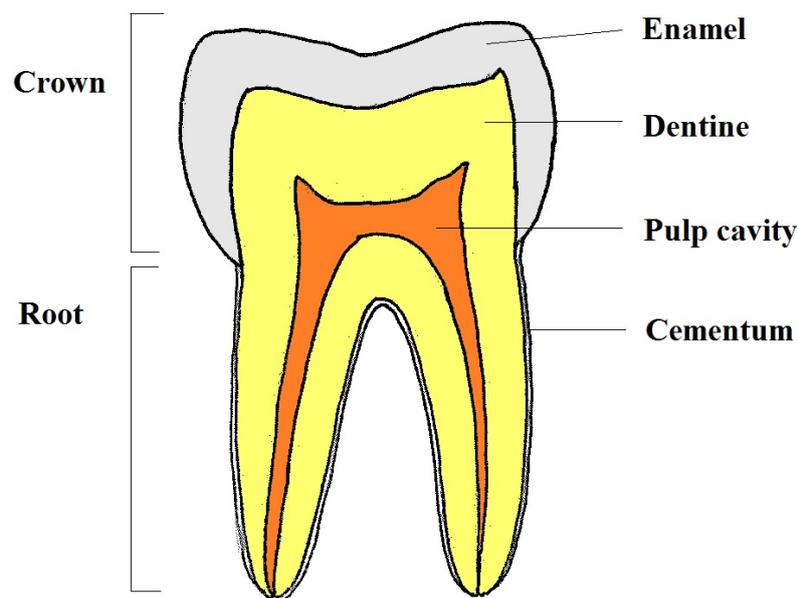


Fig. 1.1. A schematic picture of the tooth structure

1.1.2.2 Enamel hydroxyapatite dissolution in early caries

Early enamel caries is observed clinically as a white spot caused by acids from bacterial metabolism in dental plaque.

A reaction occurs back and forth between precipitation and dissolution

when hydroxyapatite (HA) contacts with water (Borggreven et al., 1986; Dawes, 2003). A very small amount of HA dissolves, with calcium, phosphate and hydroxyl ions released. The releasing continues until the water is saturated, with equal rates of dissolution and mineral precipitation. Saliva and plaque fluid contain calcium, phosphate and hydroxyl ions; therefore, only when the pH is reduced to less than the critical pH, usually 5.5, enamel dissolution occurs. The critical pH is lower when more calcium and phosphate are present in saliva or plaque fluid, as the ion levels may vary among different individuals.

The solubility of enamel increases in acid. On one hand, when $[H^+]$ increases in an acid solution, $[OH^-]$ is removed to form water. On the other hand, the lower the pH, the lower is the concentration of PO_4^{3-} in saliva or plaque fluid. The inorganic phosphate can be present in other forms, like H_3PO_4 , $H_2PO_4^-$ and HPO_4^{2-} , with various proportions depending on the pH value. As the concentrations of both OH^- and PO_4^{3-} reduces, the amount of calcium also reduces to keep the saliva and plaque fluid saturated (Dawes, 2003). This is the process of enamel HA dissolution in acid.

1.1.3 Dental plaque

1.1.3.1 Dental plaque formation

Dental caries is the result of demineralization of the enamel due to acid production from the fermentation of dietary carbohydrates by cariogenic bacteria species in dental plaque (van Houte, 1994). Dental plaque is a structurally and functionally organized biofilm formed on tooth surface. It is a diverse community of bacteria, embedded in an extracellular slime layer (Marsh, 2004) . When the bacteria attach to a surface and to each other, they cluster together to form sessile, mushroom-shaped biofilm attached to the surface with a narrow base (JS, 2003).

Distinct stages in plaque formation include:

(1) The acquired pellicle formation. The acquired enamel pellicle (AEP) is a thin film that forms on tooth surfaces when exposed to the oral environment. The AEP is composed predominantly of salivary proteins, and also of non-salivary-derived proteins, carbohydrates, and lipids (Siqueira et al., 2012). The salivary proteins, such as α -amylase, carbonic anhydrase 6, and cystatin S which present a great affinity to hydroxyapatite initiate the pellicle formation process *via* electrostatic interactions (Hay, 1973). Thereafter, the salivary proteins aggregate saliva to AEP by means of protein-protein interactions, for example neutrophil defensin 1 and lysozyme. The salivary glycoproteins (e.g. those containing sialic acid, phosphate, or sulfate),

immunoglobulins (IgA, IgG, IgM) and enzymes as well as blood group-reactive substances have also been detected in pellicles (Houte, 1982). The pellicle thickness increases from 100 to 1,000 nm within 30 to 90 minutes, depending on its location within the oral cavity (Hannig, 1999). AEP is involved in the lubrication of tooth surfaces, and also plays an important role in the regulation of mineral homeostasis, host defense and microbial colonization *via* the selectivity for bacterial adherence (Hannig and Joiner, 2006; Lendenmann et al., 2000).

(2) Bacteria adherence to tooth surface (attachment). The members of the mutans group of streptococci, such as *Streptococcus mitis*, *Streptococcus sanguinis* and *Streptococcus oralis* are considered to be the first colonizers in dental plaque (Li et al., 2004). Actinomyces are also found in early stages of dental plaque formation (Li et al., 2004).

It has been suggested that plaque formation consisted of two processes that involved separate mechanisms (Busscher et al., 1986; Cowan et al., 1986). The first step is reversible by macroscopic surface properties, involving lower-affinity association between cell and pellicle. In this phase, the initial loose association of bacteria with surfaces results from attraction by van der Waal's forces. As the organisms come closer to the surface, they are repelled by the negative electrostatic charges possessed by most natural surfaces and by

most bacteria. The second step involves irreversible attachment by microscopic and molecular interactions. In the phase of loose association, the bacteria are frequently located approximately 10 nm from the surface. It is believed that the adhesins on the filamentous appendages of the bacteria can bridge this space to form hydrogen, hydrophobic, or other types of bonds (Gibbons, 1984). Bacterial adhesins possess lectin-like or hydrophobic properties, and they are frequently present in filamentous surface appendages, such as pili or fimbriae (Gibbons, 1984). The adhesins bind to complementary components on host tissues.

H.J. Busscher and A.H. Weerkamp proposed the hypothesis on the mechanism of bacterial adhesion to solid substrata (Fig. 2.2) (Busscher and Weerkamp, 1987). Specific interactions are defined as the interactions which occur over extremely short distances allowing specific ionic, hydrogen and chemical bonds. Non-specific interactions are defined as interactions due to overall surface properties as charge or surface free energy.

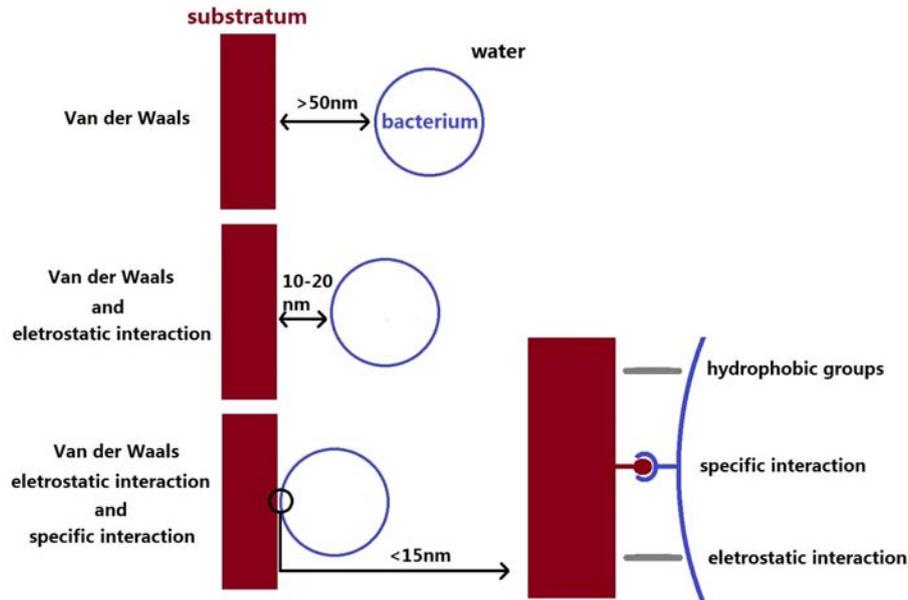


Fig. 1.2. Schematic representation of interactions involved in bacterial adhesion to solid substrata. Adapted with kind permission from John Wiley & Sons, Inc. (Busscher and Weerkamp, 1987).

When separation distances are more than 50 nm, only attractive Van der Waals forces operate. When at separation distances between 10-20 nm, interactions occur due to electrostatic repulsion. In this state, adhesion is reversible, but adhesion changes gradually to less reversible or essentially irreversible. Due to the role of hydrophobicity and hydrophobic surface component, water films between the interacting surfaces are removed at the end, which enables specific short-range interactions to occur. When separation distances are less than 1.5 nm, at this extremely short distance the energy barrier has been overcome and specific interactions occur, leading to an irreversible bonding.

(3) Co-adhesion of late colonizers to early colonizers.

This stage also involves specific adhesin-receptor interactions (often involving lectins) among bacteria leading to an increase in the diversity of the plaque. The unusual morphological structures, such as corn-cobs and rosettes are formed in this stage (Kolenbrander, 2000). This increased bacteria density on the tooth surface results in adhesive interactions between different types of bacteria, leading to the enhancing or inhibitory effects of bacteria upon each other (Houte, 1982).

(4) Multiplication and biofilm formation. Bacterial cell division leads to confluent growth. The complex extracellular matrix made up of soluble and insoluble glucans, fructans and heteropolymers is formed. Eventually, a three-dimensional spatially and functionally organized biofilm is constructed. The biofilm structure is biologically active and retains nutrients, water and key enzymes (Allison, 2003). Studies suggested that the extracellular glucan enhances the accumulation of bacteria in dental plaque (Houte, 1982).

(5) Detachment. Bacteria can respond to environmental changes and detach from surfaces. For example, some enzymes may hydrolyze the fimbria-associated adhesins that anchor cells to the surface (Cavedon and London, 1993). An endogenous enzyme, termed surface protein-releasing enzyme, was reported to be responsible for the detachment of *Streptococcus mutans* biofilm (Lee et al., 1996).

1.1.3.2 Dental plaque structure and components

Dental plaque has an open architecture with channels traversing throughout the biofilm to the tooth surface (Auschill et al., 2001; Wood et al., 2000). Bacterial vitality varies in the biofilm, with the most viable bacteria present in the central part of plaque, and lining the voids and channels (Auschill et al., 2001). The open architecture combined with the synthesis of a matrix comprised of a diverse range of exopolymers, creates a complex environment in dental plaque. Uneven patterns of penetration of radiolabelled fluoride, sucrose and phosphate in plaque have been reported (Robinson et al., 1997). The gradients (nutrients, pH, oxygen) develop are critical to microbial growth; the gradients in pH are also responsible for enamel demineralization. Considerable heterogeneity in pH has been demonstrated over relatively short distances (Vroom et al., 1999). The heterogenic environment enables micro-organisms to co-exist in plaque biofilms. This may explain how organisms with opposing metabolic requirements grow at the same site (Marsh, 2004).

Dental plaque is a diverse community of micro-organisms, embedded in an extracellular matrix of polymers. Molecular studies using 16S rRNA amplification have demonstrated the diversity of the resident oral microflora,

both in health and disease (Marsh, 2004; 2005). It is estimated that less than 50% of bacteria cells in dental plaque can be cultured in the laboratory (Wade, 2002). There are still a lot of unculturable bacteria as the *in vitro* culture condition may not allow these bacteria grow. The composition of dental plaque also varies on different anatomical surfaces, such as fissures, approximal and smooth surfaces, gingival crevice (Bowden et al., 1975; Theilade et al., 1982).

In dental plaque, 10–20% dry weight is made up of glucan while approximately 1-2% comprise of fructan. These proportions vary depending in part on the duration since the last food intake. Dental plaque also harbors approximately 40% dry weight protein (mostly derived from bacteria and saliva). Dental plaque *in situ* contains approximately 80% water. Variable amounts of lipid, Ca, P, Mg and F are also detected in dental plaque (Bowen and Koo, 2011).

The matrix of polysaccharide appears to increase following exposure to sucrose. The primary sources of extracellular polysaccharide (EPS) in dental plaque are products from the interaction of glucosyltransferases (Gtfs) and fructosyltransferases (Ftfs) with sucrose and starch hydrolysates (Vacca-Smith et al., 1996).

1.1.4 Hypotheses explaining the role of plaque bacteria in caries etiology

Once the dental plaque is established, the resident microflora is relatively stable over time (microbial homeostasis) (Marsh, 2004). The resident microflora plays an important role in the development of the physiology of the host, and reduces the chance of infection by acting as a barrier to colonization by exogenous species ('colonization resistance') (Marsh, 2004). However, homeostasis can break down if there is a substantial change to the habitat that disrupts this normal balance and drives selection of components of the microflora (Marsh et al., 2011).

In 1890, Miller proposed the "chemico-parasitic" theory that oral microorganisms can break down dietary carbohydrates to acids which dissolve hydroxyapatite and release free calcium and phosphates (Miller, 1890). After that, three hypotheses have been proposed: Specific plaque hypothesis, non-specific plaque hypothesis and ecological hypothesis.

1.1.4.1 Specific plaque hypothesis and non-specific plaque hypothesis

The specific plaque hypothesis was proposed by Walter J. Loesche in 1979 (Loesche, 1979). This hypothesis states that only a few of the many species found in dental plaque biofilm were actively involved in etiology of

caries. The evidence at the time strongly implicated mutans streptococci (MS) as the main etiological agent. Over time, as more studies identified that caries lesions developed in the absence of mutans streptococci, an alternative view on the role of plaque in caries lesion development was introduced. The nonspecific plaque hypothesis proposed that disease was the outcome of the overall activity of the total plaque microflora (as opposed to specific species).

1.1.4.2 Ecological hypothesis

Recently, an extended caries ecological hypothesis explains the relationship between the composition of dental plaque and caries process (Marsh, 1994). In this hypothesis, dental plaque is a dynamic microbial ecosystem in which non-mutans streptococci and *Actinomyces* are the key players for maintaining dynamic stability. The ecological hypothesis proposes that dental caries is the consequence of an imbalance in the resident microflora due to ecological pressure. Possible ecological pressures for caries include a sugar-rich diet, conditions of low pH, or low saliva flow (Marsh, 2006). In healthy situation, the potentially cariogenic bacteria may be found naturally in dental plaque, but present as a small proportion in the total microflora of dental plaque. With a conventional diet, non-MS and *Actinomyces* are clinically insignificant. These bacteria can produce acids from carbohydrate foods. However, the temporary decreased pH is easily returned to neutral level by homeostatic mechanisms in

the plaque (Marsh, 1999). This is a natural pH cycle, which occurs numerous times daily. The processes of de- and re-mineralization are in equilibrium. If the balance is broken down, for example the frequency of fermentable carbohydrate intake increases, the plaque spends more time below the critical pH (5.5) for enamel demineralization. The low pH environment favors the proliferation of non-MS and *Actinomyces*, leading to a microbial shift to a more acidogenic microflora. Once the acidic environment has been established, the proportion of aciduric bacteria such as MS and lactobacilli increase and act actively, sustaining an environment characterized by “net mineral loss”. Hence, high proportions of MS and/or other aciduric bacteria may be considered biomarkers of rapid caries development (Chhour et al., 2005; Nyvad and Kilian, 1990). The ecological hypothesis highlights the critical role played by the changes of the oral environment in caries as shown in Fig. 1.3.

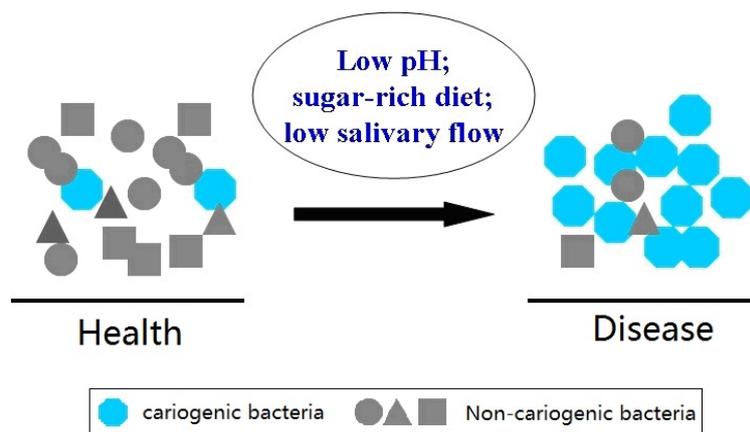


Fig. 1.3. Schematic representation of the relationship between the microbial composition of dental plaque in health and disease. Cariogenic bacteria (blue) present in low numbers in plaque. Under some major ecological pressure the pathogens out compete non-cariogenic (grey) and achieve the levels needed for disease to occur. Possible ecological pressures for caries include a

sugar-rich diet, conditions of low pH, or low saliva flow. Adapted with kind permission from BioMed Central (open access) (Marsh, 2006).

1.2 Mutans streptococci and *Streptococcus mutans*

1.2.1 Mutans streptococci (MS)

Mutans streptococci (MS) refer to mutans group of streptococci. Mutans group includes *Streptococcus mutans*, *Streptococcus rattus*, *Streptococcus cricetus*, *Streptococcus sobrinus*, *Streptococcus downei* and *Streptococcus macacae* (Kawamura et al., 1995). A phylogenetic tree for 34 species of the genus *Streptococcus* is shown in Fig. 1.4.

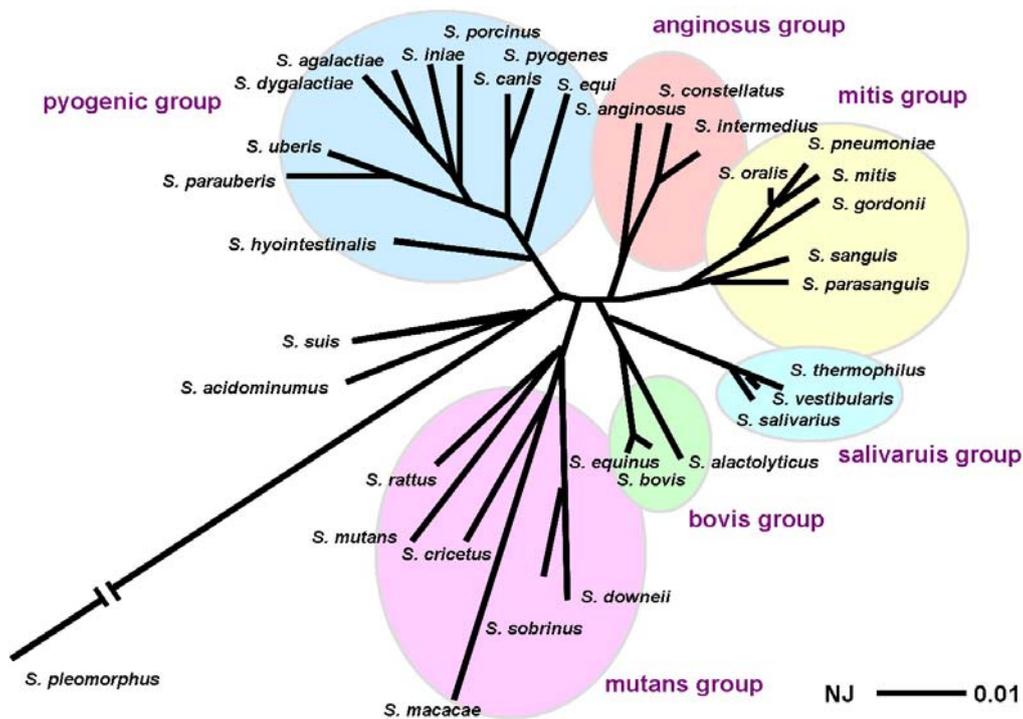


Fig. 1.4. Phylogenetic relationships among 34 *Streptococcus* species. Distances were calculated by the neighbor-joining (NJ) method. S.

pleomorphus was located far from other species, so its distance is indicated with an ellipsis; its true distance from the junction was 0.16944. Adapted with kind permission from Society for General Microbiology (Kawamura et al., 1995).

Mutans streptococci (MS) have been identified as the major pathogens for dental caries due to their strong acidogenic and aciduric potentials (Takahashi and Nyvad, 2011). They are frequently isolated from caries lesions, and are able to produce intra- and extracellular polysaccharides that facilitate microbial adherence on teeth (Hamada and Slade, 1980). The central role of MS in the initial stage of dental enamel caries has been studied, revealing a strong positive statistical association of mutans streptococci with inception or incidence of carious lesions (Tanzer et al., 2001). A systematic review in 2006 summarized that presence of mutans streptococci, both in plaque or saliva of young caries-free children, was associated with a considerable increase in caries risk (Thenisch et al., 2006). Another systematic review in 2010 also concluded that mutans streptococci levels are a strong risk indicator for early childhood caries (ECC) (Parisotto et al., 2010).

However, MS are neither a unique causative agent for white spot lesions, nor a main determinant of the acidogenicity of plaque. It was found that non-MS were dominant in the dental plaque at both of the healthy sites and white spot lesions, while MS were present at low and similar levels in plaque of healthy and white spot sites. However, the acidogenicity of plaque was

significantly greater at white spot lesions than at clinically healthy sites (Sansone et al., 1993). It was suggested that acidogenic and aciduric bacteria other than MS, including “low-pH’ non-MS and *Actinomyces* were responsible for the initiation of caries (Sansone et al., 1993; van Houte, 1994; van Houte et al., 1996). In the ecological hypothesis, non-MS and *Actinomyces* are the key players for maintaining dynamic stability.

1.2.2 Prevalence and taxonomy of *Streptococcus mutans*

Streptococcus mutans was first described by J Kilian Clarke (Clarke, 1924). In 1924 Clarke isolated the organisms from human carious lesions and named them *S. mutans*, as they were more oval than round on Gram stain and thus appeared to be a mutant form of a *Streptococcus* (Clarke, 1924). Maclean (1927) confirmed Clarke’s observation, and Onisi and Nucolls (1958) also reported micro-organisms from the deeper part of the lesion which resembled *S. mutans* (Edwardsson, 1968). In the 1960s, different *Streptococcus* species were isolated from carious lesion (Carlsson, 1968; Edwardsson, 1968; Guggenheim, 1968) and caused a transmissible infection in rodent models (Fitzgerald and Keyes, 1960; Keyes, 1968).

S. mutans is a member of MS group which also includes *S. rattus*, *S. cricetus*, *S. sobrinus*, *S. downei*, *S. macacae*. In 1970, 70 mutans streptococci

were characterized into five serotypes (a-e) (Bratthall, 1970). After that, three serotypes (f, g, h) were identified. In 2004, some non-c/e/f *S. mutans* strains were detected and named as a novel serotype k. Serotype k strains can generate antisera which are reactive with the antigens extracted from serotype c/e/f (Nakano et al., 2004). Serotype k was recently designated as the ninth serotype of mutans streptococci. *S. mutans* (serotype c, e and f) was detected in human (Beighton et al., 1981; Perch et al., 1974).

1.2.3 Virulence factors of *S. mutans*

Among the acidogenic and aciduric species, *S. mutans* is commonly found in the human oral cavity and significantly contribute to dental caries (Hudson and Curtiss, 1990). It is a strong acid producer which creates an acidic environment, thus increasing the risk for caries. It can also adhere to enamel salivary pellicle and to other plaque bacteria. *S. mutans* is able to form extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS) in the presence of sucrose, which is an important factor for *S. mutans* biofilm formation (Forssten et al., 2010).

1.2.3.1 Adherence

The adhesion of *S. mutans* in dental plaque is achieved *via* two ways:

sucrose-independent and sucrose-dependent adhesion. The attachment of *S. mutans* to tooth surface may be initiated *via* sucrose-independent adhesion to salivary components within the acquired enamel pellicle, while the colonization establishment may be mediated primarily *via* sucrose-dependent adhesion (Banas, 2004).

(1) Sucrose-independent adhesion

Sucrose-independent adhesion of *S. mutans* is thought to be profoundly associated with the cell surface protein antigen I/II (PAc, also known as adhesive P1, encoded by *spaP*) binding to the salivary glycoproteins (Koga et al., 1990), which is required for the initial attachment of *S. mutans* to the saliva-coated tooth surface. The interaction of PAc with fluid phase salivary agglutinin mediates aggregation of *S. mutans*, whereas adsorption of salivary agglutinin to solid surfaces provides a site for initial adhesion of the organism (Ahn et al., 2008). PAc is composed of several domains, including an N-terminal signal sequence, an alanine-rich repeat region (A-region), a proline-rich repeat region (P-region), and an anchor region. A-region has a strong relationship with adhesion to tooth surfaces, while the P-region has a high affinity for PAc (Matsumoto-Nakano et al., 2008).

LytR is a homologue of a regulator of autolysin activity in *Bacillus*

subtilis (Lazarevic et al., 1992). It was demonstrated that LytR played an important role in sucrose-independent attachment to polystyrene surfaces in *S. mutans* (Wen and Burne, 2002; Yoshida and Kuramitsu, 2002).

Another surface-associated protein, wall-associated protein A (WapA), is a well-studied human vaccine candidate (Russell et al., 1995; Russell and Johnson, 1987); however, the function of this protein remains controversial. Douglas & Russell reported that antibodies to this antigen do not interfere with sucrose induced aggregation (Douglas and Russell, 1982). Harrington and Russell also found that the wapA mutant *S. mutans* had no effect on sucrose-dependent adherence to surface (Harrington and Russell, 1993). However, Qian and Dao reported that the wapA mutant *S. mutans* strain GS-5 resulted in a significant decrease in sucrose-dependent adherence to surfaces (Qian and Dao, 1993). Zhu *et al.* suggested that WapA was involved in sucrose-independent cell–cell aggregation and biofilm formation (Zhu et al., 2006).

(2) Sucrose-dependent adhesion

Compared with sucrose-independent adhesion, the mechanism of sucrose-dependent biofilm formation is well understood. *S. mutans* synthesizes glucan from sucrose, which is one of the most important virulence of *S.*

mutans (Hamada and Slade, 1979). Glucan formation allows the bacteria to firmly attach to the tooth surface (Kuramitsu, 1993; Yamashita et al., 1993) and form a biofilm. The ability of glucan to facilitate adhesion of *S. mutans* may be due to hydrogen bonding of the glucan polymers to both the salivary pellicle and the bacteria (Rolla, 1998). Moreover, the diffusion of acid produced by the acidogenic bacteria from fermentable carbohydrate in the dental plaque is retarded by the gelatinous glucan, as an insoluble matrix for plaque. It eventually leads to enamel demineralization. The central role of glucans in sucrose-dependent adhesion has been confirmed in the research on dental plaque development and the etiology of dental caries (Loesche, 1986; Yamashita et al., 1993).

Glucan synthesis is mediated *via* the enzymic activity of three glucosyltransferases (GtfB, GtfC and GtfD). GtfB synthesizes mainly water-insoluble glucans (85%) with alpha-1,3-linkages (also called mutan); GtfC produces a mixture of soluble (with mostly alpha-1,6-linkages) and insoluble glucans; GtfD forms water-soluble glucans (70%) with alpha-1,6-glycosidic linkages that resembles dextran (Bowen and Koo, 2011). Both types of polymers contribute to sucrose-dependent colonization and caries, but the water-insoluble glucan may be more important for smooth surface caries (Banas, 2004).

Gtf binds to pellicles. Active Gtf was detected on HA disks within 1 minute of placing them in mouth. Sucrose rinsing enhances the amount of Gtf detected, possible due to the adherence of Gtf to glucan formed *in situ* (Scheie et al., 1987; Vacca Smith and Bowen, 2000). Gtf adsorbs to saliva-coated HA (sHA) disks (Steinberg, 1996; Venkitaraman et al., 1995), but poorly binds to uncoated HA and loses much of its activity (Schilling and Bowen, 1988; Vacca-Smith and Bowen, 1998). Although all the three Gtfs can bind to sHA, their affinity differ greatly (Vacca-Smith and Bowen, 1998). GtfC has the greatest affinity for sHA, while GtfD displays lowest affinity.

Gtfs also have the ability to bind to many oral bacteria, including those that do not synthesize Gtfs (Hamada et al., 1978; McCabe and Donkersloot, 1977; Vacca-Smith and Bowen, 1998). Furthermore, the enzyme retains its activity when adsorbed to bacteria, thereby converting non-Gtf producers into glucan formers (Vacca-Smith and Bowen, 1998).

The simultaneous synthesis of glucans by surface-adsorbed Gtfs is essential for the establishment of a biofilm matrix. GtfCs secreted by *S. mutans* are incorporated into pellicle. GtfBs are adsorbed on bacterial surfaces of both *S. mutans* and other microorganisms that do not produce Gtfs (e.g. *Actinomyces* spp.). Surface-adsorbed GtfB and GtfC utilize sucrose to synthesize insoluble and soluble glucans. The soluble glucans formed by GtfD serve as primers for

GtfB enhancing the overall synthesis of exopolysaccharides. The glucan molecules provide binding sites on surfaces for *S. mutans* (and other microorganisms) mediating bacterial clustering and adherence to the tooth enamel. Furthermore, other bacteria adsorbed Gtf also become glucan producers, binding to tooth and microbial surfaces by the same mechanisms. This model could explain the rapid formation and accumulation of highly cohesive-adherent plaque in the presence of sucrose, as shown in Fig. 1.5 (Bowen and Koo, 2011).

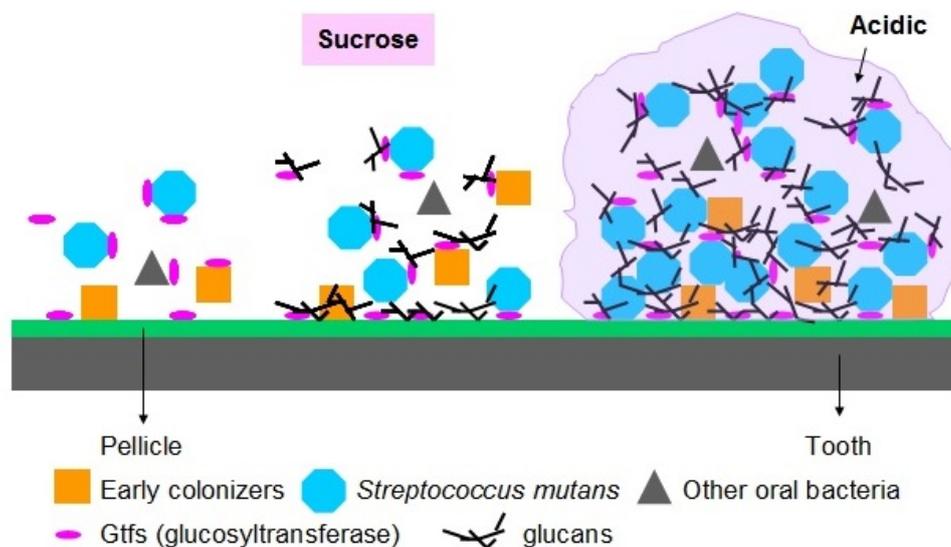


Fig. 1.5. Model of Gtf-glucan-mediated bacterial adherence. Adapted from Bowen and Koo, 2011 (open access) (Bowen and Koo, 2011).

Another group of proteins called glucan-binding proteins (i.e. GbpA, -B, -C and -D) also play important roles in subsequent cell–cell aggregation and biofilm development (Douglas and Russell, 1982; Shah and Russell, 2004;

Smith and Taubman, 1996).

Besides the proteins and enzymes that contribute to sucrose-dependent adhesion, other proteins are also involved in the metabolism of sucrose, glucans or carbohydrates, and these are considered as the potential virulence factors, such as fructosyltransferase (Ftf), fructanase (FruA), extracellular dextranase (DexA), and proteins responsible for intracellular polysaccharide accumulation (Dlt1-4) (Banas, 2004). Ftf catalyzes the synthesis of fructans and perhaps work as an energy reserve. FruA may break down fructans for energy use. DexA perhaps contributes to glucan synthesis or the breakdown of glucans. Dlt1-4 accumulates the intracellular polysaccharide and works as energy reserve (Banas, 2004).

1.2.3.2 Acidogenicity

S. mutans can produce lactate, formate, acetate, and ethanol *via* a complete glycolytic pathway. The amounts of these fermentation products depend on the growth conditions, but lactate is the major product when glucose is abundant. Deficient lactate dehydrogenase (LDH) strain showed reduced cariogenicity and the absence of LDH is lethal for *S. mutans* (Banas, 2004).

It is generally thought that the acidogenicity of *S. mutans* contributes to ecological changes in the dental plaque, which favors more acidogenic and acid-tolerant species colonization. This cariogenic flora will reduce plaque pH to lower levels, and the recovery to a neutral pH will be prolonged (Banas, 2004). Sustained plaque pH below the critical pH 5.5 is crucial for the demineralization of enamel and the development of dental caries (Barron et al., 2003). The critical pH refers to the pH value at which a solution is just saturated with respect to the mineral. If the pH of the solution is less than the critical pH, the solution is unsaturated. Hence, the mineral will tend to dissolve until the solution becomes saturated (Dawes, 2003).

Stephan Curve was first described in 1944 by RM Stephan (R.M., 1944). He observed a quantitative difference in the intensity and duration of the acidity produced by carbohydrates after sucrose challenge on the teeth of both caries-free and caries-active individuals. The plotted dental plaque pH was named as Stephan curve which displays the pH change after 10% glucose rinse (Fig. 1.6). Before the application of glucose, the pH values of most areas were around neutrality. After glucose rinse, the pH value dramatically drops within the first few minutes. This drop in pH was greatest and lasted for the longest time in the caries-active cases. The pH gradually recovered to the neutrality after that.

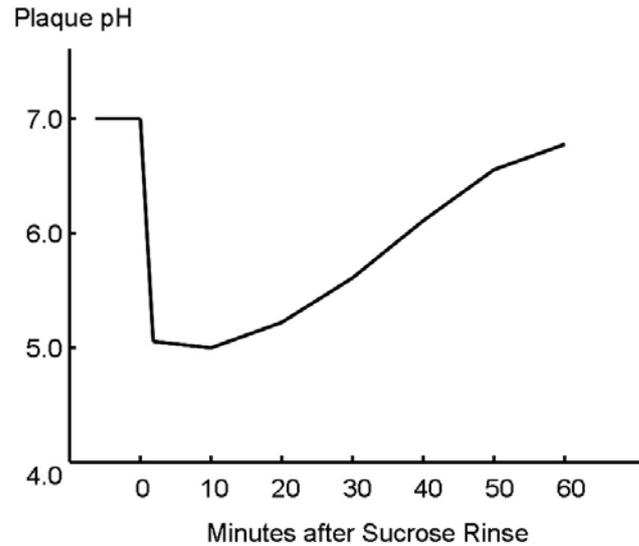


Fig. 1.6. Stephan curve of dental plaque before and after sucrose rinse.

Three features highlighting the acidogenicity of dental plaque are the lowest pH reached, recovery time, and area under the curve (AUC) which represents the degree of demineralization occurred (Preston and Edgar, 2005). Lowest pH refers to the minimum pH attained after sugar rinsing; recovery time refers to the time below the critical pH; and AUC refers to the curve area below the critical pH of 5.5. The characteristics of Stephan curve can be used to assess the cariogenicity of different food or drinks and the acidogenic properties of dental plaque (Imfeld T, 1980; Preston and Edgar, 2005). In 1985, the assessment methods for the relationship between foods and dental caries were discussed among the cariologists. Plaque pH modeling has been proposed (Preston and Edgar, 2005). Furthermore, it was suggested that the frequency of acidogenic episodes may be more important in caries progression, than the acidogenicity of plaque that develops during sugar exposure (Dong et

al., 1999).

The acidogenicity of biofilm is associated with the age of biofilm. Imfeld T investigated the intraplaque acid formation *in vivo* (Imfeld T, 1980). They reported that the rate and amount of acid formation increased with the age of the biofilm both in adolescent or old adult by comparing 2, 3, 4, 5, and 6-day-old biofilms.

1.2.3.3 Acid tolerance

Another capacity of *S. mutans* besides acidogenicity is aciduricity, also named acid tolerance. *S. mutans* can grow even at the low pH 4.4. An F1F0-ATPase proton pump primarily mediates the acid tolerance of *S. mutans*, with adaptation of changes in gene and protein expression, which is called acid tolerance response (ATR) (Banas, 2004). The ATR is induced under acidic conditions and active between pH 5-5.5. Studies showed that more than sixty proteins were involved in this response. Many of them appeared within the first 30 minutes after acid shock, whereas full induction occurred after 90-120 minutes (Lemme et al., 2010).

The low pH would induce a higher survival rate of *S. mutans* cells in biofilm. McNeill K *et al.* found that *S. mutans* biofilms were highly resistant

to acid killing and the percentage of live cells (averaged 43.4% from 1d to 7d) was much higher than the planktonic and dispersed cells in the biofilm (0.0002-0.2% survivors) (McNeill and Hamilton, 2003).

The synthesis of water-insoluble glucan and the formation of biofilm may aid the acid-tolerance of *S. mutans*. *S. mutans* grew better in biofilm than in planktonic culture under acid stress (McNeill and Hamilton, 2003). The speed of acid diffusion through *S. mutans* cells concentrate was related to the quantity of water-insoluble glucan produced by *S. mutans* (Hata and Mayanagi, 2003). The diffusion of hydronium ions was fastest in wild type *S. mutans* grown in sucrose, compared with wild type bacteria grown in glucose and mutants deficient in *gtfB*, *gtfC*, *gtfD* and fructosyltransferase. Glucan is able to increase the porosity of the cell masses causing a quick drop in pH, reaching harmful levels, and a delayed recovery of pH (Van Houte et al., 1989).

In conclusion, the major virulence factors of *S. mutans* are the ability to utilize sucrose to promote adhesion and accumulation, the acidogenicity, and acid tolerance.

1.3 *Lactobacillus*

Genus *Lactobacillus* plays an important role in human and animal

gastrointestinal tract as well as in the production and spoilage of many foods, feeds and beverages. Presently, there are more than 80 species described (Badet and Thebaud, 2008). Some of them have been found in the oral cavity.

1.3.1 *Lactobacillus* in saliva

Lactobacilli are absent in the oral cavity of newborns. They appear during the first year of life. It was observed that this species was present in 50% of newborns during their first year (McCarthy et al., 1965). The rate of salivary lactobacilli varied in children among different studies from 40% to 100% (Carlsson et al., 1975; Klock and Krasse, 1977; Kohler et al., 1984). In older subjects, the lactobacilli rate tends to increase (Fure, 2003; Percival et al., 1991). Most of the studies were only quantitative.

Many studies have been carried out to investigate the link between salivary lactobacilli and caries. Some have shown a strong correlation between lactobacilli counts and root caries (Beighton et al., 1991; Emilson et al., 1988; Ravald et al., 1986; Van Houte et al., 1990). Studies of Sullivan *et al.* and Motisuki *et al.* suggested that the presence of streptococci and lactobacilli in dental plaque was not a better indicator of carious activity than their counts in saliva (Motisuki et al., 2005; Sullivan et al., 1996). A low level of salivary *Lactobacillus* counts seems to indicate a low carious activity (Bowden, 1997;

Kingman et al., 1988; Schroder and Edwardsson, 1987). The identified lactobacilli species in saliva is listed in Table 1.1.

Table 1.1: Species of lactobacilli identified in saliva

Lactobacilli identified	References
<i>L. rhamnosus</i>	Tennpaisan and Dahlen (Teanpaisan and Dahlen, 2006) Koll-Klais <i>et al.</i> (Koll-Klais et al., 2004) Nacy (Nancy and Dorignac, 1992)
<i>L. casei</i>	Tennpaisan and Dahlen (Teanpaisan and Dahlen, 2006) Smith <i>et al.</i> (Smith et al., 2001) Botha (Botha, 1993) Nacy (Nancy and Dorignac, 1992)
<i>L. plantarum</i>	Tennpaisan and Dahlen (Teanpaisan and Dahlen, 2006) Botha (Botha, 1993) Koll-Klais <i>et al.</i> (Koll-Klais et al., 2004) Nacy (Nancy and Dorignac, 1992)
<i>L. acidophilus</i>	Tennpaisan and Dahlen (Teanpaisan and Dahlen, 2006) Smith <i>et al.</i> (Smith et al., 2001) Botha (Botha, 1993) Koll-Klais <i>et al.</i> (Koll-Klais et al., 2004) Nacy (Nancy and Dorignac, 1992)
<i>L. fermentum</i>	Tennpaisan and Dahlen (Teanpaisan and Dahlen, 2006) Smith <i>et al.</i> (Smith et al., 2001) Koll-Klais <i>et al.</i> (Koll-Klais et al., 2004) Nacy (Nancy and Dorignac, 1992)
<i>L. salivarius</i>	Tennpaisan and Dahlen (Teanpaisan and Dahlen, 2006) Smith <i>et al.</i> (Smith et al., 2001) Koll-Klais <i>et al.</i> (Koll-Klais et al., 2004) Botha (Botha, 1993) Nacy (Nancy and Dorignac, 1992)
<i>L. brevis</i>	Smith <i>et al.</i> (Smith et al., 2001) Botha (Botha, 1993)
<i>L. delbrueckii</i>	Smith <i>et al.</i> (Smith et al., 2001) Koll-Klais <i>et al.</i> (Koll-Klais et al., 2004)

<i>L. gasseri</i>	Nacy (Nancy and Dorignac, 1992) Botha (Botha, 1993) Koll-Klais <i>et al.</i> (Koll-Klais <i>et al.</i> , 2004)
<i>L. paracasei</i>	Koll-Klais <i>et al.</i> (Koll-Klais <i>et al.</i> , 2004)
<i>L. oris</i>	Koll-Klais <i>et al.</i> (Koll-Klais <i>et al.</i> , 2004)
<i>L. crispatus</i>	Koll-Klais <i>et al.</i> (Koll-Klais <i>et al.</i> , 2004)

1.3.2 *Lactobacillus* in dental plaque

Lactobacilli have been reported less detectable in dental plaque than in saliva (Hintao *et al.*, 2007; Motisuki *et al.*, 2005; Nancy and Dorignac, 1992).

Table 1.2 shows the species of lactobacilli identified in dental plaque.

Table 1.2: Species of lactobacilli identified in dental plaque

Lactobacilli identified	References
<i>L. rhamnosus</i>	Carlsson <i>et al.</i> (Carlsson <i>et al.</i> , 1975) Nancy <i>et al.</i> (Nancy and Dorignac, 1992) Ahumada <i>et al.</i> (M. C. Ahumada, 1999) Botha <i>et al.</i> (Botha <i>et al.</i> , 1998)
<i>L. casei</i>	Carlsson <i>et al.</i> (Carlsson <i>et al.</i> , 1975) Meiers and Schachtele (Meiers and Schachtele, 1984) Milnes and Bowden (Milnes and Bowden, 1985) Nancy <i>et al.</i> (Nancy and Dorignac, 1992) Ahumada <i>et al.</i> (M. C. Ahumada, 1999) Botha <i>et al.</i> (Botha <i>et al.</i> , 1998)
<i>L. plantarum</i>	Meiers and Schachtele (Meiers and Schachtele, 1984) Milnes and Bowden (Milnes and Bowden, 1985) Nancy <i>et al.</i> (Nancy and Dorignac, 1992) Ahumada <i>et al.</i> (M. C. Ahumada, 1999) Botha <i>et al.</i> (Botha <i>et al.</i> , 1998)
<i>L. acidophilus</i>	Carlsson <i>et al.</i> (Carlsson <i>et al.</i> , 1975) Meiers and Schachtele (Meiers and Schachtele, 1984) Milnes and Bowden (Milnes and Bowden, 1985)

	Nancy <i>et al.</i> (Nancy and Dorignac, 1992)
	Botha <i>et al.</i> (Botha et al., 1998)
<i>L. fermentum</i>	Carlsson <i>et al.</i> (Carlsson et al., 1975)
	Milnes and Bowden (Milnes and Bowden, 1985)
	Nancy <i>et al.</i> (Nancy and Dorignac, 1992)
	Ahumada <i>et al.</i> (M. C. Ahumada, 1999)
	Botha <i>et al.</i> (Botha et al., 1998)
<i>L. salivarius</i>	Milnes and Bowden (Milnes and Bowden, 1985)
	Nancy <i>et al.</i> (Nancy and Dorignac, 1992)
	Botha <i>et al.</i> (Botha et al., 1998)
	Schüpbach <i>et al.</i> (Schupbach et al., 1996)
<i>L. paracasei</i>	Ahumada <i>et al.</i> (M. C. Ahumada, 1999)
<i>L. brevis</i>	Milnes and Bowden (Milnes and Bowden, 1985)
	Botha <i>et al.</i> (Botha et al., 1998)
<i>L. confusus</i>	Botha <i>et al.</i> (Botha et al., 1998)
<i>L. delbrueckii</i>	Nacy (Nancy and Dorignac, 1992)
<i>L. gasseri</i>	Botha <i>et al.</i> (Botha et al., 1998)

Beighton *et al.* found in 3-4 years old children, lactobacilli were isolated from dental plaque in 54% of children with caries and in 7% of children without caries (Beighton et al., 2004). Matee *et al.* compared the number of lactobacilli isolated from dental plaque of children with rampant caries and without caries. They found that the streptococci and lactobacilli counts were 100-fold higher in caries group in comparison to the non-caries group (Matee et al., 1992).

1.3.3 *Lactobacillus* and caries

Lactobacilli are capable of producing acids, growing and surviving in acidic environment. These bacteria have a fermentative metabolism via two ways. Some species use homolactic fermentation and produce only lactic acid;

some species use heterolactic fermentation and produce lactic acid, CO₂, acetic acid or ethanol. Both result in an acidification of the environment (Badet and Thebaud, 2008).

The association between lactobacilli and caries was reported by Goadby in 1899, according to Owen (Owen, 1949). However, it was not until the late 1950s that experimental evidence clearly established the fundamental role of these bacteria in dental caries and in pulp and periapical disease (Hemmens et al., 1946). Nowadays, studies demonstrated that a wide range of *Lactobacillus* species were isolated from carious lesions, especially in root caries and deep dentinal caries (Ayna et al., 2003; Beighton and Lynch, 1995; Brown et al., 1986; Callaway et al., 2013; Preza et al., 2008). However, there is no evidence for a uniquely oral species of *Lactobacillus* that has evolved to exploit the oral cavity as a habitat, in the way that some streptococci have done. They are now considered secondary invaders rather than initiators of the caries process (Tanzer et al., 2001).

Nevertheless, lactobacilli are prevalent in fermented foods, especially probiotic foods which are beneficial for intestinal health. Hence, our oral cavity is constantly exposed to *Lactobacillus* species. Many *in vitro* studies have demonstrated that probiotic lactobacilli had inhibitory effects on the growth of mutans streptococci (Hasslof et al., 2010; Ishihara et al., 1985;

Meurman et al., 1995; Silva et al., 1987; Simark-Mattsson et al., 2007; Sookkhee et al., 2001). Clinical trials also suggested the salivary mutans streptococci counts decreased after the consumption of probiotic products (Twetman and Keller, 2012). Some currently successful probiotic *Lactobacillus* strains are originally isolated from human body (Saarela et al., 2000). It was suggested that a probiotic strain could function better in a similar environment to where it was originally isolated from, e.g. human GI-tract (Saarela et al., 2000). Furthermore, some studies have been isolating probiotic *Lactobacillus* strain from oral cavity (Maldonado et al., 2012; Yang et al., 2013).

Hence, the different roles that different oral *Lactobacillus* species play in oral cavity need to be further investigated.

1.4 Preventive therapies for caries

1.4.1 Fluoride

In the last five decades, the most widely used agent for dental caries prevention is fluoride. Since the early findings of the protective effects of fluoride present in drinking water upon caries incidence and prevalence over 70 years ago, intensive research has been conducted to determine the benefits,

safety, as well as the delivery ways (Pessan et al., 2011). Now fluoride is recognized as the main factor responsible for the decreasing prevalence in caries, which is observed worldwide (Bratthall et al., 1996). The mechanisms include inhibition of demineralization, enhancement of remineralization, and antimicrobial effect. However, high concentration of fluoride is toxic and causes fluorosis (Ismail and Hasson, 2008). Fluorosis is characterized by dental mottling and skeletal manifestations such as crippling deformities, osteoporosis, and osteosclerosis (Barbier et al., 2010). The presence of fluoride-resistant strain of *S. mutans* may also limit the long term clinical effect of fluoride (Hoelscher and Hudson, 1996).

1.4.2 Non-fluoride therapies

Chlorhexidine

It is generally accepted that chlorhexidine (CHX) digluconate is an antiplaque and anti-gingivitis agent (Matthijs and Adriaens, 2002). Chlorhexidine inhibits the growth of mutans streptococci which are associated with the development of caries lesions (Marsh, 1993; Twetman, 2004), but its use as anti-caries agent remains controversial in clinical studies (Twetman, 2004; Zhang et al., 2006). The main clinical problem with the use of chlorhexidine is the difficulty of suppressing *S. mutans* for a long time (Autio-Gold, 2008). Chlorhexidine also has shown side effects, such as a

yellow-brown staining of the teeth with a mouthrinse, and an altered taste sensation (Fardal and Turnbull, 1986; Flotra, 1973).

Xylitol

Xylitol is a five-carbon sugar alcohol (polyol), having the same sweetening property as sucrose (Mickenautsch and Yengopal, 2012). Xylitol is believed to have an 'active' anti-cariogenic property, as it can resist fermentation by bacteria into acids, and it can also inhibit growth, metabolism, as well as polysaccharide production of mutans streptococci. A systemic review concluded that the addition of xylitol to existing fluoride regimes was beneficial in the prevention of caries. However, the evidence contains a high risk of bias and may be limited by confounder effects (Mickenautsch and Yengopal, 2012). In addition, xylitol-based caries prevention has been claimed to be expensive (Soderling, 2009).

Ozone Technology

The antimicrobial ability of ozone gas (O₃) is well known (Burleson et al., 1975; Dyas et al., 1983). However, the evidence to support the use of ozone gas to prevent caries and to enhance remineralization of enamel is limited. A systematic review concluded that although laboratory studies have shown antimicrobial effects of ozone application, a strong level of efficacy has not been achieved in *in vivo* studies (Rickard et al., 2004).

Laser

Laboratory studies have shown that laser can be used to modify the chemical composition of tooth enamel to render it less soluble and more resistant to demineralization (Hsu et al., 2008; Vlacic et al., 2007; Walsh, 1997a; b). However, there are no reports as yet of *in vivo* studies testing the efficacy of these lasers in preventing caries or reducing caries progression.

Caries Vaccine

Salivary IgA antibodies have shown inhibitory effects against MS, by interfering with sucrose-independent and sucrose-dependent attachment, and accumulation on tooth surface, as well as possible inhibition on their metabolic activities (Russell et al., 1999). Strategies of mucosal vaccination have been developed to induce high levels of salivary antibodies that can achieve anti-caries effect, such as polyclonal and monoclonal antibodies to AgI/II (PAc), GTFs, glucan-binding proteins, GbpA, GbpB and GbpC, and Serotype-specific polysaccharide antigens (Koga et al., 2002). Studies have demonstrated the feasibility of inducing protective immunity against mutans streptococci and the subsequent development of dental caries in many animal models, and furthermore, decreased MS colonization in oral cavity was also achieved in few human studies (Koga et al., 2002; Russell et al., 1999; Russell et al., 2004). The main potential adverse effect of immunization is the possible

cross-reaction of the antibodies with normal tissues (Koga et al., 2002).

Replacement Therapy

Replacement therapy involves the use of a harmless effector strain which can colonize in the host's microflora to outcompete the growth of a particular pathogen (Anusavice, 2005). *S. mutans* strain BCS3-L1 is a genetically modified strain designed for use in replacement therapy to prevent dental caries. It has reduced acidogenic potential due to its low LDH activity (Hillman et al., 2000). However, there are questions which require further research considerations with regards to the effector strain (Anusavice, 2005). These issues include its genetic stability, any potential significant selective advantage in colonization *in vivo*, and possibility to control its spread within the population. Further clinical trials need to be carried out to determine the potential benefits and side effects of this therapy.

Probiotics

Probiotic bacteria are used to treat and prevent a broad range of human disease or conditions, like diarrhea, allergic reactions and gastroenteritis. Probiotics have been shown to be potentially useful in childhood respiratory infection, dental caries, against nasal pathogens and arthritis (Goldin and Gorbach, 2008). Their use in caries prevention relates to the attempts at the replacement or displacement of cariogenic bacteria in the oral cavity.

1.5 Probiotics: a promising preventive therapy

1.5.1 What are probiotics?

Probiotics have a long history. The earliest records indicate that humans took ‘soured milks’ as long as 2000 years ago. However, the real nutritional values were first researched seriously by Metchnikoff. In 1907, Ilya Mechnikow, a Nobel laureate, found that lactic acid bacteria were responsible for the long life spans of Bulgarian peasants, who consumed large quantities of yogurt containing lactic acid bacteria (Twetman and Stecksén-Blicks, 2008). The beneficial effects of probiotics have been of interest since then.

The antibacterial activity associated with cultures of lactobacilli has already been reported for *Lactobacillus acidophilus*, *Lactobacillus lactis*, and *Lactobacillus helveticus* more than 50 years ago (Vincent et al., 1959). The antagonism between intestinal lactobacilli and certain enteric bacteria responsible for post-irradiation infections in the rodents have been reported (Vincent et al., 1959). In *in vitro* experiments, definite inhibition of growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* was observed when these organisms were streaked on liver veal agar plates grown with lactobacilli isolated from rat intestine (Vincent et al., 1955). Vincent *et al.* extracted the

crude lactocidin which was active in the presence of serum, showing a broad antibacterial spectrum (Vincent et al., 1959).

In 1965 Lilley and Stillwell first introduced the term probiotic. The word is derived from the Greek and means *for life* to describe substances secreted by one microorganism to stimulate the growth of another, as an antonym to the term antibiotic (Lilly and Stillwell, 1965). An expert panel commissioned by Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Reid et al., 2003). Hull identified the first probiotic species *Lactobacillus acidophilus* in 1984, and Later in 1991, Holcomb identified *Bifidobacterium bifidum*, as stated in Saraf’s review (Saraf et al., 2010). The most important source of probiotics is the bacteria in yogurt and fermented milk products. The majority of probiotic bacteria belong to the genera *Lactobacillus*, *Bifidobacterium*, *Propionibacterium* and *Streptococcus*. It was stated by the FAO and WHO in 2001 that there is adequate scientific evidence to indicate that there is potential for probiotic foods to provide health benefits and that specific strains are safe for human use (FAO, 2001). The criteria for considering certain product should be: non toxic and non pathogenic preparation; beneficial effects; withstand gastrointestinal juice; good shelf life; replace and reinstate the intestinal microflora (Saraf et al.,

2010).

1.5.2 Effects of probiotic on human general health

Diarrhea

Numerous clinical trials have been done to demonstrate the effects of probiotics on diarrhea, with most of them being carried out in infants or children (Allen et al., 2010; Bernaola Aponte et al., 2010). Several systemic reviews have reached the same conclusion that a beneficial effect of probiotics was consistent across the different diarrhea outcomes (Allen et al., 2010; Gawronska et al., 2007; Szajewska and Mrukowicz, 2001; Van Niel et al., 2002). A number of studies reported the ability of probiotics to reduce the frequently observed intestinal adverse effects and diarrhea associated with the clinical use of antibiotics (Hempel et al., 2012; Johnston et al., 2007; Johnston et al., 2011). However, a recent randomized clinical trial, with around 3000 old patients recruited, identified that a multi-strain preparation of lactobacilli and bifidobacteria was not effective in prevention of antibiotic associated diarrhea or *Clostridium difficile* diarrhea (Allen et al., 2013).

Allergic reactions

Several studies have demonstrated the modification of allergic reactions with *Lactobacillus* strain GG (Isolauri et al., 2000; Kalliomaki et al., 2001;

Kalliomaki et al., 2003; Majamaa and Isolauri, 1997; Rautava et al., 2002). Another study reported that the infants with atopic eczema given probiotic supplemented formulas (*Lactobacillus* GG or *Bifidobacterium animalis* Bb12) also showed a significant improvement in the skin condition 2 months later (Isolauri et al., 2000).

Other infections

Some studies suggested that probiotics treatment could reduce *Helicobacter pylori* infections (Canducci et al., 2000; Felley et al., 2001). Probiotics also showed beneficial effect on gastro-intestinal infections (Biller et al., 1995; Gorbach et al., 1987; Reid and Burton, 2002), urogenital infections (Reid and Bruce, 2003), upper respiratory tract infections (Hao et al., 2011), surgical infections (Gan et al., 2002; Rayes et al., 2002), inflammatory bowel disease (Brigidi et al., 2001; Venturi et al., 1999), as well as bacterial vaginosis in women (Reid and Bocking, 2003).

Immune system

Dietary consumption of *Bifidobacterium lactis* HN019 and *L. rhamnosus* HN001 in randomized, placebo-controlled human studies showed measurable enhancement of immune parameters in the elderly (Arunachalam et al., 2000; Sheih et al., 2001). Probiotic lactobacilli were also found to differentially stimulate interleukin-12 and tumour necrosis factor- α (TNF- α) production in

dendritic cell (Christensen et al., 2002). The increases in the proportion of total, helper (CD4+) and activated (CD25+) T lymphocytes and natural killer cells in blood were reported for the elderly volunteers (63 ± 84 years) who took a milk supplemented with *B. lactis* HNO 19 for 3 weeks (Gill et al., 2001). Long-term studies are needed to see if these changes translate into clinical benefit.

Cancer

In vitro studies with *L. rhamnosus* GG and bifidobacteria and an *in vivo* study with *L. rhamnosus* GG, LC-705 and a *Propionibacterium* species showed a decrease in availability of carcinogenic aflatoxin in the lumen (El-Nezami et al., 2000; Oatley et al., 2000). Definitive clinical conclusions require efficacy studies in humans.

B. R. Goldin and S. L. Gorbach summarized the present and future clinical applications of probiotics in Table 1.1 (Goldin and Gorbach, 2008).

Table 1.3 Present and future clinical applications of probiotics

Applications with strong evidence

Gastroenteritis

Acute

Antibiotic associated

Applications with substantial evidence of efficacy

Allergic reactions, specifically atopic dermatitis

Applications that have shown promise

Childhood respiratory infection

Dental caries

Nasal pathogens
Relapsing *Clostridium difficile*-induced gastroenteritis (prevention)
Inflammatory bowel disease
Potential future applications
Rheumatoid arthritis
Irritable bowel syndrome
Cancer (prevention)
Ethanol-induced liver disease
Diabetes
Graft-versus-host disease

Adapted with kind permission from Oxford University Press (Goldin and Gorbach, 2008).

1.5.3 Effects of probiotic strains on oral health

1.5.3.1 Inhibitory effects on growth of oral pathogenic bacteria

The studies for the interaction between lactobacilli and oral bacteria started in 1980's. K Ishihara *et al.* tested the inhibition effect of heated water-soluble extracts of *Lactobacillus fermentum* and *Lactobacillus salivarius* which were isolated from healthy humans feces on 16 strains of *Streptococcus mutans* (serotypes from a to g) (Ishihara et al., 1985). The extracts slowed down or even completely inhibited the growth of *S. mutans* with higher *Lactobacillus* extracts concentration. M Silva *et al.* also investigated the antimicrobial substance from a human *Lactobacillus* strain isolated from healthy person stool specimens (Silva et al., 1987). The findings showed that the strain GG was inhibitory against strains of *E. coli*,

Streptococcus, *Pseudomonas*, *Salmonella*, *Bacteroides fragilis*, *Clostridium* and *Bifidobacterium*.

Meurman JH *et al.* demonstrated that *Lactobacillus* GG inhibitory substance extract suppressed the growth of *Streptococcus sobrinus* without interfering with the cellular ultrastructure (Meurman *et al.*, 1995). Supernatant of lactic acid bacteria isolated from healthy oral cavities of Thai volunteers inhibited the growth of oral pathogenic bacteria, such as *S. mutans*, *Staph. aureus*, *P. gingivalis*, *C. albicans* (Sookkhee *et al.*, 2001). A recent study showed that some lactobacilli strains from human saliva had inhibitory effects on the growth of *S. mutans* and *S. sobrinus*, including clinical isolates and reference strains (Simark-Mattsson *et al.*, 2007). Lactobacilli isolated from saliva and plaque from children and adolescents with or without caries lesions were reported significantly inhibiting the growth of both test strains of mutans streptococci and the subject's autologous mutans streptococci *in vitro* (Simark-Mattsson *et al.*, 2007). This effect was more pronounced in caries-free subjects. Eight strains of commercial probiotics lactobacilli from fruit drinks, yogurt, chewing gum or fermented milk also demonstrated the suppression on oral mutans streptococci and candida (Hasslof *et al.*, 2010).

1.5.3.2 Clinical trials of probiotics and dental caries

Clinical studies have been carried out to determine whether probiotics are able to prevent or decrease caries. More than twenty studies describing human interventions by probiotic bacteria with caries-related microbiological endpoints in saliva were reported. All utilized parallel arms with intervention and a placebo/control, or a crossover design. A variety of vehicles and modes of delivery for administration of lactobacilli and/or bifidobacteria was used, including milk, straw, tablet, chewing gum, yogurt, ice cream, powder, lozenges and drops. The probiotic treatment duration varied from 10 days to nearly 2 years. The participants include different age groups, involving 1 month babies, 1-6 years preschool and 6-12 years school old children, 12-17 years old adolescents, 18-35 years old adults and 58-84 years old people. The clinical trials focus on probiotic strains *L. rhamnosus* GG, *L. reuteri*, *L. rhamnosus* LB21, *L. paracasei* and Bifidobacterium. Statistically significant reductions of mutans streptococci in saliva were reported in most of these studies. Some studies also reported the reduction of yeast in oral cavity. However, unchanged mutans streptococci and increased or unchanged salivary counts of lactobacilli were also reported in a few studies. Table 1.4 summarizes the controlled clinical studies to date in the oral cavity with lactobacilli-derived probiotics.

Table 1.4 Summary of clinical studies in the oral cavity with probiotics

First author	Sample size, age, design	Vehicle, time	Strains	Oral outcome
Nase(Nase et al., 2001)	594, 1-6, RCT	Milk, 7 months	<i>L. rhamnosus</i> GG	Salivary MS decreased
Ahola(Ahola et al., 2002)	74, 18-35, RCT	Cheese, 3 weeks	<i>L. rhamnosus</i> GG	Salivary MS and yeast decreased
Nikawa(Nikawa et al., 2004)	40, 20, RCT	Yoghurt, 2 weeks	<i>L. reuteri</i>	Salivary MS decreased
Montalto(Montalto et al., 2004)	35, 24-33, RCT	Liquid and Capsules, 45 days	<i>L. sporogens, L. bifidum, L. bulgaricus, L. termophilus, L. acidophilus, L. casei, L. rhamnosus</i>	Salivary MS no difference
Çaglar(Caglar et al., 2005)	21, 21-24 crossover	Yogurt, 2 weeks	Bifidobacteria	Salivary MS decreased
Çaglar(Caglar et al., 2006)	120, 21-24, RCT	Straw, tablet, 3 weeks	<i>L. reutei</i>	Salivary MS decreased
Çaglar(Caglar et al., 2007)	80, 21-24 RCT	Chewing gum, 3 weeks	<i>L. reuteri</i>	Salivary MS decreased
Çaglar(Caglar et al., 2008b)	24, 20 crossover	Ice cream, 20 days	Bifidobacterium	Salivary MS decreased
Çaglar(Caglar et al., 2008a)	20, 20 RCT	Lozenge, 10 days	<i>L. reuteri</i>	Salivary MS decreased
Cildir(Cildir et al., 2009)	24, 12-16 Crossover	Yogurt, 4 weeks	Bifidobacterium	Salivary MS decreased
Stecksén-Blicks(Stecksén-Blicks et al., 2009)	248, 1-5, RCT	Milk, 21 months	<i>L. rhamnosus</i> LB21	Relatively low levels of MS, but not significant difference
Lexner(Lexner et al., 2010)	18, 13-17, RCT	Milk, 2 weeks	<i>L. rhamnosus</i> LB21	Salivary MS no difference
Jindal(Jindal et al., 2011)	150, 7-14, RCT	Powder, 14 days	<i>L. rhamnosus</i> and	Salivary MS decreased

Singh(Singh et al., 2011)	40, 12–14, Crossover	Ice cream, 10 days	Bifidobacterium and <i>L. acidophilus</i>	Salivary MS decreased
Ferrazzano(Ferrazzano et al., 2011)	84, 12-18, RCT	Yogurt, 2 weeks	<i>L. bulgaricus</i> and <i>S. thermophilus</i>	Salivary MS decreased
Chuang(Chuang et al., 2011)	78, 20-26, RCT	Tablets, 2 weeks	<i>L. paracasei</i>	Salivary SM decreased
Aminabadi(Aminabadi et al., 2011)	105, 6-12, RCT	Yogurt, 5 weeks	<i>L. rhamnosus</i> GG	Salivary SM no difference
Petersson(Petersson et al., 2011)	160, 58-84, RCT	Milk, 15 months	<i>L. rhamnosus</i> LB21	Salivary MS no difference
Cildir(Cildir et al., 2012)	19, 4-12, Crossover	Drops, 25 days	<i>L. reuteri</i>	Salivary MS no difference
Keller(Keller et al., 2012)	62, 19–35, RCT	Lozenge, 6 weeks	<i>L. reuteri</i>	Salivary MS no difference
Glavina(Glavina et al., 2012)	25, 6-10, Crossover	Yoghurt, 14 days	<i>L. rhamnosus</i> GG	Salivary MS decreased
Taipale(Taipale et al., 2012)	106, 1 month, RCT	Tablets, 23 months	Bifidobacterium	Salivary MS no difference
Juneja(Juneja and Kakade, 2012)	40, 12-15, RCT	Milk, 3 weeks	<i>L. rhamnosus</i>	Salivary MS decreased
Marttinen(Marttinen et al., 2012a)	13, 25.3, Crossover	Tablets, 2 weeks	<i>L. reuteri</i>	Salivary MS no difference

MS: mutans streptococci; SM: *Streptococcus mutans*; RCT: randomized controlled trial; crossover = randomized crossover trial.

There are 4 randomized controlled trials with caries as primary endpoint have been reported.

A randomized, double-blind, placebo-controlled intervention study was designed to examine whether milk containing *Lactobacillus rhamnosus* GG (LGG) has an effect on caries and the risk of caries in children, when

compared with normal milk (Nase et al., 2001). After 7 months consumption, less dental caries in the LGG group and lower mutans streptococcus counts were found. LGG reduced the risk of caries significantly, particularly in the 3- to 4-year-old children.

Stecksén-Blicks *et al.* investigated the effect of 21 months consumption of milk supplemented with probiotic lactobacilli and fluoride on dental caries in preschool children (Stecksén-Blicks et al., 2009). It was concluded that daily consumption of probiotic milk and fluoride reduced caries with a prevented fraction of 75% (caries increment prevented in the intervention group compared to the control group), which was higher than expected from previous trials with fluoridated milk (Yeung et al., 2005). However, in this study the levels of mutans streptococci (MS) and lactobacilli were relatively low, without significant difference among groups. This finding may be a result of the modified sampling technique different with tablets consumption, in which no chewing preceded the collection.

Another study by Petersson focused on the effects of probiotic milk and fluoride on root caries in the elderly (Petersson et al., 2011). The findings displayed beneficial effects (root caries reversal) in all experimental groups which consumed supplemented milk (Fluoride or/and *L. rhamnosus* LB21) for 15 months. The intervention suggested that probiotic treatment might be a

proper adjunct to fluoride in preventing and controlling the caries process. No severe adverse or side-effects were reported from any of the clinical trials.

The recently published study is the follow-up of the previous RCT with 106 infants involved receiving *Bifidobacterium animalis* subsp. *lactis* BB-12, xylitol or sorbitol tablets from the age of 1–2 months to 2 years. The previous study suggested that MS colonization percentages of the children at the age of 2 years were rather low, and the early administration of BB-12 did not result in permanent oral colonization of this probiotic or significantly affect MS colonization in the children (Taipale et al., 2012). The current study continued data collection using clinical examinations and questionnaires at the age of 4. The results showed that administration of BB-12 in infancy does not seem to increase or decrease the occurrence of caries by 4 years of age in a low-caries population (Taipale et al., 2013).

Although the trials have apparent limitations and the mechanisms of action were not explained clearly, the link to general health and the possible caries prevention benefit are too interesting to be ignored.

1.5.4 Mechanisms

1.5.4.1 Antibacterial activity

The mechanisms underlying the antibacterial activity of probiotic *Lactobacillus* strains appears to be multi-factorial. Probiotic bacteria produce a variety of substances including organic acids, hydrogen peroxide and bacteriocins. pH also plays an important role in the inhibitory effect. In addition, blocking of adhesion sites, co-aggregation and stimulation of immunity also have been proposed as the mechanisms (Fig. 1.7).

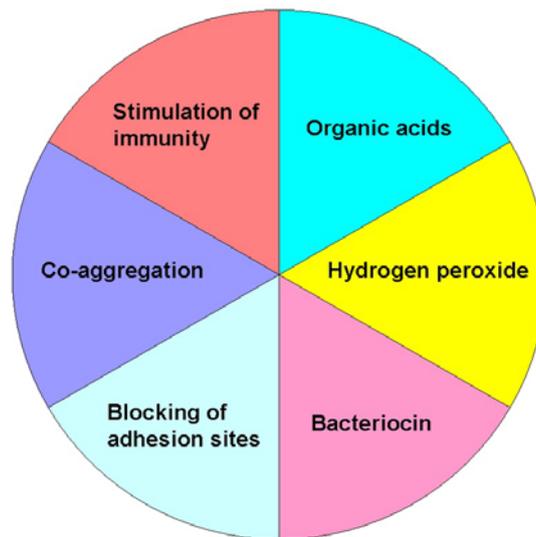


Fig. 1.7. Mechanisms of antibacterial effects of *Lactobacillus*

(1) Organic acids

Lactobacilli produce lactic acid and some other organic acids, which are known to have antimicrobial activity (Taniguchi et al., 1998). A study showed that the inhibition on growth and sporulation of *Bacillus cereus* were strongly

related to the initial rate of lactic acid production (Rosslund et al., 2005). The strains that produced lactic acid fastest inhibited *B. cereus* best. The authors suggested that the contribution from other antimicrobial metabolites (bacteriocins, hydrogen peroxide and ethanol) seemed to be only secondary. Alakomi *et al.* showed that the lactic acid acted as a permeabilizer of the outer membrane of gram-negative pathogens, thus increasing their susceptibility to antimicrobial molecules by allowing these molecules to penetrate the bacteria (Alakomi et al., 2000). Cadieux PA *et al.* supported lactic acid playing a major role in uropathogenic inhibition by lactobacilli (Cadieux et al., 2009). They observed no growth in concentrations above 30 mM lactic acid and an almost 70% growth reduction in as little as 10 mM, since previous report showed 24 hour cultures of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 produced approximately 45 and 35 mM lactic acid, respectively (Fayol-Messaoudi et al., 2005). Lin WH *et al.* also agreed with the above findings (Lin et al., 2009b). They found that *Lactobacillus* strains LGG and R1 showed stronger bactericidal activity against both the type strain and clinical isolates of *Helicobacter pylori*. The concentrations of lactic acid and acetic acid of these 2 strains were also higher than the other LAB strains studied in this report. Hence, lactic acid and acetic acid were believed to contribute to the antibacterial activity (Lin et al., 2009b).

However, some *Lactobacillus* strain with fairly weak acid production,

such as *L. reuteri* ATCC 55730, also proved to be effective against both mutans streptococci and candida. This indicates that other inhibitory substances may be involved (Hasslof et al., 2010). Meurman JH *et al.* observed that the inhibition obtained by the *Lactobacillus* extract on *S. sobrinus* was stronger than that found with plain lactic acid in the growth medium controls (Meurman et al., 1995). This finding also supports the hypothesis that although lactic acid alone does inhibit bacterial growth, the *Lactobacillus* extract contains other substances with even stronger action. Sookkhee S suggested that the antimicrobial activity could be partly due to organic acids, because the antimicrobial effect was more active at acidic pH than at alkali pH (Sookkhee et al., 2001). After adjusting the supernatant fluids from these cultures to pH 7.0, there was a slight reduction (10-20%) of the antimicrobial activity. This indicated that organic acids were not the only antimicrobial substances. Fayol-Messaoudi D *et al.* provided evidence that the killing effect of probiotic *Lactobacillus* strains against *S. enterica* serovar Typhimurium resulted mainly from the strain-specific non-lactic acid molecules present in their cell free culture supernatant (CFCS) (Fayol-Messaoudi et al., 2005). However, they also suggested that the higher antibacterial activity of probiotic *Lactobacillus* strains that produced L-lactic acid could be related in part to the greater proportion of L-lactate in the CFCS than in strains producing D,L-lactic acid. Hence, the antibacterial activity of probiotic *Lactobacillus* strains that leads to the killing of bacterial pathogens

may be due to a synergistic action of lactic acid and the secreted non-lactic acid molecules.

(2) Hydrogen peroxide

Hydrogen peroxide has been reported to be responsible for the inhibitory activity of streptococci (Tano et al., 2003a). It was shown in Sookkhee S's study that the antimicrobial activity of the oral isolates was reduced after treatment with catalase, and their growing colonies demonstrated a blue pigment around the colonies on the medium supplemented with tetramethylbenzidine and horseradish peroxidase (Sookkhee et al., 2001). These results suggested that the isolates could produce hydrogen peroxide which is another type of antimicrobial substance. Pridmore RD *et al.* also demonstrated the ability of *Lactobacillus johnsonii* NCC 533 as well as eight different *L. johnsonii* strains and *L. gasseri* to produce H₂O₂ when resting cells were incubated in the presence of oxygen (Pridmore et al., 2008). The culture supernatant containing NCC 533-produced H₂O₂ was effective in killing *Salmonella enterica* serovar Typhimurium SL1344 *in vitro*.

(3) Bacteriocin

Sookkhee S *et al.* suggested a significant reduction in inhibitory activity

after treatment with trypsin and pepsin (Sookkhee et al., 2001). They proved that the additional type of antimicrobial substances were bacteriocins, which are proteinaceous antimicrobial agents. de Carvalho AA *et al.* partially purified the inhibitory substance of *Lactobacillus* isolate from Italian sakami (de Carvalho et al., 2006). They found the inhibitory substance maintained activity when treated with catalase or incubated with trypsin, while it showed much less inhibitory activity when treated with proteinase K and papain. The activity was maintained at temperatures up to 100°C, but decreased if the crude extract was autoclaved. Furthermore, the activity in the culture supernatants was not significantly affected by various pH values (2.0-10.0). The antagonistic activity of isolate PD 6.9 was suggested to be due to bacteriocin production. Abo-Amer AE tested 63 strains of *Lactobacillus acidophilus* isolated from Egyptian home-made cheese (Abo-Amer, 2007). He found only 8 strains demonstrated inhibitory activity against spoilage microorganisms and pathogens. *Lactobacillus acidophilus* AA11 produced higher antimicrobial activity with a wide range of inhibition. The inhibitory substance was identified as a bacteriocin, designated acidocin AA11. In Awaisheh and Ibrahim's report, cell-free neutral supernatant of human lactic acid bacteria (LAB) isolates had the highest antibacterial activity against different pathogenic strains, followed by RTE-VPMP (ready-to-eat vacuum-packaged meat products) LAB isolates, and fermented vegetables isolate (Awaisheh and Ibrahim, 2009). Antibacterial activity of neutral supernatant is a strong

confirmation of bacteriocin presence and production, since all sources of antibacterial effects of extracted supernatants other than bacteriocins (i.e., H₂O₂, lactic acid) were eliminated. The bacteriocin was sensitive to proteolytic enzymes and retained full activity at 100°C, could be extracted from the culture supernatant fluids with n-butanol. The 12% SDS-PAGE analysis of 40% ammonium sulphate precipitated agent showed two peptides with molecular weights of ~ 36kDa and ~ 29kDa.

Lin PP *et al.* presented the opposite results. They investigated the inhibitory effect of LAB-CFCS towards EAggEC strains (Lin et al., 2009a). The treatment with different enzymes did not affect the antagonistic activity. This result suggested that the antimicrobial compounds might not have proteinaceous nature. This finding contradicts with the other reports, which may be due to the different *Lactobacillus* strains they studied.

(4) Blocking of adhesion sites

Pioneering studies by Reid and co-workers have demonstrated that selected *Lactobacillus* strains of urovaginal origin have adhesive properties so that they could inhibit the colonization of uropathogens with uroepithelial cells (Bruce and Reid, 1988; Chan et al., 1985; Reid et al., 1985; Reid et al., 1987). The same mechanism of action has subsequently been proposed (Conway et

al., 1987). However, this property is strain specific. Tuomola and Salminen found that the four most adhesive strains tested to the Caco-2 cell line were *L. casei* (Fyos), *L. johnsonii* La1, *L. rhamnosus* LC-705 and *L. casei rhamnosus* GG (Tuomola and Salminen, 1998). Heat-killed *L. acidophilus* LB bacteria were also found to adhere as efficiently as the live strain to both undifferentiated and differentiated human intestinal epithelial Caco-2 cells. A study reported that several species of *Bifidobacterium* spp. produced a proteinaceous molecule or molecules(s) with a molecular weight around or over 100 000 Da and a neutral isoelectric point, which enabled the competitive binding to ganglioside GM1 (asialo-GM1 or GA1), a common bacterium-binding structure (Fujiwara et al., 1997).

Although it is believed that the maximum probiotic effect is achieved if the organisms adhere to intestinal mucosal cells, it is unclear that exogenously administered probiotics really do this in human bodies. They may pass through into the faeces without having adhered or multiplied. A few studies have been conducted to investigate the persistence of probiotic strains in the gut and their colonization. *L. casei rhamnosus* strain GG could adhere *in vivo* to the colonic epithelia of most people after consumption of probiotic LGG product (Alander et al., 1997; Alander et al., 1999). Similarly, *L. johnsonii* strain La1 and *L. casei* strain Shirota ingested by adult patients were able to survive intestinal transit (Donnet-Hughes et al., 1999).

(5) Co-aggregation

A novel, specific co-aggregation was found between *L. paracasei* or *L. rhamnosus* and mutans streptococci *in vitro* (Lang et al., 2010). The co-aggregation by these specific lactobacilli was characterized as heat stable and protease-resistant, and lectin-independent. It was operational over a wide pH range, unaffected by whole saliva, but calcium dependent. Twetman *et al.* proved that the probiotic strains they tested showed co-aggregation abilities with the oral pathogens (Twetman et al., 2009). The co-aggregation was strain specific and dependent on time. *S. mutans* GS-5 exhibited a significantly higher ability to co-aggregate with all the probiotic strains than the other mutans streptococci and *E. coli* in their study. The results demonstrated different abilities of lactobacilli-derived probiotic bacteria to co-aggregate with selected oral streptococci. Another study also demonstrated that some commercially available probiotic *Lactobacillus* strains (*L. plantarum*, *L. paracasei*, *L. rhamnosus* GG, *L. reuteri* and *L. acidophilus*) had an ability to co-aggregate with the MS strains isolated from human oral cavity (Keller et al., 2011).

Hence, it is speculated that the co-aggregation between lactobacilli and mutans streptococci blocks the binding of mutans streptococci to exposed

teeth or plaque biofilm, so that caries risk is reduced by the clearance of planktonic mutans streptococci from the mouth by swallowing (Lang et al., 2010).

(6) Stimulation of immunity

Probiotic bacteria affect the intestinal luminal environment, epithelial and mucosal barrier function, and the mucosal immune system. They display their effects on numerous cell types involved in the innate and adaptive immune responses, such as epithelial cells, dendritic cells, monocytes/macrophages, B cells, T cells and NK cells (Hart et al., 2004; Kaila et al., 1992; Lammers et al., 2002; Miettinen et al., 1998; Takeda et al., 2006). Some studies have shown that probiotics can induce regulatory cytokine IL-10 and suppress proinflammatory cytokines, such as TNF, in the mucosa of patients with ulcerative colitis and pouchitis (Borruel et al., 2002; Pathmakanthan et al., 2004; Ulisse et al., 2001).

Not all *Lactobacillus* and *Bifidobacterium* species are equally beneficial; each may have individual mechanisms of action that are dependent on host characteristics. Different bacteria may have dominant effects in different genetic backgrounds and in diseases that vary in their pathogenesis.

1.5.4.2 Effects on biofilm formation and acidogenicity of oral bacteria

(1) Biofilm formation

Ze Zhang T Wen *et al.* (Wen *et al.*, 2010) reported that biofilm formation by *S. mutans* was significantly decreased when grown with *Streptococcus sanguinis*, but was modestly increased when co-cultivated with *Lactobacillus casei*, compared to mono-species biofilm. Real Time Polymerase Chain Reaction (PCR) analysis showed that the expression of *spaP*, *gtfB* and *gpbB* decreased significantly when *S. mutans* was co-cultivated with *L. casei*.

It was reported that oral bacterium S11 isolated from human saliva showed 99.5% similarity with *Lactobacillus fermentum*. This strain and its culture supernatant significantly inhibited the formation of the insoluble glucan produced by *S. mutans* Ingbritt without inhibiting the multiplication of *S. mutans* Ingbritt. The adherence onto cuvette walls was also inhibited (Chung *et al.*, 2004).

Eva M. Söderling *et al.* found all of the probiotic lactobacilli that tested inhibited the biofilm formation of the clinical isolates of mutans streptococci (Söderling *et al.*, 2011a). The biofilm formation of the reference strains of MS was also inhibited by the lactobacilli, but *L. plantarum* and *L. reuteri* PTA

5289 showed a weaker inhibition in comparison to *L. reuteri* SD2112 and *L. rhamnosus* GG. The antimicrobial activity against *S. mutans* was pH-dependent as the supernatant with higher pH showed less inhibitory effects.

(2) Acid production

Keller and Twetman found that in the presence of *L. reuteri*, dental plaque produced less lactic acid in comparison to *L. plantarum* and the blank control (Keller and Twetman, 2012a). However, in their clinical study, no significant difference in lactic acid concentration was found between groups with and without probiotic lactobacilli tablets taken for 2 weeks.

Marttinen *et al.* investigated thirteen volunteers who used tablets containing *L. rhamnosus* GG or a combination of *L. reuteri* SD2112 and PTA 5289 for 2 weeks (Marttinen *et al.*, 2012a). All the subjects had good oral hygiene with low DMFT (decayed, missing, filled teeth) score. They found there was no difference of the lactic acid production and MS levels in plaque suspension between the baseline and the end of probiotic treatment period.

1.5.5 Safety of lactobacilli used as probiotic agents

Lactobacillus species are generally considered to be nonpathogenic since they have a long history of safe use in food. Although some lactobacilli might cause endocarditis in immunosuppressed patients, none of these infections were related to lactobacilli used in probiotic food products (Alvarez-Olmos and Oberhelman, 2001). However, it is not clear if the pathogenic *Lactobacillus* strains isolated from patients with endocarditis are indigenous or ingested strains (Sipsas et al., 2002). *Lactobacillus* species are also a rare cause of liver abscesses (Rautio et al., 1999). Both endocarditis and liver abscess due to *Lactobacillus* species are extremely rare conditions. A study reported no relation between *Lactobacillus* strains isolated from patients with bacteremia and strains used in the food industry (Saxelin et al., 1996). It was suggested that for some immunocompromised patients and patients with intestinal bleeding, probiotic ingestion may or may not have beneficial results (Reid, 2002).

1.5.6 Cariogenicity of probiotic *Lactobacillus*

Probiotic lactobacilli and bifidobacteria are considered to be non-pathogenic and studies have demonstrated the potential beneficial effect of probiotic bacteria on oral health. However, some species of lactobacilli are

thought to be associated with the development of dental caries as they are also acidogenic bacteria.

A study examined the cariogenicity of the probiotic bacterium *Lactobacillus salivarius* in rats (Matsumoto et al., 2005). The rats were infected with *L. salivarius* and/or *Streptococcus mutans*. The results showed that *L. salivarius* became established in the oral cavity of rats and induced significant level of dental caries. The caries scores of rats infected with both *S. mutans* and *L. salivarius* were significantly higher than those infected with either *L. salivarius* or *S. mutans* alone. It was concluded that *L. salivarius* possesses an inherent cariogenic activity following adherence to the tooth surface. Another study investigated *L. reuteri* strains ATCC PTA 5289 and ATCC 55730. The authors found strain ATCC PTA 5289 adhered on saliva-coated hydroxyapatite and formed detectable biofilm, but strain ATCC 55730 was poor in both adhesion and biofilm formation (Jalasvuori et al., 2012). The amount of dissolved calcium from hydroxyapatite correlated with bacterial growth rate and the final pH of the growth medium. It was concluded that probiotic lactobacilli are likely to differ in their behaviour and cariogenic potential. The possible cariogenicity of probiotic lactobacilli should be further investigated.

1.6 Yakult[®] and *Lactobacillus casei* Shirota

1.6.1 Invention of Yakult[®]

Yakult[®] is a probiotic milk-like product made by fermenting a mixture of skimmed milk with a special strain of bacterium *Lactobacillus casei* Shirota. It was created by Japanese scientist Minoru Shirota (Sako, 2010). In 1930 he succeeded in culturing a strain of lactic acid bacteria which benefited human health in suppressing harmful bacteria within the intestines. This rod-shaped gram positive bacterium was named *Lactobacillus casei* strain Shirota. Shirota then began working together with supporters to make a drink incorporating the strain. This led to the development of Yakult[®]. It was first sold in Japan in 1935 and presently popular in 31 countries with daily consumption by 6 million people globally (Yakult, 2012).

Standard Yakult[®] contains (Yakult-Australia):

- (1) Sugar (sucrose, dextrose) to balance sourness with sweetness.
- (2) Skimmed milk powder
- (3) Natural flavours
- (4) Live *Lactobacillus casei* Shirota strain, 6.5 billion per 65 ml bottle
(concentration of 10^8 cfu/ml)
- (5) Water

1.6.2 Effects of *L. casei* Shirota on general health

1.6.2.1 *In vitro* and *in vivo* studies

(1) Immune modulation

Lactobacillus casei Shirota (LcS) was reported that it enhanced the gene expression involving defense/immune functions and lipid metabolism (Shima et al., 2008). Daily intake of *Lactobacillus casei* strain Shirota provided a positive effect on NK-cell activity (Matsuzaki et al., 2005; Takeda and Okumura, 2007). The involvement of induced interleukin (IL)-12 may be responsible for the enhanced NK cell activity (Takeda et al., 2006). Administration of LcS has also been shown to induce the production of several cytokines, such as Interferon- γ (IFN- γ), IL-1 β and TNF- α (Matsuzaki, 1998). A recent study demonstrated that LcS activated cytotoxic lymphocytes preferentially in both the innate and specific immune systems, which suggested that LcS could potentiate the destruction of infected cells in the body (Dong et al., 2010).

Sabine Wagnerberger *et al.* also used a mouse model and found the dietary intake of LcS protects against the onset of fructose-induced

non-alcoholic fatty liver disease through mechanisms involving an attenuation of the Toll-like receptor-4-signalling cascade in the liver (Wagnerberger et al., 2013). A report in 1998 suggested that the oral administration of LcS was able to modify the humoral and cellular immune responses to type II collagen, and these modifications could result in the reduction of the development of type II collagen-induced arthritis in DBA/1 mice (Kato et al., 1998). A study explored the immunomodulatory effects of probiotics on allergen-specific allergic reactions in an allergy mouse model (Lim et al., 2009). The authors found the oral administration of heat-killed LcS could effectively down regulate the pre-existing Th-2 allergic responses and pulmonary inflammatory responses, and therefore possibly treat the allergic respiratory diseases.

(2) Anti-infectious effects

LcS has demonstrated anti-infectious activity against multi-drug resistant *Salmonella enterica* serovar Typhimurium DT104 (DT104) (Asahara et al., 2011), while the heat-killed LcS was not protective against the infection, suggesting that the metabolic activity of lactobacilli is important. LcS inhibited *Helicobacter pylori* growth in an *in vitro* study (Cats et al., 2003). Further more, Sgouras *et al.* using a mouse model displayed a significant reduction in the levels of *H. pylori* colonization in the antrum and body mucosa *in vivo* in the LcS treated group (Sgouras et al., 2004). This reduction

was accompanied by a significant decline in the associated chronic and active gastric mucosal inflammation.

Waard *et al.* carried out a study investigating the effect of viable *Lactobacillus casei* Shirota on enteric pathogen *Listeria monocytogenes* in Wistar rats (de Waard et al., 2002). Orally administered LcS was able to enhance host resistance against oral *L. monocytogenes* infection. In the gastrointestinal tract, as well as in the spleen and liver, *L. monocytogenes* numbers were reduced. It was concluded that the enhancement of this anti-*Listeria* activity might be partially due to the increased cell-mediated immunity.

(3) Anti-carcinogenic effects

A study demonstrated intrapleural administration of LcS into tumour-bearing mice has been shown to effectively inhibit the growth of tumour cells in the thoracic cavity and to significantly prolong survival time (Matsuzaki, 1998). It was also reported that LcS inoculated into mice induced protection against *T. spiralis* adult worms and an increase in the production of IgA anti-*T. spiralis* (Martinez-Gomez et al., 2009).

1.6.2.2 Clinical trials

Yakult[®] has been used as probiotic therapy for gastrointestinal diseases, such as constipation, frequent defecation and diarrhea.

Several clinical trials have evaluated the efficacy and safety of using *L. casei* Shirota for the treatment of constipation. Most of these studies demonstrated that consumption of LcS milk reduced the incidence of hard or lumpy stools, or showed a beneficial effect on gastrointestinal symptoms of patients with chronic constipation (Koebnick et al., 2003; Sakai et al., 2011). One study controversially found that *L. casei* strain Shirota did not alleviate constipation severity or stool frequency, consistency, and quantity when compared with control (Mazlyn et al., 2013). The function of *L. casei* Shirota improving irregular defecation frequency and stool quality and increasing the intrinsic bifidobacteria in healthy individuals with soft stool has also been demonstrated (Matsumoto et al., 2010). Take together, LcS is a probiotic strain that has intestine-conditioning activity in people who tend to get constipated, as well as those with diarrhea-like symptoms.

LcS has also been evaluated for beneficial effect on acute diarrhea. A double-blind, randomized and controlled field trial involving 3758 children aged 1–5 years was conducted in India to evaluate the effect of probiotic drink

(contain *L. casei* Shirota) on acute diarrhea (Sur et al., 2011). The level of diarrhea protective efficacy for the probiotic drink was 14%, compared with nutrient drink.

Volunteers treated with LcS showed a significant reduction in levels of antigen induced IL-5, IL-6 and IFN- γ production compared with volunteers supplemented with placebo. Meanwhile, levels of specific IgG increased and IgE decreased in the probiotic group. These data showed that probiotic supplementation modulated immune responses in allergic rhinitis (Ivory et al., 2008).

A recent clinical trial showed that daily consumption of a fermented milk drink containing LcS had no statistically or clinically significant effect on the protection against respiratory symptoms (Van Puyenbroeck et al., 2012), although it was suggested in animal study that the oral administration of *L. casei* Shirota could effectively reduce the allergen-specific allergic reactions, and possibly treat the allergic respiratory diseases (Lim et al., 2009).

1.6.3 Effects of *L. casei* Shirota on oral health

Lactobacillus casei Shirota (LcS) is a well researched probiotic strain, widely used as a dietary supplement for reduction of functional and infectious

gut diseases. However, its potential benefits on oral health have not been sufficiently investigated.

The lactobacilli species are related to dental caries progression and highly identified in deep caries dentin (Maltz et al., 2002). As LcS also has acidogenic and aciduric features, LM Lima *et al.* compared the adhesion of two probiotics microorganisms (*Lactobacillus casei* Shirota and *Lactobacillus acidophilus*) to an artificial dentin caries after 48 hours culture (Lima et al., 2005). It was found that the amount of *L. acidophilus* in the artificial caries dentin was significantly more than that of *L. casei* Shirota. The authors suggested that further investigations must be carried out to identify the cariogenic potential of *L. casei* Shirota, since Yakult[®] is a sweetish drink with a low pH that favors caries development.

Lodi C.S. *et al.* carried out a study on plaque pH and tooth demineralization using Yakult[®] (Lodi et al., 2010). Ten volunteers wore devices containing bovine dental enamel blocks. The appliances were treated with Yakult[®], another fermented milk Batavito[®] or 20% sucrose solution for 5 minutes 8 times every day. The results showed that the ionic concentration (F, Ca and P) in the biofilm in Batavito[®] group was significantly higher than Yakult[®] and 20% sucrose groups, with no difference between the latter two. The two fermented milk groups did not differ significantly but had smaller

mineral loss than control group. All treatments decreased the pH of dental biofilm and promoted enamel demineralization. The acidogenicity results were different with the studies on other probiotic species. Keller and Twetman found that in the presence of *L. reuteri*, dental plaque produced less lactic acid compared with the blank control (Keller and Twetman, 2012a). Marttinen *et al.* found no difference in the lactic acid production in plaque suspension before and after *L. reuteri* tablet intake (Marttinen *et al.*, 2012a). Further studies need to be carried out to evaluate the effects of different probiotic strains on acidogenicity of dental plaque and tooth demineralization.

A. Haukioja *et al.* investigated the binding of 17 *Lactobacillus* (including LcS) and 7 *Bifidobacterium* strains to hydroxyapatite and microtitre wells coated with human saliva and human buccal epithelial cells (Haukioja *et al.*, 2006). They found a large variation in binding to saliva-coated surfaces and buccal epithelial cells, but all the *Lactobacillus* and *Bifidobacterium* strains survived in saliva for the 24 hours tested. LcS showed low binding to saliva-coated microtiter wells, but high binding to saliva-coated hydroxyapatite beads.

There is no concrete evidence of the effect of *L. casei* Shirota on oral bacteria and caries risk so far. A recent study in our research group (unpublished data) showed the beneficial effect of Yakult on dental plaque.

Fifteen volunteers consumed the fermented milk Yakult® 100ml every day for 1 week. Before and after the period, the dental plaque resting pH and Stephan curve were recorded and plotted. *S. mutans* counts in the dental plaque before and after the 1-week consumption were also analyzed. The results showed that the Stephan curve changed significantly, which demonstrated that the acidogenicity of dental plaque decreased after Yakult consumption. Furthermore, *S. mutans* counts in dental plaque reduced after probiotic treatment. The result indicated that Yakult® could have potential cariostatic effect and the probiotic milk treatment might decrease caries risk.

1.7 Research Gaps

Most of the current therapies for caries prevention have limitations and/or side effects. Probiotic therapy may offer a promising alternative as different probiotic species have demonstrated potential cariostatic effects. However, to the best of our knowledge, no study has been conducted to investigate the effects of the widely consumed probiotic milk Yakult® and the strain *L. casei* Shirota on oral pathogenic bacterium *Streptococcus mutans*. Furthermore, the exact mechanisms of the cariostatic effects of probiotic strains remain unclear.

1.8 Aim of this study

This study was designed to investigate the effects of the fermented probiotic milk Yakult[®] and the probiotic strain *Lactobacillus casei* Shirota on oral cariogenic bacterium *Streptococcus mutans*, and the possible mechanisms involved. It may shed the light on the clinical application of this non-fluoride high-compliance “sweet” therapy (probiotic drink) to prevent caries formation, particularly in children.

1.9 Objectives of this study

(1) To investigate the effects of Yakult[®] on *S. mutans* biofilm formation and acidogenicity.

(2) To investigate the effects of *L. casei* Shirota on *S. mutans* biofilm formation, acidogenicity and the lesion depth of enamel demineralization underneath biofilm.

(3) To investigate the effects of *L. casei* Shirota on *S. mutans* growth, EPS production, biofilm structure and virulence genes expression in biofilm.

(4) To investigate the effects of the supernatant of *L. casei* Shirota on growth of *S. mutans* and to characterize the inhibitory compounds.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2:

Materials and Methods

PART 1: Effects of Yakult[®] on *S. mutans* biofilm

In this part, the biofilm-on-tooth (BOT) model was built in order to investigate the effects of Yakult[®] on *Streptococcus mutans* biofilm formation and acidogenicity. The biofilms were distributed into three groups to be treated with Yakult[®], acidic milk and acidic PBS, separately. Confocal laser scanning microscope (CLSM) and micro-touch method were used to evaluate the biofilm thickness and acidogenicity.

2.1.1 Tooth sample preparation

Six extracted human third molars (approved by National University of Singapore – Institutional Review Board: 07-275) were collected. These teeth were observed under stereomicroscope (Olympus SZ-CTV, Japan) to ensure there was no caries or demineralization. The teeth were stored in 0.1% thymol at 4°C before use. Eighteen blocks with intact enamel surface were cut from the crowns of the teeth by a micromotor handpiece (NSK Volvere Vmax, Japan). Each block was 2 mm thick. These specimens were sterilized by autoclaving at 121°C and 15psi for 15 minutes (min), and then dried overnight.

The blocks were applied with red nail varnish twice, leaving a 3 mm x 3 mm exposed enamel surface window and air-dried overnight.

2.1.2 Bacteria culture and biofilm-on-tooth (BOT) model

S. mutans ATCC 25175 (purchased from the American Type Culture Collection) was grown to the mid-exponential phase in Brain Heart Infusion (BHI) (Biomed, US) broth. The bacteria were collected by centrifugation at 8000 rpm for 10 min at 4°C and washed with PBS twice, then suspended in BHIS (BHI supplemented with 1% sucrose) in a 24-well culture plate at a final concentration of 1×10^8 cfu/ml (2 ml in each well).

Fresh simulated human saliva was collected from 10 healthy volunteers who had abstained from food within 8 hour (h). The collected saliva was sterilized through 0.22 µm-pore-size Millex GS filter units (Millipore, Billerica, Massachusetts, USA), and stored at -20°C prior to use.

The BOT model was modified according to Arnold WH (Arnold et al., 2001; Arnold et al., 2006). The tooth blocks were incubated in the above thawed human saliva at 37°C for 1 h for coating with the acquired pellicle. After incubation, the blocks were put into above *S. mutans* culture in a 24-well plate and cultured in 5% CO₂ atmosphere at 37°C for 24 h (Fig. 2.1).

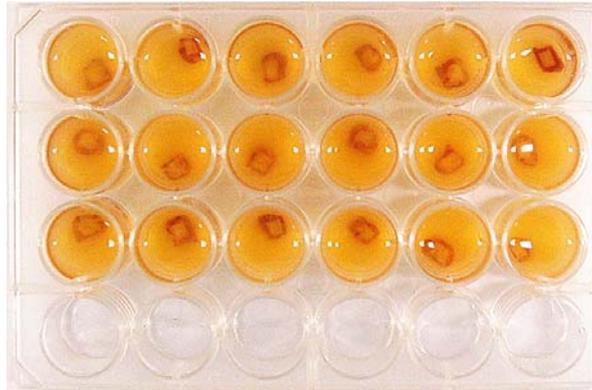


Fig. 2.1. Tooth blocks incubated with *S. mutans* in BHIS in a 24-well plate.

2.1.3 Experimental treatments

The pH of Yakult[®] (Yakult Singapore Pte. Ltd., Singapore) was measured using pH meter (model 370, Orion Research, Inc, USA). Commercial fresh milk (Meiji fresh milk, Singapore) and sterilized phosphate-buffered saline (PBS) were adjusted to the measured pH of Yakult[®] by adding sterilized 5M lactic acid. Yakult[®], acidic milk and acidic PBS were stored in 4°C.

The tooth blocks were randomly distributed to three groups (named as Yakult, milk and PBS groups) with six blocks in each group after 24 h culture. They were removed from the 24-well plate, immersed in 2 ml Yakult[®], acidic milk or acidic PBS respectively, and incubated at 37°C for 30 min. After incubation, the tooth blocks were gently rinsed with sterilized distilled water for 5 seconds, and put back into the previous 24-well plate in which broth was

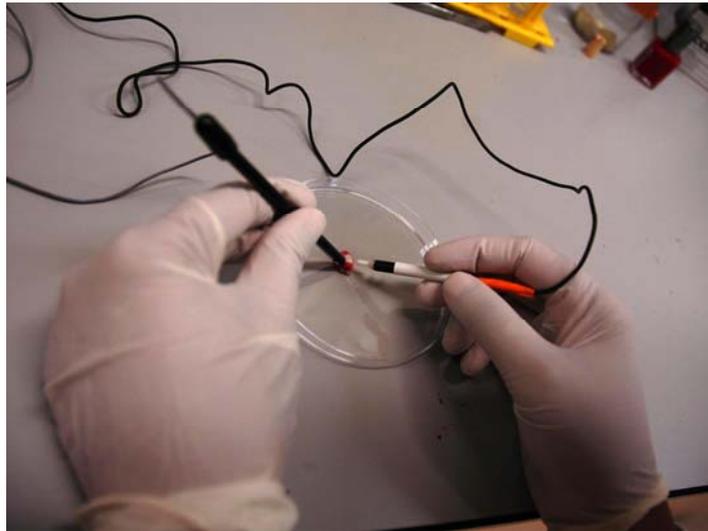
refreshed. The plate was continuously cultured in the CO₂ incubator at 37°C. The above treatments were repeated once every day. The biofilm was cultured for 7 days (d).

2.1.4 Biofilm morphology and acidogenicity

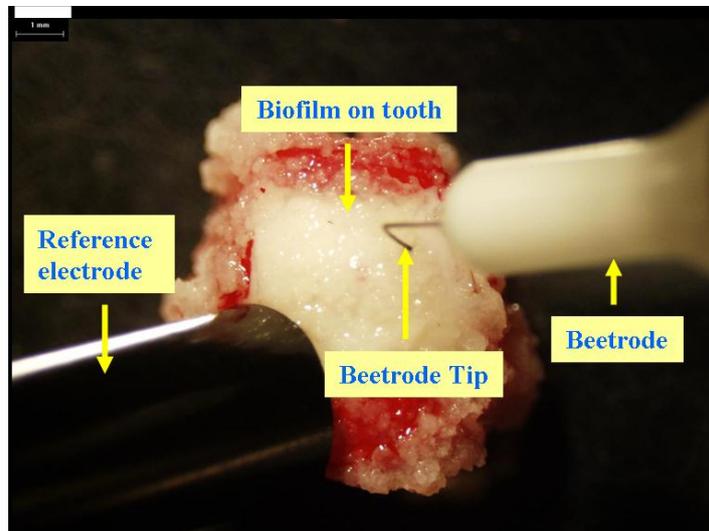
After the 7-day treatment, the tooth blocks were taken out from *S. mutans* culture. The pH of tooth-biofilm interface was measured using the micro-touch method (Fig. 2.2 & Fig. 2.3). A microelectrode Beetrode (NMPH3, World Precision Instruments Inc., Sarasota, Florida, USA) with a 100 µm sensing tip was connected to a pH meter (model 370, Orion Research, Inc, USA). Readings were obtained from three random sites in each biofilm sample.



Fig. 2.2. Micro-pH meter set up. The Beetrode and reference electrode were connected to the pH meter *via* the Bee-cal offset device.



(a)



(b)

Fig. 2.3. Biofilm pH measurement using micro-touch method. (a) Biofilm pH was measured using a microelectrode (Beetrode) connected to a pH meter equipped with a reference electrode. (b) The Beetrode tip was inserted into the biofilm, reaching to the tooth surface-biofilm interface.

2.1.5 Biofilm thickness

Live and Dead dye were prepared following the instruction of the Live/Dead *BacLight* Bacterial Viability Kit (Invitrogen, Eugene, Oregon, USA), gently dropped on biofilm surface and incubated in dark for 15 minutes.

After incubation, the biofilm was observed under inverted confocal laser scanning microscope (CLSM) (Olympus FluoView FV1000, Japan). Images were acquired with UPLAPO 20x NA: 0.70 objective lens. Filters were set to 488 nm for SYTO 9 to detect live cells and 543nm for PI to detect dead cells. The Z dimension was set as 2µm/slice. Each biofilm was scanned at the area not closed to the edge of the field. As the tooth surface was curved, the area selected to be scanned was in the center of the visible field. The biofilm thickness was calculated according to the slice number in Z stack.

The experiment design is shown in Fig. 2.4.

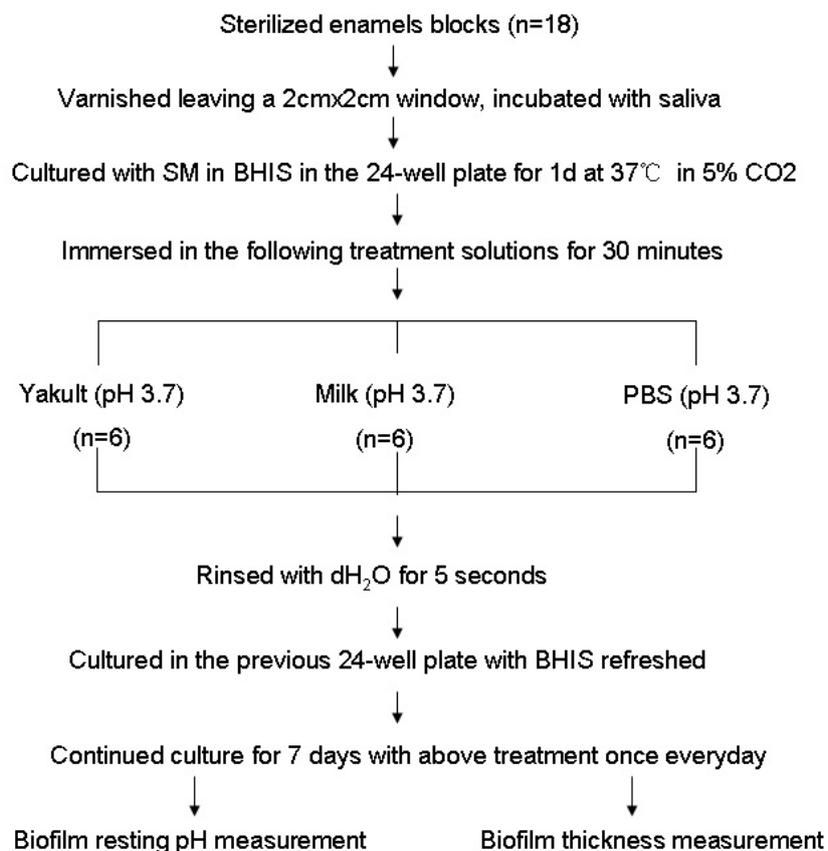


Fig. 2.4. Experimental flow chart of the effects of Yakult® on *S. mutans* biofilm.

2.1.6 Statistical analysis

Data were analyzed by using ANOVA with Tukey post hoc test (SPSS 20).

The significance level was set as 0.05.

PART 2: Effects of *L. casei* Shirota on *S. mutans* biofilm

L. casei Shirota is the probiotic bacteria in Yakult. In order to evaluate the effects of *L. casei* Shirota on *S. mutans* biofilm formation and acidogenicity, biofilm-on-tooth (BOT) model and Stephan curve *in vitro* model were employed. The tooth demineralization underneath biofilm was also measured under polarized light microscope (PLM) to evaluate the relationship between acids formed on tooth surface and tooth demineralization.

2.2.1 Stephan curve *in vitro* model

2.2.1.1 Tooth preparation

Tooth blocks were prepared and coated with stimulated human saliva using aforementioned methods.

2.2.1.2 Bacteria culture and biofilm formation

S. mutans ATCC 25175 cells in mid-exponential phase were collected as described in 2.1.2. The cell suspension was cultured in 6 ml BHIS in a 6-well culture plate with inoculation of 1×10^8 cfu/ml. The saliva-coated tooth blocks were incubated in above *S. mutans* culture at 37°C at 5% CO₂ for 7 days. Fresh BHIS was replaced everyday.

2.2.1.3 Stephan curve plotting

After 7 days of incubation, the pH value of tooth-biofilm interface was recorded by the aforementioned micro-touch method. The tooth blocks were then immersed in the artificial saliva (20 mmol/l KCl, 10 mmol/l NaH₂PO₄, 0.3 mmol/l CaCl₂, 0.03 mmol/l MgCl₂) (Lagerlof et al., 1984) until the biofilm pH was neutralized as in oral cavity. The artificial saliva used here was to mimic the buffering system in human saliva and was easy to prepare. The buffering capacity of artificial saliva was similar to stimulated human saliva as tested in our pilot study. The tooth blocks were immersed in 10% sucrose for 1 min. The pH of tooth-biofilm interface was measured every 2 min until it reached the lowest pH. The blocks were then re-immersed in the artificial saliva and the pH was recorded until it was neutralized. To prevent biofilm from drying, the samples were kept in a humidity chamber before pH

measurement. After the time and duration for pH measurement were optimized, artificial Stephan curve was plotted.

2.2.1.4 Statistical analysis

The experiment was repeated twice in triplicate. Data were analyzed using 2-sample *t*-test (SPSS 20.0) after the Levene's test confirming the homogeneity of variance. The statistical significance was set as $P < 0.05$.

2.2.2 Effects of *L. casei* Shirota on *S. mutans* biofilm and enamel demineralization

2.2.2.1 Bacteria culture

L. casei Shirota YC-13 used in this study was isolated from a commercial probiotic drink Yakult® (Yakult Singapore Pte. Ltd., Singapore) using De Man, Rogosa, Sharpe (MRS) agar (Becton, Dickinson and Company, Sparks, Maryland, USA). Its identification was confirmed by strain specific primers (Fujimoto et al., 2008). *S. mutans* ATCC 25175 (SM) and *L. casei* Shirota (LcS) were cultured overnight in BHI and MRS broth individually. The cells were grown to the mid-exponential phase, followed by centrifuge with 8000rpm for 10 min at 4°C. The pellets was washed twice with PBS, and

suspended in cold PBS prior to use.

2.2.2.2 Tooth blocks preparation and biofilm culture

The tooth blocks were prepared as aforementioned and incubated in human saliva for 1h at 37°C. The blocks were randomly distributed into two groups (n=4): SM group and SM-LcS group. In SM group, the blocks were cultured with 1×10^8 cfu/ml *S. mutans* in 6 ml BHIS in a 6-well plate. In SM-LcS group, the blocks were cultured with *S. mutans* and *L. casei* Shirota (both 1×10^8 cfu/ml). The tooth blocks were incubated at 37°C at 5% CO₂ for 7 days. Fresh BHIS was replaced everyday.

2.2.2.3 Biofilm morphology and acidogenicity

After 7 days of incubation, the biofilm morphology was observed under the stereomicroscope. Three pH readings of tooth-biofilm interface of each sample were recorded using the micro-touch method, as well as the pH of the spent culture and the biofilm formed on the bottom of the 6-well plates. Artificial Stephan curves were plotted to enumerate the lowest pH and the recovery time, together with the area under curve (AUC) (area below the critical pH 5.5) using Image-pro software (Image Pro International, InC, USA).

2.2.2.4 Lesion depth

The tooth samples were sectioned using the hard tissue microtome (Series 1000 Deluxe, Scientific Fabrications, USA) after the removal of the biofilm and varnish. The lesion image was captured under polarized light microscope (PLM) (Olympus BX51, Japan). Three representative enamel lesions with 200 μm width in each image were selected to calculate the average lesion area by using Image-pro. The average lesion depth was calculated by the lesion area divided by 200 μm .

2.2.2.5 Statistical analysis

The experiments were repeated once with similar results. Data were analyzed using 2-sample *t*-test (SPSS 20.0) after the Levene's test confirming the homogeneity of variance. The statistical significance was set as $P < 0.05$.

2.2.3 Effects of other bacteria on *S. mutans* biofilm

In order to further evaluate the effects of probiotic strains on SM biofilm, and to exclude the nutrition depletion in co-culture, *S. sanguinis*, one of the first colonizers in oral cavity, and a commonly used probiotic strain *L.*

rhamnosus GG were harbored as controls.

2.2.3.1 Bacteria culture

S. mutans (SM), *S. sanguinis* ATCC 10556 (SS) (purchased from the American Type Culture Collection), *L. casei* Shirota (LcS) and *L. rhamnosus* GG (LGG) (donated by Professor Meurman JH, Faculty of Dental Medicine, University of Helsinki) at mid-exponential phase were collected, washed and suspended in cold PBS prior to use.

2.2.3.2 Tooth preparation and biofilm culture

The enamel blocks with a 3 mm x 3 mm window were prepared and coated with stimulated human saliva as aforementioned. The blocks were randomly distributed into SM mono-culture, SM-SS, SM-LcS and SM-LGG co-culture groups (n = 3). For SM group, the blocks were cultured with *S. mutans* of 1×10^8 cfu/ml in 6 ml BHIS in a 6-well plate. For SM-SS, SM-LcS, SM-LGG group, the blocks were cultured in the same inoculation of *S. mutans* and *S. sanguinis*, *L. casei* Shirota or *L. rhamnosus* GG, respectively. The blocks were cultured at 37°C at 5% CO₂ for 7 days. Fresh BHIS was replaced everyday.

2.2.3.3 Biofilm morphology and acidogenicity

After 7 days of incubation, the biofilm formed on tooth surface was observed under the stereomicroscope. Three random pH readings of tooth-biofilm interface were recorded. The artificial Stephan curves were plotted as described above.

2.2.3.4 Statistical analysis

Data were analyzed by using ANOVA with Tukey post hoc test (SPSS 20). The significance level was set as 0.05.

PART 3: Modes of action of *L. casei* Shirota on *S. mutans*

To understand the action of *L. casei* Shirota (LcS) in SM-LcS co-culture, the growth of SM and LcS in both biofilm and liquid culture, as well as biofilm structure and EPS production, were investigated in this part. In stead of using BOT model, we cultured biofilms on cover slip and 6-well plate to exclude the bias of variation of tooth size. The dynamic changes of biofilm during 1-7 days culture were analyzed under confocal laser scanning microscope (CLSM), including SM and LcS biovolumes, biofilm thickness and acidogenicity.

2.3.1 Effects of *L. casei* Shirota in biofilm culture within 24 h

2.3.1.1 Biofilm culture

S. mutans (SM) and *L. casei* Shirota (LcS) cells at mid-exponential were collected and cultured with 6 ml BHIS in a 6-well plate in three groups (SM mono-culture group, LcS mono-culture group, and SM-LcS co-culture group, n = 3). The inoculation of *S. mutans* and *L. casei* Shirota was 1×10^8 cfu/ml. To avoid the bias of bacteria counting on tooth surface with size variation, biofilms were cultured on a piece of sterilized cover slip in the well. Bacteria counting were facilitated as the biofilms were easily removed from the glass surface (Hyde et al., 1997). The biofilms were cultured at 5% CO₂ at 37°C for 7 days. Fresh BHIS was replaced everyday.

2.3.1.2 Bacteria counting

The biofilms on the cover slip were washed with PBS twice to remove the unattached bacteria. The biofilms were then scraped from the cover slip, and suspended in 15 ml PBS. The suspension was serially diluted and cultured on the selective agar plates. Mitis salivarius (MS) agar (Becton, Dickinson and Company) and MRS agar were used for *S. mutans* and *L. casei* Shirota culture

respectively. Colony forming units for *S. mutans* and *L. casei* Shirota in mono-culture and co-culture biofilms were determined. Data were presented as mean \pm SD of 3 independent experiments performed in triplicate.

2.3.1.3 Biofilm structure under SEM

SM and SM-LcS 24 h biofilms were fixed in 2.5% glutaraldehyde (GA) overnight at 4°C. The biofilms were washed in PBS for 10 min with two changes. After fixing in 1% Osmium tetroxide (OsO₄, pH 7.4) for 2 h, the biofilms were washed in PBS and dehydrated through an ascending ethanol series (50%, 75%, 95% and absolute). The biofilms were coated with a thin layer of gold and sent to the scanning electronic microscope (SEM) (Philips XL30 FEG, FEI Company, Holland) for observation.

2.3.1.4 Quantification of EPS

The 24 h biofilms were incubated with 10 mg/ml fluorescein isothiocyanate-concanavalin A (FITC-conA) (Sigma-Aldrich, St. Louis, Missouri, USA) at room temperature for 15 min. After incubation, they were rinsed with dH₂O, and stored at -20°C prior to the CLSM examination. Images were acquired using the inverted CLSM with UPLAPO 20x NA: 0.70 objective lens. Filters were set to 488 nm for FITC to detect EPS. The EPS

production was determined by *bioImage_L* v2.1 software (Luis E. Chávez de Paz, Sweden).

2.3.1.5 Statistical analysis

Data were analyzed using 2-sample *t*-test (SPSS 20.0) after the Levene's test confirming the homogeneity of variance. The statistical significance was set as $P < 0.05$.

2.3.2 Effects of *L. casei* Shirota in liquid culture within 24h

2.3.2.1 Bacteria culture

Mid-exponential phase *S. mutans* (SM) and *L. casei* Shirota (LcS) cells were collected using aforementioned method. SM mono-culture and SM-LcS co-culture were incubated in 6 ml BHIS in a polypropylene centrifuge tube at 37°C in 5% CO₂ for 24 h respectively. The same inoculation of 1×10^8 cfu/ml as that in above biofilm culture part (2.3.1) was used, in order to investigate the action of planktonic LcS in comparison with that in biofilm.

2.3.2.2 Bacteria counting

During the incubation, 0.1ml culture in both groups was taken out at 0h, 4h, 6h, 8h and 24h. After serial dilution, it was spread on the selective MS and MRS agar plates. The agar plates were cultured at 37°C for 2 days.

2.3.2.3 Statistical analysis

Data were presented as mean \pm SD of two independent experiments in triplicate and analyzed by 2 samples t test (SPSS 20).

2.3.3 Dynamic changes in biofilm

To observe the dynamic changes of SM and LcS in mono-culture and co-culture biofilms during days 1-7, FISH technique was applied and the biofilms were observed under CLSM. However, since the stimulated auto-fluorescence of enamel surface interfered with the fluorescence signal of biofilms in the pilot study, we chose not to use the BOT model. As SM biofilm could not grow more than 3 days on glass surface, a discovery made in our pilot study, we finally employed biofilm on 6-well plate model. The effects of LcS on SM biofilm was achieved using this model.

2.3.3.1 Effects of LcS on 6-well plate model

Biofilm culture

Mid-exponential phase of SM, *S. sanguinis* (SS), LcS and *L. rhamnosus* GG (LGG) were collected. SM mono-culture biofilm and SM-LcS, SM-SS, SM-LGG co-culture biofilms were cultured for 7 days. For SM group, *S. mutans* was cultured in 6ml BHIS with inoculation of 1×10^8 cfu/ml in the 6-well plate. For SM-SS, SM-LcS, SM-LGG group, the inoculation of SM and SS, LcS and LGG were identical to that of the SM group. Fresh BHIS was replaced everyday.

Morphology observation and acidogenicity measurement

After 7 days of incubation, the spent culture was taken out from the wells and the biofilm morphology was observed. The pH of the spent culture and the biofilm was measured using the micro-touch method.

Biofilm thickness

The bottoms of the 6-well plate covered with biofilm were cut with the biofilm intact. LIVE/DEAD dye (Invitrogen, Eugene, Oregon, USA) was applied on biofilm surface before incubation in dark for 15 min. The biofilms were observed under inverted confocal laser scanning microscope (CLSM) (Olympus) as described in 2.1.5. The biofilm thickness was calculated according to the slice number in Z stack.

Statistical analysis

Data were analyzed by using ANOVA with Tukey post hoc test (SPSS 20).

The significance level was set as 0.05.

2.3.3.2 Dynamic Changes in biofilm assessed by fluorescence *in situ* hybridisation (FISH)

Biofilm culture

SM and SM-LcS biofilms were cultured in 6-well plates as described above. In total, seven 6-well plates were prepared with each one composed of SM group and SM-LcS group (n = 3). After 1 day, one plate was taken out from the incubator and named as Day 1 (D1) biofilm. D2-7 biofilms were collected accordingly.

Morphology observation and acidogenicity assessment

The D1-D7 biofilm were observed after the spent culture was removed from the well. The pH of biofilm and spent culture was measured. The experiment was repeated once. Data were shown as mean \pm SD of the two independent experiments performed in triplicate.

Sample fix and pretreatment

The biofilms were gently rinsed with ice cold PBS twice, then fixed with

4% PFA (Paraformaldehyde) at 4°C overnight. After washing with PBS, the well bottoms covered with biofilm were cut from the plate with the biofilm intact. The biofilm cells were permeabilized with lysozyme solution (Sigma-Aldrich, St. Louis, Missouri, USA; 70,000 U/ml, 100 mM Tris-HCl, pH 7.5) for 15 min at 37°C. The biofilms were then dehydrated with 50%, 80% and 96% ethanol for 3 min and left to dry at room temperature.

Hybridization

The synthesized oligonucleotide probes were purchased from Integrated DNA technologies, Singapore. The SM probe was MUT590 (5'-Cy5-ACTCCAGACTTTCCTGAC-3'), and the LcS probe was Lcas467 (5'-56-FAM-CCGTCACGCCGACAACAG-3') (Quevedo et al., 2011). FISH was performed using a modification of the methods described in previous studies (Frojd et al., 2011; Klug et al., 2011; Marttinen et al., 2012b; Quevedo et al., 2011; Thurnheer et al., 2001). Briefly, cells on each well were exposed to 20 µl hybridization buffer containing 0.9 M NaCl, 20 mM Tris-HCl buffer, pH 7.5, with 0.01% sodium dodecyl sulfate (SDS) and 30% formamide, with 5 ng/µl MUT590 and 15 ng/µl Lcas467. The biofilms were put in a 6-well plate, covered with a piece of paper-towel wet with 2 ml of hybridization buffer to equilibrate humidity before the plate was capped. After that, the 6-well plate was incubated at 46°C for 2 h. After hybridization the biofilms were washed twice in washing buffer (280 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl

buffer, pH 7.5, with 0.01% SDS) and incubated in washing buffer at 48°C for 15 min. The hybridization and washing were carried out in dark. The biofilms were washed in dH₂O, and stored in -20°C prior to observation under CLSM.

Bacteria detection under CLSM

The biofilms were laid on the cover slip with the surface against the glass. Images were acquired using the inverted CLSM with PLAPO 60xWLSM NA: 1.00 objective lens. Filters were set to 488 nm for FAM to detect *L. casei* Shirota and 633 nm for Cy5 to detect *S. mutans*. The Z dimension was set as 1 µm/slice. The bacteria biovolumes were determined by *bioImage_L* v2.1.

Statistical analysis

Data were analyzed using 2-sample *t*-test (SPSS 20.0). The statistical significance was set as $P < 0.05$.

2.3.4 Gene Expression Analysis using Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNAs from the SM and SM-LcS biofilms (days 1-4) were extracted using the Hybrid RTM RNA extraction kit (GeneAll, Seoul, Korea). RNAs were treated with DNase I (Promega, Wisconsin, USA) prior to reverse transcription according to the manufacturer's protocol. cDNAs were

synthesised using the iScript reverse transcription supermix (Bio-Rad, Hercules, California, USA). The resulting cDNAs were subjected to qRT-PCR using the SYBR Green PCR kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). The primers used were *gtfB* (forward: 5'-AGCAATGCAGCCAATCTACAAAT-3'; reverse: 5'-ACGAACTTTGCCGTTATTGTCA-3'), *gtfC* (forward: 5'-AAAGCAACGGATACAGGGGA-3'; reverse: 5'-CTCTGTCATTGGTGTAGCGC-3'), *ldh* (forward: 5'-CTTCCTCGTTGCTGCTAACC-3'; reverse: 5'-TGGCATGAGACCATACTGCA-3'), and 16S rRNA (forward: 5'-CCTACGGGAGGCAGCAGTAG-3'; reverse: 5'-CAACAGAGCTTTACGATCCGAAA-3'). The expression of the respective mRNAs was normalised to the relative abundance of the housekeeping gene 16S rRNA (Livak and Schmittgen, 2001).

After performing Levene's test to confirm the homogeneity of variance, data were analysed using a 2-sample *t*-test (SPSS 20.0) and analysis of variance (ANOVA) with Tukey's post-hoc test. Statistical significance was set at $p < 0.05$.

PART 4: Effects of *L. casei* Shirota supernatant on *S. mutans*

Yakult[®] is fermented probiotic milk which contains a suspension of live *L. casei* Shirota in a sugary skimmed milk-based medium. In addition to the investigation of the action of *L. casei* Shirota, it was important to evaluate the effects of its supernatant on *S. mutans*.

2.4.1 *L. casei* Shirota growth kinetics

L. casei Shirota overnight culture was adjusted to optical density (OD) 0.05 at 600 nm in MRS and started to culture for 72 h. A volume of 100 µl *L. casei* Shirota culture was taken at different time points within 72 h. After serial dilution and plating on MRS agar plate, colonies forming units (cfu) was counted. The OD value of *L. casei* Shirota culture was measured, as well as the pH value of *L. casei* Shirota supernatant.

2.4.2 Inhibition assay

Overnight cultured *L. casei* Shirota was grown for 12, 24, 30, 36, 48 and 54 hours in MRS broth at 5% CO₂ at 37°C. The cell free culture supernatant (CFCS) were obtained by centrifugation at 6,000 rpm for 30 min at 4°C. CFCS were sterilized through 0.22 µm-pore-size Millex GS filter units. *L. casei*

Shirota CFCS (2 ml) was mixed with 10 ml of *S. mutans* in BHI (0.4×10^8 cfu/ml) at 37°C, and incubated for 8 h. MRS (pH was adjusted to that of the stationary phase of CFCS using 1 M HCl) was used as a control. The OD value at 600 nm was measured every 2 h by a spectrophotometer (UV-1700, Shimadzu, Japan). Data were expressed as mean \pm SD of three independent experiments.

2.4.3 Characterization of the inhibitory compounds

pH sensitivity

L. casei Shirota CFCS (pH 3.7) (2 ml) collected at 30 h time point was mixed with 10 ml of *S. mutans* in BHI (0.4×10^8 cfu/ml). The neutralized CFCS which was adjusted to 7.0 by 1M NaOH was also mixed with above *S. mutans*. Two groups of MRS (pH 3.7 and pH 7.0) were served as the controls. All the four groups were incubated at 37°C for 12 h in a 5% CO₂ incubator. A volume of 0.1 ml culture was collected every 2 h and cultured on the BHI agar plates after serial dilution. *S. mutans* colony forming unit was counted. Data were shown as mean \pm SD of three independent experiments.

Heat sensitivity

L. casei Shirota CFCS at 30 h was autoclave-sterilized. Fresh CFCS and autoclaved CFCS, both 2 ml, were mixed into 10 ml of *S. mutans* culture

(0.4×10^8 cfu/ml) respectively. The growth of *S. mutans* with fresh CFCS or autoclaved CFCS was assessed every 2 h by measuring optical density over a 10 hour duration. MRS (pH was adjusted to 3.7 with 1M HCl) was served as the control group. Data were shown as mean \pm SD of two independent triplicate experiments.

Stability

L. casei Shirota CFCS at 30 h was stored at 4°C for 3 months. Fresh CFCS and stored CFCS were added into *S. mutans* culture using the method described above. MRS (pH 3.7) was used as the control group. The optical density was measured every 2 h. Data were shown as mean \pm SD of two independent triplicate experiments.

2.4.4 Antimicrobial activity of *L. casei* Shirota CFCS crude extract

L. casei Shirota CFCS was collected after centrifugation (4000 rpm, 30 min), extracted with equal volume of ethyl acetate. The crude extract (hydrophobic phase) was dried using a rotary evaporator, then dissolved in methanol (200 fold concentrated). MRS broth was also extracted using the same method. The inhibition effect of *L. casei* Shirota CFCS crude extract was determined by agar well diffusion test. Agar wells (5 mm diameter) made in BHI plates were inoculated with 100 μ l of *S. mutans* (0.4×10^8 cfu/ml). A

volume of 5 µl CFCS crude extract, MRS crude extract and Ampicillin (200 mg/l) were dropped into the wells. The agar plates were incubated at 37°C for 24 h.

2.4.5 Statistical analysis

Data were processed with SPSS20 and subjected to One-way ANOVA and Tukey post hoc test. The level of significance was set at $P < 0.05$.

CHAPTER 3

RESULTS

Chapter 3:

Results

PART 1: Effects of Yakult® on *S. mutans* biofilm

3.1.1 Biofilm morphology

The 7-day-old biofilm in the Yakult group was thinner with a smooth surface, while in the milk and PBS group, the biofilm was obviously thick with a rough surface (Fig. 3.1).

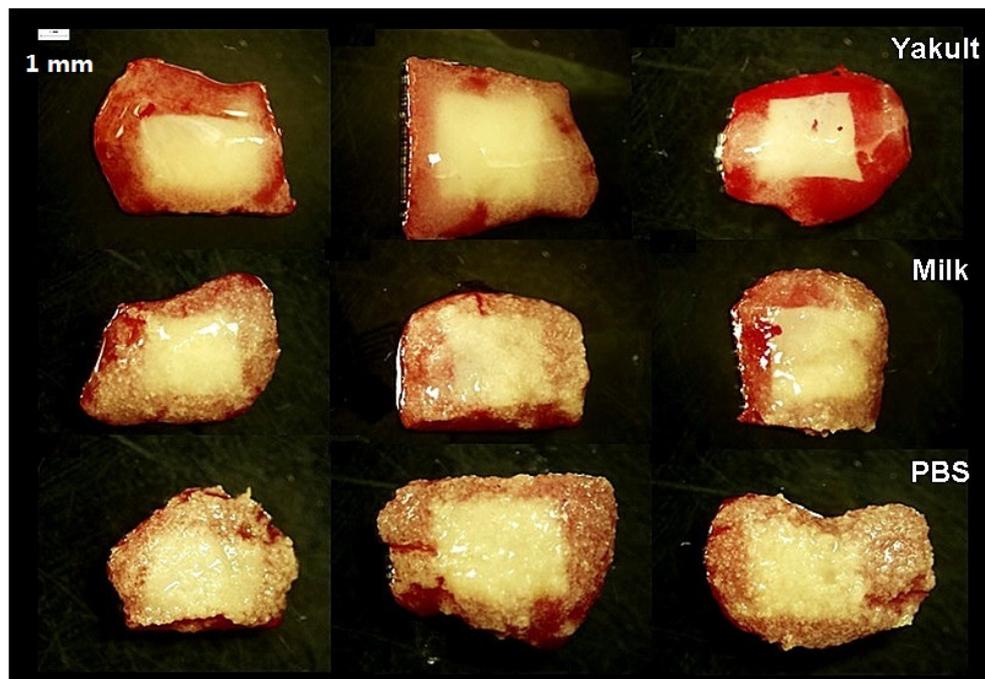


Fig. 3.1. Images of 7-day-old *S. mutans* biofilms on tooth surfaces after daily treatment with Yakult, PBS or milk.

3.1.2 Tooth-biofilm interface pH

The pH of Yakult was 3.7. The milk and PBS were adjusted to pH 3.7 by adding lactic acid before treatment. After treatment, the tooth-biofilm interface pH in the Yakult, milk and PBS groups were measured as shown in Table 3.1. The pH in the Yakult group was 4.62 ± 0.04 , significantly higher than those in the milk and PBS group, which was 4.45 ± 0.04 and 4.47 ± 0.03 respectively (both $P < 0.001$), while there was no significant difference between the milk and PBS group ($P > 0.05$). Biofilm acid production (concentration of $[H^+]$) in the Yakult group was significantly less than that in the milk and PBS group by 33.52% and 29.62% respectively.

Table 3.1 Tooth-biofilm interface pH (Mean \pm SD) in Yakult, milk and PBS groups

Measurement	Groups (n = 6)		
	Yakult	Milk	PBS
Tooth-biofilm interface pH	4.62 ± 0.04^a	4.45 ± 0.04^b	4.47 ± 0.03^b
$[H^+]$ in biofilm (1×10^{-5} mol/L)	2.40 ± 0.19^a	3.61 ± 0.37^b	3.41 ± 0.28^b

Different letters indicate statistically significant difference for each measurement ($P < 0.001$). The experiment was repeated once with similar results.

3.1.3 Biofilm thickness

Biofilm thickness of the Yakult group was $32.67 \mu\text{m}$, significantly thinner than milk group ($52.67 \mu\text{m}$) and PBS group ($54.67 \mu\text{m}$) (both $P < 0.05$) as

shown in Table 3.2. The biofilm in the Yakult group was 47.63% and 50.85% thinner than those in the milk and PBS groups respectively.

Table 3.2 Biofilm thickness (μm) (mean \pm SD) in Yakult, milk and PBS groups

Measurement	Groups (n = 6)		
	Yakult	Milk	PBS
Biofilm thickness	29.33 \pm 8.07 ^a	56.00 \pm 15.90 ^b	59.67 \pm 22.03 ^b

Different letters indicate statistically significant difference ($P < 0.05$). The experiment was repeated once with similar results.

PART2: Effects of *L. casei* Shirota on *S. mutans* biofilm

3.2.1 Stephan curve model of *S. mutans* biofilm

The 7-day-old biofilms were taken out from the plate and neutralized in the artificial saliva to mimic the situation in oral cavity. After 30 min, the pH was stable at about 6-7 (Fig. 3.2). The pH value gradually dropped after 1 min of 10% sucrose rinse until it reached the lowest point and remained stable between 20-30 min (Fig. 3.3). When the biofilm was re-immersed in the artificial saliva, the pH increased and eventually stabilized at neutral pH (Fig. 3.4). Hence, the typical artificial Stephan curve was plotted (Fig. 3.5).

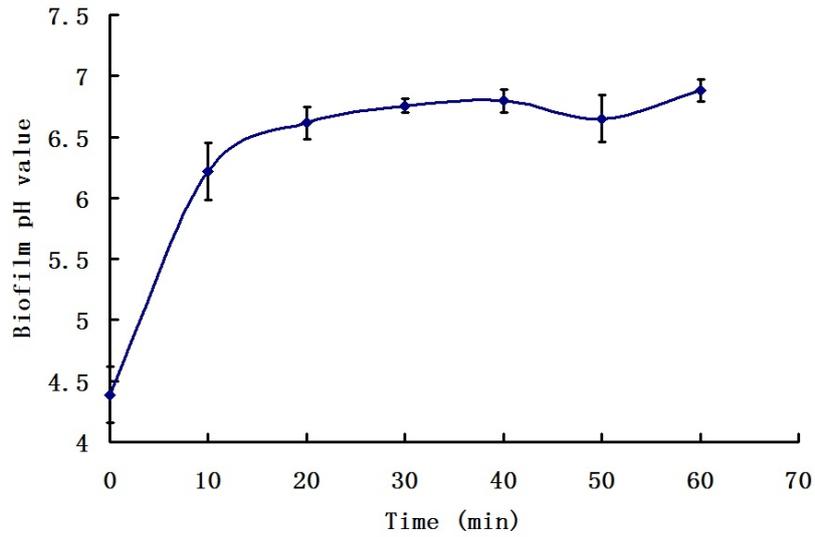


Fig. 3.2. Recovery of the tooth-biofilm interface pH in artificial saliva. The pH was neutralized and stabilized at 6-7 after 30 minutes (n=3). The experiment was repeated twice with similar results.

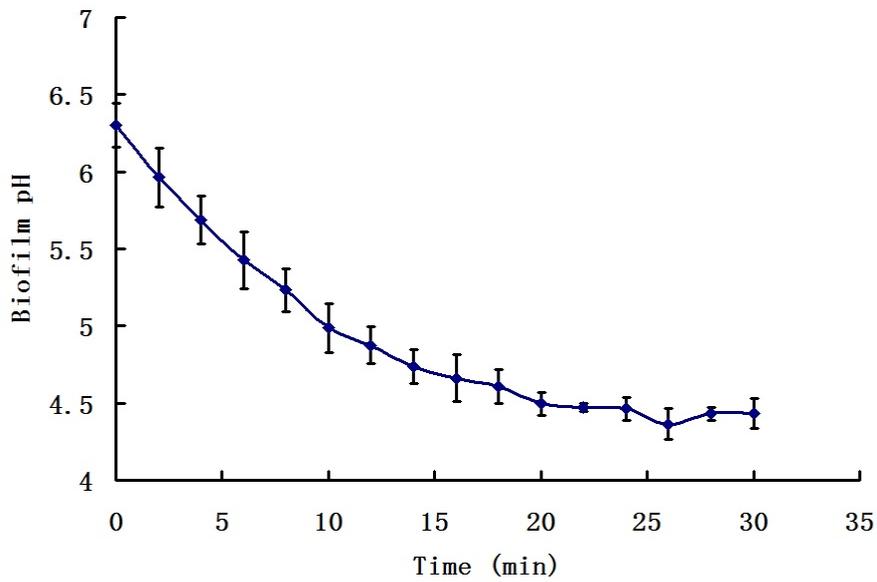


Fig. 3.3. Decrease in tooth-biofilm interface pH after 1-min of 10% sucrose rinse. The pH reached to a lowest point and was stable between 20-30min (n=3). The experiment was repeated twice with similar results.

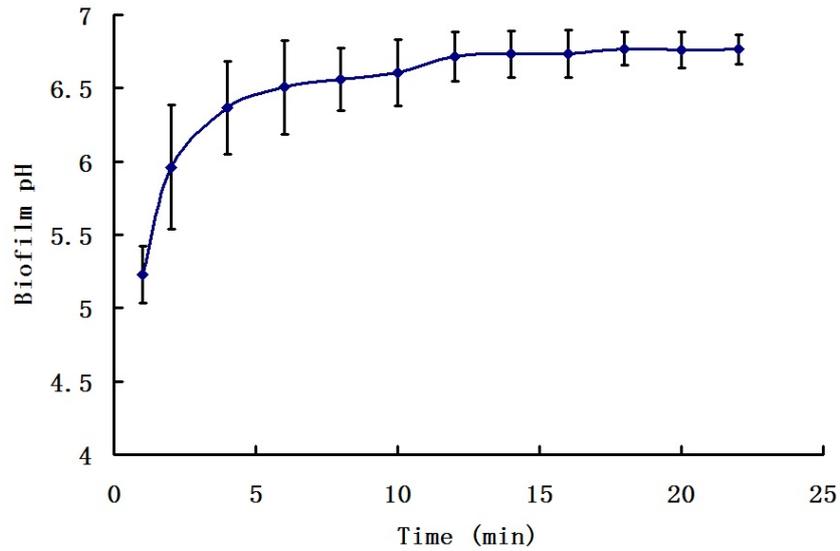


Fig. 3.4. Recovery of tooth-biofilm interface pH in artificial saliva after sucrose challenge. The pH was recovered and reached stabilization at neutral pH within 10 minutes (n=3). The experiment was repeated twice with similar results.

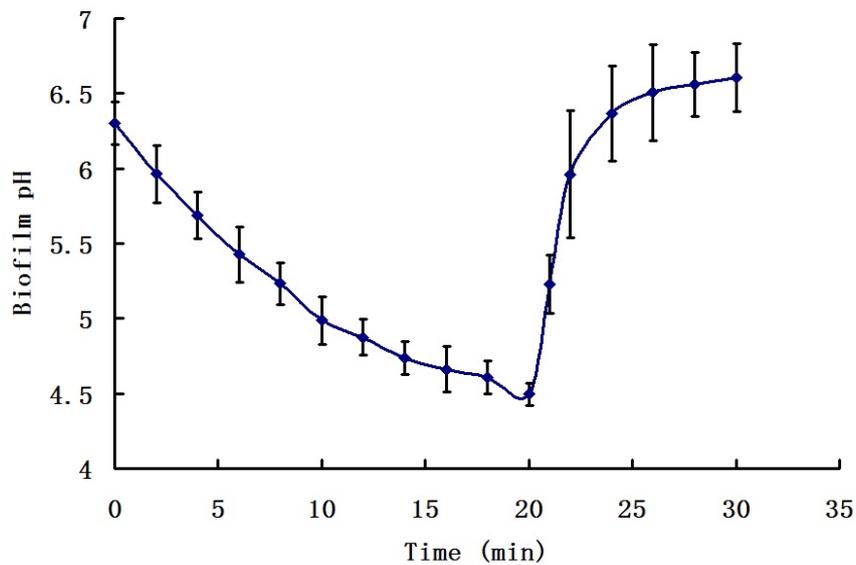


Fig. 3.5. Artificial Stephan curve of 7-day-old *S. mutans* biofilm. The tooth-biofilm interface pH dropped gradually to the lowest point after sucrose rinse at 20 min, and dramatically increased to the neutral pH within 10 min when re-immersed in artificial saliva. The experiment was repeated once with similar result (n=3).

3.2.2 Effects of *L. casei* Shirota on *S. mutans* biofilm and enamel demineralization

3.2.2.1 Biofilm morphology

As shown in Fig. 3.6, biofilms in the SM group on the tooth blocks were thick with rough surfaces, while biofilms in the SM-LcS group were thinner with smooth surfaces.

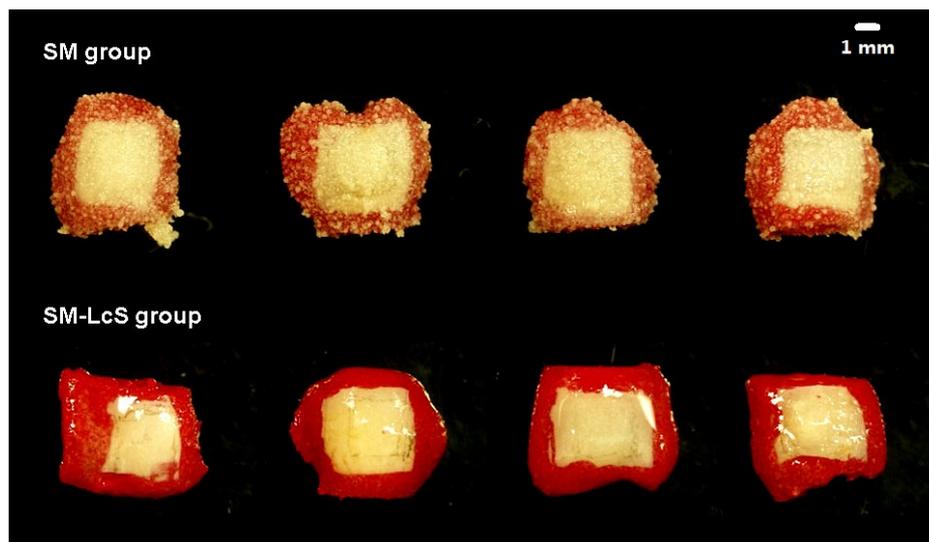


Fig. 3.6. SM and SM-LcS biofilms on tooth surface after 7 days culture. SM mono-culture biofilms displaying rough surfaces were thicker than the smooth SM-LcS co-culture biofilms.

3.2.2.2 Biofilm acidogenicity

The tooth-biofilm pH of 7-day-old SM biofilm and SM-LcS biofilm were listed in Table 3.3. The pH of SM biofilm was 4.25 ± 0.03 , significantly lower

than that of SM-LcS biofilm which was 4.73 ± 0.04 ($P < 0.001$). Significant differences were also found between the spent culture of the SM and SM-LcS groups ($P < 0.001$), and between the pH of the biofilm formed on the bottom of the 6-well plates in the two groups ($P = 0.001$). The acid production (concentration of $[H^+]$) accumulated on the tooth surface dramatically decreased by 66.66% when *L. casei* Shirota was added into *S. mutans* biofilm.

Table 3.3 pH values of tooth-biofilm interface, spent culture and biofilms formed on plates (mean \pm SD)

Measurements	Groups (n = 4)		P
	SM	SM-LcS	
Tooth-biofilm interface pH	4.25 ± 0.03	4.73 ± 0.04	< 0.001
$[H^+]$ in biofilm on tooth surface (1×10^{-5} mol/L)	5.64 ± 0.44	1.88 ± 0.16	< 0.001
Spent culture pH	4.34 ± 0.04	4.75 ± 0.01	< 0.001
pH of biofilm on the 6-well plate	4.10 ± 0.10	4.39 ± 0.08	0.001

3.2.2.3 Stephan curves

The tooth-biofilm interface pH of the SM and SM-LcS biofilms increased to 6.62 ± 0.11 and 6.59 ± 0.14 respectively when neutralized in artificial saliva. After sucrose rinsing, the pH value gradually dropped and reached the lowest point after 20 minutes. The lowest pH was 4.90 ± 0.10 in the SM group, lower than the 5.46 ± 0.19 in the SM-LcS group ($P = 0.002$). The pH returned back to 6.32 ± 0.03 and 6.95 ± 0.22 in the SM and SM-LcS group respectively after immersion in the artificial saliva for 9 min. The biofilm pH changes from day

1 to 7 during culture and the Stephan curves were plotted as shown in Fig. 3.7. The recovery time in the SM-LcS group (1.75 ± 2.02 min) was 86% shorter than that in the SM group (12.75 ± 1.55 min) ($P < 0.001$). The AUC in the SM-LcS group was significantly lower than that in the SM biofilm by 94.25% ($P = 0.012$) (Table 3.4).

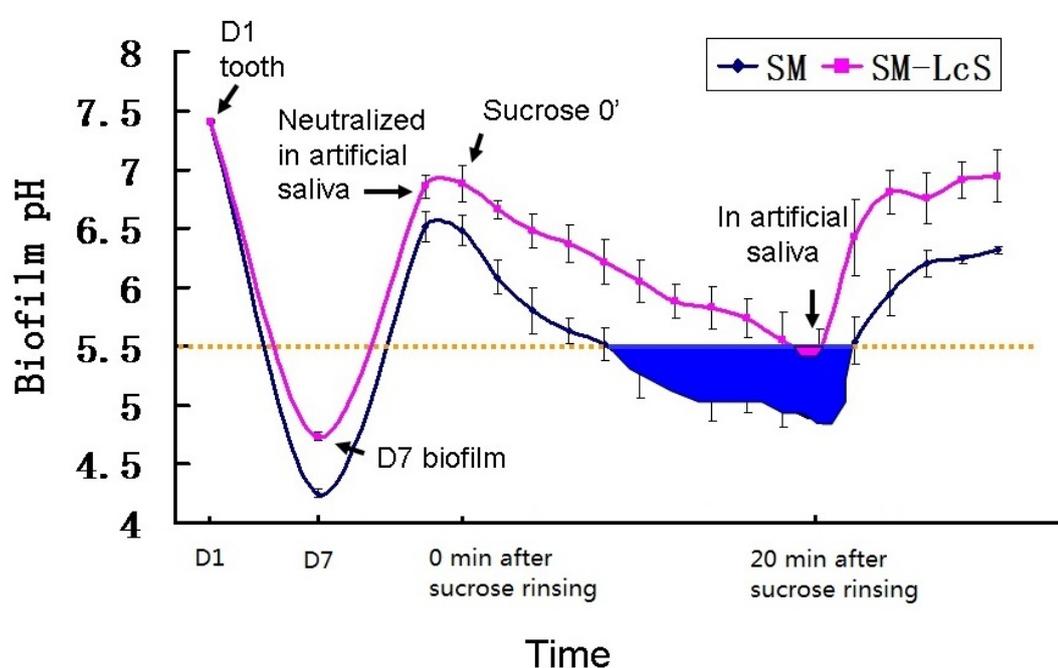


Fig. 3.7. Comparison of biofilm pH changes and Stephan curves of SM and SM-LcS biofilms. On Day 1 (D1), the tooth samples were incubated in media with same neutral pH. The biofilm pH dropped to below 5 on Day 7 (D7), and recovered back to neutral in artificial saliva. After 1 min sucrose rinsing, the Stephan curves were plotted. The pink area under pH 5.5 indicates the area under curve (AUC) of the SM-LcS biofilm. The total area of blue and pink under pH 5.5 indicates the AUC of the SM biofilm. The experiment was repeated once with similar results ($n = 4$).

Table 3.4 Comparison of the features of Stephan curves between SM and SM-LcS biofilms (mean \pm SD)

Outcome	Groups (n = 4)		<i>P</i>
	SM	SM-LcS	
Lowest pH	4.90 \pm 0.10	5.46 \pm 0.19	0.002
Recovery time (min)	12.75 \pm 1.55	1.75 \pm 2.02	< 0.001
AUC	22417.25 \pm 8159.95	1289.00 \pm 1750.32	0.012

The experiment was repeated once with similar results.

3.2.2.4 Lesion depth

The lesion was formed underneath biofilm due to the acid production accumulated on the tooth surface. There was no obvious lesion underneath the varnish as shown in Fig. 3.8. The lesion appeared as a dark layer under the enamel surface. Fig. 3.9 showed the lesion boxes selected for the calculation of lesion depth. The enamel lesion was $83.11 \pm 10.91 \mu\text{m}$ deep in the SM-LcS group, significantly less than that in the SM group which was $128.25 \pm 3.65 \mu\text{m}$ ($P = 0.002$) (Fig. 3.10, table 3.5). The lesion reduction was 35.20%.

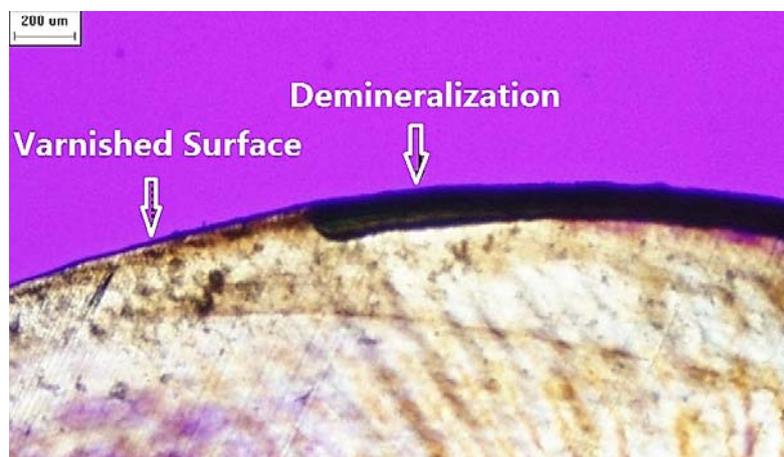


Fig. 3.8. Tooth section with enamel lesion observed under PLM. The teeth were sectioned (120 μm thick) after the removal of biofilm and varnish. The

lesion appeared as a dark layer underneath the exposed enamel surface, while no lesion underneath the varnished enamel surface.

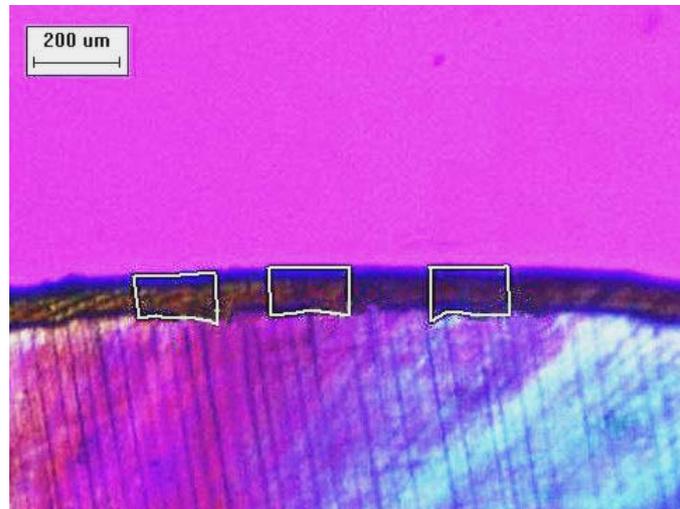


Fig. 3.9. Representative boxes selected in the enamel lesion of the tooth section. The boxes were 200 μm in width.

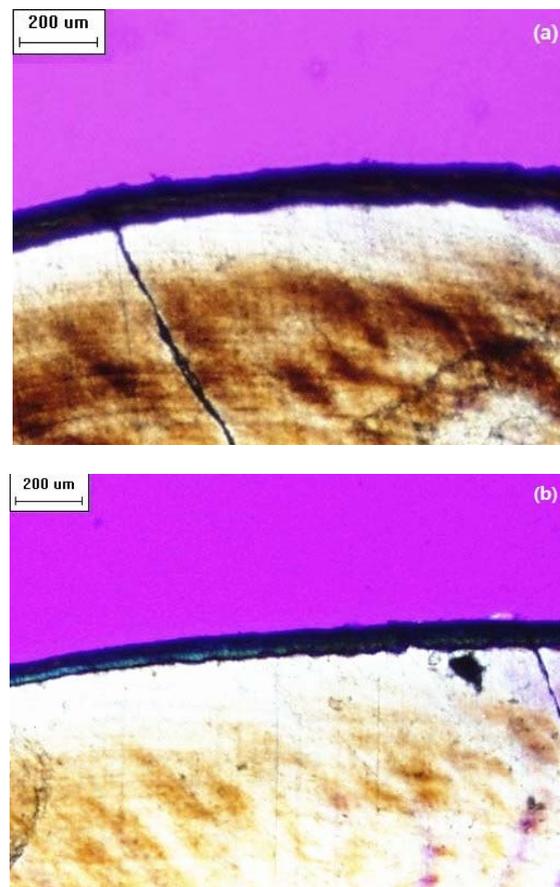


Fig. 3.10. Enamel lesions under the SM biofilm (a) and the SM-LcS biofilm

(b). The lesion appeared as a dark layer on the tooth surface. The lesion under the SM biofilm was significantly deeper than that under the SM-LcS biofilm ($P = 0.002$).

Table 3.5 Enamel lesion depth underneath the SM and SM-LcS biofilms (mean \pm SD)

Outcome	Groups (n = 4)		Percentage of lesion reduction (%) *	P
	SM	SM-LcS		
Lesion depth (μm)	128.25 \pm 3.65	83.11 \pm 10.91	35.20%	0.002**

* Percentage of lesion reduction was expressed as the ratio of the difference to the value of SM group.

** Homogeneity of variance was confirmed by the Levene's test ($P > 0.05$)

The experiment was repeated once with similar results.

3.2.3 Effects of other bacteria on the acidogenicity of *S. mutans* biofilm

3.2.3.1 Biofilm morphology

As shown in Fig. 3.11, the biofilms in the SM and SM-SS groups were thicker than those in the SM-LcS and SM-LGG groups. SM and SM-SS biofilms displayed rough surfaces, while SM-LcS and SM-LGG biofilms showed smooth surfaces.

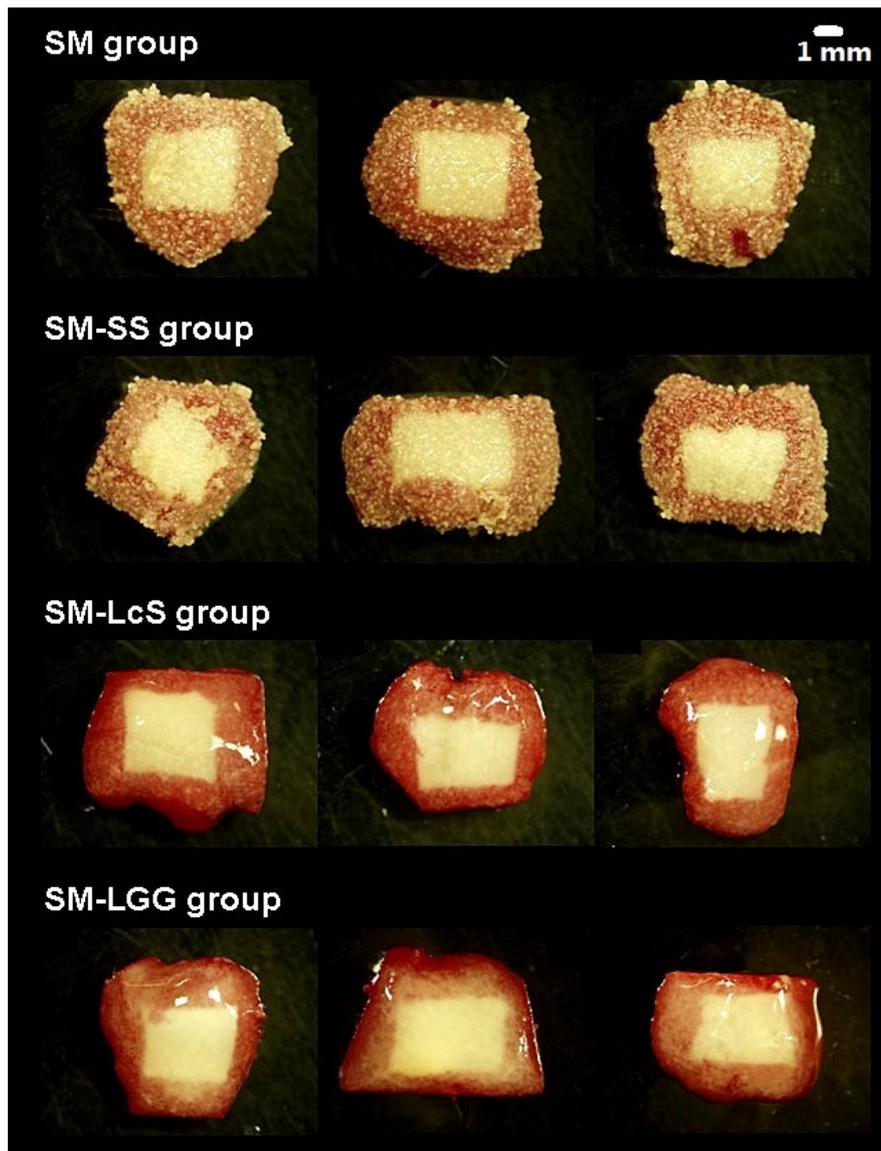


Fig. 3.11. SM, SM-SS, SM-LcS, SM-LGG biofilms formed on the tooth surface after 7 days culture.

3.2.3.2 Acidogenicity

The tooth-biofilm pH values of the four groups of biofilms and the lowest pH values after sucrose rinse are shown in Table 3.6. The tooth-biofilm interface pH in the SM and SM-SS groups were significantly lower than those in the SM-LcS and SM-LGG groups. The pH value of the SM biofilm was not

statistically different from that of the SM-SS biofilm ($P > 0.05$). Similarly, the pH value of the SM-LcS biofilm was also not different from that of the SM-LGG biofilm ($P > 0.05$).

Table 3.6 Tooth-biofilm interface pH and the lowest pH after sucrose rinse in different co-culture biofilms

Outcome (mean \pm SD)	Groups (n = 3)			
	SM	SM-SS	SM-LcS	SM-LGG
Tooth-biofilm interface pH	4.48 \pm 0.02 ^a	4.45 \pm 0.04 ^a	4.83 \pm 0.05 ^b	4.58 \pm 0.04 ^b
Lowest pH	5.32 \pm 0.18 ^a	5.42 \pm 0.01 ^a	5.87 \pm 0.03 ^b	5.77 \pm 0.22 ^b

Different letters indicate statistically significant difference for each outcome ($P < 0.01$)

The Stephan curves of the four groups of biofilms are shown in Fig. 3.12. The Stephan curve of the SM biofilm was similar to that of the SM-SS biofilm. The SM-LcS and SM-LGG biofilms also had the similar curve shape. The lowest pH of the SM and SM-SS groups were 5.32 \pm 0.18 and 5.42 \pm 0.01 respectively, while the SM-LcS and SM-LGG groups had significantly higher lowest pH values of 5.87 \pm 0.03 and 5.77 \pm 0.22 respectively. The SM-LcS and SM-LGG biofilm pH did not drop below 5.5 after 1 min of sucrose rinsing; hence it was not meaningful to calculate the recovery time and AUC. Nevertheless, the SM-LcS and SM-LGG biofilms showed the decreased acidogenicity than the SM and SM-SS biofilms.

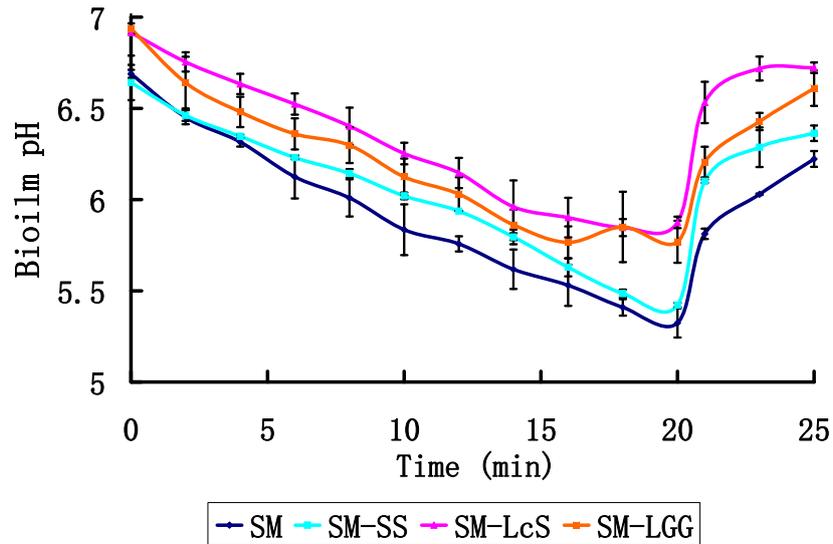


Fig. 3.12. Stephan curves of the 7-day-old SM, SM-SS, SM-LcS and SM-LGG biofilms. The experiment was repeated once with similar results (n = 3).

PART 3: Modes of action of *L. casei* Shirota on *S. mutans*

3.3.1 Effects of *L. casei* Shirota on *S. mutans* biofilm within 24 h

3.3.1.1 Bacteria counting

S. mutans and *L. casei* Shirota counts (cfu/ml) in the 24h SM mono-culture and SM-LcS co-culture biofilms are shown in Fig. 3.13. *S. mutans* counts in the co-culture showed an increasing trend compared to those in the mono-culture biofilm, but the difference was not significant ($P = 0.371$). *L. casei* Shirota hardly formed mono-culture LcS biofilm. Its counts in the co-culture biofilm was significantly greater than that in the mono-culture LcS

biofilm ($P = 0.001$).

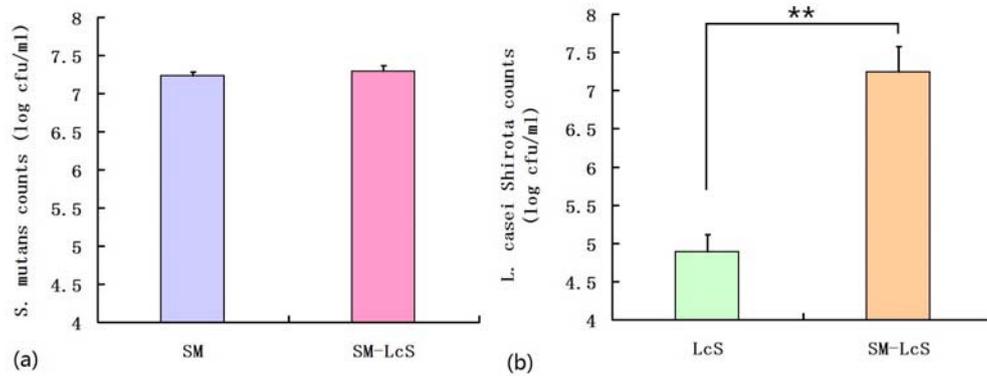


Fig. 3.13. *S. mutans* and *L. casei* Shirota counts in the mono-culture and co-culture biofilms. (a) *S. mutans* counts in SM and SM-LcS biofilms. (b) *L. casei* Shirota counts in LcS and SM-LcS biofilms. The experiment was performed 3 times in triplicate (** $P < 0.01$).

3.3.1.2 Biofilm structure under SEM

As shown in Fig. 3.14, the SM biofilm was firm with more extracellular matrix in the biofilm, while the SM-LcS biofilm displayed less extracellular matrix.

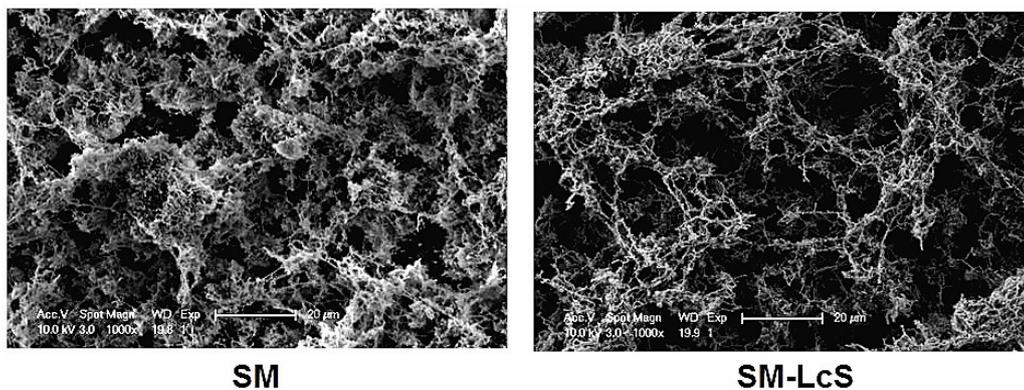


Fig. 3.14. SEM images of the 24h SM and SM-LcS biofilm. Images were obtained at a 1,000 x magnification.

3.3.1.3 EPS production

The SM biofilm contained more EPS matrix than the SM-LcS biofilm. EPS clustered to form microcolonies in the SM biofilm, while the formation of microcolonies was impaired in the SM-LcS biofilm, with sparse and scattered EPS (Fig. 3.15a). The quantification of the biovolume of the stained EPS using a CLSM indicated that the EPS production in the SM-LcS biofilm was significantly lower than that in the SM biofilm ($P = 0.037$) (Fig. 3.15b).

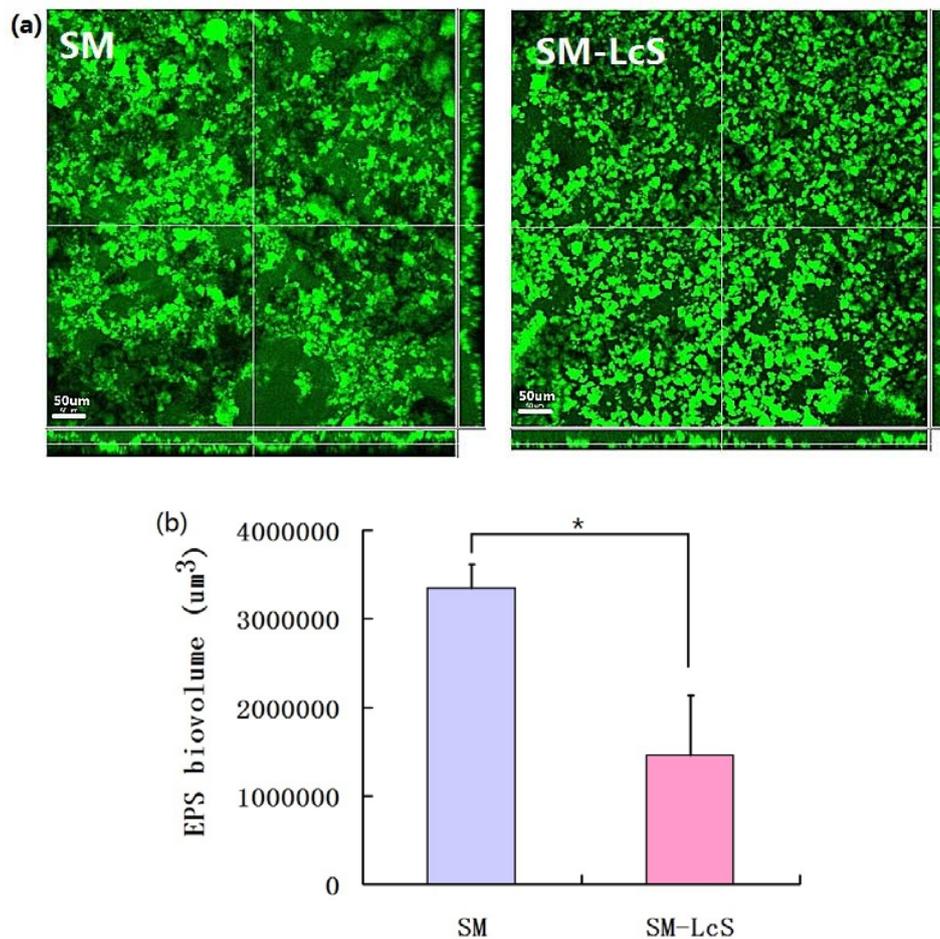


Fig. 3.15. EPS in the 24 h SM and SM-LcS biofilms. (a) EPS in the biofilms were stained with FITC-conA (green). (b) Staining EPS biovolume was

quantified (n=9) (* $P < 0.05$).

3.3.2 Effect of *L. casei* Shirota on *S. mutans* liquid culture within 24 h

In order to investigate if *L. casei* Shirota acted differently in liquid culture in comparison to biofilm culture, SM and LcS were co-cultured in a centrifuge tube, which prevented biofilm formation, with the same inoculation as in previous biofilm culture. As shown in Fig. 3.16, there was no difference of *S. mutans* cfu between the SM mono-culture and SM-LcS co-culture during 0 – 24 h (all $P > 0.05$).

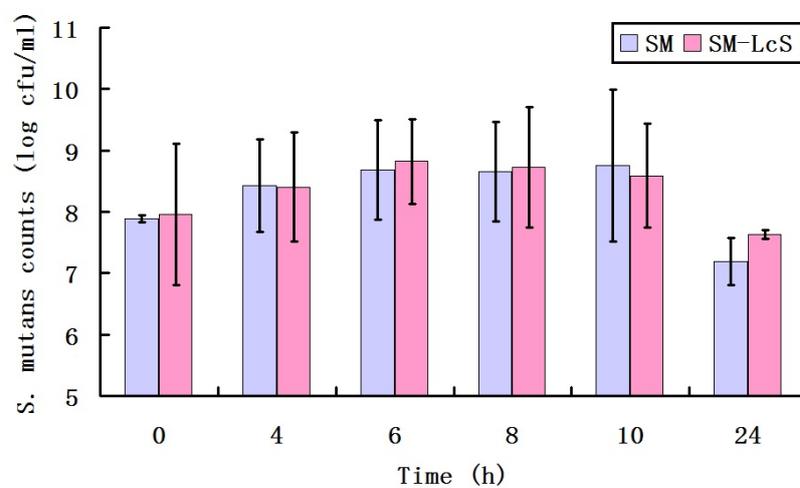


Fig. 3.16. *S. mutans* growth in the SM and SM-LcS liquid culture within 24 h. Data were expressed as the mean \pm SD (log cfu/ml) of two independent experiments in triplicate.

3.3.3 Dynamic changes in biofilms

3.3.3.1 Effects of LcS on 6-well plate model

The biofilms in the SM and SM-SS groups showed rough surfaces, while the SM-LcS and SM-LGG biofilms were smooth as shown in Fig. 3.17.



Fig. 3.17. SM mono-culture biofilm and SM-SS, SM-LcS, SM-LGG co-culture biofilms on the 6-well plates.

The pH values of the four groups of biofilms are shown in Table 3.7. SM-LcS and SM-LGG biofilms presented significantly higher pH values than SM and SM-SS biofilms. No difference was found between the SM and SM-SS groups, and between the SM-LcS and SM-LGG groups (both $P > 0.05$).

Table 3.7 Biofilm pH in the SM, SM-SS, SM-LcS and SM-LGG groups (mean \pm SD)

Measurement	Groups (n = 3)			
	SM	SM-SS	SM-LCS	SM-LGG
Biofilm pH	4.251 \pm 0.037 ^a	4.210 \pm 0.047 ^a	4.429 \pm 0.020 ^b	4.413 \pm 0.034 ^b

Different letters indicate statistically significant difference ($P < 0.01$)

SM and SM-SS biofilms were thicker than the SM-LcS and SM-LGG biofilms. The thickness is shown in Fig. 3.18.

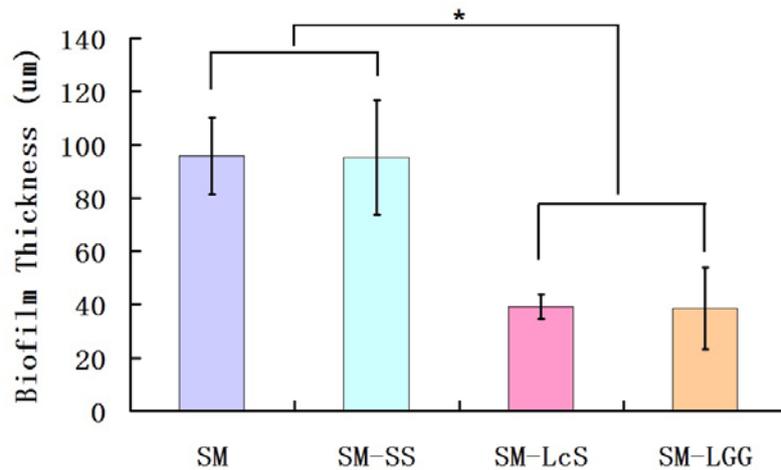


Fig. 3.18. Biofilm thickness in the SM, SM-SS, SM-LcS and SM-LGG groups (n = 3) (* $P < 0.05$).

3.3.3.2 Dynamic changes in biofilms assessed by fluorescence *in situ* hybridisation (FISH)

3.3.3.2.1 Acidogenicity

The pH values of the biofilms and their changes over a 7 day period are shown in Fig. 3.19. The pH of the SM biofilm was higher than that of the SM-LcS biofilm after 1 day culture, but the difference was not significant ($P > 0.05$). The pH of the D2 SM biofilm continuously increased while the D2 SM-LcS biofilm pH was the same as that of the D1 biofilm ($P < 0.05$).

However, at D3 and onwards, the SM biofilm pH was lower than that of the SM-LcS biofilm (all $P < 0.05$).

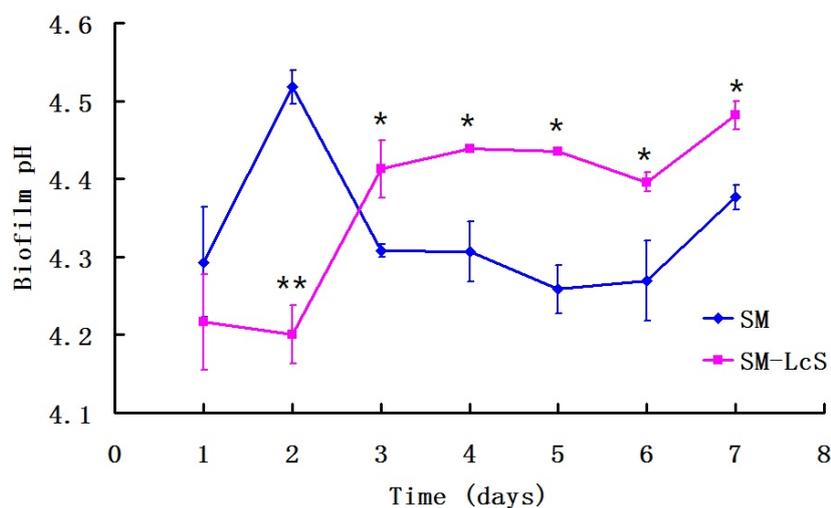


Fig. 3.19. Dynamic changes of pH in SM and SM-LcS biofilms from days 1 to 7. Data were mean \pm SD of two independent experiments in triplicate. * $P < 0.05$; ** $P < 0.01$ when SM-LcS was compared to SM.

3.3.3.2.2 Biovolumes of *S. mutans* and *L. casei* Shirota

S. mutans was detected by the specific probe MUT590 which showed red color. *L. casei* Shirota was labeled by Lcas467 showing green color. Fig. 3.20 showed the D1 and D7 SM and SM-LcS biofilm 3D structure under CLSM.

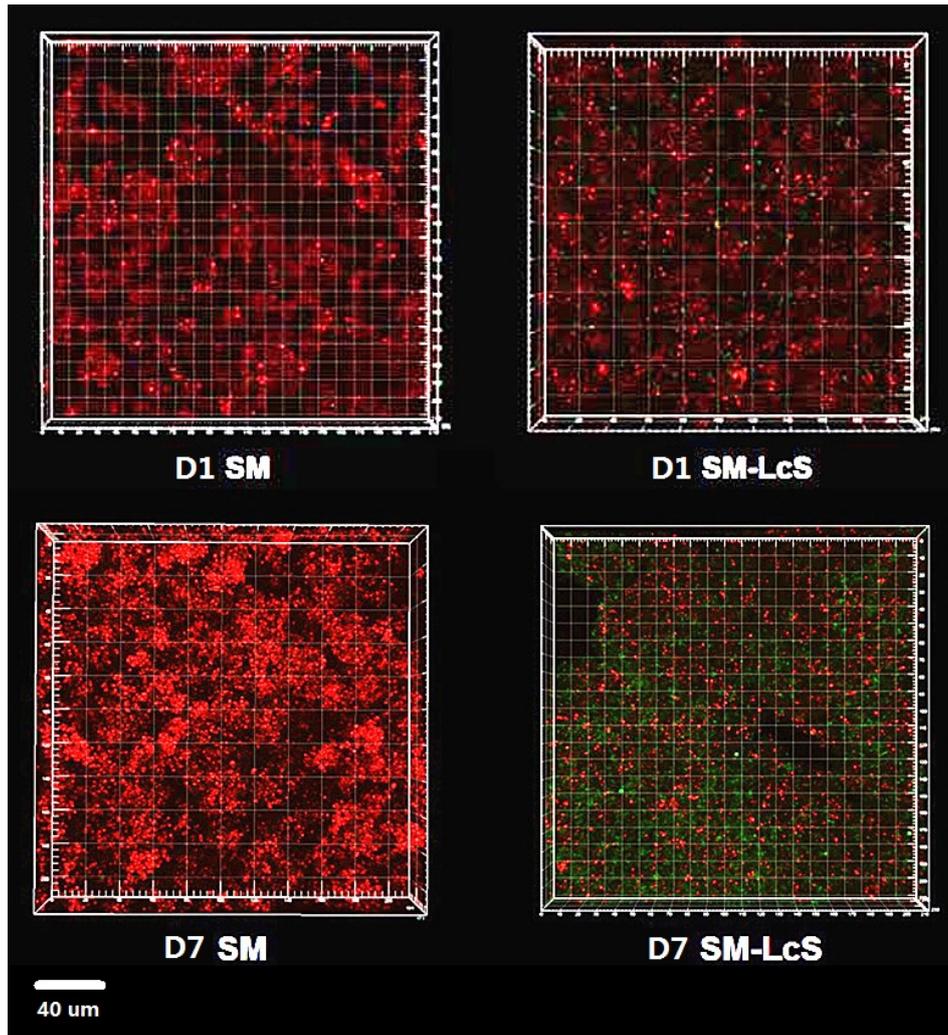


Fig. 3.20. D1 and D7 SM and SM-LcS biofilms under CLSM (Z projectional view). *S. mutans* was labeled by Cy5 showing the red color, and *L. casei* Shirota was labeled by FAM showing green light.

The biovolume change of *S. mutans* during 1-7 day culture is shown in Table 3.8 and Fig. 3.21. On the first day, *S. mutans* in the SM-LcS co-culture biofilm was a little more than that in the SM mono-culture biofilm, but the difference was not significant ($P = 0.414$). However, *S. mutans* in the D2 SM-LcS biofilm was significantly greater than that in the D2 SM biofilm ($P = 0.039$). From D3, *S. mutans* in the mono-culture SM biofilm gradually increased while that in the co-culture SM-LcS biofilm remained at a similar

level to that in the first two days.

Table 3.8 *S. mutans* biovolume in the D1-7 SM and SM-LcS biofilms (n=6)

Days	<i>S. mutans</i> biovolume (μm^3)		<i>P</i>
	SM biofilm	SM-LcS biofilm	
1	746 ± 358	1515 ± 1418	0.414
2	757 ± 305	2152 ± 740	0.039
3	2633 ± 463	2037 ± 781	0.348
4	5040 ± 2382	1379 ± 1103	0.145
5	6136 ± 3288	1127 ± 567	0.135
6	11120 ± 4339	789 ± 491	0.052
7	12121 ± 3672	2685 ± 1718	0.006

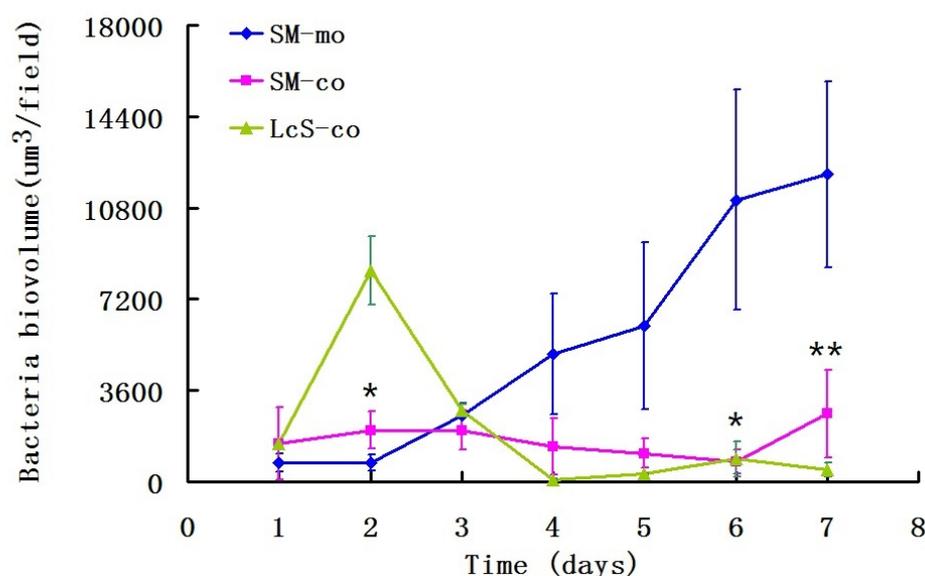


Fig. 3.21. Dynamic changes of *S. mutans* and *L. casei* Shirota biovolumes in biofilms. SM-mo: SM in SM mono-culture biofilm; SM-co: SM in SM-LcS co-culture biofilm; LcS-co: LcS in SM-LcS co-culture biofilm (n=6). * $P < 0.05$, ** $P < 0.01$ when SM-co was compared to SM-mo.

3.3.4 Gene expression analysis using qRT-PCR

The expression of *gtfB*, *gtfC*, and *ldh* in SM significantly decreased in the presence of LcS on D1, D3, and D4 (all $P < 0.05$) (Fig. 3.22). However, the

changes in the expression of *gtfB*, *gtfC*, and *ldh* on D2 did not reach statistical significance (all $P > 0.05$).

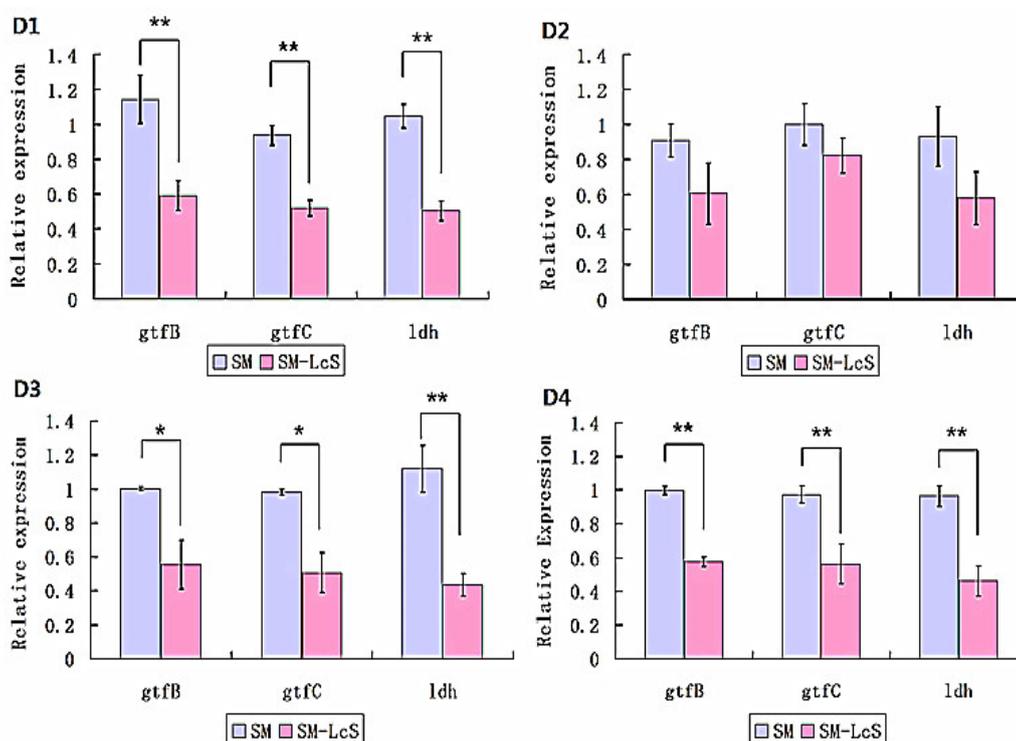


Fig. 3.22. Effects of LcS on the expression of *gtfB*, *gtfC* and *ldh* in SM in biofilm. Gene expression was determined by qRT-PCR in days 1-4 SM and SM-LcS biofilms. Expression of the respective mRNAs was normalized to the relative abundance of the housekeeping gene 16S rRNA. The experiment was performed twice in triplicate. Data were expressed as mean \pm SD (* $P < 0.05$, ** $P < 0.01$).

PART4: Antimicrobial effect of *L. casei* Shirota supernatant

3.4.1 *L. casei* Shirota growth kinetics

As shown in Fig. 3.23 (a), the exponential phase of *L. casei* Shirota

growth curve was from the 4th hour to the 24th hour, and the stationary phase was from the 24th hour to the 48th hour. Cells death was after the 48 h culture. Fig. 3.23 (b) is the optical density curve of *L. casei* Shirota culture, which was in accordance with the growth curve in Fig. 3.23(a). The pH values of *L. casei* Shirota CFCS at the different time points are shown in Fig. 3.24. The pH of CFCS at 12 h (the exponential phase) was 4.3, and it remained at 3.7 when the cells grew to the stationary phase (after 24h).

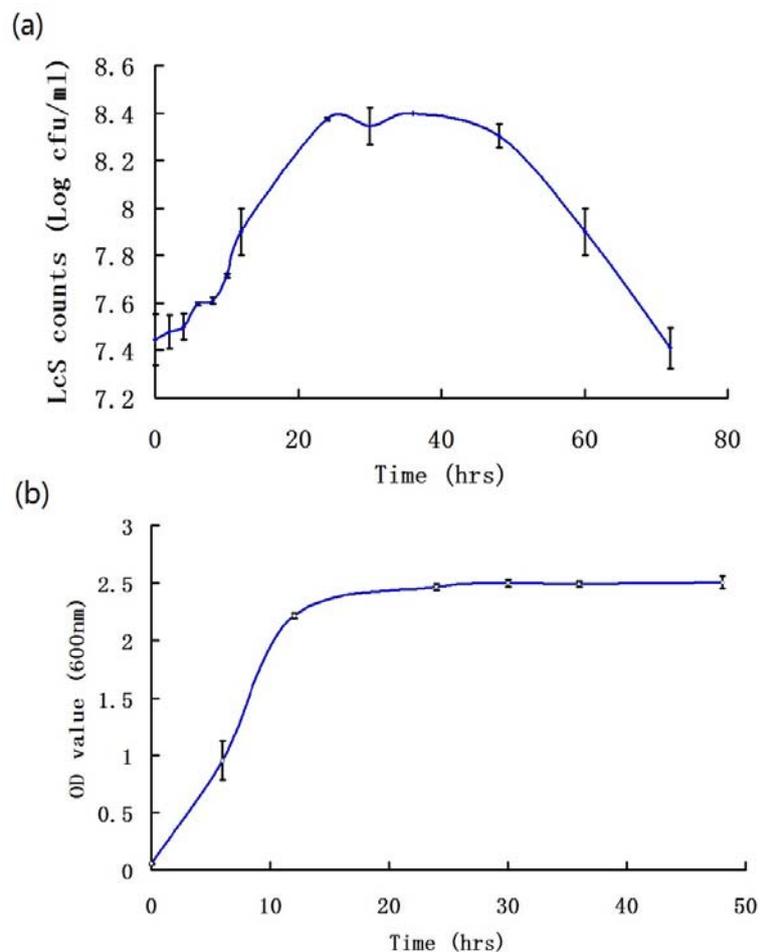


Fig. 3.23. Growth curve of *L. casei* Shirota. (a) *L. casei* Shirota cfu in 0- 72 hours culture. (b) *L. casei* Shirota OD values in 0-50 h culture. Data were shown as mean \pm SD of two independent experiments performed in triplicate.

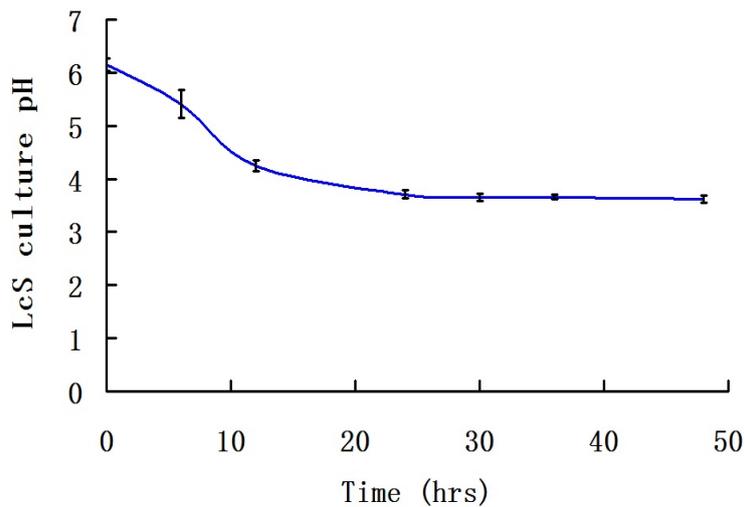


Fig. 3.24. pH curve of *L. casei* Shirota culture. Data were shown as mean \pm SD of three independent experiments in triplicate.

3.4.2 Inhibition assay

L. casei Shirota CFCS at different growth phase (12 h, 24 h, 30 h, 36 h, 48 h, and 54 h) were collected and cultured with *S. mutans*. As pH at 12 h CFCS was 4.3, and pH at 24 – 54 h CFCS were 3.7, MRS was adjusted to pH 3.7 with 1 M HCl (named MRS-HCl), served as a control. Fig. 3.25 illustrated that the 12 h CFCS did not show inhibitory effect on *S. mutans* growth, compared with the control MRS-HCl group ($P > 0.05$). However, CFCS after the stationary phase (24 h, 30 h, 36 h, 48 h and 54 h CFCS) significantly inhibited the growth of *S. mutans* (all $P < 0.01$). Meanwhile, there was no difference among the above CFCS groups after stationary phase ($P > 0.05$).

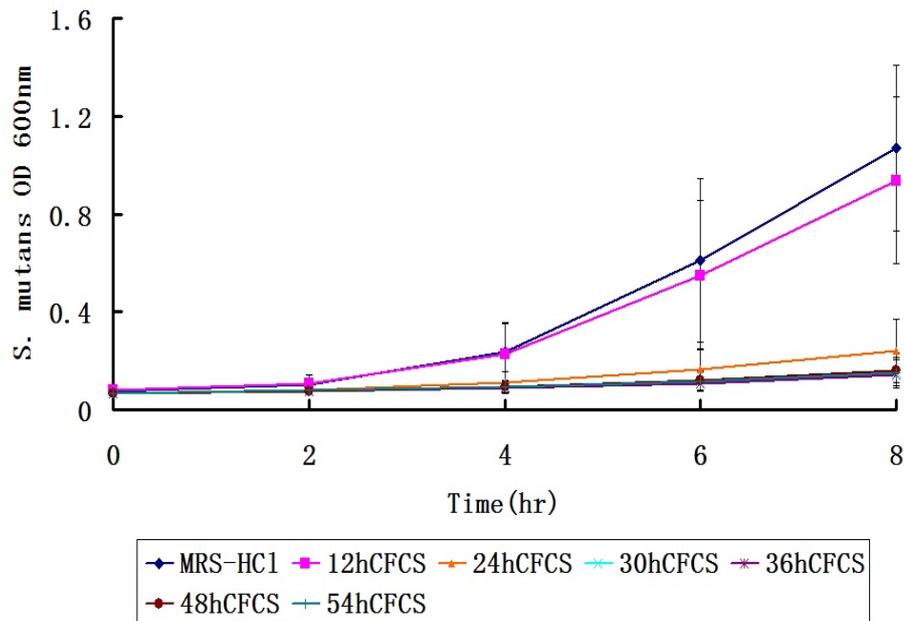


Fig. 3.25. Antimicrobial effect of *L. casei* Shirota CFCS. *L. casei* Shirota CFCS collected at different time points was cultured with *S. mutans* for 8 hours. Data were shown as mean \pm SD of three independent experiments.

3.4.3 Characterization of the inhibitory compounds

pH sensitivity

Acidic *L. casei* Shirota CFCS significantly inhibited the growth of *S. mutans*, while neutralized CFCS did not (Fig. 3.26). No significant difference was found among MRS 3.7 (pH was adjusted to 3.7 with 1 M HCl), MRS 7 and CFCS 7 (pH was adjusted to 7 with 1 M NaOH) groups (all $P > 0.05$).

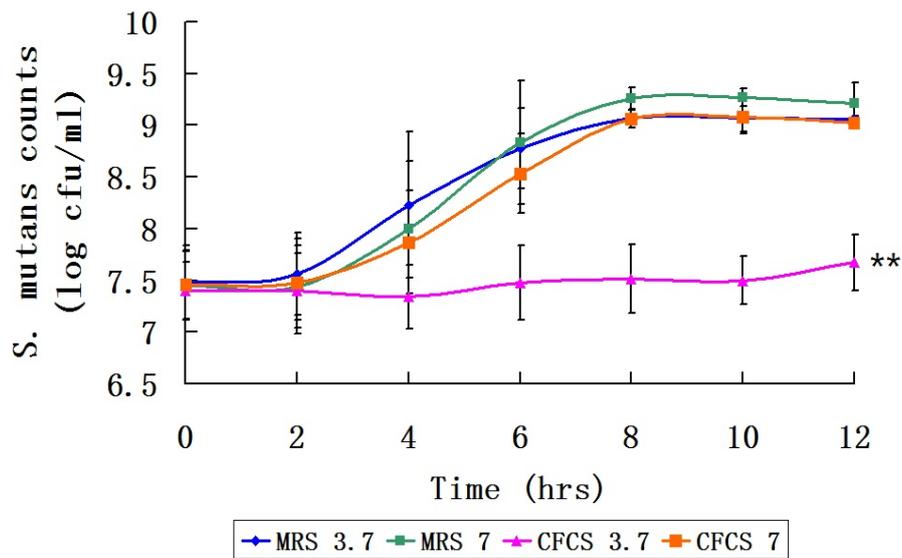


Fig. 3.26. Effect of pH on the antimicrobial activity of *L. casei* Shirota CFCS. Data were shown at mean \pm SD of three independent experiments in triplicate. (** $P < 0.01$)

Heat resistance

The pH of the autoclaved *L. casei* Shirota CFCS was 3.7, same as that of the fresh CFCS. We found that both autoclaved CFCS and fresh CFCS significantly inhibited the growth of *S. mutans* in comparison to the control MRS-HCl group (all $P < 0.01$) (Fig. 3.27). Moreover, no difference was found between autoclaved CFCS and fresh CFCS ($P > 0.05$). This result indicated that the antimicrobial effect of *L. casei* Shirota CFCS was heat resistant.

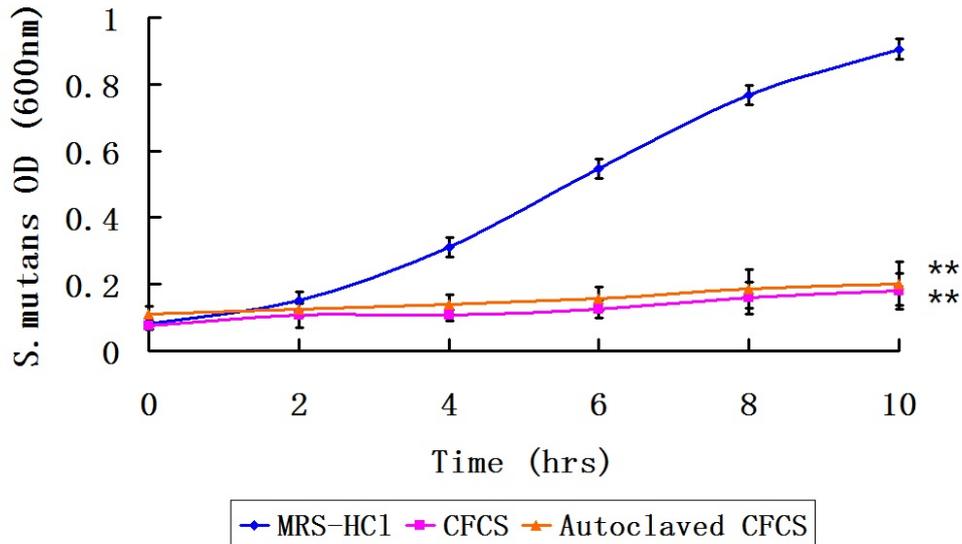


Fig. 3.27 Effect of heat on the antimicrobial activity of *L. casei* Shirota CFCS. Data were shown as mean \pm SD of two independent experiments performed in triplicate (** both $P < 0.01$ when compared to MRS-HCl).

Stability

It is shown in Fig. 3.28 that there was no significant difference between stored *L. casei* Shirota CFCS (3 months) and fresh CFCS ($P > 0.05$). Both of them exhibited the inhibitory effect on *S. mutans* growth ($P < 0.01$).

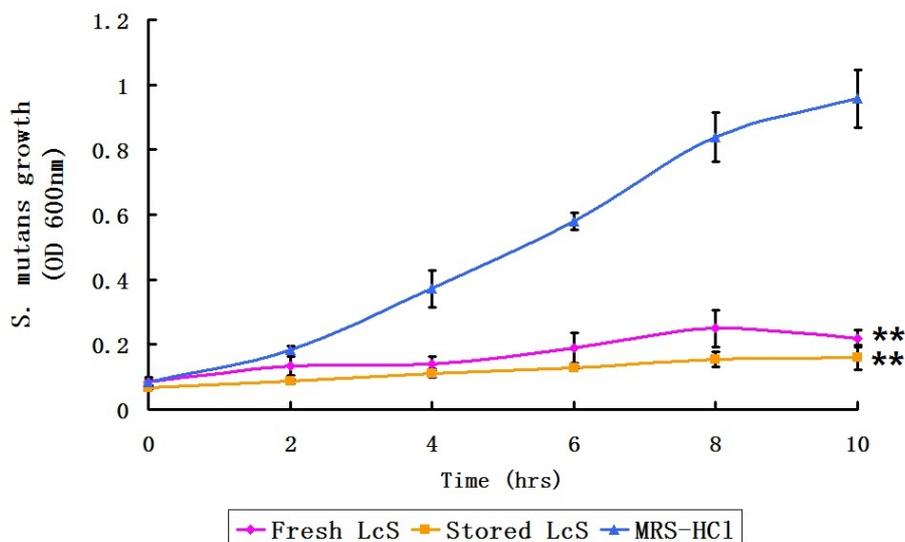


Fig. 3.28. Stability of the antimicrobial activity of *L. casei* Shirota CFCS. Data

were shown as mean \pm SD of two independent experiments performed in triplicate (** $P < 0.01$ when compared to MRS-HCl).

3.4.4 Antimicrobial activity of *L. casei* Shirota CFCS crude extract

L. casei Shirota CFCS crude extract showed inhibition zone (19.5 ± 1.32 mm) on *S. mutans* BHI agar plate while MRS crude extract did not show any effect (Fig. 3.29).

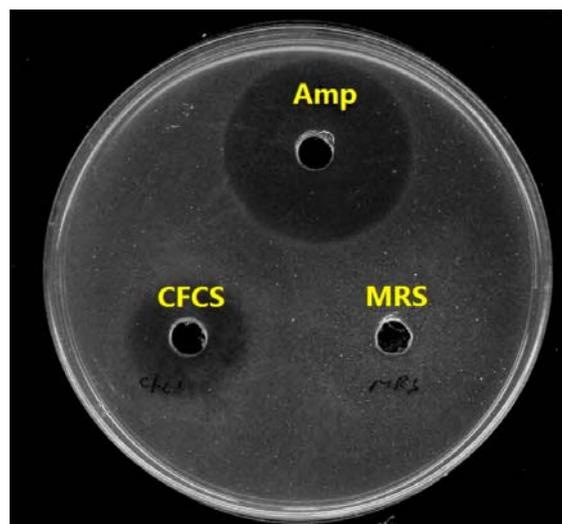


Fig. 3.29. Agar inhibition test for *L. casei* Shirota CFCS crude extract. Inhibition zones were found around CFCS crude extract and Ampicillin (Amp).

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Chapter 4:

Discussion and Conclusions

4.1 Discussion

4.1.1 *S. mutans* biofilm-on-tooth (BOT) model

S. mutans is one of the most cariogenic bacteria in oral cavity due to its high acidogenicity, acid tolerance and its ability to utilize sucrose to form biofilm. Hence, it is important to investigate the effects of probiotic milk on *S. mutans* biofilm. In our study, *S. mutans* biofilm-on-tooth (BOT) model was developed. *S. mutans* biofilm was cultured on tooth surface which was coated with human saliva. Our model was further built on that of Arnold WH's study (Arnold et al., 2001; Arnold et al., 2006) except the changes of the size and shape of tooth blocks. The BOT model was validated in this study, showing the difference of biofilm formation and acidogenicity upon different treatments. Therefore, this model is sufficiently sensitive to evaluate the anti-cariostatic effect of Yakult® in terms of inhibition on *S. mutans* biofilm.

4.1.2 Probiotic drink and the cariostatic effect

Different types of milk have been studied for their effects on biofilm

formation, composition, acidogenicity, acid tolerance and tooth demineralization, including infant formula and fluoride milk. An *in situ* study was conducted with 11 volunteers wearing palatal appliance containing human enamel slab treated with different types of formulas (milk-based and soy-based) 8 times everyday (de Mazer Papa et al., 2010). The results showed that the infant formula induced the enamel mineral loss with decreased biofilm pH, especially when added with sucrose. The fermentation of the carbohydrates present in the formula resulted in acid production that decreased biofilm pH, which led to tooth demineralization. Fluoride milk has demonstrated anti-caries effect by increasing the biofilm pH (Pratten et al., 2000), decreasing acid tolerance (Neilands et al., 2012) of biofilm, and reducing *Streptococcus mutans* count in biofilm (Pratten et al., 2000). Fluoride milk also inhibited the demineralization formed by *S. sobrinus* biofilm on tooth surface (Arnold et al., 2006). Taken together, milk as a common nutrient may increase the bacteria metabolism and the subsequent demineralization, but milk supplemented with fluoride exhibits anti-caries effect.

Probiotic milk is the milk supplemented with the probiotic strains. There are more than 20 clinical trials demonstrating that consumption of probiotic products, including probiotic milk or yogurt and others, decreased the mutans streptococci in dental plaque and saliva, as summarized in literature review. The study of probiotics on acid production of dental plaque is limited. Keller

and Twetman found that in the presence of *L. reuteri* dental plaque produced less lactic acid compared with that in presence of *L. plantarum* and the blank control, thus this effect was species dependent (Keller and Twetman, 2012a). However, in their clinical study, there was no significant difference in lactic acid concentration between the groups with and without probiotic lactobacilli tablets treatment for 2 weeks. Marttinen *et al.* investigated 13 volunteers who consumed probiotic tablets for 2 weeks, also found no difference on acid production in plaque between the experimental and control groups (Marttinen *et al.*, 2012a). From the above two studies, the short term probiotic treatment did not appear to affect acidogenicity of dental plaque.

In Part 1 of this study, the *S. mutans* biofilm was treated with Yakult[®] for 30 minutes every day to mimic the duration of effective contact with the oral cavity during and after drinking. The biofilm acidogenicity in the Yakult group decreased, displaying a higher resting pH value, which suggested the possible cariostatic effect of this probiotic drink (Table 3.1). The more pronounced effect revealed in *in vitro* studies compared to those in the clinical trials could be due to the complex dental plaque microflora and the oral environment. The interesting phenomenon observed in this study was the effect on biofilm formation. *S. mutans* biofilm in the PBS group was significantly thicker (51%) than that in the Yakult group (Table 3.2). The decreased acidogenicity was related to the decreased biofilm thickness which suggested the less bacteria

counts in the biofilm.

Probiotic *Lactobacillus* strains produce lactic acid, which leads to the low pH of probiotic drinks. In this study, the fresh milk and PBS were adjusted to 3.7 (the same pH of Yakult®) by adding lactic acid. Hence, the inhibitory effect of Yakult® may not be due to its low pH alone to suppress *S. mutans* growth. The difference between the Yakult group and acidic milk group also suggested that the cariostatic effect of Yakult® could not be due to the main nutrient in the probiotic milk, but largely caused by the probiotic strain *L. casei* Shirota. To date, there is no study reported the potential effects of *L. casei* Shirota on *S. mutans*. Our study suggested the cariostatic effect of Yakult® on *S. mutans* may possibly be due to the probiotic strain *L. casei* Shirota.

4.1.3 Artificial Stephan curve in BOT model

In Part 1 of this study, it was demonstrated that the treatment of probiotic milk Yakult® could inhibit *S. mutans* biofilm formation and decrease biofilm acidogenicity possibly due to the probiotic strain *L. casei* Shirota. In order to verify the speculation, the effect of *L. casei* Shirota on the biofilm acidogenicity of *S. mutans* was evaluated in Part 2. The classic Stephan curve profiling the pH of dental plaque in response to sugar is often used to assess the acidogenicity of dental plaque (Imfeld T, 1980; Preston and Edgar, 2005).

As aforementioned, the *in vitro* BOT model was used in this part.

The characteristic Stephan curve has been of interest for nearly 70 years (R.M., 1944). However, there are only a few studies reporting the *in vitro* Stephan curve model so far (Lagerlöf et al., 1984; Macpherson et al., 1991; Macpherson and Dawes, 1991). These studies had investigated the salivary buffering capacity in artificial plaque after the sucrose rinse, using an apparatus in which artificial saliva flowed over the plaque. In our study the artificial Stephan curve was plotted on BOT model to observe the pH lowering potential and trend. To prevent the biofilm drying in air, the tooth sample covered with biofilm was kept in a humidity chamber before pH measurements. The findings showed that it is feasible to profile the “artificial Stephan curve” of 7-day-old biofilm using the aforementioned methodology. In our model, the Stephan curve is composed of a slow pH decreasing phase and a fast increasing phase before reaching the neutral. Although it is different from the classic Stephan curve which has a fast decreasing and a slow increasing phase (R.M., 1944), this special characteristic of our BOT model makes it easy to quantify the acidogenicity of different biofilms.

4.1.4 Acidogenicity of dental plaque and caries formation

The acidogenicity of dental plaque is critical for caries development.

Some studies have demonstrated that the initial pH of plaque from the “white spot” areas was lower than that from the “sound surface” after a sucrose rinse (Lagerlöf et al., 1985; Lingström et al., 2000; Sansone et al., 1993; Van Houte et al., 1991). Furthermore, the plaque pH was found to be significantly different between high caries risk subjects and low caries risk subjects (Cagetti et al., 2011; Lingström et al., 2000). The relationship between acidogenic potential of human dental plaque and its microbial composition remains a complex issue. It is indicated that increasing cariogenicity is usually accompanied with significant changes in the plaque microbial composition favoring highly acidogenic and aciduric bacteria, especially the predominance of mutans streptococci (MS) and lactobacilli (Marsh, 2006). However, Fejerskov *et al.* reported that there was no difference in the pH response after sucrose rinse on sound surfaces of caries active and inactive Kenya children, but significant differences in the Stephan curves between active occlusal caries lesions and sound occlusal surfaces were found (Fejerskov et al., 1992). Some studies reported no difference in the plaque pH responses (Stephan curve) between sound and carious root surfaces (Aamdal-Scheie et al., 1996), or no differences between caries active and inactive subjects (Sansone et al., 1993). These seemingly controversial data may be due to the way plaque pH was measured and how caries active individuals were defined. As every tooth of the same individual may exhibit different caries risk, a high caries risk patient may have a low risk tooth and/or site-specific biofilm, such as the lower

anterior incisors. In this study, we found that the pH of the SM-LcS biofilm was higher than that of the SM biofilm (Table 3.3). Moreover, the Stephan curve of SM-LcS biofilm was significantly different with that of SM biofilm (Fig. 3.7). Not only the lowest pH, but the recovery time and area under curve, suggested the decreased acidogenicity of SM-LcS biofilm in comparison with SM biofilm (Table 3.4). Subsequently, the enamel lesion under the SM-LcS biofilm was significantly less than that under the SM biofilm (Table 3.5). The results further substantiate the association between biofilm acidogenicity and enamel lesion development.

4.1.5 *Lactobacillus* and oral pathogenic bacteria

Some studies have shown the inhibitory effect of lactobacilli against *S. mutans*, *S. sobrinus* and candida (Hasslof et al., 2010; Simark-Mattsson et al., 2009; Sookkhee et al., 2001). This inhibitory effect could be due to a variety of antibacterial substances produced by *Lactobacillus* strains, including organic acids (e.g. lactic acid) (Taniguchi et al., 1998), hydrogen peroxide (Sookkhee et al., 2001; Tano et al., 2003b) and bacteriocins (de Carvalho et al., 2006; Sookkhee et al., 2001). Furthermore, it was reported that *L. rhamnosus* GG, *L. reuteri* and *L. plantarum* greatly inhibited *S. mutans* biofilm formation *in vitro*, probably through the decreased adhesion or viable counts of *S. mutans* (Söderling et al., 2011b). A few clinical studies have illustrated that MS counts

decreased in saliva and caries risk decreased after consumption of lactobacilli-derived probiotics, as reported in a review (Twetman and Keller, 2012). However, one conceivable risk of probiotic lactobacilli application to oral health is caries development, which may be promoted by lactic acids produced by lactobacilli. Only a few studies have investigated the acid production in biofilm by probiotic treatment. Interestingly, Keller *et al.* found that in the presence of *L. reuteri* dental plaque produced less lactic acid compared with that in the presence of *L. plantarum* and the blank control, and thus this effect was species dependent (Keller and Twetman, 2012b). However, in their clinical study, no significant difference in lactic acid concentration between groups with and without consumption of probiotic lactobacilli tablets, supporting the findings of Marttinen's (Marttinen *et al.*, 2012a). Marttinen *et al.* reported there was no difference of the lactic acid production and MS levels in plaque suspension at the baseline and the end of probiotic tablet taking period. In their study, all the subjects had good oral hygiene with low DMFT (decayed, missing, filled teeth) score. The caries risk level of the subjects may account for the above conflicting observations between laboratory and clinical results.

It is quite interesting that the current successful probiotic lactobacilli are originally isolated from human body (Saarela *et al.*, 2000). It was suggested that a probiotic strain could function better in a similar environment to where

it was originally isolated from, e.g. human GI-tract (Saarela et al., 2000). However, the association between oral lactobacilli and caries was reported, especially in root caries and deep dentinal caries (Ayna et al., 2003; Beighton and Lynch, 1995; Brown et al., 1986; Callaway et al., 2013; Preza et al., 2008). They are now considered secondary invaders rather than initiators of the caries process (Tanzer et al., 2001). The contradiction of probiotic effect and cariogenic effect of lactobacilli highlights the need for more research to evaluate the different roles of different oral *Lactobacillus* species play in oral cavity.

4.1.6 Effects of *L. casei* Shirota on *S. mutans* biofilm formation and acidogenicity

L. casei Shirota is a special *Lactobacillus* strain isolated from Yakult[®], a sweet acidic probiotic milk. It has been demonstrated the anti-infectious activities, such as preventing diarrhea, protecting intestinal epithelial and immune cells from virus infection, and modulating immune response (Ivory et al., 2008; Sur et al., 2011). However, only a few studies have reported the interaction of Yakult[®] or *L. casei* Shirota with oral bacteria or caries so far. One study compared the effect of Yakult[®] and 20% sucrose solution treatment on enamel demineralization. Less mineral loss was found in the Yakult group, although both of the two treatments reduced biofilm pH (Lodi et al., 2010).

The other study revealed that *L. casei* Shirota had some but low colonizing potential on artificial carious dentin surface (Lima et al., 2005). These studies suggested the possible cariogenic potential of Yakult® and *L. casei* Shirota. However, in our study (Part 2), although *S. mutans* and *L. casei* Shirota are both acid-producing bacteria, the co-culture biofilm counter-intuitively exhibited a higher resting pH and less acidogenicity than those of the *S. mutans* mono-culture biofilm (Table 3.3). These results are in line with Keller's findings, and demonstrated the cariostatic effect of the specific probiotic bacteria. The pH of spent culture and the biofilm formed on the bottom of 6-well plate also showed significant difference between the SM and SM-LcS groups (Table 3.3), supporting the inhibitory effect of LcS on SM biofilm acid production. As *L. casei* Shirota can barely form biofilm on the tooth surface, it is impossible to establish the 7d LcS mono-culture group as the control. However, the comparison of SM biofilm with SM-SS and SM-LGG biofilms verified the cariostatic effect of probiotic strains. *S. sanguinis* is one of the first colonizers in oral cavity, so the co-culture of *S. mutans* and *S. sanguinis* was used as a control. We also used LGG as another control as *L. rhamnosus* GG is a widely used probiotic strain. The cariostatic effect of LGG has been demonstrated in several clinical trials, showing the decreased salivary mutans streptococci and less caries prevalence (Ahola et al., 2002; Glavina et al., 2012; Nase et al., 2001). Our results (Fig. 3.11 & Table 3.6) revealed that both the SM and SM-SS biofilms formed thicker biofilm and

displayed higher acidogenicity than SM-LcS and SM-LGG biofilms. Hence, it is concluded that the inhibitory effect of *L. casei* Shirota may not be due to the decreased nutrient for co-culturing, but the special cariostatic effect of probiotic strains. The significant difference in acid production of *S. mutans* biofilm with and without *L. casei* Shirota has shed light on the cariostatic mechanisms of the probiotic drink. In conclusion, *L. casei* Shirota is promising in reducing the acidogenicity of *S. mutans* biofilm, and thus inhibiting enamel demineralization.

4.1.7 Effects of *L. casei* Shirota on *S. mutans* growth within 24 hours

In order to investigate the effect of *L. casei* Shirota on *S. mutans* adherence, *S. mutans* and *L. casei* Shirota colonies forming units (cfu) in 24h mono-culture and co-culture biofilm were evaluated in Part 3. The results showed that *S. mutans* increased a little when *L. casei* Shirota was co-cultured in the biofilm, but the difference was not significant (Fig. 3.13a). *L. casei* Shirota did not inhibit *S. mutans* growth in biofilm. On the contrary, *L. casei* Shirota counts significantly increased in the co-culture biofilm than in the *L. casei* Shirota mono-culture biofilm (Fig. 3.13b). *L. casei* Shirota hardly form biofilm, but *S. mutans* produces GTFs which can bind to other microorganisms (Hamada et al., 1978; McCabe and Donkersloot, 1977; Vacca-Smith and Bowen, 1998). Thus *L. casei* Shirota also became “glucans producer”, which

enhanced its adherence into biofilm. These findings are in accordance with our observation for *S. mutans* counts in D1 biofilm using FISH technique (Table 3.8 & Fig. 3.21). The same finding was also found in the liquid co-culture, showing no difference of *S. mutans* counts when cultured with *L. casei* Shirota within 24 h (Fig. 3.16). Taken the results together, *L. casei* Shirota can not inhibit *S. mutans* growth both in biofilm and liquid culture within the first 24 hours.

4.1.8 Effects of *L. casei* Shirota on *S. mutans* EPS production

Although *S. mutans* counts in the 24 h SM-LcS co-culture did not change significantly in comparison to SM mono-culture, the biofilm structure appeared differently under SEM. The density of extracellular matrix appeared less than that in the mono-culture biofilm (Fig. 3.14). The finding was confirmed by the EPS analysis using the EPS-staining dye (FITC-conA) which can specifically detect mannose and glucose. EPS clustered to form microcolonies in the SM biofilm, while the formation of microcolonies was impaired in the SM-LcS biofilm, with sparse and scattered EPS (Fig. 3.15a). The quantification of the biovolume of the stained EPS indicated that the EPS production in the SM-LcS biofilm was significantly lower than that in the SM biofilm (Fig. 3.15b). This result is attributable to the decreased expression of *gtfB* and *gtfC* in SM due to the presence of LcS in co-culture (Fig. 3.22). A

previous study investigating a dual species biofilm also reported that the expression of *gtfB* was significantly decreased when *S. mutans* UA159 was co-cultured with *L. casei* 4646 (Wen et al., 2010),

EPS is composed of mostly glucans synthesized by microbial glucosyltransferases (Gtfs) (Paes Leme et al., 2006). It was reported by J. Xiao and H. Koo that EPS were closely associated with microcolonies throughout the biofilm development process (Xiao and Koo, 2010). They found EPS were detected between saliva-coated hydroxyapatite (sHA) surface and microcolonies, within, surrounding and covering the microcolonies, and also bridging microcolonies. The presence of EPS is essential for the initial formation and in maintaining the three-dimensional structure of the biofilm. H. Koo *et al.* reported that the *S. mutans* strains with defective *gtfB* gene or the *gtfB* and *gtfC* genes hardly form microcolonies on sHA surfaces (Koo et al., 2010). GtfCs secreted by *S. mutans* are incorporated into pellicle. GtfBs are adsorbed on bacterial surfaces of both *S. mutans* and other microorganisms that do not produce Gtfs (e.g. *Actinomyces* spp.). Surface-adsorbed GtfB and GtfC utilize sucrose to synthesize insoluble and soluble glucans. GtfB synthesizes insoluble alpha-1,3-linkages, allowing vertical growth of the microcolonies and contributes to the increasing biofilm thickness (Koo et al., 2010).

In our study, the 24 h EPS in the SM-LcS biofilm was significantly less

than that in the SM biofilm. The decreased EPS production may be one of the important roles of *L. casei* Shirota on *S. mutans* biofilm growth during 7 days culture as it affected *S. mutans* microcolonies formation and biofilm architecture. However, although EPS production in the 24 h SM-LcS biofilm was decreased, the *S. mutans* counts did not significantly change within 24 hours, in comparison to the SM mono-culture biofilm. Our findings are similar with Chung's study (Chung et al., 2004). Chung *et al.* reported *L. fermentum* and its culture supernatant significantly inhibited the formation of the insoluble glucan produced by *S. mutans* Ingbritt without inhibiting the multiplication of *S. mutans* Ingbritt.

4.1.9 FISH technique in biofilm analysis

To further investigate the effect of *L. casei* Shirota on *S. mutans* biofilm after 24 hour, the dynamic change from D1 to D7 was observed using fluorescence in situ hybridization (FISH) technique. Instead of using BOT model, we used biofilm on 6-well plate model because the auto-fluorescence of enamel surface was detected under CLSM in the pilot study, which interfered the fluorescence signals of SM and LcS probes. With this model, the dynamic change of *S. mutans* biofilm acidogenicity and formation during days 1-7 in the presence of *L. casei* Shirota was observed, as well as *S. mutans* and *L. casei* Shirota biovolumes.

FISH technique offers qualitative and quantitative monitoring of single cells by binding oligonucleotide probes to their complementary target sequences (Manz, 1999). FISH in combination with CLSM and digital image analysis became an important approach for identification and localization of microorganisms (Manz, 1999). The primary structure of 16S and 23S rRNA are highly conserved and the sequences are usually selected as target ribosomes (Korber et al., 1999). Synthetic oligonucleotides probes can be labeled directly at the 5' end with different fluorochromes. The most widely distributed fluorochromes include fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC), other rhodamine stains, the blue fluorescing aminomethylcoumarin (AMCA), and, more recently, cyanine dyes (Cy Dye) (Manz, 1999). Enzyme-labeled oligonucleotides and Hapten-labeled oligonucleotides can also be used, but they are limited by the larger molecular size of the antihapten-antibody or the oligonucleotide-enzyme conjugate, which might be restricted to penetrate biofilm matrices and cell walls of certain target cells (Manz, 1999). Compared with immunofluorescence, FISH is an inexpensive, precise and straightforward labeling technique. The selection and production of the oligonucleotide probes is faster and much cheaper than the generation and production of monoclonal antibodies. Moreover, the finding of genera specific rRNA target sequences for probe production is fairly simple, while the

production of genera-specific antibodies is difficult as antibodies tend to target the level with structure of more restricted expression (Thurnheer et al., 2001). In our study, the probes were designed according to the previous studies on oral biofilm. These studies employed FISH and CLSM technique to identify different bacteria in dental plaque (Klug et al., 2011; Quevedo et al., 2011; Thurnheer et al., 2001), to detect and enumerate the oral bacteria with the effect of different treatments (Marttinen et al., 2012b). In our study, *S. mutans* and *L. casei* Shirota labeled with Cy5 and FAM respectively were successfully identified in the CLSM images.

4.1.10 Dynamic changes during days 1-7 biofilm culture

As shown in Table 3.8 and Fig. 3.21, SM growth was not inhibited by the presence of LcS on day 1. Thus, the total number of acid-producing bacteria in co-culture was higher than that in mono-culture. Nevertheless, the acid production in the co-culture biofilm did not increase significantly on day 1 (Fig. 3.19). This result could be due to the decrease in the expression of *ldh*, which is the gene encoding a lactate dehydrogenase associated with acidogenicity, in SM during co-culture (Fig. 3.22). On day 2, the LcS numbers increased significantly compared with those on day 1, and the biofilm of SM in co-culture proliferated more rapidly than that in mono-culture (Fig. 3.19). As Gtfs bind not only to SM but also to other bacterial surfaces (Bowen and

Koo, 2011), the above phenomenon may be due to the increasing amounts of Gtfs, which bind to the surfaces of both SM and LcS, thereby providing further bacterial attachment sites during biofilm formation. With increasing numbers of two acid-producing bacteria on day 2, the pH of the co-culture biofilm significantly decreased compared with the pH of the mono-culture biofilm (Fig. 3.19). Although *gtfB*, *gtfC*, and *ldh* expressions decreased in the presence of LcS at most of the time points examined, on day 2, the expression of these genes was not significantly reduced (Fig. 3.22). Low pH has been reported to induce the expression of *gtfB*, *gtfC*, and *ldh* in SM (Li and Burne, 2001). The low pH in the co-culture biofilm on day 2 may have masked the inhibitory effects of LcS on these genes, resulting in the lack of significant changes. As EPS also functions as a barrier that limits acid diffusion in biofilms (Koo et al., 2013), the decreased EPS production may cause easy and rapid diffusion of acid from the co-culture biofilms. Thus, due to the decreased EPS production and *ldh* expression, the SM-LcS biofilm exhibited a higher pH than the SM biofilm on days 3-7 (Fig. 3.19).

Our results showed that the presence of the acid producing bacteria LcS in the SM biofilm did not lower the biofilm pH in co-culture. In contrast, the increase in pH was likely due to the inhibitory effects of LcS on *gtfB*, *gtfC*, and *ldh* expression and subsequent biofilm formation of SM. These results provide a mechanistic explanation for the results obtained by Keller and

Twetman, who showed that dental plaque produced less lactic acid when cultured with *L. reuteri in vitro* (Keller and Twetman, 2012a). Based on all the aforementioned data, it is clear that LcS exhibits cariostatic properties that may be different from those of other probiotic strains with bactericidal effects on mutans streptococci (e.g., *L. paracasei* and *L. reuteri*) (Hasslof et al., 2010).

4.1.11 Inhibitory effect of *L. casei* Shirota supernatant

Yakult[®] is fermented probiotic milk which contains live *L. casei* Shirota and its supernatant. In addition to investigate the action of *L. casei* Shirota, it was important to evaluate the effects of its supernatant on *S. mutans*.

Studies have proven that probiotic *Lactobacillus* strains had inhibitory effect on the growth of oral bacteria, such as *S. mutans*, *S. sobrinus*, *Staph. aureus*, *P. gingivalis*, and *C. albicans* (Hasslof et al., 2010; Simark-Mattsson et al., 2007; Sookkhee et al., 2001). Although in our study *L. casei* Shirota did not inhibit *S. mutans* growth in both liquid culture and biofilm culture within 24 hours, it was of interest to examine whether *L. casei* Shirota supernatant also had the antibacterial effect. Our results (Part 4) evaluated the inhibitory effect of LcS cell free culture supernatant (CFCS) collected at different growth phases. The 12 h CFCS did not show inhibitory effect on *S. mutans* growth, in

comparison with the control MRS-HCl group. However, CFCS after the stationary phase (24 h, 30 h, 36 h, 48 h and 54 h CFCS) significantly inhibited the growth of *S. mutans* (Fig. 3.25). These results suggested that the antimicrobial compounds in LcS CFCS were mainly produced in the stationary phase or later.

4.1.12 Inhibitory compounds produced by *L. casei* Shirota

A variety of substances including organic acids, hydrogen peroxide and bacteriocins produced by lactobacilli have been reported effective against pathogenic bacteria. This study characterized the inhibitory compounds in *L. casei* Shirota CFCS.

Our study showed that the antimicrobial activity of *L. casei* Shirota CFCS was more active at the acidic pH than at the neutralized pH. The result proved that acid played an important role in this effect, which was in agreement with the following reports. Cadieux PA *et al.* investigated the inhibitory effect of *Lactobacillus* spent cell-free supernatant on uropathogenic strains of *E. coli* growth, and reported that the effect was pH-dependent, as neutralized spent cell-free supernatant caused no change (Cadieux et al., 2009). Lin WH also observed the lost of the inhibitory activity of *Lactobacillus* strain GG spent cell-free supernatant or strain R1 spent cell-free supernatant against *H. pylori*

when the supernatant was neutralized (Lin et al., 2009b). Lin PP reported that the pH-neutralized LAB-CFCS (*L. salivarius* MM1, *L. acidophilus* RY2, and *L. paracasei* En4) did not inhibit the growth of enteroaggregative *E. coli* (EAggEC) strains (Lin et al., 2009a). However, S. sookkhee's study showed that there was a slight reduction (10-20%) of the antimicrobial activity after neutralizing the supernatant of lactobacilli which were isolated from oral cavity (Sookkhee et al., 2001). The results of the different studies converged to show that the antibacterial activity was pH-dependent and active in an acid pH, although there was no agreement whether the effect of the neutralized lactobacilli supernatant was completely abolished. The disagreement may be due to the different *Lactobacillus* strains used in their studies and other potential confounders.

A large number of bacteriocins have been isolated and characterized from lactic acid bacteria. For example, the lantibiotic nisin is produced by different *Lactococcus lactis* spp (Savadogo et al., 2009). These bacteriocins are of small size, proteinaceous antibacterial compounds. Three defined classes of bacteriocins have been established: the lantibiotics; the small heat stable non-lantibiotics; large heat labile bacteriocins (Savadogo et al., 2009). Some bacteriocins from lactobacilli were also identified (Jimenez-Diaz et al., 1993). The investigators suggested that the antimicrobial activity of the *Lactobacillus* strain they studied was caused by a heat-stable proteinaceous substance which

could be new bacteriocins (Abo-Amer, 2007; de Carvalho et al., 2006). Our findings indicated the antimicrobial substance from *L. casei* Shirota CFCS was heat stable, suggesting that the antimicrobial compounds might not be the heat liable bacteriocin.

L. casei Shirota CFCS crude extract showed the inhibition zone on *S. mutans* agar plate in our study, while MRS crude extract did not. This result confirmed that *L. casei* Shirota CFCS crude extract contained the inhibitory substance which was active in the low pH environment as the CFCS crude extract was acidic while MRS crude extract was neutral. The inhibitory substance extracted from hydrophobic phase of the supernatant contributes for the further purification of the inhibitory compounds.

Our results revealed that the cell free supernatant of *L. casei* Shirota strongly inhibited the growth of oral pathogenic bacteria *S. mutans*. This result supports the promising cariostatic effect of probiotic drink (e.g. Yakult®). The active compounds were proven active in the acidic environment and exhibited heat stable and storage stable properties. However, the inhibitory compounds may need to be further purified and studied.

4.1.13 Limitations

This *in vitro* study has investigated the effects of Yakult[®] and *L. casei* Shirota on *S. mutans* biofilm formation and acidogenicity, as well as the potential mechanisms involved. However, dental plaque is a complex microbial community established through continuous dynamic interaction between several hundreds of species and the host factors, including salivary pH, concentration of peroxidases, enamel surface energy, and individual diets. The findings in this study, focusing only on *S. mutans*, may not be able to forecast the possible effects of *L. casei* Shirota on multi-species biofilm in the mouth, and related mechanisms beyond the scope of this study (e.g. quorum sensing). Secondly, the sustainability and/or continuous development of probiotic effect after the consumption of probiotic drinks remain largely unknown.

4.1.14 Future work

Currently, our team are compiling and analyzing the results from three clinical studies investigating the short-term effects of Yakult[®] consumption on dental plaque, including one study employing a next generation sequencing platform to characterize the oral microbiome before, and after Yakult[®] consumption.

The investigation of cariostatic mechanisms of *L. casei* Shirota at the molecular level is on-going, together with the further analysis and

identification of the inhibitory compounds.

The synergetic effect of the probiotic drink with fluoride and laser may also be further evaluated, as laser has been employed to alter the surface energy and affect the adhesion force of first colonizers.

4.2 Conclusions

This study revealed the cariostatic effect of the probiotic drink Yakult[®] on *S. mutans* and the possible mechanisms. The probiotic strain *L. casei* Shirota inhibits the virulence gene expression and thus reduces biofilm formation and acidogenicity in SM *in vitro*. The inhibitory compounds produced by *L. casei* Shirota may also contribute to the cariostatic effect. Therefore, the clinical cariostatic effects of Yakult[®] and *L. casei* Shirota warrant further study. This study provided the understanding of cariostatic effect of probiotics and shed the light on the clinical application of this non-fluoride high-compliance “sweet” therapy (probiotic drink) to prevent the caries formation, particularly in children.

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