THE EFFECT OF PREBIOTICS ON *Lactobacillus acidophilus* DSMZ 20079 FOR INHIBITING GROWTH OF *Streptococcus mutans*

SANTICHAI NUNPAN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science Degree in Dentistry (Operative Dentistry)
March 2018

Thesis entitled "The effect of prebiotics on *Lactobacillus acidophilus* DSMZ 20079 for inhibiting growth of *Streptococcus mutans*"

by Mr. Santichai Nunpan

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Sciences in Dentistry (Operative Dentistry) of Naresuan University

Oral Defense Committee

..... Chair

(Professor Rawee Teanpaisan, Ph.D.)

..... Advisor

(Kornchanok Wayakanon, Ph.D.)

..... Co – Advisor

(Chatrudee Suwannachart, Ph.D.)

..... Internal Examiner

(Assistant Professor Suttipalin Suwannakul, Ph.D.)

Approved

.....

(Associate Professor Paisarn Muneesawang, Ph.D.)

Dean of the Graduate School

3 [illegible] 2018

ACKNOWLEDGEMENT

I would like to thank to my thesis advisors Dr.Kornchanok Wayakanon and Dr.Chatrudee Suwannachart from Thailand Institute of Scientific and Technological Research for the worth suggestion in the laboratory techniques and paper works.

I would like to thank to Dr.Marut Yanaranop and Dr.Pormsuda Nhowchai for statistic consultation.

I am also thankful to laboratory staffs in the Faculty of Dentistry, Naresuan University; Mrs.Suthimas Yuangyong, Mrs.Kusuma Jamdee and Mr.Thanakon Boonkerd for laboratory supports.

I would like to acknowledge The National Research Council of Thailand for the granted FY2017 Thesis Grant for Master Degree Student.

Finally, I would like to specially thank to my family especially my mom for helping to get through all problems during study.

Santichai Nunpan

Title	THE EFFECT OF PREBIOTICS ON *Lactobacillus acidophilus* DSMZ 20079 FOR INHIBITING GROWTH OF *Streptococcus mutans*
Author	Santichai Nunpan, B.Sc., D.Ds.,
Advisor	Kornchanok Wayaknon, D.Ds., M.Sc., Ph.D.
Co - Advisor	Chatrudee Suwannachart, B.Sc., M.Sc., Ph.D.
Academic Paper	Thesis M.Sc. in Dentistry (Operative Dentistry), Naresuan University, 2017
Keywords	Probiotics, Prebiotics, Dental caries

ABSTRACT

Dental caries is a consequence of the physiological imbalance of tooth mineral contents under dental biofilm. The key pathogen in the dental caries is *S. mutans*. Probiotics is a beneficial microorganism which is well-known for caries prevention. Moreover, the non-digestible oligo-saccharides namely prebiotics might be able to enhance the growth and function of probiotics.

The objective of this study is to evaluate the enhancing efficacy of probiotic (*Lactobacillus acidophilus*) on the growth rate of *S. mutans*. *S. mutans* A32-2 and *L. acidophilus* TISTR 2365^T = DSMZ 20079^T were co-cultured in the de Man, Rogosa and Sharpe (MRS) media supplemented with different concentrations of GOS (v/v) and FOS (w/v) at 1%, 2%, 3%, 4% and 5%. The co-culturing in non-supplemented MRS medium were performed as the control group. The efficacy of GOS and FOS was determined from the growth rate of *S. mutans* and *L. acidophilus* along with the fatty acids analysis.

In single culture, the growth rates of *S. mutans* significant decreased in 3% FOS ($p=0.032$). But the growth rate of *L. acidophilus* were not affected ($p>0.05$).

When *S. mutans co*-culture with *L. acidophilus*, the growth rates of *S. mutans* significantly decreased in 3% GOS ($p=0.040$), 4% GOS ($p=0.034$) and 5% GOS ($p=0.034$). For the co-culture in FOS, the growth rate of *S. mutans* significant decreased in all concentrations of FOS compared to the control group ($p<0.05$). There was no significant difference in the growth rate of *L. acidophilus* in all concentrations of both

FOS and GOS ($p>0.05$). The proportion between the unsaturated and saturated cellular fatty acids increased when grown in the prebiotics-supplemented medium.

In the conclusion, the growth rate of *S. mutans* was significantly retarded when co-cultured with *L. acidophilus* in 3-5 % GOS and 1-5 % FOS. Hence, *L. acidophilus* with the specific prebiotics concentrations could suppress the growth of *S. mutans*.

LIST OF CONTENTS

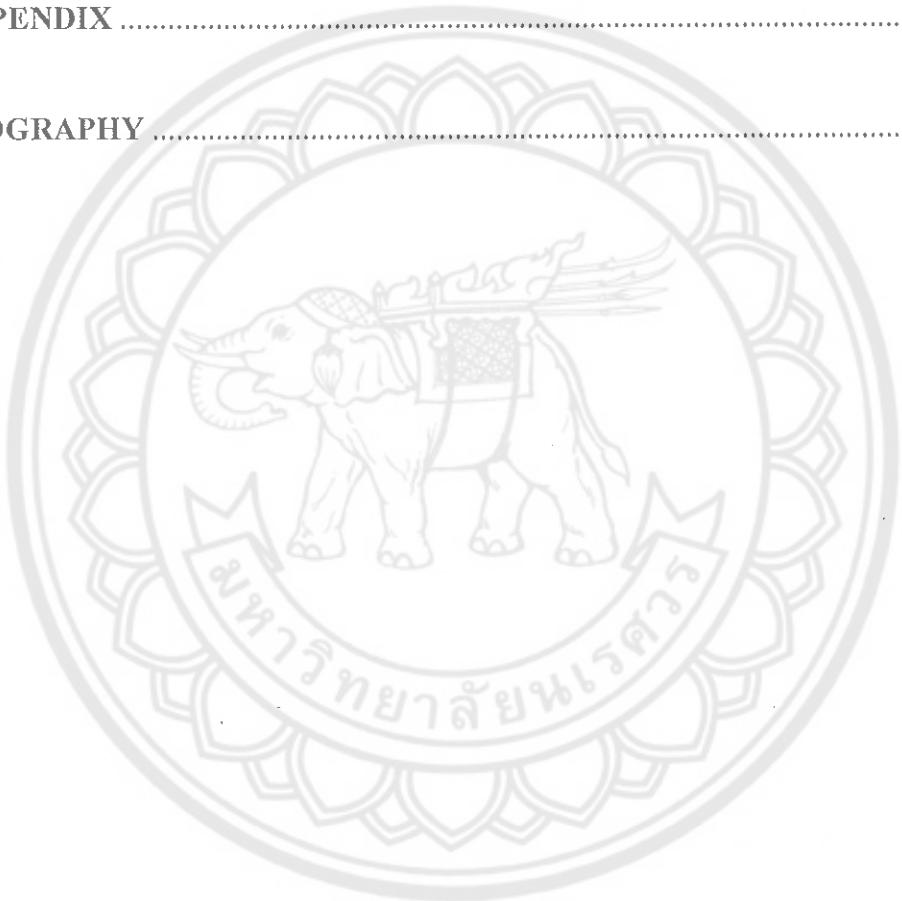
LIST OF CONTENTS (CONT.)

Chapter **Page**

LIST OF CONTENTS (CONT.)

Chapter **Page**

LIST OF CONTENTS (CONT.)



LIST OF TABLES

LIST OF TABLES (CONT.)

LIST OF FIGURES

ABBREVIATIONS

α	=	Alpha
ATCC	=	American Type Culture Collection
ATPase	=	Adeninylpyrophosphatase
β	=	Beta
BHI	=	Brain Heart Infusion
^0C	=	Degree Celsius
Ca^{2+}	=	Divalent Calcium
CFU/ml	=	Colony forming unit per millimeter
CO_2	=	Carbon dioxide
DSMZ	=	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
e.g.	=	Example
EMP	=	Embden-Mayerhof-Pannas
EPS	=	Exopolysaccharides
F_1-F_0-ATPase	=	F-ATPases /ATP synthases
FAs	=	Fatty acids
Fe^+	=	Ferrous
$FeCl_2$	=	Ferrous dichloride
FID	=	Flame Ionization detector
FOS	=	Fructo-oligosaccharides
FTFs	=	Fructosyltransferase
g	=	Gram
g/l	=	Gram per litter
GC	=	Gas chromatography
GOS	=	Galacto-oligosaccharide
G + C	=	Guanine base plus Cytosine base
GlcNAc	=	N-acetyl glucosamine
GPH	=	Galactoside-pentose-hexuronide
GTFs	=	Glucosyltransferase

ABBREVIATIONS

α	=	Alpha
ATCC	=	American Type Culture Collection
ATPase	=	Adeninylpyrophosphatase
β	=	Beta
BHI	=	Brain Heart Infusion
^0C	=	Degree Celsius
Ca^{2+}	=	Divalent Calcium
CFU/ml	=	Colony forming unit per millimeter
CO_2	=	Carbon dioxide
DSMZ	=	Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures
e.g.	=	Example
EMP	=	Embden-Mayerhof-Pannas
EPS	=	Exopolysaccharides
F_1-F_0-ATPase	=	F-ATPases /ATP synthases
FAs	=	Fatty acids
Fe^+	=	Ferrous
$FeCl_2$	=	Ferrous dichloride
FID	=	Flame Ionization detector
FOS	=	Fructo-oligosaccharides
FTFs	=	Fructosyltransferase
g	=	Gram
g/l	=	Gram per litter
GC	=	Gas chromatography
GOS	=	Galacto-oligosaccharide
G + C	=	Guanine base plus Cytosine base
GlcNAc	=	N-acetyl glucosamine
GPH	=	Galactoside-pentose-hexuronide
GTFs	=	Glucosyltransferase

ABBREVIATIONS (CONT.)

H^+	=	Protonated hydrogen ion
H^+/ATPase	=	Proton pump Adeninylpyrophosphatase
H_2	=	Hydrogen gas
HCl	=	Hydrochloric acid
hr^{-1}	=	Per hour
$lb/inch^2$	=	Pound per inch square
IMO	=	Isomalto-oligosaccharides
KH_2PO_4	=	Potassium dihydrogen phosphate
K_2HPO_4	=	Dipotassium hydrogen phosphate
K^+	=	Potassium ion
L. acidophilus	=	*Lactobacillus acidophilus*
LTA	=	Lipoteichoic acid
µm	=	Micro-meter
MIS	=	Microbial Identification System
ml	=	Milliliter
mg	=	Milligram
mg/ml	=	Milligram per milliliter
µm	=	Micro-meter
Mc	=	Culture-based medium without bacteria
Mur/NAc	=	N-acetyl muramic acids
$MgSO_4.7H_2O$	=	Magnesium Sulfate Heptahydrate
$MnSO_4.4H_2O$	=	Manganese(II) Sulfate Tetrahydrate
MRS	=	de Man, Rogosa and Sharpe
N	=	Normality-gram equivalent weight of a solution in a solution
NaOH	=	Sodium hydroxide
NDOs	=	Non-digestible oligosaccharides
N_2	=	Nitrogen gas
Na^+	=	Sodium ion

ABBREVIATIONS (CONT.)

NaCl	=	Sodium chloride
OH	=	Hydroxide
OD	=	Optical density
ω	=	Omega
%	=	Percentage
PTS	=	Phosphoenolpyruvate: phosphotransferase system
P value	=	Probability value
pH	=	Power of hydrogen ion concentration
rRNA	=	Ribosomal Ribonucleic acid
SCCs	=	Short-chain carbohydrates
Spp.	=	Species
S. mutans	=	*Streptococcus mutans*
ST	=	Supernatant from the co-culture
tert	=	Tertiary
TISTR	=	Thailand Institute of Scientific and Technological Research Culture Collection
TYE	=	Tryptic Soy broth plus yeast extract and trace element
TSB	=	Tryptic Soy Broth
Trk	=	Tyrosine kinases
Th_{reg}	=	T lymphocyte helper regulatory
Th_0	=	T lymphocyte helper 0
Th_1	=	T lymphocyte helper 1
Th_2	=	T lymphocyte helper 2
Th_{12}	=	T lymphocyte helper 12
Th_{17}	=	T lymphocyte helper 17
UFAs	=	Unsaturated fatty acid
WTA	=	Wall teichoic acid

ABBREVIATIONS (CONT.)

v/v	=	Volume by volume
w/v	=	Weight by volume
XOS	=	Xylo-oligosaccharides

CHAPTER I

INTRODUCTION

Oral microorganisms composing commensal and pathogenic microorganisms ordinarily live as an equilibrium community. They colonize on oral tissue in a complex architecture named bacterial biofilm (Fejerskov, Kidd, & Nyvad, 2008, p. 4). When the biofilm becomes exposed to carbohydrate substrates especially sucrose, the metabolically organic acids are generated from the acid-producing bacteria. These acid products subsequently attack and dissolve tooth mineral. Moreover, extracellular polysaccharides (EPS) are also produced from the carbohydrate fermentation. The EPS are extracellularly released and accumulated in the bacterial community, and they also protect the microorganism from the savage environment (Fejerskov et al., 2008, p. 4). The acid-producing bacteria related to dental caries are *S. mutans, Actinomyces* spp. and *Lactobacillus* spp. The *S. mutans* plays a major role in the initial step of dental caries whereas *Actinomyces* spp. have been commonly isolated and highly associated with smooth surface caries. Within deep carious lesion, plaque fluid is low in pH and oxygen. These environment supports the growth of *Lactobacillus* spp. (Caufield, Schon, Saraithong, Li, & Argimon, 2015, pp. 110S-118S).

An occurrence of dental carious lesions requires a period of time for the acid, produced from the carbohydrate fermentation by bacteria, to dissolve the inorganic components of tooth structure. According to this procedure, the imbalance of these factors introduces the process of dental caries. Several studies attempt to interrupt the colonization of *S. mutans* with probiotics which are beneficial living microorganism. Probiotics have an influence on both microorganisms and host cells in several mechanisms that include specific antimicrobial substances production, host gene modulation expression and pathogen exclusion (Nair, & Takeda, 2011, pp. 87- 109). They consist of several genera such as *Lactobacillus* spp., *Bifidobacterium* spp. and *Saccharomyces* spp. (Anusha, Umar, Basheer, & Baroudi, 2015, pp. 43-47).

Lactobacillus spp. produce several kinds of bacteriocins, which are antimicrobial peptides specific to different pathogens (Dobson, Cotter, Ross, & Hill, 2012, pp. 1-6). Several *In vitro* studies showed that *Lactobacillus* spp. produce different kinds of antimicrobial components such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins and adhesion inhibitors against *Streptococcus* spp. (Hasslof, Hedberg, Twetman, & Stecksen-Blicks, 2010, p. 18).

Unlike bacteria pathogens, probiotics and the beneficial microorganism are able to utilize non-digestible substances called prebiotics. These substances are unable to be digested by human or pathogenic bacteria. They are used for selectively stimulating the growth and activities of beneficial microorganisms in colons (Gibson, Probert, Loo, & Rastall, 2004, pp. 259-275). After probiotics consume prebiotics, they increase in number and produce several bacteriocins in order to suppress the pathogens' activities.

The most common prebiotics are non-digestible polysaccharides such as lacto-sucrose, galacto-oligosaccharides (GOS) , fructo-oligosaccharides (FOS) and isomalto-oligosaccharides (Sabater-Molina, Larque, Toorella, & Zamora, 2009, pp. 315-328). However, only GOS, FOS and inulin have been tested and received all requirements for prebiotics (Roberfroid, 2007, pp. 830s-837s; Shigwedha, Hal, Jia, Sichel, & Zhang, 2016, pp. 41-57).

Regarding the benefits of probiotics and prebiotics, their synergisms named synbiotics have been popular and applied in health promotion products. However, there have few studies in the synbiotic effect on oral microorganisms.

Research aim

The aim of this study was to evaluate the competitive growth of *Lactobacillus acidophilus* (TISTR 2365^T = DSMZ 20079^T = ATCC 4356^T (with *Streptococcus mutans* (A32-2) after received galacto-oligosaccharides (GOS and fructo-oligosacharides (FOS) in various concentrations (1%, 2%, 3%, 4% and 5%).

Research hypothesis

1. Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) are able to enhance the growth rate of *L. acidophilus* both in single culture and co-culture with *S. mutans*.

2. Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) are able to decrease the growth rate of *S. mutans* both in single culture and co-culture with *L. acidophilus*.

3. Different prebiotic concentrations have different effects on growth rate of *L. acidophilus* and *S. mutans*.

4. Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) have an influence on cellular fatty acids redistribution of *S. mutans* and *L. acidophilus*.

Research scope

Due to the ability of *Lactobacillus* spp. on growth competition with *S. mutans*, the efficacy of the prebiotics on the growth rate of *L. acidophilus* was observed in this study. *S. mutans* was accounted as the cariogenic bacteria. *L. acidophilus* was treated with two prebiotics which are Fructo-oligosaccharides (FOS) and Galacto-oligosaccharides (GOS) with concentration of 1%, 2%, 3%, 4% and 5% (v/v, w/v). The efficiency of probiotic on growth competition of bacteria was determined by the growth rate of them.

Research significance

L. acidophilus in this study is commensal microorganism commonly isolated from human. The ability to suppress growth of *S. mutans* might enhanced by different kinds of prebiotics. If the intensification is effective, prebiotics are able to be applied in various oral-care products for dental caries prevention.

CHAPTER II

LITERATURE REVIEW

Dental caries is a consequence from physiological imbalance of tooth mineral components under dental biofilm (Fejerskov et al., 2008, p. 4). The disease is driven by the interaction among host, oral microorganisms and carbohydrate. For the oral microorganisms, they normally colonize in a human oral cavity soon after birth and generally coexist in a complex community form. These microorganisms adhere to oral tissue through acquired pellicle coated on tooth surface and oral mucosa. Firstly, they approach dental tissue with non-specific adhesion like van der Waals' s attractive force. For the selective binding, they recognize and bind to pellicle glycoprotein such as sialic acids residual, statherin or proline–rich protein (Fejerskov et al., 2008, p. 4; Mahajan, Singh, Kashyap, Kumar, & Mahajan, 2013, p. 765434). The pioneer bacteria are mainly *Streptococcus* spp. such as *S. oralis*, *S. mitis*, *S. sanguinis*, *S. parasanguinis*, *S. gordonii* including *Actinomyces* spp. , *Veillonella*, *Gemella*, *Abiotrotrophia*, *Granulicatella* and *Haemophilus* spp. (Fejerskov et al., 2008, p. 4; Lamont, & Jenkinson, 2010, pp. 34-35). After attached to oral tissues, microorganisms start colonizing and increasing the complexity until plaque community stable. This heterogenic community is called dental plaque or dental biofilm.

The dental biofilm is ecology of oral commensal and pathogenic microorganisms in an equilibrium condition. When the equilibrium has been shifted due to either the environment or the microorganisms themselves, the roles of pathogenic bacteria are dominant. In high caries risk patients, their common habit is the high consumption of the sugary food. This clearly influences on the environment of dental plaque ecology by promoting the virulence of the cariogenic bacteria particularly the amount of metabolic acid products such as lactic acids, succinic acid or acetic acid. These acids dissolve the component of tooth substrate leading to the dental caries.

The microorganisms play an important role in tooth dissolution are acidogenic bacteria i.e. *S. mutans*, *Actinomyces* spp. and *Lactobacillus* spp. Even though *S. mutans* is a normal flora, but they are turning to be a dental caries initiator after received fermentable sugar and produced acids (Bente, & Ole, 1987, pp. 287 - 296).

Streptococcus mutans

S. mutans is a normal flora oral cavities and upper respiratory tract. It was first isolated from human carious lesion by Clark in 1942 (Clarke, 1924, pp. 141-147) and now is recognized as a vital cause of human caries and associated bacterial endocarditis (Banas, 2004, pp. 1267-1277; McGhie, Hutchison, Nye, & Ball, 1977, pp. 456-458) . *S. mutans* belongs to *Streptococcaceae* family which is catalase-negative and non-spore forming. It is characterized as facultative anaerobic, Gram-positive, cocci or spherical to ovoid in short or medium chain, non-motile bacteria. *S. mutans* normally grow within a temperature range from 20 – 42 °C. It uses several kinds of carbohydrates such as sucrose, mannose, glucose, maltose, mannitol, raffinose, ribose and trehalose. It also produces α-Galactosidase for lactose dissociation, but it cannot produce α-L-fructosidase, β-D- fructosidase enzymes decrease Robinson, Carl, A., & Patel, 2000, pp. 2117-2127).

S. mutans requires the complex medium such as tryptic soy and brain heart infusion and 5% CO_2. The acids are commonly produced along with the growth of *S. mutans* and that makes the pH of the dental plaque decrease (Robinson, Carl, A., & Patel, 2000, pp. 2117-2127).

As in the human oral cavities, the number of *S. mutans* ranges from undetectable to 10^6-10^7 colony forming units/ milliliter (CFU/ml). It is recognized as the commensal bacteria and accounted for 2% of *Streptococcus* spp. of the initial colonizers. When biofilm receive sugar, it rapidly multiply and produces acids. The virulent characteristics of *S. mutans* are versatile colonization, biofilm formation, acid production and acid tolerance (Figure 1).

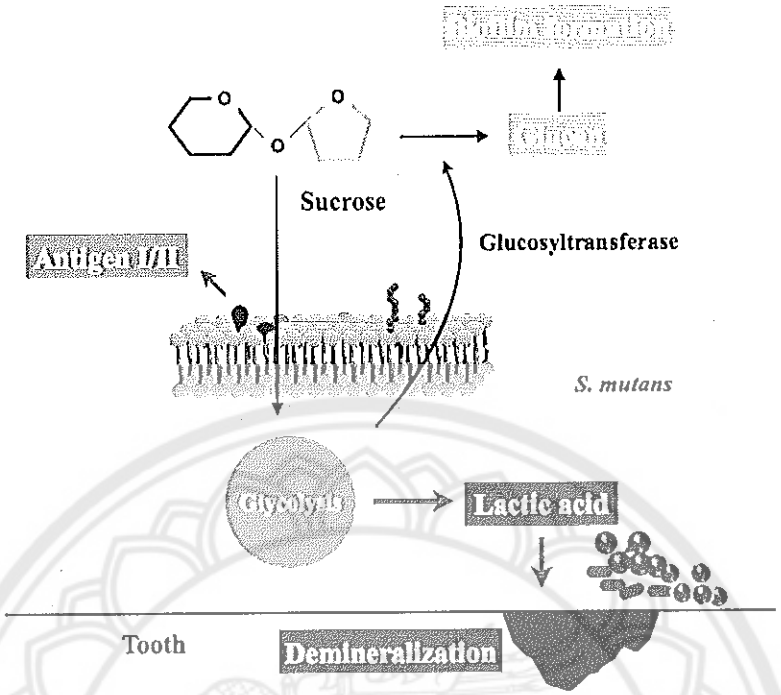


Figure 1 Virulence factors of *S. mutans*

Adhesion and biofilm formation of *Streptococcus mutans*

The biofilm is an organized structure of the poly-microbial species embedded in an organic matrix-derived salivary glycoprotein and the extracellular bacterial products (Saini, Saini, & Sharma, 2011, pp. 71-75). The formation and the maturation of the oral biofilm require the interaction between the primary and secondary colonizers. As the primary colonizer, *S. mutans* firstly adhere to the acquired pellicle coated tooth through the sucrose-independent and sucrose-dependent mechanisms.

For the sucrose-independent adhesion, *S. mutans* poses the antigen I/ II surface proteins on the cell wall. These antigens contain alanine-rich and proline-rich domains responsible for binding to the salivary agglutinin, the salivary pellicle components and other bacteria in the dental plaque (Petersen, Assev, van der Mei, Busscher, & Scheie, 2002, pp. 249-256).

For the sucrose-dependent adhesion, *S. mutans* produces glucosyltransferase (GTFs) enzyme for sucrose digestion. The GTFs cleave sucrose into free-fructose and glucose homopolymer (Glucan). These glucan polymers are found in both water-insoluble (α-1,3 –glycosidic bond) and water-soluble (α-1,6 –glycosidic bond) forms.

The glucan are able to attached to *S. mutans* and salivary pellicle through hydrogen bond (Banas, 2004, pp. 1267-1277). The insoluble-glucan facilitates the adhesion of the second colonizer, stabilizes the biofilm and protects the bacteria from host defend mechanisms (Banas, 2004, pp. 1267-1277; Lamont, & Jenkinson, 2010, pp. 34-35; Nair, & Takeda, 2011, pp. 87- 109; Robinson et al., 2000, pp. 2117-2127; Zeng, & Burne, 2013, pp. 833-843).

Acidogenicity of *Streptococcus mutans*

As described above, all species in *Streptococcaceae* family can assimilate wide range of carbohydrates (Robinson et al., 2000, pp. 2117-2127). Sucrose is considered as the most important cariogenic sugar. The sucrose fermentation by *S. mutans* results in the weak organic acids such as lactic acid, formic acid and acetic acid. When the acids are generated especially the lactic acid, the pH of the dental plaque drops to a critical point. Without the procedure of the acid elimination, the sustained acidic condition is favor for tooth demineralization and aciduric/acidogenic bacteria colonization (Banas, 2004, pp. 1267-1277).

Acid-tolerance of *Streptococcus mutans*

According to Bender et al. in 1985, the growth activity of *S. mutans* are inhibited at pH 4.4 (Bender, Thibodeau, & Marquis, 1985, pp. 90-95). Their ordinarily metabolic products from the carbohydrate fermentation are weak acids. *S. mutans* has been adapted mechanism to maintain the intracellular pH to survive in the acidic condition.

Unsaturated fatty acids (UFAs) in the cell membrane of *S. mutans* increased after acid challenged. The UFAs provoke the membrane proton translocating ATPase (H^+/ATPase) which export H^+ to the extracellular area (Baker, Faustoferri, & Quivey, 2017, pp. 107-117; Banas, 2004, pp. 1267-1277). Besides H^+ / ATPase, potassium transporter, Trk system, is also upregulated following the acid shock. This pump uptakes and incorporates potassium ions for an electrical balance of *S. mutans* (Baker et al., 2017, pp. 107-117; Gong et al., 2009, pp. 3322-3332). A study demonstrated a capability of the glucan to retain metabolic proton. These could pre-condition the bacterium for further acid-stress (Guo, McLean, Lux, He, & Shi, 2015, pp. 18015).

Within the mature biofilm, the water-insoluble glucan covered on the bacterial colony resulting in the acid retention. This ecology favors the colonization of the acidophilic bacteria, especially *Lactobacillus* spp. (Nyvad, & Kilian, 1990, pp. 267 - 272). In low pH condition, *Lactobacillus* spp. increased but with the smaller number than *S. mutans* (Loesche, Stephen, Earnest, & Burt, 1984, pp. 765-772). They are normally isolated from the surface of the deep caries (Byun et al., 2004, pp. 3128-3136).

***Lactobacillus* species**

Lactobacilli group composed over 25 unique species. They appear as Gram-positive, non-spore forming, rod shape, and size approximately 0.5 -1.2 x 1-10 μm. According to the glucose assimilation, they are categorized as the homofermenter and the heterofermenter microorganisms as in Table 1 (Robinson, Carl A, & Patel, 2000, pp. 1134 - 1157). For the homofermenter, they produce lactic acid as a majority of their products from glucose. The heterofermenter can produce other acids apart from lactic acid such as carbon dioxide, acetic acid or ethanol. (Nair, & Takeda, 2011, pp. 87- 109; Robinson et al., 2000, pp. 1134 - 1157). The optimal growth temperature is between 30 – 40 ^{0}C enhanced by 5% CO_2 at pH 5.5 – 5.8. In addition, *Lactobacillus* species produce several kinds of bacteriocins which effect on the specific strains in the complex microbial biofilm (Table 2).

Table 1 *Lactobacillus* species classification

Group I Obligate Homofermenter	*Lactobacillus acidophilus*	*Lactobacillus helveticus*
	Lactobacillus delbrueckii	*Lactobacillus salivarius*
Group II Facultative Homofermenter	*Lactobacillus plantarum*	*Lactobacillus casei*
	Lactobacillus sake	*Lactobacillus curvatus*
Group III Obligate Heterofermenter	*Lactobacillus brevis*	*Lactobacillus cellbiosus*
	Lactobacillus buchneri	

Source: Robinson et al., 2000, pp. 1134 - 1157

Table 2 Bacteriocins of *Lactobacillus* species

Bacteriocins	Organisms	Sensitive strains
Lactacin B	*L. acidophilus*	*L. delbrueckii, L. helveticus*
Lactacin F	*L. acidophilus*	*L. fermentum, Staphylococcus aureus, Enterococcus faecalis*
Brevicin 37	*L. brevis*	*Pediococcus dannosus, Leuconostoc oenos*
Lactacin A	*L. delbrueckii*	*L. delbrueckii* subsp. *lactis*
Helveticin J	*L. helveticus*	*L. helveticus, L. delbrueckii* subsp. *bulgaricus*
Sakacin A	*L. sake*	*Carnobacterium piscicola, Listeria monocytogenes*
Plantarincin A	*L. plantarum*	*Lactococcus lactis, Enterococcus faecalis*
Gassericin A	*L. gasseri*	*L. acidophilus, L. brevis*

Source: Robinson et al., 2000, pp. 1134 – 1157

Lactobacillus acidophilus

L. acidophilus are normally isolated from gastrointestinal tract of humans and animals. They are found in feces of milk-fed infants and elderly person consuming high milk, lactose or dextrin diets. *L. acidophilus* are able to ferment several kinds of hexose. Regarding glucose assimilation, they are classified as homofermenter. *L. acidophilus* preferred the microaerophilic condition however it can be survived in aerobic condition. Without oxygen condition, it requires a mixture of 5% CO_2, 10% H_2, and 85% N_2. *L. acidophilus* is a fastidious microorganism required an enriched media with amino acids and vitamins (peptone, tryptone or yeast/ beef extracts), sorbitan monooleate (Tween 80) , sodium acetate and magnesium salt stimulating bacterial growth. The suitable commercial media are de Man, Rogosa and Sharpe (MRS) (Robinson et al., 2000, pp. 1134 - 1157).

Carbohydrate metabolism of *Lactobacillus acidophilus*

L. acidophilus mainly produces lactic acid (>85%) after ferment hexose in Embden-Mayerhof-Pannas (EMP) pathway. According to Watson et al, *L. acidophilus* ATCC4356^T (DSMZ20079^T) utilized glucose, lactose, lactulose, fructo-oligosaccharide

(FOS), galacto-oligosaccharide (GOS) and inulin (Watson et al., 2013, pp. 1132-1146), but it cannot use gluconate or pentose (Robinson et al., 2000, pp. 1134 – 1157). *L. acidophilus* produced several antimicrobial substances such as lactic acid, hydrogen peroxide and various bacteriocins (Table 3).

Table 3 Bacteriocins production by *Lactobacillus acidophilus*

Producers	Bacteriocins		
L. acidophilus	Lactacin B	Acidocin A	Acidocin 8912
	Lactacin F	Acidocin B	Acidocin
	Acidophilucin A	Acidocin J1229	JCM1132

Source: Robinson et al., 2000, pp. 1134 - 1157

As described above, *L. acidophilus* is so called probiotic. *In vitro* studies showed that *Lactobacillus* spp. strain GG produced different antimicrobial components such as organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins, and adhesion inhibitors against *Streptococcus* spp. (Hasslof et al., 2010, p. 18; Silza, Jacobus, Deneke, & Gorbachi, 1987, pp. 1231-1233). According to Kojima et al. (2015), *Lactobacillus* spp. could inhibit insoluble-glucan formation of *S. mutans* (Kojima, Ohshima, Seneviratne, & Maeda, 2015, pp. 27-32). Thus, *Lactobacillus* spp. can be used as a potential probiotic.

Probiotics bacteria

Probiotics are defined as living microorganism providing benefits to human (Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report, 2001, pp. 1-56). It was first introduced in early 20th century by Elie Metschnikoff. Eighty-four years later, Hull and colleagues introduced *Lactobacillus acidophilus* as the first probiotic specie (Anusha et al., 2015, pp. 43-47). Probiotics are traditionally used to modulate bacterial balance in digestive system. The beneficial effects are maintaining balance between commensal

microorganism and pathogens of intestinal microflora, regulating the intestinal immune system and reinforcing the intestinal barrier (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013, pp. 1-16). From previous studies showed that consumption approximately $10^7 - 10^9$ CFU/ml of *Lactobacillus* spp. was adequate to modulate the benefit to intestine microflora (Charteris, Kelly, Morelli, & Collins, 1998, pp. 759-768; Fernández, Boris, & Barbés, 2003, pp. 449-455; Pitino et al., 2012, pp. 57-63). In oral cavity, probiotics must first accumulate and attach to oral tissues. After that, they formed a protective barrier preventing pathogenic microorganism colonization. Finally, the number of them increased for survive and produced effective capacities (Teughels, Van Essche, Sliepen, & Quirynen, 2008, pp. 111-147).

Mechanism of action of probiotics

After probiotics adhere to host tissues, they form biofilm as protective barrier preventing oral pathogen colonization and their toxin (Nair, & Takeda, 2011, pp. 87-109). According to several previous studies, probiotics could form a barrier preventing pathogen colonization, competing in growth with oral pathogenic microorganisms and eliminating pathogens' toxin by decreasing pH, secreting bactericidal proteins, stimulating defensing production and proteolytic binding proteins (Comelli, Guggenheim, Stingele, & Neeser, 2002, pp. 218-224; Nair, & Takeda, 2011, pp. 87-109). Moreover, they could alter host immune responses through regulating T-cells and macrophage functions (Nair, & Takeda, 2011, pp. 87- 109). The probiotics mechanism of action is shown in Figure 2.

Lactobacillus spp. and *Bifidobacterium* spp. are the most famous probiotics. These bacteria are found in fermented dairy products and play a part of gastrointestinal normal flora (Stamatova, & Meurman, 2009, pp. 329 - 338). The less common probiotics are *Streptococcus* spp., *Escherichia* spp., *Bacillus* spp. and *Saccharomyces* spp. (Bowen, 2011, pp. 78 - 82; Stamatova, & Meurman, 2009, pp. 329 - 338). According to the study of Michael in 2001, *Streptococcus thermophilus* had also been used as probiotics to enhance digestion of lactose intolerant subjects (Vrese, Stegelmann, Bernd Richter, Laue, & Schrezenmeir, 2001, pp. 421S-429S).

Regarding the benefits of probiotics, they are now drawn more attention in caries modulation. A number of researchers try to develop novel methods for caries treatment as described in Table 4.

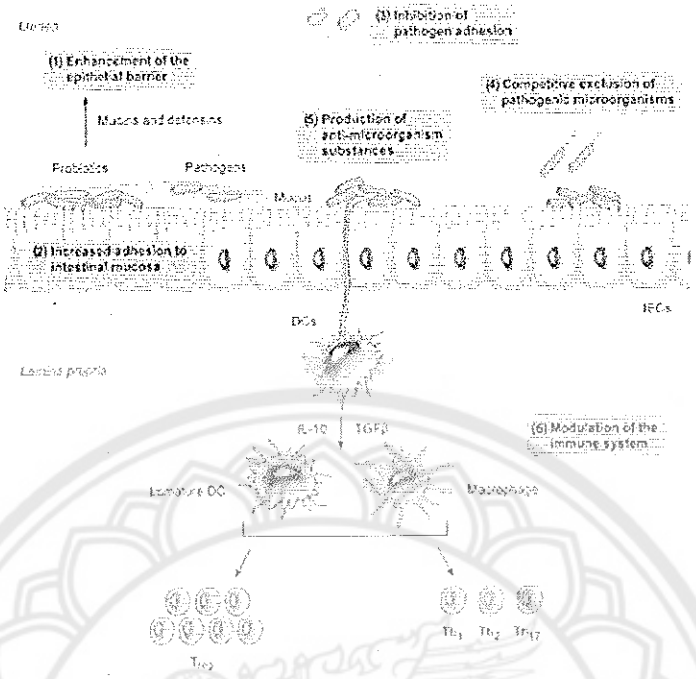


Figure 2 The mechanism of action of probiotics in the intestine

Source: Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012, pp. 160 -174

Note: The mechanism of action of probiotics including 1) Enhancement of epithelial barrier by stimulating tight junction signaling and preventing cytokine-induced epithelial damage secretion and promoting mucins secretion to maintained epithelial integrity. 2) Increase adhesion to intestinal mucosa by stimulating mucous secretion which is involved with probiotics adhesion and pathogens adhesion. 3-4) Competitive exclusion of pathogenic microorganisms, antimicrobial substances inhibit pathogenic microorganism and deplete essential nutrients. 5) Production antibacterial peptides against other bacteria 6) Modulation immune systems through innate (dendritic cell, monocyte, macrophage) and adaptive immunities (B and T lymphocyte) such as stimulate Toll like receptor on dendritic cells, subsequently the differentiation of regulatory T- lymphocyte (Th_0 and Th_{reg}) to control the inflammation and inhibit T-lymphocyte (Th_1, Th_2, Th_{17}) functions.

Table 4 Probiotics and dental caries modulation

	Study design	Strain	Vehicle	Frequency	Concentration	Results
Nase et al. (Nase et al., 2001, pp. 412- 420)	Double blind Randomized	*L. rhamnosus* GG	Milk	± 250 ml, 5 days/week for 7 months	> 5 x 10^5 CFU/ml	Significantly reduced calculated caries-risk score
Ahola et al. (Ahola et al., 2002, pp. 799-804)	Double blind Randomized	*L. rhamnosus* GG *L. rhamnosus* LC 705	Cheeses	15 g., 5 times/day for 3 weeks	> 1 x 10^7 CFU/g	Decreased *S. mutans* count after 6 weeks in prebiotic group
Nikawa et al. (Nikawa et al., 2004, pp. 219-223)	Double blind two-way crossover	*S. thermophilus* *L. bulgaris* *L. reuteri*	Yogurt	Once daily for 2 weeks	ND	Significant decreased *S. mutans* count
Chuang et al. (Chuang, Huang, Ou-Yang, & Lin, 2011, pp. 471-476)	Double blind Randomized	*L. paracesei*	Oral tablet	3 times/ day after meal for 2 weeks	3 x 10^8 CFU/ml	Significant decreased *S. mutans* count after 2 weeks in prebiotics group

Source: Teughels et al., 2008, pp. 111-147

Host status is one of the most important factor influences on the occurrence of dental caries. The frequency of fermentable carbohydrate intake leads to greater acid production. Multiple acids production and retention create a niche for acidophilic and acidogenic microorganisms (Banas, 2004, pp. 1267-1277). However, there are novel indigestible substances by pathogenic bacteria. The one of these substances called prebiotic.

Prebiotics

Prebiotics are defined as a non-digestible food ingredient that beneficially affects the hosts by selectively stimulating growth and activities of the microorganisms (Gibson et al., 2004, pp. 259-275). The prebiotics are used to optimize the bowel functions by reducing the number of non-beneficially intestinal microorganisms (Akter, Wu, Memon, & Moshin, 2015, pp. 733-741; Jia, Li, Zhao, & Nicholson, 2008, pp. 123-129). The common prebiotics are inulin, fructo-oligosaccharides (FOS), mannan-oligosaccharides, galacto-oligosaccharides (GOS) and arabinogalactans. The non-digestible oligosaccharides (NDO) are well known as the prebiotics. The NDOs are the short-chain carbohydrates (SCCs) with low molecular weight and unable to be digested by the human enzymes except some strains of intestinal microorganisms (Quigley, Hudson, & Englyst, 1999, pp. 381-390). When the cariogenic microorganisms expose to the NDOs, there are neither lactic acid nor extracellular polysaccharide products. Therefore, the NDOs are popular in used as the cariogenic sugar substitution (Crittenden, & Playne, 1996, pp. 353-361).

The oligosaccharides are the carbohydrate polymer containing between 3 and 10 monosaccharides. They are made from one to three different types of the monosaccharides such as fructose, galactose, glucose or xylose (Mussatto, & Mancilha, 2007, pp. 587-597). The example of the oligosaccharides are galacto-oligosaccharides (GOS) (Lamsal, 2012, pp. 2020-2028), fructo-oligisacharides (FOS) (Sabater-Molina et al., 2009, pp. 315-328), isomalto-oligosaccharides (IMO) and xylo-oligosaccharides (XOS). The GOS and the FOS have been tested in the *in vivo* studies, they fulfill all requirements for the prebiotics (Roberfroid, 2007, pp. 830S-837S). The structure of those oligosaccharides are shown in Figure 3.

The prebiotics can be extracted from natural or synthesized from the disaccharides (Mussatto, & Mancilha, 2007, pp. 587-597; Saad et al., 2013, pp. 1-16). They must be able to resist the gastric acid or the mammalian hydrolysis enzymes, and they should not be absorbed. The prebiotics are fermented by the intestine microorganisms resulting in stimulating the growth and activities of those intestinal bacteria (Gibson et al., 2004, pp. 259-275; Saad et al., 2013, pp. 1-16).

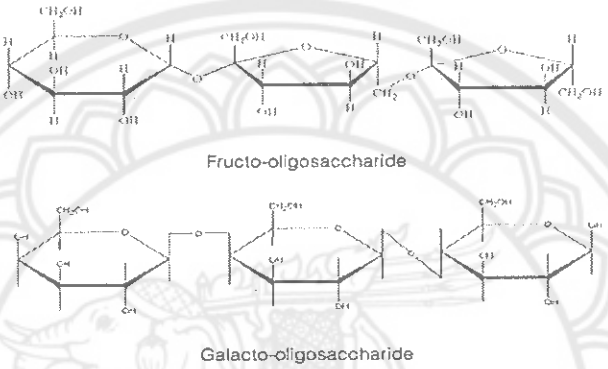


Figure 3 The molecular structure of oligosaccharides

Source: Mathews, hold, & Ahern, 2000

Fructo-oligosaccharides (FOS)

The fructo-oligosaccharides (FOS) is carbohydrate polymers consisting of 3 - 9 molecules of the fructose (Bornet, Brouns, Tashiro, & Duvillier, 2002, pp. S111-120; Bruggencate, Bovee-Oudenhoven, Lettink-Wissink, Katan, & Meer, 2006, pp. 70-74) linking with either β(2→1) or α(1→2) glycosidic bonds and terminated by the glucose units (Figure 3) (Bornet et al., 2002, pp. S111-120; Crittenden, & Playne, 1996, pp. 353-361; Oliveira et al., 2011, pp. 305-311). The Human amylases (α-, β- amylases) and the pancreatic juice can only digest α(1→2) the internal bond linkage (Campbell, Smith, & Peters, 2005), but cannot digest β(2→1) the fructo-oligosaccharides (Spiegel, Rose, Karabell, Frankos, & Schmitt, 1994, pp. 389-394). The *in vivo* experiments, the FOS is fermented by the intestine microorganisms and produced CO_2 and the short chain fatty acids (SCFAs) especially the propionic and butyric acids (Hosoya, Norimasa, Dhorranintra, & Hidaka, 1988, pp. 67-74) to against

the acid-sensitive pathogens (Bruggencate et al., 2006, pp. 70-74). Therefore, the FOS is considered as an alternative prebiotic.

For *L. acidophilus* metabolism, FOS was transported into cell via ATP-binding cascade (ABC) transporters of the MsmEFGK family (Barrangou, Altermann, Hutkins, Cano, & Klaenhammer, 2003, pp. 8957-8962; Barrangou et al., 2006, pp. 3816-3821). FOS was then hydrolyzed by BfrA or BrfB fructosidase enzyme to receive fructose (Barrangou et al., 2003, pp. 8957-8962; Ehrmann, Korakli, & Vogel, 2003, pp. 0391-0397)

The enzymes in the β-fructosidase superfamily hydrolyzed these β-linkage including the β-D-fructosidic linkage (Naumoff, 2001, pp. 66-76). *S. mutans* carry *fruA* gene that is encoded for FruA protein. The *fruA* gene is detected in *S. mutans* serotype c, e, and f strain. These enzymes hydrolyze β-(2,6)- and β-(2,1)-linked sugars (Burne, & Penders, 1992, pp. 4621-4632). After FruA is released from bacterial cell, it soon accumulates on cell wall via LPxTG motif (Burne, & Penders, 1992, pp. 4621-4632). The FruA enzyme liberates fructose from carbohydrate polymers, fructans, inulin, sucrose and raffinose (Burne, Wen, Chen, & Penders, 1999, pp. 2863-2871; Hartemink, Quataert, van Laere, Nout, & Rombouts, 1995, pp. 551-557; Russell, Aduse-Opoku, Sutcliffe, Tao, & Ferretti, 1992, pp. 4631-4637). Free fructose is then subsequently use in cell metabolism (Burne, & Penders, 1992, pp. 4621-4632). Hence, the FOS is considerable as the potentially cariogenic polysaccharide similar to the sucrose (Hartemink et al., 1995, pp. 551-557; Moynihan, 1998, pp. 209-218).

The fructo-oligosaccharides (FOS) is synthesized from sucrose by the β-fructofuranosidase or from the enzymatic hydrolysis of the inulin by the inulinase (Bornet et al., 2002, pp. S111-120; Crittenden, & Playne, 1996, pp. 353-361) and it is also produced from oral *Streptococcus* spp. with the fructosyl transferase.

Galacto-oligosaccharides (GOS)

The galacto-oligosaccharides (GOS) is defined as the oligosaccharide comprising 2-8 saccharides with the glucose terminus as shown in Figure 3 (Boler, & Fahey, 2012, pp. 13-26). It is produced from the transgalactosylation of the lactose with the β-D-galactosidase in the lactose-rich substrates especially milk, milk whey, or a mixture of both (Figure 4) (Boler, & Fahey, 2012, pp. 13-26; Torres, Gonçalves, Teixeira, & Rodrigues, 2010, pp. 438-454). In the commercial products, the β(1→6)

linkage formation of the galactosidase is obtained from *Aspergillus oryzae* and *Streptococcus thermophilus*. Whereas the β(1→4) linkage formation of the galactosidase is obtained from *Cryptococcus lauentii* and *Bacillus circulans* (Sako, Mastumoto, & Tanaka, 1999, pp. 69-80).

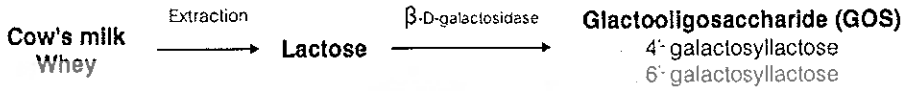


Figure 4 The galacto-oligosaccharides synthesis

Source: Boler, & Fahey, 2012, pp. 13-26; Torres et al., 2010, pp. 438-454

The GOS is widely used as the prebiotic, since it cannot be digested by the mammalian enzymes. In addition, the GOS provides benefits to the hosts by stimulating the growth of selected members of the gut microbiota (Boler & Fahey, 2012, pp. 13-26; Torres et al., 2010, p. 438-454). It has more efficiency to enhance the growth of the intestinal *Bifidobacteria* than FOS (Wei et al., 2015, pp. 158-168). Therefore, GOS is a potential prebiotic because it improved growth of many *Lactobacillus* spp. In addition, its acid metabolites is lower when compared with glucose and lactose (Hernandez-Hernandez et al., 2012, pp. 355-361).

According to the benefits of probiotics and prebiotics, several studies try to improve the spectrum of the oral microbial modulation by combining the probiotics with the prebiotics (Table 5). This combination is called the synbiotics. In 1995, Gibson and Roberfroid were first introduced the concept of synbiotics (Gibson et al., 2004, pp. 259-275) as a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival of the microorganisms in the gastrointestinal tract. The recent *in vitro* study showed that *L. acidophilus* cultured with conjac glucomanan was able to inhibit *S. mutans* growth (Tester, & Al-Ghazzewi, 2011, pp. 234 - 237).

Since *S. mutans* and *L. acidophilus* are the Gram-positive bacteria. Both strains have a thicker outermost wall (15- 80 nm) (Todar, 2008, unpage) enclosed intracellular organelles compare with Gram-negative bacteria. The boundary

compartments of cell including lipid membrane and peptidoglycan. The peptidoglycan is the oligomer of disaccharide peptide units of N-acetyl muramic acids (Mur/NAc) and N-acetyl glucosamine (GlcNAc). The oligomers are jointed with β- (1→ 4) linkage to form glycan strand (Rogers, Perkins, & Ward, 1980, pp. 72-104). The modification of bacterial wall is a critical determinant of their survival. The essential wall modifications are wall teichoic acid (WTA) and lipoteichoic acid (LTA). The anionic wall teichoic acid is extended from bacterial peptidoglycan, while lipoteichoic acid is originated from glycolipid in membrane (Vollmer, 2008, pp. 287-306). The modified wall is determining cell activity in many circumstances. With the thick and rigid of peptidoglycan, bacterial can maintain shape and morphology of bacteria (Malanovic, & Lohner, 2016, pp. 936-946). WTA and LTA have a critical role in preventing of bacterial autolysis. The ester bond of repeating of D-alanyl esters monomer provide an ionic site for autolysin binding site, resulting in autolysin inactivation (Wecke, Madela, & Fischer, 1997, pp. 2953-2960). Since WTA and LTA are poly anion structure. They have an affluence binding site for metal ions for cellular utilization. In the year 1994, Rose et al. determined the capacity of LTA binding with divalent Calcium (Ca^{2+}) in oral streptococcus. The result indicated that the 30% of LTA bind to Ca^{2+} while the whole call were saturated with Ca^{2+}. Moreover, they also found the more abundant in LTA secretion during oral streptococcus growing in sucrose (Rose, Hogg, & Shellis, 1994, pp. 1742-1747). This might be one example to indicate the LTA function in cation homeostasis. In addition, peptidoglycan occupied the site for cell surface protein for bacterial activity such as LPXTG protein that is a substrate for sortase A. After LPXTG and sortase A reaction, the surface proteins were combined with peptidoglycan cell wall. These anchored proteins subsequently involved in cell activity corresponding to their function. The known function of sortase A including bacterial adhesion to organ tissue (Kharat, & Tomasz, 2003, pp. 2758-2765), inhibit biofilm formation (Wallock-Richards et al., 2015, pp. 10483-10485) etc. According to Soon-Nang-Park et al., oleic acid can inhibit peptidoglycan synthesis in *S. mutans* UA159 by down regulated (52-85%) the gene *PBP2a, PBP2b*, and *PBP2X* expression (Park, Ahn, & Kook, 2015, pp. 613-617).

Beneath the peptidoglycan layer, cell membrane occupied this space. The cell membrane is composed 60% of proteins and 40% phospholipid. The phospholipid contains 2-fatty acids that attached to glycerol phosphate group. The cell membrane plays important roles in cell stability including regulated cellular permeability, the location of trans port system, peptidoglycan production and location of cellular enzymes (Todar, 2008).

Bacterial fatty acids (FAs) normally incorporated as the structural components of cells such as cell membrane, and lipid storage components e. g. inclusion body. The primary function of bacterial fatty acids is turning membrane to hydrophobicity (Carson, & Daneo-Moore, 1980, pp. 1122-1126). These fatty acids were synthesized with enzymatic reaction in type II fatty acid biosynthesis pathway. They could be hydrolyzed with the enzymatic reaction and liberated free fatty acids (FFAs). FFAs are used for cellular metabolism and gene regulation (Andrew, P. Desbois, & Valerine, J. Smith, 2010, pp. 1629-1642; Rustan, & Drevon, 2001, pp. 1-7). FAs with carbon atoms less than 8 are classified as short chain fatty acid while those with more than 16 carbon atoms are long chain fatty acids. If carbon double bond (C=C) is presented in the structural molecule, it is the unsaturated FA. For saturated fatty acids, carbon atoms are bond each other by single bonds (C-C) (A. P. Desbois, & V. J. Smith, 2010, pp. 1629-1642).

The common fatty acids in the living microorganism are palmitic acid (16:0), stearic acid (18:0), myristic acid (14:0), oleic acid (18:1 ω9c) and palmitoleic acid (16:1 ω7c) (Rustan, & Drevon, 2001, pp. 1-7). Moreover, the fluidity of cell membrane is determined by percentage of unsaturated fatty acid. (Boyaval, Corre, Dupuis, & Roussel, 1995, pp. 17-29; Greenway, & Dyke, 1979, pp. 233-245; Speert, Wannamaker, Gray, & Clawson, 1979, pp. 1202-1210; Wang, & Johnson, 1992, pp. 624-629). However, the bacteria membrane is able to redistribute the fatty acids response to adverse environment (Guerzoni, Lanciotti, & Cocconcelli, 2001, pp. 2255-2264; Streit, Delettre, Corrieu, & Beal, 2008, pp. 1071-1080)

Table 5 The enhancement of prebiotics on probiotics

	Probiotics	Prebiotics	Results
Kondepudi *et al.* (Kondepudi, Ambalam, Nilsson, Wadström, & Ljungh, 1012, p. 489-497)	*Bifidobacterium* spp.	Galacto-oligosaccharide, Lactose, Fructo-oligosaccharides	Enhance growth rates of *B. breve, B. longum, B. pseudocatenulatum*
Depeint *et al.* (Depeint, Tzortzis, Vulevic, I'Anson, & Gibson, 2008, p. 785-791)	*Bifidobacterium bifidum*	Mixture of Galacto-oligosaccharide and novel galacto-oligosaccharides	Increased Bifidobacterium population ratio compared with placebo
Kojima *et al.* (Kojima et al., 2015, p. 27-32)	*Lactobacilli* group	Arabinose, xylose and xylitol	Only *Lactobacillus* exhibited growth in present of arabinose, xylose and xylitol (compared with *C. albicans and S. mutans*)

Source: Teughels et al., 2008, pp. 111-147

CHAPTER III

RESEARCH METHOD

This chapter described experimental methods in this study. It is divided to be the culturing media and prebiotics preparations, microorganism preparation, the efficacy of prebiotic on competitive growth of *L. acidophilus* and *S. mutans* determination, data collection and data analysis.

Culturing media and prebiotic preparation

1. Culturing media preparation

The culturing media in this study were 3.7% (w/v) Brain Heart Infusion broth plus 10% yeast extract (BHI, Difco, USA), 5.5% (w/v) de Man, Rogosa and Sharpe (MRS, Difco, USA) and Tryptic Soy Broth (10% (w/v) TSB plus tryptone, 5% (w/v) yeast extract, 1.33% (w/v) KH_2PO_4, 2.66% (w/v) K_2HPO_4, 0.01% (w/v) $MgSO_4.7H_2O$, 0.01% (w/v) $FeCl_2$, 0.01% (w/v) $MnSO_4.4H_2O$, 0.01% (w/v) NaCl and 0.2% glucose) (TYE, Difco, USA) (Hamilton, & Bowden,1982, pp. 255-262). For an agar preparation, 1.5 % (w/v) of agar powder was added into the culturing broth. All prepared media were encountered a sterilization in an autoclave at 121 ^{0}C, pressure 15 lb/inch^2, for 15 minutes. The BHI and MRS were chosen for *S. mutans* and *L. acidophilus* revival cultivation, respectively.

2. MRS media supplemented with prebiotics preparation

The prebiotics in this experiment were fructo-oligosaccharide (FOS) (Bornnet corporation Co., Ltd., Bangkok, Thailand) and galacto-oligosaccharides (GOS) (Bornnet corporation Co., Ltd., Bangkok, Thailand). They were prepared in the different concentrations of 1%, 2%, 3%, 4% and 5% (v/v) for GOS-supplemented media and 1%, 2%, 3%, 4% and 5% (w/v) for FOS-supplemented media in the MRS broth. These prepared solutions were filter-sterilized with Millipore vacuum filter (Millipore Stericup™, with 0.22 μm, Capitol Scientific)

Microorganism preparations

The microorganisms in this study were *Streptococcus mutans* A32-2 and *Lactobacillus acidophilus* TISTR 2365^T = DSMZ 20079^T = ATCC4356^T (DSMZ stands for the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, TISTR also stands for TISTR Culture Collection, Thailand Institute of Scientific and Technological Research and ATCC stands for American Type Culture Collection).

S. mutans A32-2 in this experiment is a clinical strain which was represented the cariogenic pathogen. A loop full of *S. mutans* was inoculated in 10 ml of BHI broth (Difco, USA) at 37°C in 5% CO_2 for 18 hours. The overnight cultures (16-18 hours) were then inoculated on BHI agar by using streak plate technique. Then a single colony was transferred to another fresh media allow them to growth effectively.

The type strain of *L. acidophilus* (TISTR 2365^T = DSMZ 20079^T) that was revived via using MRS broth medium (Difco, USA) and incubated overnight at 37 ^{0}C with 5% CO_2 for 16-18 hours. It was performed with the same fashion as cariogenic microorganism.

Determination the efficacy of prebiotics on competitive growth of *S. mutans* and *L. acidophilus* in co-culture

The inhibition on the growth of the cariogenic bacteria was observed from the broth dilution method to determine the competitive growth of the two-interested microorganism (Reller, Weinstein, Jorgensen, & Ferraro, 2009, pp. 1749-1755). This section composed of three consecutive parts: 1) optimization the culturing media and determination the number of bacteria from growth curve; 2) optimization the proportion of *S. mutans* and *L. acidophilus*; 3) determination the capacity of *L. acidophilus* to inhibit growth of *S. mutans* in co-culture cultivation which was supplemented with prebiotics.

1. Optimization of the culturing media and determination the growth curve of *S. mutans* and *L. acidophilus*

The aim of this part was to determine the appropriate culturing media for *S. mutans* and *L. acidophilus*. The candidate culturing media were BHI, TYE and MRS.

Firstly, 100 µl of either *S. mutans* or *L. acidophilus* was inoculated individually into 20 ml of difference cultured medium. This was the starting point of growth curve. The number of microorganisms was determined by measuring the optical density (OD) at 600 nm along with 10- fold serially dilution method (Figure 5) for colony counting at 0, 1,3, 6, 8, 10, 12, 16, 18, 24 and 48 hours.

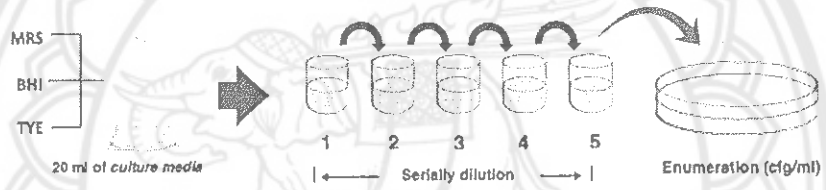


Figure 5 The determination of the number of microorganisms by 10-fold serially dilution method

2. Optimization of the appropriated proportion of *S. mutans* and *L. acidophilus*

S. mutans and *L. acidophilus* at the mid-log phase were grown together in the MRS medium with various proportions. The proportion of them were varies to 1:5, 1:10, 1:20 and 1:40 (v/v). The individual *S. mutans* and *L. acidophilus* suspension in the mid-log phase were prepared following the previously described proportion and centrifuged at 4,500 rpm for 10 minutes for collecting the cell pellets. Then bacterial pellets were reconstituted and mixed in the MRS broth. The number of each species in the mixed cultures were observed at 0, 1, 3, 6, 8, 12, 16, 18, 24 and 48 hours by colony counting with the same method as section 3.1 (Figure 6). The proportion which has an equal number of *S. mutans* and *L. acidophilus* will be selected for the next experiment.

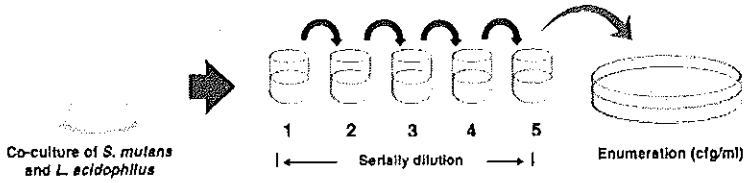


Figure 6 The mixed bacterial culture evaluation in MRS medium

Effect of the prebiotics on *L. acidophilus* on inhibiting growth of *S. mutans*

The inhibitory effect of *L. acidophilus* (TISTR2365^{T}= DSMZ20079^{T} on *S. mutans* (A32-2) after treatment with prebiotics was investigated in this section.

The proportion of 1:20 between *S. mutans* and *L. acidophilus* was selected for this experiment. They were co-cultured in difference kinds of prebiotics (FOS and GOS) in various concentrations (1% , 2% , 3% , 4% and 5%) in MRS broth. The culturing media with no prebiotics were the control groups. The number of *S. mutans* and *L. acidophilus* were determined at 0, 3, 6 and 12 hours with the same method as section 3.1 (Figure 7). The growth rate of both *S. mutans* and *L. acidophilus* were determined.

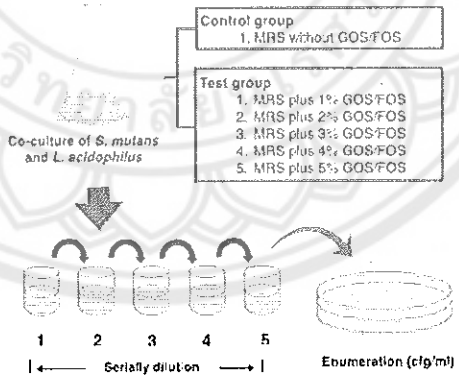


Figure 7 The co-cultured of *S. mutans* and *L. acidophilus* in MRS supplemented with different concentrations of prebiotics (GOS and FOS)

Fatty acids analysis

The cellular fatty acids of bacterial culture were identified with gas chromatography (Agilent 7890B Gas chromatograph) , MIDI Sherlock^{TM} microbial identification system (MIDI, 2012, p. 1-166). 0.06 g of bacterial pallets and 0.5 ml of supernatant from the bacterial culture were mixed with 1 ml of reagent 1 (3. 75N NaOH in deionized water and methanol) and vortex for 10 seconds. The solutions were heated in 100 ^{0}C water for 25 minutes followed by cooled down in the ice bucket. For the methylation, 2 ml of reagent 2 (6.0 N HCl in methanol) was added into the cold sample and vortexed for 10 seconds. The samples were continuously incubated in 80^{0}C water for 10 minutes and suddenly put in the ice bucket. For the extraction, 1.25 ml of reagent 3 (200 ml of hexane in 200 ml of methyl tert-butyl ether) was added and consecutively mixed for 10 minutes. The upper layer solution was collected and mixed with the reagent 4 (0.3N NaOH). The mixture was mixed for 5 minutes. The supernatant was collected for the fatty acids analysis. The 10 µl of each extracted sample was injected into phenyl methyl silicone silica capillary column (HP-ultra2, size 25 m. x 0.2 mm. x 0.33 mm., column flow of 1 ml/min.). The fatty acids fractions were detected with Flame Ionization detector (FID). The operating system conditions must be strictly concern (inlet temperature of 250 ^{0}C, FID detector temperature of 300 ^{0}C, oven temperature of 170-250 ^{0}C).

Research variables

1. **Independent variable**
   - 1.1 Concentrations and types of prebiotics
   - 1.2 Specie of probiotic
   - 1.3 Specie of cariogenic pathogen
2. **Dependent variable**
   - 2.1 Growth rate of *S. mutans* and *L. acidophilus*
   - 2.2 Anti-*S. mutans* activity of probiotic
3. **Confounding variables**
   - 3.1 Growth phase of bacteria
   - 3.2 Initial number of microorganisms

3.3 Growing conditions

3.4 Culturing media

Research Instruments

1. Microorganisms

1.1 *Streptococcus mutans* (A32-2)

1.2 *Lactobacillus acidophilus* (ATCC4356^{T}=TISTR2365^{T}= DSMZ20079^{T})

2. Prebiotics

2.1 Fructo-oligosaccharides (FOS) (Bornnet corporation Co. , Ltd. , Thailand)

2.2 Galacto-oligosaccharides (GOS) (Bornnet corporation Co. , Ltd., Thailand)

3. Culturing media

3.1 deMand Rogosa Sharpe (MRS) (Difco Laboratories Inc., Detroit, MI, USA)

3.2 Brain heart infusion (BHI) (Difco Laboratories Inc. , Detroit, MI, USA)

3.3 Tryptic Soy Broth plus yeast extract and trace elements (TYE)

4. Equipments

4.1 Biological safety cabinet; Class II, type A - (Labconco, Kansas, MO)

4.2 Culture incubator (Thermo electron corporation, Marietta, USA)

4.3 Autoclave (Sturdy industrial Co., Ltd, New Taipei city, Taiwan)

4.4 Disposable inoculation loops 10 µl (Biologix Group Ltd, Changzhou, China)

4.5 Pipette (BRAND GMBH + CO KG, USA)

4.6 S1 Pipet Filler (Thermo Fisher scientific, Beijing China)

4.7 Laboratory weighing (Mettle Toledo, Switzerland)

4.8 Spectrophotometer (Thermo electron corporation, Marietta, USA)

4.9 GC-MIDI (Agilent 7890B Gas chromatography, Sherlock Microbial Identification System Version 6.2)

Data Collection

This experiment was performed triplicate to investigate the best concentrations of either GOS or FOS to enhance growth of *L. acidophilus* to inhibit *S. mutans*. The viable count and growth rate of *S. mutans* and *L. acidophilus* were determined. For the bacterial growth rate at any two-point in the growth curve, they were estimated by using the formula which modified from Widdle' s formula by substituted number of bacterial instead of optical density (Widdel, 2007, pp. 1-11)

$$\text{Growth rate } (h^{-1}) = \frac{\ln N_t - \ln N_0}{t - t_0}$$

This formula could be transformed to the logarithm by the following equation.

$$\text{Growth rate } (h^{-1}) = \frac{2.303\ (\log N_t - \log N_0)}{t - t_0}$$

ln: Natural logarithm (2.303)

N_t: Number of bacteria at the interested time point (CFU/ml)

N_0: Number of bacteria at the initial time of interested time interval (CFU/ml)

t: Duration of observing time

t_0: Initial time point (time point "0")

Data Analysis

The number and growth rate of *S. mutans* and *L. acidophilus* were analyzed to present the ability of prebiotics to enhance the efficacy of *L. acidophilus* to suppress growth of *S. mutans* compared with the non-treated ones. All data were firstly test for the normal distribution. The Kruskal-Wallis test and Mann-Whitney U Test were used. The data was statistically evaluated with a Kruskal-Wallis and Mann-Whitney test ($p<0.05$).

Plan of study

The experiment would be following as described in the table below (Table 6).

Table 6 Plan of study

Experiment	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Materials and instruments preparation	←	→										
Probiotic, pathogen and prebiotics preparation		←	→									
Determination the efficacy of prebiotics-treated probiotic			←	—	—	→						
Data analysis and interpretation							←	—	→			
Writing thesis									←	—	→	
Publications										←	—	→

CHAPTER IV

RESULTS

Growth curve of microorganisms

L. acidophilus and *S. mutans* were grown individually in the BHI, TYE and MRS media to find the most suitable culture media for both strains. The number of them was collected by colony counting along with the optical density measurement (OD) at 600 nm which is the common wave length for bacterial enumeration (Hazan, Que, Maura, & Rahme, 2012, pp. 259-259). Moreover, at the wave length of 500- 600 nm., the variation in bacterial size would be diminished (Koch, 1970, pp. 252-259). However, the optical density reflected both death and alive cells. So, the colony counting was the main procedure to estimate the number of bacteria.

S. mutans grew for 14 hours and 10 hours in the BHI and TYE media, respectively (Figure 8). Alternatively, it grew through the growth phase in the MRS culturing media. The log phase in the MRS ranged from the 3rd – 12th hours. This finding reflects the non-fastidious characteristic of *S. mutans* which could be grow in most enriched media.

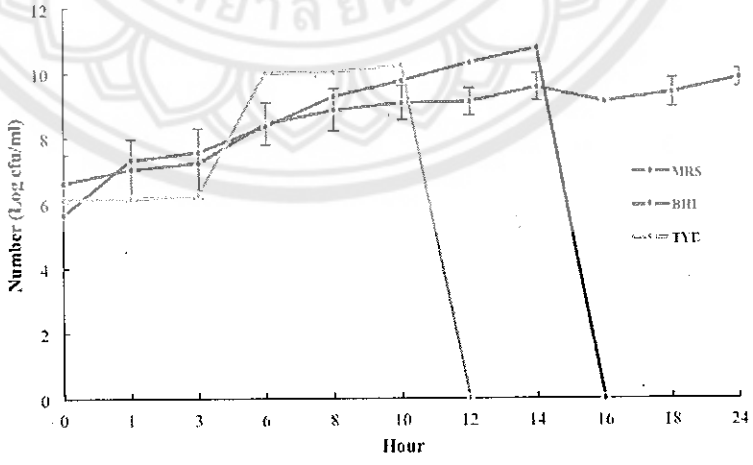


Figure 8 The growth patterns of *S. mutans* in MRS, BHI and TYE media

L. acidophilus grew in the TYE during the first three hours and 1 hour in the BHI medium. It grew in the MRS throughout the experiment which was similar to that of *S. mutans*. The log phase ranged from the 3rd – 12th hours of incubation period. This implied that *L. acidophilus* could not grow well in the TYE and BHI media (Figure 9). Since *L. acidophilus* is a fastidious microorganism which need more specific nutrients for growth and metabolism.

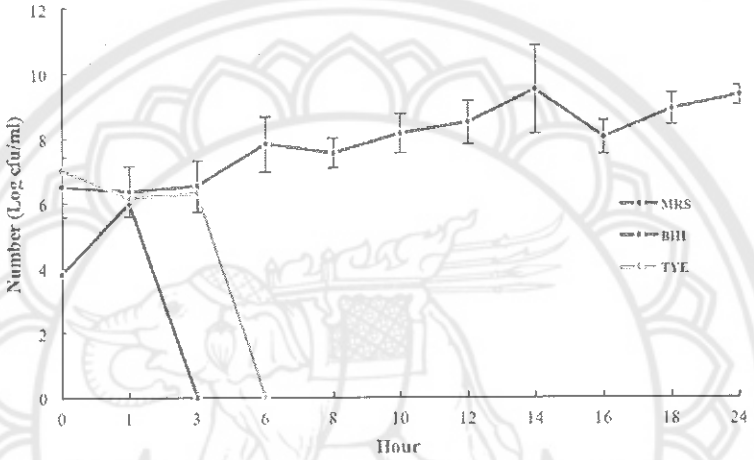


Figure 9 The growth patterns of *L. acidophilus* in MRS, BHI and TYE

Sine *L. acidophilus* could only grow in MRS, so the triplicated growth curve of *S. mutans* and *L. acidophilus* were carried on MRS media. The average number of them (log cfu/ml ± SD) were plotted in growth pattern graph. The results showed that both *L. acidophilus* and *S. mutans* multiplied well in the MRS medium. The average growth rate in log phase (time interval between 3rd-12th hour) of *S. mutans* (0.491 hr^{-1}) was higher than *L. acidophilus* (0.481 hr^{-1}) (Figure 10). The growth pattern of *L. acidophilus* slightly fluctuated in the log phase (at the 3rd -14th hours) which the number of *S. mutans* deviated from the trend line at the hour of the 7th. Alternatively, *S. mutans* grew consistently through the log and stationary phases. Therefore, MRS medium was chosen for their co-culture.

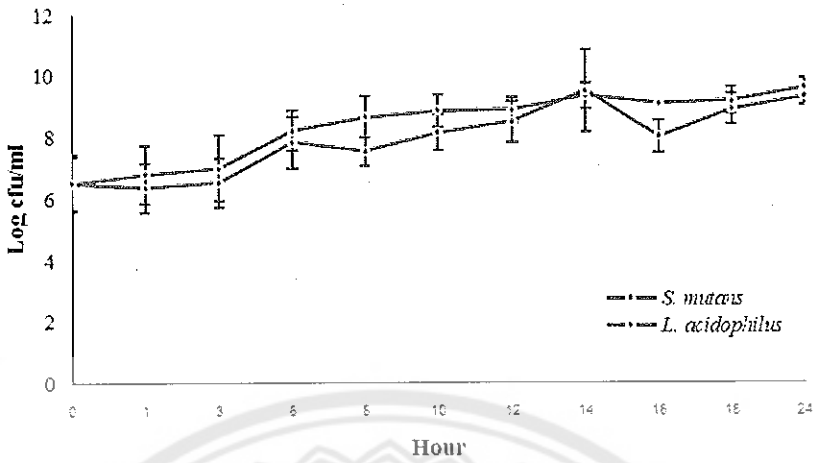


Figure 10 The growth patterns of *S. mutans* and *L. acidophilus* in MRS

Optimization of the appropriated proportion of *S. mutans* and *L. acidophilus*

Since the growth rate of *S. mutans* (0.491 hr^{-1}) slightly higher in MRS compared with *L. acidophilus* (0.481 hr^{-1}). And at the same optical density (0.6 at wave length of 600 nm.), the number of *S. mutans* is 4.25×10^9 cfu/ ml and *L. acidophilus* is 5.0×10^7 cfu/ml. The equal number of both bacteria needed to be set up at the beginning of the experiment. According to the growth curve of *S. mutans* and *L. acidophilus*, the proportion between *S. mutans* and *L. acidophilus* was varied to 1:5, 1:10, 1:20 and 1:40 volume by volume. In the proportion of 1:5 (Figure 11) and 1:10 (Figure 12), the number of *S. mutans* started to be higher than *L. acidophilus*. The viable count of *S. mutans* and *L. acidophilus* was equaled at the 24^{th} and the 16^{th} hours for the proportions 1:5 and 1:10 respectively.

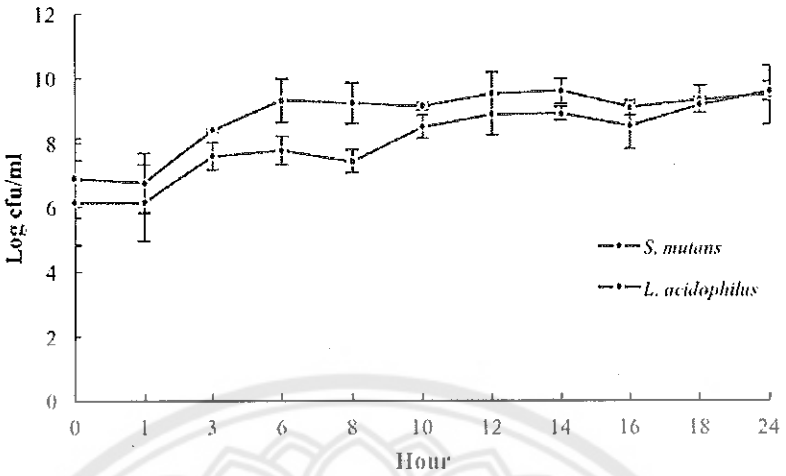


Figure 11 The growth patterns of *S. mutans* and *L. acidophilus* in the proportion of 1:5 in the MRS medium

Note: At the beginning, the number of *S. mutans* was higher than *L. acidophilus*. After 16 hours, the number *of L. acidophilus* slightly increased and got equal at the 24th hours. At the hour 24th, pH of culture media drops to 4.02. This might create the niche for *L. acidophilus* propagation

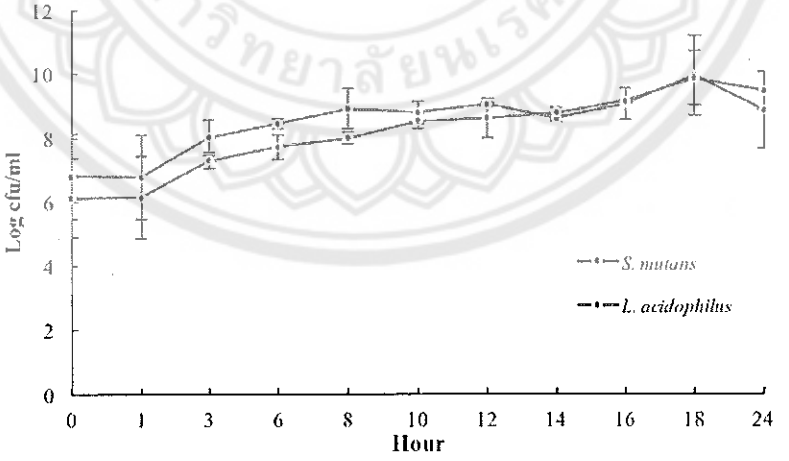


Figure 12 The growth patterns of *S. mutans* and *L. acidophilus* in the proportion of 1: 10 in the MRS medium

Note: *S. mutans* and *L. acidophilus* had the similar growth patterns. The numbers of them was equal after 12 hours. After that the numbers of *L. acidophilus* was greater than *S. mutans*

In the proportion 1:20, the viable count of *S. mutans* and *L. acidophilus* was close to each other (Figure 13). After 9 hours, the number of *S. mutans* decreased and less than *L. acidophilus* ever since.

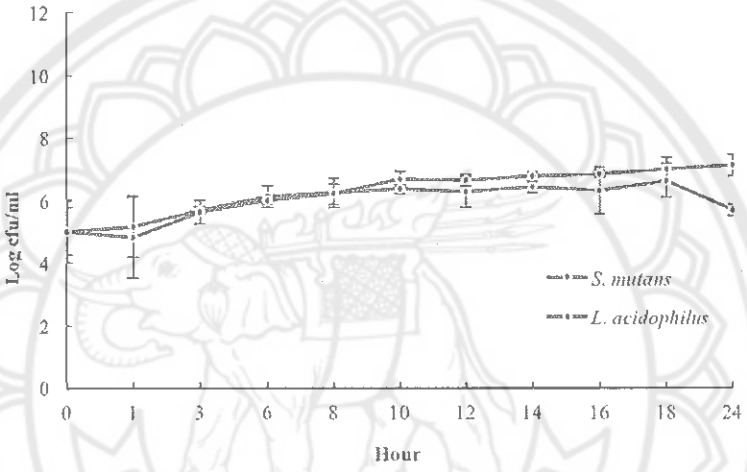


Figure 13 The growth patterns of *S. mutans* and *L. acidophilus* in the proportion of 1: 20 in the MRS medium

Note: The number of *S. mutans* and *L. acidophilus* were equal at the beginning of the growth phase. They had the similar growth rate and growth pattern. After 8 hours of incubation, the number *L. acidophilus* was higher than *S. mutans* through the experiment

For the proportion of 1: 40 (Figure 14) , *L. acidophilus* was higher than *S. mutans* at the beginning. This might due to the numbers of *L. acidophilus* exceed the numbers *S. mutans*. Hence, the proportion 1:20 was selected for determination of the prebiotics efficacy.

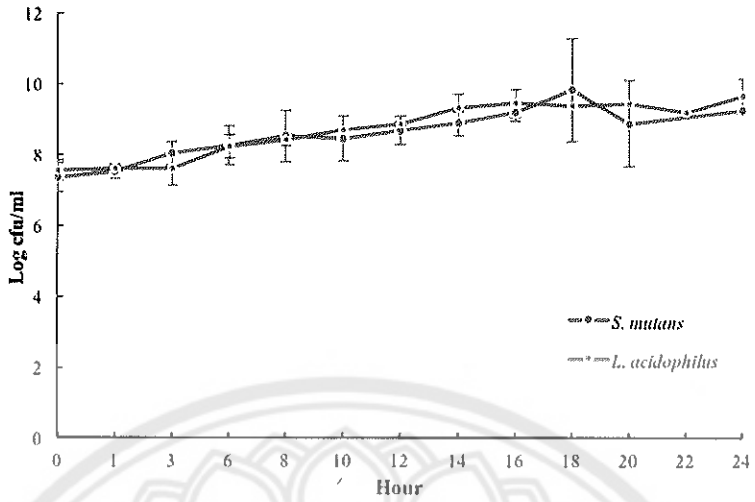


Figure 14 The growth patterns of *S. mutans* and *L. acidophilus* in the proportions of 1:40 in the MRS medium

Note The number of *L. acidophilus* was greater than *S. mutans* at the beginning of experiment. After 1 hour, the number of them was fluctuated along the experiment

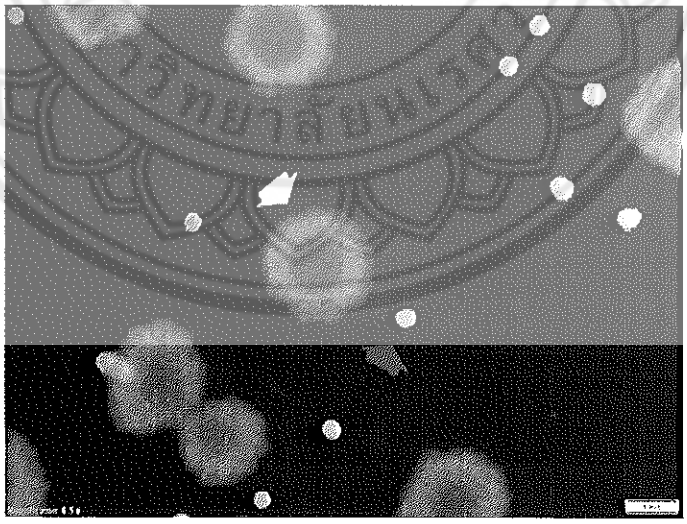


Figure 15 The colony of *S. mutans* (red arrow) and *L. acidophilus* (white arrow)

Note: *S. mutans* was a round, convex, mucoid and yellowish white colony and *L. acidophilus* showed a large round, plaque type, and corrugate border colony

Effect of the prebiotics on the *L. acidophilus* on inhibition growth of *S. mutans*

According to the previous data, *S. mutans* and *L. acidophilus* at the proportion 1:20 was selected for the co-cultured in the MRS medium as the control group. The different concentrations of prebiotics, (1%, 2%, 3%, 4% and 5% of GOS (v/v) and FOS (w/v)) (Kondepudi et al., 1012, pp. 489-497; Roberfroid, 2007, pp. 830S-837S), were supplemented into the MRS co-culture medium. *S. mutans* and *L. acidophilus* were individually grown along with the co-cultured. The growth rates of each strains were observed and the results was show in the table 7.

1. The growth rate of S. *mutans* in the prebiotics

The growth rates of S. *mutans both in single culture and co*-culture were deviated from the normal distribution. Kruskal-Wallis test was then used to determine the growth rate alteration among them. When S. *mutans* was individually grown in the prebiotics-free condition, the growth rates were 0.9921 ± 0.14 hr^{-1} (Table 7). When supplemented with the different concentrations of GOS, the growth rate in all prebiotics concentrators were similar to the control group which no statistic difference ($p=0.285$).

For the co-cultivation of *S. mutans and L. acidophilus, S. mutans* grew with the lower rate compared to the individual culture in almost all concentrations of prebiotics including the control group which did not have the prebiotics. When the co-cultures were supplemented with GOS, the growth rate in the 3 % GOS (0.3775 ± 0.06 hr^{-1}) was significantly decreased compared the control (0.9623 ± 0.17 hr^{-1}) ($p = 0.040$). When the concentration of GOS was increased to 4% and 5% , the growth rate of *S. mutans* significantly decreased when compared to that of the control ($p < 0.05$). The maximum growth rate was found in 2% GOS and the minimum growth rate was found in 3% GOS. Furthermore, the growth rate of *S. mutans in 2%* GOS (1.0272 ± 0.01 hr^{-1}) was significantly higher than that of the 5% GOS (0.5491 ± 0.09 hr^{-1}) ($p = 0.034$). In addition, the growth rate of 1% GOS was not statistic different from control ($p > 0.05$).

The growth rate of single culture of *S. mutans* significantly decreased when compared to the control in 3% FOS ($p = 0.032$), while 1% FOS, 2% FOS, 4% FOS and 5% FOS were not statistic different ($p > 0.05$). When FOS was added into to co-culture, the growth rate of *S. mutans* significant decreased in 1% FOS ($p = 0.034$),

2% FOS ($p = 0.034$), 3% FOS ($p = 0.034$), 4% ($p = 0.034$) and 5% ($p = 0.034$) when compared to the control. The minimum growth rate was found in 3% FOS both on single-culture ($0.2998 \pm 0.11\ h^{-1}$) and co-culture ($0.2281 \pm 0.12\ h^{-1}$).

In the FOS-supplemented medium, the growth rates of *S. mutans in the co-*culture condition was lower than the single cultivation in all concentrations of FOS. The growth rates of *S. mutans* significantly decreased in all concentrations of FOS compared to the control group ($p = 0.034$). The minimum growth rate was appeared in 3% FOS ($0.2281 \pm 0.12\ h^{-1}$) which corresponded to the single culture in FOS.

The growth rates between the single-cultured and co-cultured conditions in the same concentrations were compared with Mann-Whitney U Test due to the non-homogenized data. In 5% GOS, the growth rate of single culture significant higher than co-culture ($p = 0.025$).

Table 7 The growth rate of *S. mutans* in prebiotics in single cultured and co- cultured at 6 hours

	GOS (% v/v)		FOS (%w/v)	
Prebiotic concentration	Single cultured	Co-cultured	Single cultured	Co-cultured
Control (MSR)	$0.9921 \pm 0.14^{A,a}$	$0.9623 \pm 0.17^{A,a}$	$0.9921 \pm 0.14^{X,x}$	$0.9623 \pm 0.17^{X,x}$
1%	$1.0058 \pm 0.13^{A,a}$	$0.8391 \pm 0.19^{A,B,C,a}$	$0.5419 \pm 0.01^{X,x}$	$0.5209 \pm 0.09^{Y,x}$
2%	$0.9099 \pm 0.29^{A,a}$	$1.0272 \pm 0.01^{B,a}$	$0.6467 \pm 0.07^{X,x}$	$0.5683 \pm 0.10^{Y,x}$
3%	$0.7104 \pm 0.02^{A,a}$	$0.3775 \pm 0.06^{C,a}$	$0.2998 \pm 0.11^{Y,x}$	$0.2281 \pm 0.12^{Z,x}$
4%	$1.1588 \pm 0.37^{A,a}$	$0.4672 \pm 0.12^{C,a}$	$0.5604 \pm 0.07^{X,x}$	$0.5724 \pm 0.06^{Y,x}$
5%	$0.9070 \pm 017^{A,a}$	$0.5491 \pm 0.09^{C,b}$	$0.7123 \pm 0.06^{X,x}$	$0.3429 \pm 0.07^{Z,x}$

Note: The statistics comparison was separately tested within the same type of prebiotics. The parameters were evaluated by Kruskal-Wallis test ($p < 0.05$), follow by Mann-Whitney U Test ($p < 0.05$). The uppercases indicated the significant difference within the same columns. The lowercases indicated the significant difference within same rows for the same type of prebiotics

2. The growth rate of *L. acidophilus* in prebiotics

Due to the non-homogenized of variance, the growth rates comparison of *L. acidophilus* in FOS were determined with Kruskal-Wallis test ($p< 0.05$), followed by Mann-Whitney U Test ($p< 0.05$).

Without prebiotics, the single culture *L. acidophilus* grew with the rate $0.6446 \pm 0.06\ hr^{-1}$. When increased the concentration of GOS, the growth rate of *L. acidophilus* insignificant different from the control ($p=0.088$), and the growth rate among 5 concentrations were also not difference (Table 8).

In the co-culture condition, the growth rates of *L. acidophilus* in all concentrations of GOS were insignificantly different from the control groups ($p> 0.05$). For the same concentration of prebiotics, there was no different in their growth rates between the single cultured and the co-cultured ($p> 0.05$) Table 8.

Table 8 The growth rate of *L. acidophilus* in prebiotics in single cultured and co- cultured at 6 hours

	GOS (% v/v)		FOS (%w/v)	
Prebiotic concentration	Single cultured	Co-cultured	Single cultured	Co-cultured
Control (MSR)*	$0.6446 \pm 0.06^{A,a}$	$0.5743 \pm 0.30^{A,a}$	$0.6446 \pm 0.06^{X,x}$	$0.5743 \pm 0.30^{X,x}$
1%	$0.3379 \pm 0.01^{A,a}$	$0.7895 \pm 0.26^{A,a}$	$0.4703 \pm 0.29^{X,x}$	$0.4322 \pm 0.20^{X,x}$
2%	$0.4301 \pm 0.19^{A,a}$	$0.7691 \pm 0.39^{A,a}$	$0.5287 \pm 0.23^{X,x}$	$0.6357 \pm 0.08^{X,x}$
3%	$0.4065 \pm 0.04^{A,a}$	$0.6268 \pm 0.18^{A,a}$	$0.4802 \pm 0.07^{X,x}$	$0.6580 \pm 0.13^{X,x}$
4%	$0.5049 \pm 0.27^{A,a}$	$0.6583 \pm 0.25^{A,a}$	$0.6848 \pm 0.12^{X,x}$	$0.4625 \pm 0.13^{X,x}$
5%	$0.6796 \pm 0.08^{A,a}$	$0.5806 \pm 0.07^{A,a}$	$0.3546 \pm 0.01^{X,x}$	$0.3971 \pm 0.09^{X,x}$

Note: The statistics comparison was separately tested within the same type of prebiotics. The parameters were evaluated by Kruskal-Wallis test ($p< 0.05$), follow by Mann-Whitney U Test ($p< 0.05$). The uppercases indicated the significant difference within the same columns. The lowercases indicated the significant difference within same rows for the same type of prebiotics

3. The pH of culture medium

Organic acids are one of the common metabolic products from *S. mutans* and *L. acidophilus* while the lactic acid as the majority. Both of them can survive in the acidic condition. To determine the influence of pH on growth rates of *S. mutans* and *L. acidophilus* (at the hour 6th). Therefore, the pH of culturing medium at the hour 6th was observed along with the viability counting in the co-cultured and single cultured conditions. The initial pH of the prebiotics-supplemented MRS media was 6.5 ± 0.2. The pH was ranged from 5 to 6.21 (Table 9). In the single cultured condition, pH of FOS-supplemented medium was generally slightly higher than GOS-supplemented medium within both *S. mutans* and *L. acidophilus* while in the co-cultured condition, there was no difference in the pH between GOS- and FOS-supplemented media. In addition, pH of *S. mutans* culture (pH =5.9-6.2) seemed to be higher than either single *L. acidophilus* (pH = 5-5.4) culture or co-culture (5.1-5.6).

Table 9 The pH of *S. mutans* and *L. acidophilus* cultivation media

	Single cultured				Co-cultured	
	S. mutans		*L. acidophilus*		*S. mutans/L. acidophilus*	
	GOS	FOS	GOS	FOS	GOS	FOS
MRS	5.98±0.12		5.19 ±0.31		5.35 ± 0.01	
1%	5.95±0.11	6.16±0.02	5.04±0.22	5.32 ± 0.33	5.53 ± 0.39	5.42±0.31
2%	6.01±0.05	6.21±0.02	5.11±0.17	5.39 ± 0.35	5.31 ± 0.57	5.40±0.30
3%	5.96±0.09	6.21±0.10	5.07±0.23	5.38 ± 0.29	5.14 ± 0.24	5.63±0.51
4%	5.94±0.05	6.16±0.10	5.00±0.20	5.34 ± 0.36	5.08 ± 0.22	5.38±0.33
5%	5.90 ±0.19	6.16±0.11	5.11± .28	5.38 ± 0.42	5.34 ± 0.48	5.36±0.35

For the study, the growth rate and pH of culture-supplemented prebiotics media were observed at 0, 3, 6, 9 and 12 hours. The growth rates of *S. mutans* and *L. acidophilus* were mainly observed at the mid-log phase of them, the 6th hour of cultivation. According to table 10, the growth rate of *S. mutans* and *L. acidophilus*

were almost peaked at the 6th hour. After that the growth of them continually decreased. For the pH of culture medium, they were continually decreased from the beginning of the experiments which had the similar trend as growth rate.

Table 10 The growth rates of *S. mutans and L. acidophilus* and pH in co-culture cultivation media in prebiotics at time 0-12 hours

Hour	Fructo-oligosaccharides																	
	0%			1%			2%			3%			4%			5%		
	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L
0	6.46	-	-	6.50	-	-	6.50	-	-	6.50	-	-	6.52	-	-	6.52	-	-
3	6.17	0.678	0.556	6.27	0.537	0.385	6.26	0.533	0.645	6.27	0.654	0.436	6.24	0.841	0.609	6.23	0.411	0.313
6	4.27	0.992	0.574	4.34	0.541	0.432	4.32	0.646	0.635	4.33	0.299	0.658	4.31	0.560	0.462	4.28	0.712	0.397
9	4.47	0.487	0.433	4.83	0.257	0.352	4.86	0.263	0.563	4.83	0.159	0.465	4.87	0.414	0.470	4.83	0.444	0.483
12	4.44	0.363	0.367	4.46	0.190	0.379	4.45	-	-	4.47	-	-	4.45	-	-	4.44	-	-
	Galacto-oligosaccharides																	
	0%			1%			2%			3%			4%			5%		
	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L	pH	GR_S	GR_L
0	6.47	-	-	6.57	-	-	6.50	-	-	6.49	-	-	6.50	-	-	6.14	-	-
3	6.01	0.614	0.437	6.00	0.642	0.709	6.26	0.611	0.494	5.95	0.523	0.572	5.97	0.742	0.332	5.89	0.594	0.644
6	5.34	0.992	0.574	5.38	0.881	0.789	4.32	0.909	0.769	5.31	0.710	0.626	5.09	0.770	0.658	5.10	0.907	0.580
9	5.25	0.405	0.454	5.17	0.421	0.534	4.86	0.378	0.539	5.06	0.445	0.610	4.60	0.331	0.488	4.58	0.244	0.419
12	4.57	0.389	0.374	4.58	0.326	0.440	4.45	0.326	0.416	4.58	0.273	0.388	4.47	0.325	0.453	4.46	0.150	0.319

GR_S: Growth rate of *S. mutans*, GR_L: Growth of *L. acidophilus*

The fatty acids analysis of *S. mutans* and *L. acidophilus*

Since the experiments were simulated the oral event which carried on culturing medium. Lots of study had stated the influence of the culturing media and the culturing conditions on both quality and quantity of fatty acids which are one of the important compositions of bacterial cells. The compositions of culturing medium particularly the acetate, glycerol, carbohydrate, lipid, and nitrogenous substances including the culturing conditions such as oxygen supply, pH, and the age of the culture affect on the fatty acids production. In this study, the fatty acids both for cellular fatty acids and secreted fatty acids were observed in co-culture at the hour 6^{th} of incubation.

1. Cellular fatty acids from *S. mutans* cultured in prebiotics-supplemented media

S. mutans and *L. acidophilus* were challenged with different concentrations of prebiotics (FOS and GOS). The alteration of the cellular fatty acids of *S. mutans* and *L. acidophilus* were evaluated by gas chromatophilic (Microbial Identification Inc. Sherlock™ Microbial Identification System (MIS).

For the *S. mutans* strain A32-2, 45.19 % of their cellular fatty acids were saturated fatty acids and 54.81 % were unsaturated fatty acids (Table 11).

When *S. mutans* was cultivated in GOS and FOS, the composition of the cellular fatty acids were shown in Table 11. The percent of unsaturated fatty acids of *S. mutans* increased when grown in the prebiotics-supplemented medium, 1% of GOS (57.43%), 1-4 % of FOS (57.66% , 58.41% , 59.29% and 57.61% respectively). The unsaturated fatty acids increased when compared to control which consisted of (11Z)-11-icosenoic acid (20:1 ω9c) and Oleic acid (18:1 ω9c). When prebiotics were added into the culture, the newly fatty acids were discovered. Those fatty acid were not found when *S. mutans* was culture in MRS media. The newly fatty acids which were increased including (10Z)-10-pentadenoic acid (15:1 ω5c), 17:1 iso ω5c, (7Z)-13-methly-7-hexadecenoic acid (17:1 anteiso ω9c), 19:1 iso I. Moreover, the common fatty acids of microorganism were also affect. The common fatty acids which were decreased including palmitic acid, stearic acid and oleic acid.

Table 11 The percentages of cellular fatty acids from *S. mutans* cultured in prebiotics-supplemented media

Prebiotics concentration (% v/v, w/v)	GOS		FOS	
	Unsaturated fatty acids	Saturated fatty acids	Unsaturated fatty acids	Saturated fatty acids
Control	54.81	45.19	54.81	45.19
1%	57.43	42.57	57.66	42.34
2%	52.47	47.53	58.43	41.57
3%	52.38	47.62	59.29	40.71
4%	51.15	48.85	57.61	42.39
5%	45.54	56.45	55.25	44.75

2. Secreted fatty acids from *S. mutans* cultured in prebiotics-supplemented media

The secreted fatty acids from *S. mutans* was labile in the cultured media. This section of the experiment analyzed the secreted fatty acids from the supernatant by the MIDI method.

Without prebiotics, the unsaturated fatty acids accounted for 75.52 % and 24.48 % for saturated fatty acids. When cultured *S. mutans* in the GOS-supplemented media, the percentages of secreted saturated fatty acid increased while the unsaturated fatty acid decreased. (Table 12).

On the contrary, the percentages of the released saturated fatty acid were decreased when cultured in the FOS-supplemented media. Meanwhile the percentages of the unsaturated fatty acid increased (Table 12).

Table 12 The percentages of secreted fatty acids from *S. mutans* cultured in prebiotics-supplemented media

Prebiotics concentration (% v/v, w/v)	GOS		FOS	
	Unsaturated fatty acids	Saturated fatty acids	Unsaturated fatty acids	Saturated fatty acids
Control	75.52	24.48	75.52	24.48
1%	75.25	24.75	75.60	24.40
2%	73.95	26.05	77.74	22.26
3%	73.43	26.57	76.19	23.81
4%	69.02	30.98	76.30	23.70
5%	75.38	24.62	75.38	24.62

3. Cellular fatty acids from *L. acidophilus* cultured in prebiotics-supplemented media

The percentages of cellular fatty acids of *L. acidophilus* before and after cultured in prebiotics-supplemented media were analyzed by using MIDI method.

In the prebiotics-free media, *L. acidophilus* composed of 27. 55% of cellular saturated fatty acids and 72.45 % of cellular unsaturated fatty acids (Table 13).

When *L. acidophilus* were grown in GOS and FOS, there was the alteration of the percentages of the cellular fatty acids. The percentages of cellular saturated fatty acid decreased when grown in both GOS- or FOS-supplemented media while the percentages of unsaturated fatty acid increased, except in the 2% GOS-supplemented media (Table 13). Because the percentage of palmitic acid (saturated fatty acid, 16:0) was 5 times to the control (8.3%).

Table 13 The percentages of cellular fatty acids from *L. acidophilus* cultured in prebiotics-supplemented media

Prebiotics concentration (% v/v, w/v)	GOS		FOS	
	Unsaturated fatty acids	Saturated fatty acids	Unsaturated fatty acids	Saturated fatty acids
Control	72.45	27.55	72.45	27.55
1%	79.00	21.00	76.04	23.96
2%	39.37	60.63	75.91	24.09
3%	77.45	22.55	76.80	23.20
4%	76.61	23.39	78.93	21.07
5%	79.33	20.67	78.53	21.47

4. Secreted fatty acids from *L. acidophilus* cultured in prebiotics-supplemented media

The secreted fatty acids from *L. acidophilus* were labile in the cultured media. They were analyzed from the supernatant by the MIDI method (Table 14).

When cultured *L. acidophilus* in the prebiotics-supplemented media, the percentages of secreted saturated fatty acids increased while the unsaturated fatty acids decreased (Table 14).

Table 14 The percentages of secreted fatty acids from *L. acidophilus* cultured in prebiotics-supplemented media

Prebiotics concentration (% v/v, w/v)	GOS		FOS	
	Unsaturated fatty acids	Saturated fatty acids	Unsaturated fatty acids	Saturated fatty acids
Control	75.22	24.78	75.22	24.78
1%	74.03	25.97	74.43	25.57
2%	73.60	26.40	75.37	24.63
3%	73.71	26.29	75.39	24.61
4%	72.95	27.05	74.90	25.10
5%	72.78	27.22	75.27	24.73

5. Cellular fatty acids from the co-cultured between *S. mutans* and *L. acidophilus* in prebiotics-supplemented media

The cell pellets from the co-cultured cultivation between *S. mutans* and *L. acidophilus* in MRS and prebiotics-supplemented media were collected and analyzed by using MIDI method.

The percentages of cellular saturated fatty acids from the mixed pellets decreased when grown in both GOS or FOS supplemented media while the percentages of unsaturated fatty acid increased (Table 15). The trend was similar to the individual culture of *L. acidophilus* (Table 13).

Table 15 The percentages of cellular fatty acids from the co-cultured between *S. mutans* and *L. acidophilus* in prebiotics-supplemented media

Prebiotics concentration (% v/v, w/v)	GOS		FOS	
	Unsaturated fatty acids	Saturated fatty acids	Unsaturated fatty acids	Saturated fatty acids
Control	79.42	20.58	79.42	20.58
1%	81.59	18.41	80.80	19.20
2%	82.36	17.64	80.96	19.04
3%	82.67	17.33	82.62	17.38
4%	84.61	15.39	79.37	20.63
5%	84.99	15.01	85.25	14.75

6. Secreted fatty acids from the co-cultured between *S. mutans* and *L. acidophilus* cultured in GOS-supplemented MRS

The trend of percentages of secreted fatty acids from the co-culture cultivation was inconsistent in all concentration of prebiotics when compared with the cultured media (Table 16). The percentages of the arachidic acid clearly increased 4-fold in the co-culture MRS medium (0.82%) compared with the cell-free medium (0.21%). When the concentration of GOS increased, the percentages of the arachidic acid also increased (Table 16).

7. Secreted fatty acids from the co-cultured between *S. mutans* and *L. acidophilus* in FOS-supplemented MRS

The percentages of secreted fatty acids from the co-cultured were fluctuated in all prebiotic concentrations when compared with the cultured media (Table 16). The percentages of the arachidic acid clearly increased in the co-culture MRS medium (0.82%) compared with the cell-free medium (0.21%). When the concentration of FOS increased, the percentages of the arachidic acid also increased (Table 16)

Table 16 The percentages Secreted fatty acids from the co-cultured between *S. mutans* and *L. acidophilus* cultured in prebiotics-supplemented MRS

Prebiotics concentration (% v/v, w/v)	GOS		FOS	
	Unsaturated fatty acids	Saturated fatty acids	Unsaturated fatty acids	Saturated fatty acids
Control	75.06	24.94	75.06	24.94
1%	75.04	24.96	71.68	28.32
2%	73.94	26.06	72.92	27.08
3%	72.79	27.21	74.00	26.00
4%	73.25	26.75	76.25	25.75
5%	74.69	25.31	73.80	26.20

CHAPTER V

DISCUSSION

Dental caries is a major oral problem around the world. *S. mutans* are the vital cariogenic bacteria due to their acidogenic and aciduric properties when metabolite sugar (Loesche, 1986, pp. 353-380). The acid products from the sugar fermentation are a cause of tooth demineralization consequently the carious lesions. *S. mutans* used in this study was A32-2. It is a clinical stain which isolated from highly active carious patients. The strain contains the fimbriae for the pathogenic capacity (Perrone, Gfell, Fontana, & Gregory, 1997, pp. 291-296; Ray, Gfell, Buller, & Gregory, 1999, pp. 400-404). The A32-2 was kindly provided from Professor Richard L. Gregory, Indiana University.

Several studies tried to interfere *S. mutans* colonization with probiotics which are beneficial living microorganism. The probiotics have several roles in curing diseases such as a protective barrier, inhibition of pathogenic microorganisms, or stimulation the host defensive mechanisms (Bermudez-Brito et al., 2012, pp. 160 -174; Nair, & Takeda, 2011, pp. 87- 109).

The probiotics also naturally found in oral cavities in small numbers and a short period of time (Ahrne et al., 1998, pp. 88-94; Haukioja, 2010, pp. 348-355). The selected probiotics must be able to 1) adhere and colonize on human tissue 2) provoke both non-specific and specific host immune responses 3) produce anti-microbial substances such as bacteriocin (Table 2) 4) compete the pathogenic microorganisms at binding sites 5) endure to gastro-intestinal digestion and resistance to host defense mechanism 6) do no harm on macro-organisms (Stamatova, & Meurman, 2009, pp. 329 - 338). According to EU Scientific Committee on Food, the recommend dose of probiotics for infant formula should be achieve 10^6 to 10^8 CFU/ml. of formula prepared as ready for consumption (SCF, 2003, pp. 153-164). For generalized used, the select probiotics should be able to indicate the safe and useful in food supplementation. The daily intake must be followed the country regulation (Binns, 2013, pp. 1-40).

In healthy person, host oral defensive mechanism is effectively function such as oral clearance. So, long-term colonization of probiotics is unlikely impossible (Yli-Knuuttila, Snall, Kari, & Meurman, 2006, pp. 129-131). Therefore, the adverse effect on oral tissue is overlooked and still not reported (Haukioja, 2010, pp. 348-355). However, Lactobacillus bacteria was associated with deep dental caries. So, patient selection maybe concerned before dental application (Byun et al., 2004, pp. 3128-3136).

Lactobacillus spp. were drawn attention in probiotics because of their ability to adhere to epithelial cell (Greene, & Klaenhammer, 1994, pp. 4487-4494; Ocaña, & Nader-Macías, 2001, pp. 265-273) and produce the bacteriocins (De Vuyst et al., 2004, pp. 93-106; Pandey, Malik, Kaushik, & Singroha, 2013, pp. 1977-1987). In Hasslof et al. study, *L. plantarum* 299v and *L. plantarum* 931 (10^3 cfu/ml) totally inhibited the growth of *S. mutans* NCTC 10449, *S. sobrinus* OMZ176 and *S. mutans* P1: 27. It also produced several kinds of antimicrobial substances against *S. mutans*. On the contrast, *L. acidophilus* La5 had no effects on *S. mutans'* growth (Hasslof et al., 2010, p. 18; Silza et al., 1987, pp. 1231-1233). *L. acidophilus* was found in oral cavities about 3 - 9% (Botha, 1993, pp. 177-181; Koll-Klais, Mandar, Leibur, & Kjaeldgaard, 2004, pp. 107-109; Teanpaisan, & Dahlen, 2006, pp. 79-83). The Lactobacillus bacteria that was used in this experiment was *Lactobacillus acidophilus* (DSMZ20079^T = TISTR2365^T= ATCC4356^T). They were reported as probiotics in the many study (Arslan et al., 2016, pp. 477-487; Ghasemi, Mazaheri, & Tahmourespour, 2017, pp. 257-263; Ortakci, & Sert, 2012, pp. 6918-6925; Resta-Lenert, & Barrett, 2003, pp. 988-997). This type strain was isolated from human body (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, 1970). *L. acidophilus* survives in an acidic condition with the pH between 5.5 – 5.8 (Robinson et al., 2000, pp. 1134 - 1157) which was lower than that of the gastrointestinal tract (pH of 6-7) (Fallingborg, 1999, p. 183-196). *L. acidophilus* was found in oral cavities about 3 - 9% (Botha, 1993, pp. 177-181; Koll-Klais et al., 2004, pp. 107-109; Teanpaisan, & Dahlen, 2006, pp. 79-83).

In this study *S. mutans* and *L. acidophilus* were co-cultured in many circumstances. The appropriate culturing condition for both bacteria was initially important to determine. According to DSMZ specification, the optimum condition for

L. acidophilus was anaerobic condition at 37 ^{0}C in Medium 232 pre-reduced (oxygen-free) (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, p. unpage). For Robinson et al., *L. acidophilus* preferred the gas mixture of 5% CO_2, 10% H_2, and 85% N_2 under oxygen-free condition, but they could be grow in aerobic condition in static culture without agitation (Robinson et al., 2000, p. 1134 - 1157) which corresponding to the study of Hasslöf et al. (Hasslöf, Hedberg, Twetman, & Stecksén-Blicks, 2010, pp. 18-18). However, Campana et al. could be cultured *L. acidophilus* under microaerophilic condition with 5% CO_2, 10% H_2, and 85% N_2 (Campana, Federici, Ciandrini, & Baffone, 2012, pp. 371-378). In Study of Knight et al., *L. acidophilus* was culture in mixture gas of N_2/ CO_2/ H_2 (90: 5: 5) (Knight, McIntyre, Craig, Mulyani, & Zilm, 2008, pp. 349-353). Alternatively, the American Type Culture Collection stated that optimum growth condition could be 5% CO_2 at 37 ^{0}C (ATCC, pp. 1-2). For cultivation of *S. mutans*, the microaerophilic condition at 37 ^{0}C was required. So, 5% CO_2, 10% H_2, and 85% N_2 at 37 ^{0}C was accounted for them. In this experiment, *S. mutans* and *L. acidophilus* were carried on 5% CO_2, at 37 ^{0}C which adopted from the ATCC specification. The result show that *S. mutans* and *L. acidophilus* could be survived in this condition.

For the *in vitro* cultivation, the culture media had influences on their growth and survivability. *L. acidophilus* grew in a short period of time in the culturing enrich medium of BHI (1 hour) and TYE (3 hours), but they could grow in the MRS medium throughout the growth phase. Normally, *L. acidophilus* is the fastidious microorganism. This strain cannot grow in only carbon source, it needs more complex nutrients such as amino acids, peptides, fatty acids esters, minerals, vitamins, oleic acids, buffering agents and polysorbates for growing and cell activity (Hébert, Raya, & Savoy de Giori, 2004, pp. 139-148; Robinson et al., 2000, pp. 1134 - 1157). All these substances are in the MRS, but not in the BHI and TYE.

Dextrose (glucose produced from corn) in the MRS is a source of energy. The glucose fermentation of *L. acidophilus* provides two molecules of the lactic acid and two molecules of the ATP from the partial oxidation of glucose (Fugelsang, & Edwards, 2007, pp. 36-38). The ATPs are used for the cell activities such as the cell division and the H^+ pump activation.

Proteose peptone and beef extract are nitrogenous and carbonaceous compounds for the proteolytic activity of *L. acidophilus*. This activity is essential for the synthesis of proteins, peptides and amino acids for bacteria growth (Hayek, & Ibrahim, 2013, p. 15). The amino acids and peptides are important in many proteolytic systems; 1) binding to the cell wall for the protein degradation; 2) transporting peptides for the intracellular uptake and 3) intracellularly metabolite peptides into smaller fragments and amino acids (Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010, p. 36). Amino acids and peptides are also obtained from yeast extract, tryptone, peptone or beef extract (Aguirre, Garro, & Savoy de Giori, 2008, pp. 976-982; Hébert, Raya, & Giori, 2004, pp. 341-345; Yu, Lei, Ren, Pei, & Feng, 2008, pp. 496-502). However, the excess yeast extract (more than 12 g/l) could suppress growth of lactic acid-produced bacilli (Oh, Rheem, Sim, Kim, & Baek, 1995, pp. 3809-3814). In this study, 10 g/l of yeast extract was added in to BHI. The result show that *L. acidophilus* could survived in BHI (supplemented with yeast extract) only for one hour. This proved that 10 g/l of yeast extract is enough to suppressed growth Lactobacillus bacteria. In addition, disagreement might due to the different strain of Lactobacillus and the method of study.

The lactic acid-produced bacteria require the oleic acid for the growth acceleration. In the MRS medium, the oleic acid was derived from Tween 80 and Tween 85 (Jenkins, & Courtney, 2003, pp. 51-57; Li et al., 2011, pp. 9138-9154). The supplemented oleic acids were up taken and cooperated into the bacterial cell membrane and converted to the cyclopropane fatty acids. The converted fatty acids are helpful for bacteria adaptation in the stressful environment (Bâati, Fabre-Gea, Auriol, & Blanc, 2000, pp. 241-247; Li et al., 2011, pp. 9138-9154).

Trace elements in the culturing medium are several kinds of metal ions. They are essential for many cell activities such as the enzyme production, the membrane transportation and as the components of cell structure (Hébert et al., 2004, pp. 139-148). Manganese (0.05 g of Manganese sulfate in MRS) is corporate into the glutamine synthase, lactate dehydrogenase and alkaline phosphatase (Fitzpatrick, Ahrens, & Smith, 2001, pp. 671-675). Magnesium (1 g of magnesium sulfate in MRS) is needed for the growth of lactic acid-produced bacteria (Hébert et al., 2004, pp. 341-

345). In addition, Ca^{2+}, Fe^{+}, K^{+} and Na^{+} are also required for enzymatic activities and nutrient transportation (Hayek, & Ibrahim, 2013, p. 15).

Different from the MRS, the BHI culturing medium is composed of the infusion of calf brain and beef heart, proteose peptone, dextrose, NaCl and Na_2HPO_4 (GmbH, 2013, p. 1-3). It lacks of essential trace element and oleic acid. While, TYE is composed of glucose, yeast extract and abundant of trace elements (K_2HPO_4, $MgSO_4.7H_2O$, $FeCl_2$ 10 mg, $MnSO_4.4H_2O$, NaCl), but it lacks of oleic acid. Both oleic acid (from tween 80) and trace element are important for growth acceleration (Hayek, & Ibrahim, 2013, p. 15; HiMedia-Laboratories, 2015, pp. 1-2; Jenkins, & Courtney, 2003, pp. 51-57). Therefore, *L. acidophilus* survived in both media but only for short period of time in this study.

S. mutans A32-2 may be required less nutrients when compared to *L. acidophilus*. *S. mutans* seem to be able to grow in almost all types of culturing media. However, after 10 hours (in TYE) and 14 hours (in BHI), *S. mutans* was receded form the culture. In several studies, BHI media was the more flavored culturing of *S. mutans* (Lin, Chen, Tu, Wang, & Chen, 2017, pp. 4175-4181; Wu et al., 2015, pp. 16-26). In some study, tryptic soy in mixture gas ($N_2/CO_2/H_2$ – 90:5:5) is another best condition for *S. mutans*. (Knight et al., 2008, pp. 349-353).

In most study, the interaction between *S. mutans* and *L. acidophilus* were carried on agar diffusion method under anaerobic condition which examined the bacterial inhibition zone of *L. acidophilus* (Wasfi, Abd El-Rahman, Zafer, & M. Ashour, 2018, p; Wu et al., 2015, pp. 16-26). For this study, *S. mutans* and *L. acidophilus* were co-cultured in culture broth under the 5% CO_2. The results show that under 5% CO_2, *L. acidophilus* cannot survive in BHI and TYE broth, but they grow well in MRS broth throughout the experiment instead. Hence when anaerobic condition was unavailable, the mixed-cultures of *S. mutans* and *L. acidophilus* might carried on MRS under 5% CO_2.

According to the growth pattern in the MRS medium (Figure 10), the log phase of *S. mutans* and *L. acidophilus* was in the duration of the 3rd and 12th hour. Their mid-log phases were in between the 6th and 8th hours (OD= 0.6). The mid-log phase of them were chosen for further study because of their energetic activity. However, at the same OD, the number of *S. mutans* was quite different from

L. acidophilus due to the difference in size of cells. *L. acidophilus* (0.5 -1.2 x 1-10 μm) (Robinson et al., 2000, pp. 1134 - 1157) are bigger than *S. mutans* (0.321 ± 0.007 μm) (Ryan, Hart, & Schiller, 1980, pp. 313-324). At OD 0.6, the total number of *S. mutans* was around 4.2 $x10^9$ CFU/ml while it was about 0.5 $x10^8$ CFU/ml for *L. acidophilus*. Thus, *S. mutans* and *L. acidophilus* were co-cultured in various proportions (volume by volume) to find the appropriated ratio to receive the equal number of cells. Although, the number of *S. mutans* greater than *L. acidophilus* for 85 times at the same OD, but the optimum ratio between *S. mutans* and *L. acidophilus* was 1:20.

L. acidophilus is normally able to suppress *S. mutans* cell activities in many circumstances. According to the double-blind cross-over controlled trial of Singh *et al.*, consuming of probiotic ice cream contained freeze-dried *L. acidophilus* La5 significantly decreased numbers of salivary mutans streptococci (Singh, Damle, & Chawla, 2011, pp. 389-394). For the *in vitro* study of Ahmed *et al.*, *L. acidophilus* inhibited 60-80 % of the *GtfB* and *LuxS* gene expressions leading to the depletion of the biofilm formation and maturation of *S. mutans* (Ahmed et al., 2014, pp. 1523-1528). Moreover, Raj et al. also demonstrated that the supernatant of *L. acidophilus* inhibited the biofilm formation of *S. mutans* (Raj, Bhati, & Bhadekar, 2015, pp. 2533-2538). *L. acidophilus* DSM 20079^T suppressed the ability of *S. mutans* ATCC35668 to adhere to the microtiter plates, and it was more efficient when *L. acidophilus* was introduced into the system before *S. mutans* (Tahmourespour, & Kermanshahi, 2011, pp. 37-40). It also has abilities to decrease the viable count of *S. mutans* (Ahola et al., 2002, pp. 799-804; Chuang et al., 2011, pp. 471-476; Nikawa et al., 2004, pp. 219-223). But in this study, we observed the growth rate of *S. mutans*, while the other previous study determined the viable count of *S. mutans*. The growth rate reflected the ability of bacteria to multiply to replace the precursor cell. The low growth rate indicated the low new bacteria, while the old one die away. So, the growth rate gives the more accurate than viable count. The growth rate of single culture of *S. mutans* in this study was 0.9921 ± 014 h^{-1}. When *S. mutans* co-cultured with *L. acidophilus,* its' growth rate decreased to 0.9623 ± 0.17 h^{-1} which was insignificant when compared to the single culture. This finding disagrees with the previous study. The results from this

experiment indicated that *L. acidophilus* DSMZ 20079^T unable to decreased the growth rate of *S. mutans* A 32-2 when co-culture under 5% CO_2 in MRS broth.

The ability of microorganism to ferment sugar is the one characters of cariogenic properties (Banas, 2004, pp. 1267-1277). *S. mutans* was able to ferment sugar and carbohydrate and provided the organic acid products to dissolved tooth mineral compartment (Banas, 2004, pp. 1267-1277). With the low cariogenic properties, non-digestible oligosaccharides that is called prebiotics were introduced to decrease the prevalence of dental caries. Not only to be able to resist to human digestive enzymes (Gibson et al., 2004, pp. 259-275), the indigestible carbohydrates are not the substrate for *S. mutans* fermentation (Grizard, & Barthomeuf, 1999, pp. 563-588).

Prebiotics are short chain carbohydrate polymers. They promote functions of the natural human bowel probiotics. The criteria for prebiotics are 1) the stability in the gastro-digestive system by resisting to gastric acid, enzyme digestion and absorption 2) the ferment ability by gut microorganisms 3) the selectively stimulating ability to the growth and the activity of bacteria which gain benefit for host (Gibson et al., 2004, pp. 259-275). Since the galacto-oligosaccharide (GOS) and the fructo-oligosaccharides (FOS) have been tested in the *in vivo* studies and they meet the requirements of the prebiotics selection criteria (Roberfroid, 2007, pp. 830S-837S). Therefore, the prebiotics, GOS and FOS were utilized as the prebiotics in this study.

This is the first study to determine the growth of *S. mutans* when co-culture with *L. acidophilus* in the prebiotics-supplemented media. In order to simulate oral situation, the 6th hour of incubation was selected for growth rates determination. From the literature reviews, *L. acidophilus* can utilize GOS as a source of nutrient by lactose permease pathway (Andersen et al., 2011, p. 17785-17790) and was hydrolyzed by β-galactosidase enzyme (Obst, Hehn, Vogel, & Hammes, 1992, pp. 209-214). For the metabolizing mechanism of GOS in *S. mutans* still obscured.

After GOS was added into the individual culture, the growth rate of *S. mutans* were not statistic different form control. So, GOS might not affected the growth of S. *mutans*. When the co-culture received GOS, the growth in 1% GOS was not different from control. In 2% GOS, the growth rate of *S. mutans* was slightly higher than 1% GOS and control (no statistic different) which deviated from the trend of growth rates.

This phenomenon might due to the experimental errors such as time slipped in experiment of each strains and human error (aspiration, mixing and counting, etc.). When the concentration was increased, the growth rate of *S. mutans* statistically affected. The growth rate of S. *mutans* in 3% GOS, 4% GOS and 5% GOS significantly decreased compared to the control. The results indicated that the growth rate of *S. mutans* started to be decreased from 3% GOS in co-culture. When the concentration of GOS increased to be 4% and 5% of GOS, the growth rates among 3% GOS, 4% GOS and 5% GOS were not statistic difference. Therefore, 3% GOS was the optimum concentration that could be decreased growth rate of *S. mutans* when co-culture with *L. acidophilus*.

Even though *L. acidophilus* can utilize GOS, but GOS was unpleasant in polymers digestion. So, the metabolizing and acid production is limited (Gänzle, 2012, pp. 116-122). In accordance to our study, the growth rates of *L. acidophilus* were not affected by GOS both for individual culture and co-culture. However, when *L. acidophilus* co-culture with *S. mutans*, the growth rates of *S. mutans* decreased. This finding indicated that GOS might enhance the cell activity of *L. acidophilus* in competitive growth with *S. mutans*, but not the growth rates.

FOS is another well-known prebiotic that was used for this study. The FOS are linked with β-(2 →1) or β-(2 →6) D-fructose units terminated with D-glucose or D-fructose. *S. mutans* can use FOS as a substrate in their energy production (Burne, & Penders, 1992, pp. 4621-4632; Burne et al., 1999, pp. 2863-2871; Hartemink et al., 1995, pp. 551-557; Russell et al., 1992, pp. 4631-4637). For *L. acidophilus* metabolism, ATP-binding cascade (ABC) transporters was responded for FOS transportation into bacterial cell (Barrangou et al., 2003, pp. 8957-8962; Barrangou et al., 2006, pp. 3816-3821). FOS was hydrolyzed within cytoplasm by BfrA or BrfB fructosidase enzyme (Barrangou et al., 2003, pp. 8957-8962; Ehrmann et al., 2003, pp. 0391-0397). According to the study, the growth rates of *L. acidophilus* in all concentrations of FOS were not different from control (without FOS supplementation). herefore, FOS was not affected the growth rate of *L. acidophilus*. Alternatively, the growth of *S. mutans* affected both in single culture and co-culture which were decreased after receive FOS. The minimum growth rate was seen in 3% FOS both in single-culture and co-culture. In co-culture, the growth rate in all concentration

deceased, while only 3% FOS in single-culture was decreased. Therefore, the efficacy of FOS to decreased growth rate of *S. mutans* was potentiated when *L. acidophilus* was presented in the culture. Moreover, the decreasing in pH of culture medium might support the decreased-growth-rate of *S. mutans* when co-culture with *L. acidophilus* in GOS- and FOS-supplemented MRS media.

GOS and FOS are resisted to gastric digestion in human (Macfarlane, Steed, & Macfarlane, 2008, pp. 305-344), The energy from GOS and FOS are 1.7 kcal/g (Watanuki, Wada, & Matsumoto, 1996, pp. 1-12) and 1.5 kcal/g (Roberfroid, 2007, pp. S13-S25) respectively. Hence, they are used as sweeteners in foods and beverage. However, the excessive intake probably resulting in abdominal discomfort, flatulence (Teuri, Korpela, Saxelin, Montonen, & Salminen, 1998, pp. 465-471) and diarrhea (Deguchi, Matsumoto, Ito, & Watanuki, 1997, pp. 13-22; Saavedra, & Tschernia, 2007, pp. S241-S246). Therefore, the recommend dose from the previous study is range from 8-15 g/day. According to EU Scientific Committee on Food, the approved dose of GOS (90%) and FOS (10%) combination for infant formula is lower than 0.8 g/100 ml (SCF, 2001, pp. 1-6). But for the general used (not infant), 2- 20 g/day is recommended (Binns, 2013, pp. 1-40).

Generally, the growth rates of *S. mutans* in co-culture were slightly lower than single culture which significant in 5% GOS. For 5% GOS, the growth rate of *S. mutans* significant decreased from 0.9070 ± 0.17 (single culture) to 0.35491 ± 0.09 (co-culture). Therefore, *L. acidophilus* might had a synergistic effect with 5% FOS to decreased growth rate of *S. mutans*.

However, the mechanism of GOS and FOS dominated on *L. acidophilus* is not identified and need further study to identify the extracted mechanism and make the conclusive evidence.

As mention earlier, the culturing media might affect on fatty acids production of bacteria. Since GOS and FOS were supplemented into the culture, then the fatty acids alteration was observed. This part of the study is the preliminary study to determine the fatty acids change after bacteria received FOS and GOS.

The unsaturated fatty acids of *S. mutans* increased when received prebiotics especially FOS. The unsaturated fatty acids were able to co-operate into cell membranes and make the cell membrane lose its stability. The electron transport

bound proteins then detach from the inner membrane and cannot be perform the energy production (Galbraith, & Miller, 1973, pp. 659-675; Peters, & Chin, 2003, pp. 117-124). Moreover, the unsaturated cellular fatty acids are associated with the fluidity and permeability of bacterial membrane (Boyaval et al., 1995, pp. 17-29; Greenway, & Dyke, 1979, pp. 233-245; Speert et al., 1979, pp. 1202-1210; Wang, & Johnson, 1992, pp. 624-629). After unsaturated fatty acids were incorporated into bacterial membrane, it became more fluidity and susceptible to the cell rupture (Boyaval et al., 1995, pp. 17-29; Carson, & Daneo-Moore, 1980, pp. 1122-1126; Greenway, & Dyke, 1979, pp. 233-245; Speert et al., 1979, pp. 1202-1210; Wang, & Johnson, 1992, pp. 624-629).

The common fatty acids in the living microorganism are palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), myristic acid ($C_{14:0}$), oleic acid ($C_{18:1\ \omega9c}$) and palmitoleic acid ($C_{16:1\ \omega7c}$) (Rustan, & Drevon, 2001, p. 1-7). According to previous studies, palmitic acid and stearic acid are saturated fatty acids and accounted for over 80% in bacterial autolysin activity inhibition (Carson, & Daneo-Moore, 1980, pp. 1122-1126). In this study, palmitic ($C_{16:0}$) acid and stearic acid ($C_{18:0}$) of *S. mutans* decreased in prebiotics-supplemented media. This might increase the susceptibility of *S. mutans* to autolysis.

According to this study, *S. mutans* A32-2 had oleic acid 19.8 % in prebiotics-free condition. After they received prebiotics, the oleic acids of bacteria increased to 20.79 - 27.93%. The oleic acid ($C_{18:1\ \omega9c}$) is unsaturated fatty acid. This acid was able to induce the lysis of the bacterial membrane (Carson, & Daneo-Moore, 1980, pp. 1122-1126). Because the double bond of unsaturated fatty acids are susceptible to oxidizing agent (Rustan, & Drevon, 2001, pp. 1-7).

By increasing of unsaturated fatty acid and decreasing of the beneficial saturated fatty acids, the stability of bacterial membrane might be decreased. Hence, FOS and GOS had an influence on the cellular fatty acids redistribution and might be promoted fragility of *S. mutans* when cultured in prebiotics-rich medium.

In animal model, short chain fatty acids (SCFAs) were produced and released after beneficial intestinal microorganisms metabolized prebiotics. The concentration of bowel SCFAs significantly increased after the mouse received GOS and FOS. The released-fatty acids might corporate in to host cell activity and in defense mechanisms

such as propionate and butyrate (Pan, Chen, Wu, Tang, & Zhao, 2009, pp. 258-263; Shigwedha et al., 2016, pp. 41-57).

For this study, *L. acidophilus* represented the beneficial bacterial that might be able to ferment GOS and FOS and released the beneficial fatty acids in to the culture medium. After GOS and FOS were added in to the single culture of *L. acidophilus*, the fatty acids were labile in the cultured media. The fatty acids are medium chain fatty acids which was released in greater amount than the control including capric acid ($C_{10:0}$), myristic acid ($C_{14:0}$), stearic acids ($C_{18:0}$), 17-methylstearic acids ($C_{19:0\ iso}$) and margaric acid ($C_{17:0}$) were released into the medium of individual culture. For the co-culture, the increasing fatty acids comprised of stearic acids ($C_{18:0}$), $C_{17:0\ OH}$, 17- methylstearic acid ($C_{19:0\ iso}$), arachidic acid ($C_{20:0}$) in GOS, while stearic acid ($C_{18:0}$), 17- methylstearic acid ($C_{19:0\ iso}$), arachidic acid ($C_{20:0}$) increased in FOS.

Free fatty acids have an antibacterial property against opportunistic and pathogenic bacteria. Several *in vitro* reviews showed the antimicrobial property of artificial free fatty acids to destroy unfavorable bacteria. The fatty acids which have anti-bacterial property on Gram-positive bacteria are capric acid ($C_{10:0}$) and myristic acid ($C_{14:0}$). Moreover, the margaric acid ($C_{17:0}$) can inhibit Gram-negative bacteria. (Andrew P. Desbois, & Valerine J. Smith, 2010, pp. 1629-1642; Pan et al., 2009, pp. 258-263).

For arachidic acid ($C_{20:0}$), it is a saturated fatty acid which derived from chain elongation of stearic acid ($C_{18:0}$) (Halver, 1980). In this study, the stearic acid in co-culture was increased. Therefore, the excess stearic acids might subsequent turn into arachidic acid.

Since dextrose and prebiotics were incorporated into the MRS medium, and *S. mutans* is able to metabolite them and finally released the acids into the culture medium. Without the medium replenishment in the culturing procedure, the accumulation of the metabolic acids in the culturing medium can create the acidic condition. According to the study, pH of culture medium continuing decreased from the 1st hour to the 12th hour. When the pH of culture media below 5.25 (at the 9th hour), the growth rates of *S. mutans* sudden double-decreased compared with the growth rate at the 6th hours. According to the study of Hamilton *et al.*, the acidic

condition has an influence on *S. mutans*. At pH 5.5 the glycolysis activity of *S. mutans* triple increased when compared to pH 6.5. But at the numbers (mg (dried weight)/ml) of *S. mutans* were not significant from the control (pH 6.5) (Hamilton, & Ellwood, 1978, pp. 434-442). In this study, the growth rate was represented for the amounts of the new generation of *S. mutans*. The results show that at pH below 5.25 (the pH at the 6th hours of culture), the numbers of *S. mutans* double decreased from the pH of 6 (the pH at the 3rd hours of culture), as in the table 10 (statistic not done). So, the decreased-growth rate of *S. mutans* might be due to acidic condition.

The pH of the culturing medium has an influence on the rearrangement of the membrane fatty acids. According to Quivey et al. study, *S. mutans* altered its membrane fatty acids during growth in the low pH condition (Quivey, Kuhnert, & Hahn, 2000, pp. 239-274). At pH 6.5, cellular fatty acids of *S. mutans* UA159 composed of 65.4% saturated fatty acids and 34.6% of unsaturated fatty acids. When pH drops to 5.5, saturated fatty acids became 53.3 % , and unsaturated fatty acids became 46.7% (Fozo, & Quivey, 2004, pp. 929-936). In this study, the percentages of the unsaturated fatty acids of *S. mutans* A32-2 were much higher than the saturated fatty acids in the acidic condition which corresponded with the former study.

Moreover, Bender et al. mentioned that the monounsaturated fatty acids of *S. mutans* were upregulation in acidic condition especially for $C_{18:1}$ and $C_{20:1}$ (Bender, Sutton, & Marquis, 1986, pp. 331-338). With the corresponding to this study, Oleic acids ($C_{18:1\ \omega 9c}$), $C_{18:1}$ and gondoic acid ($C_{20:1\ \omega 9c}$) were also increased in the experiments (at 6th hour of incubation) like Bender finding in 1986. All these increased-fatty acids are the of membrane alteration mechanism for withstanding to acidic environment. It was also discovered that F_1-F_0-ATPase proton pump of *S. mutans* was active under acidic environment. This pump has a powerful capacity to excrete the intracellular protons for maintaining their intracellular homeostasis (Kuhnert, Zheng, Faustoferri, & Quivey, 2004, pp. 8524-8528). In addition, the MRS medium contains dipotassium phosphates which functions as the buffering agent (GmbH, 2014, pp. 1-3; HiMedia-Laboratories, 2015, pp. 1-2). In this study, pH of culturing medium in prebiotics-supplemented MRS ranged between 5.08 ± 0.22 and 6.21 ± 0.02 which are higher than their acidic-pH limitation. Therefore, the viability

and metabolism of *S. mutans* (at the 6th hour of incubation) might not be interfered by acidic condition, but the ability of *S. mutans* to multiply might be retarded.

This experiment is a preliminary study to determine the efficacy of prebiotic-FOS/ GOS on probiotic *Lactobacillus acidophilus* ATCC4356^{T} = DSMZ20079T = TISTR2356^{T} to compete growth with *S. mutans* A32-2. The results show that, the growth rate of *S. mutans* significant decreased from control in co-culture with *L. acidophilus* which supplemented with GOS and FOS. But the mechanism of action is obscured. To identify the specific mechanism, the further study is required to make the statement. And to eliminate the effected of acidic condition of the growth rates of each strain, the pH of culture medium must be neutralized.

Conclusion

The prebiotics GOS and FOS have no effect on the growth rates of *L. acidophilus* TISTR 2365^{T} = DSMZ 20079^{T}= ATCC4356. The 3% FOS significant decreased growth rate of single culture of *S. mutans* A32-2. With the optimal condition, the 3%, 4%, 5% GOS and 1%, 2%, 3%, 4% and 5% FOS significantly decreased growth rate of *S. mutans* in the co-cultured with *L. acidophilus* with no effect on the growth rate of *L. acidophilus*. Therefore, *L. acidophilus* with the specific prebiotics concentration could suppress the growth of *S. mutans* A32-2.

REFERENCES

REFERENCES

Aguirre, L., Garro, M. S., & Savoy de Giori, G. (2008). Enzymatic hydrolysis of soybean protein using lactic acid bacteria. *Food Chemistry, 111*(4), 976-982.

Ahmed, A., Dachang, W., Lei, Z., Jianjun, L., Juanjuan, Q., & Yi, X. (2014). Effect of lactobacillus species on *Streptococcus mutans* biofilm formation. *Pakistan Journal of Pharmaceutical Sciences, 27*(5), 1523-1528.

Ahola, A. J., Yli-Knuuttila, H., Suomalainen, T., Poussa, T., Ahlström, A., Meurman, J. H., & Korpela, R. (2002). Short-term consumption of probiotic-containing cheese and its effect on dental caries risk factors. *Archives of Oral Biology, 47*(11), 799-804.

Ahrne, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A. E., & Molin, G. (1998). The normal lactobacillus flora of healthy human rectal and oral mucosa. *Journal of Applied Microbiololgy, 85*(1), 88-94.

Akter, N., Wu, B., Memon, A. M., & Moshin, M. (2015). Probiotics and prebiotics associated with aquaculture. *A review Fish & Shellfish Immunolgy, 45*, 733-741.

Andersen, J. M., Barrangou, R., Abou Hachem, M., Lahtinen, S., Goh, Y. J., Svensson, B., & Klaenhammer, T. R. (2011). Transcriptional and functional analysis of galactooligosaccharide uptake by lacs in *Lactobacillus acidophilus*. *Proceedings of the National Academy of Sciences of the United States of America, 108*(43), 17785-17790.

Anusha, R. L., Umar, D., Basheer, B., & Baroudi, K. (2015). The magic of magic bugs in oral cavity: Probiotic. *Journal of Advanced Phamaceutical Technology & Research, 6*(2), 43-47.

Arslan, A. A., Gocer, E. M. C., Demir, M., Atamer, Z., Hinrichs, J., & Kücükcetin, A. (2016). Viability of *Lactobacillus acidophilus* ATCC 4356 incorporated into ice cream using three different methods. *Dairy Science & Technology, 96*(4), 477-487.

American Type Culture Collection, ATCC. (n.d.). *Lactobacillus acidophilus* (atcc® 4356™) (pp. 1-2). N.P.: n.p.

Bâati, L. l., Fabre-Gea, C., Auriol, D., & Blanc, P. J. (2000). Study of the cryotolerance of *Lactobacillus acidophilus*: Effect of culture and freezing conditions on the viability and cellular protein levels. *International Journal of Food Microbiology, 59*(3), 241-247.

Baker, J. L., Faustoferri, R. C., & Quivey, R. G., Jr. (2017). Acid-adaptive mechanisms of *Streptococcus mutans*-the more we know, the more we don't. *Molecular Oral Microbiology, 32*(2), 107-117.

Banas, J. A. (2004). Virulence properties of *Streptococcus mutans*. *Frontiers in Bioscience, 9*, 1267-1277.

Barrangou, R., Altermann, E., Hutkins, R., Cano, R., & Klaenhammer, T. R. (2003). Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. *Proceedings of the National Academy of Sciences of the United States of America, 100*(15), 8957-8962.

Barrangou, R., Azcarate-Peril, M. A., Duong, T., Conners, S. B., Kelly, R. M., & Klaenhammer, T. R. (2006). Global analysis of carbohydrate utilization by *Lactobacillus acidophilus* using cdna microarrays. *Proceedings of the National Academy of Sciences of the United States of America, 103*(10), 3816-3821.

Bender, G. R., Sutton, S. V., & Marquis, R. E. (1986). Acid tolerance, proton permeabilities, and membrane atpases of oral streptococci. *Infection and Immunity, 53*(2), 331-338.

Bender, G. R., Thibodeau, E. A., & Marquis, R. E. (1985). Reduction of acidurance of streptococcal growth and glycolysis by fluoride and gramicidin. *Journal of Dental Research, 64*(2), 90-95.

Bente , N., & Ole , F. (1987). Scaining microscopy of early microbial colonization of human enamel and root surface *in vivo*. *European Journal of Oral Sciences, 95*(4), 287 - 296.

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., & Gil, A. (2012). Probiotic mechanisms of action. *Animals of Nutrition & Meatbolism, 61*, 160 -174.

Binns, N. (2013). Probiotics, prebiotics and the gut microbiota. *Ilsi Europe Concise Monograph Series, 1,* 40.

Boler, B. M. V., & Fahey, G. C. (2012). Prebiotics of plant and microbial origin. In T. R. Callaway & S. C. Ricke (Eds.), *Direct-fed microbials and prebiotics for animals: Science and mechanisms of action* (pp. 13-26). New York: Springer.

Bornet, F. R. J., Brouns, F., Tashiro, Y., & Duvillier, V. (2002). Nutrional aspects of short-chain fructooligosaccharides: Natural occurrence, chemistry, physiology and health implications. *Digestive and Liver Disease, 34*(2), 111-120.

Botha, S. J. (1993). Oral lactobacilli isolated from teenage orthodontic patients. *Journal of Dental Association of South Africa, 48*(4), 177-181.

Bowen, D. M. (2011). Prevention of root caries. *The Journal of Dental Hygiene, 85*(2), 78 - 82.

Boyaval, P., Corre, C., Dupuis, C., & Roussel, E. (1995). Effects of free fatty acids on propionic acid bacteria. *Lait, 75*(1), 17-29.

Bruggencate, S. J. M. T., Bovee-Oudenhoven, I. M. J., Lettink-Wissink, M. L. G., Katan, M. B., & Meer, R. v. d. (2006). Dietary fructooligosaccharides affect intestinal barrier function in healthy men. *The journal of nutrition, 136*(1), 70-74.

Burne, R. A., & Penders, J. E. (1992). Characterization of the *Streptococcus mutans* gs-5 frua gene encoding exo-beta-d-fructosidase. *Infection and Immunity, 60*(11), 4621-4632.

Burne, R. A., Wen, Z. T., Chen, Y. Y., & Penders, J. E. (1999). Regulation of expression of the fructan hydrolase gene of streptococcus mutans gs-5 by induction and carbon catabolite repression. *Journal of Bacteriology, 181*(9), 2863-2871.

Byun, R., Nadkarni, M. A., Chhour, K.-L., Martin, F. E., Jacques, N. A., & Hunter, N. (2004). Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *Journal of Clinical Microbiology, 42*(7), 3128-3136.

Campana, R., Federici, S., Ciandrini, E., & Baffone, W. (2012). Antagonistic activity of *Lactobacillus acidophilus* ATCC4356 on the growth and adhesion/invasion characteristics of human campylobacter jejuni. *Current Microbiology, 64*(4), 371-378.

Campbell, P. N., Smith, A. D., & Peters, T. J. (2005). *Biochemistry illustrated biochemistry and molecular biology in th post-genomic era*. China: Harcount.

Carson, D. D., & Daneo-Moore, L. (1980). Effects of fatty acids on lysis of *Streptococcus faecalis*. *Journal of Bacteriology, 141*(3), 1122-1126.

Caufield, P. W., Schon, C. N., Saraithong, P., Li, Y., & Argimon, S. (2015). Oral lactobacilli and dental caries: A model for niche adaptation in humans. *Internatioal & American Association for Dental Research, 94*(9), 110S-118S.

Charteris, Kelly, Morelli, & Collins. (1998). Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract. *Journal of Appiedl Microbiology, 84*(5), 759-768.

Chuang, L.-C., Huang, C.-S., Ou-Yang, L.-W., & Lin, S.-Y. (2011). Probiotic *Lactobacillus paracesei* effect on cariogenic bacterial flora. *Clinical oral investigation, 15*, 471-476.

Clarke, J. K. (1924). On the bacterial factor in the ætiology of dental caries. *British journal of experimental pathology, 5*(3), 141-147.

Comelli, E. M., Guggenheim, B., Stingele, F., & Neeser, J.-R. (2002). Selection of dairy bacterial strains as probiotics for oral health. *Europe an Journal ofOral Sciences, 110*(3), 218-224.

Crittenden, R. G., & Playne, M. J. (1996). Production, properties and applications of food-grade oligosaccharides. *Trend in Food Science & tecnology, 7*, 353-361.

De Vuyst, L., Avonts, L., Neysens, P., Hoste, B., Vancanneyt, M., Swings, J., & Callewaert, R. (2004). The lactobin a and amylovorin l471 encoding genes are identical, and their distribution seems to be restricted to the species lactobacillus amylovorus that is of interest for cereal fermentations. *International Journal of Food Microbiology, 90*(1), 93-106.

Deguchi, Y., Matsumoto, K., Ito, A., & Watanuki, M. (1997). Effects of β1-4 galacto-oligosaccharides administration on defecation of healthy volunteers with constipation tendency. *The Japanese Journal of Nutrition and Dietetics, 1,* 13-22.

Depeint, F., Tzortzis, G., Vulevic, J., I'Anson, K., & Gibson, G. R. (2008). Prebiotic evaluation of a novel galacto-oligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* ncimb 41171, in healthy humans: A randomized, double-blind, crossover, placebo-controlled intervention study. *American Journal of Nutrition, 87*, 785-791.

Desbois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. *Applied Microbiology Biotechnology, 85*(6), 1629-1642.

Dobson, A., Cotter, P. D., Ross, R. P., & Hill, C. (2012). Bacteriocin production: A probiotic trait? *Applied and Environmental Microbiology, 78*(1), 1-6.

Ehrmann, M. A., Korakli, M., & Vogel, R. F. (2003). Identification of the gene for β-fructofuranosidase of bifidobacterium lactis dsm10140t and characterization of the enzyme expressed in escherichia coli. *Current Microbiology, 46*(6), 0391-0397.

Fallingborg, J. (1999). Intraluminal ph of the human gastrointestinal tract. *Danish Medical Bulletin, 46*(3), 183-196.

Fejerskov, O., Kidd, E., & Nyvad, B. (2008). Dental caries the disease and its clinical management (2nd ed., p. 4). Singapore: Blackwell Munksgaard

Fernández, M. F., Boris, S., & Barbés, C. (2003). Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. *Journal of Applied Microbiology, 94*, 449-455.

Fitzpatrick, J. J., Ahrens, M., & Smith, S. (2001). Effect of manganese on lactobacillus casei fermentation to produce lactic acid from whey permeate. *Process Biochemistry, 36*(7), 671-675.

Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report, F. (2001). *Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria.* Retrieved November 22, 2015, from http://apps.who.int/iris/bitstream/handle/10665/43411/WHO_TRS_935_eng.pdf;jsessionid=2AF2B3212A828A81C6F0135EEE07ED4D?sequence=1

Fozo, E. M., & Quivey, R. G. (2004). Shifts in the membrane fatty acid profile of streptococcus mutans enhance survival in acidic environments. *Applied and Environmental Microbiology, 70*(2), 929-936.

Fugelsang, K. C., & Edwards, C. G. (2007). Wine microbiology. In K. C. Fugelsang & C. G. Edwards (Eds.), (2nd ed., pp. 36-38). N.P.: Springer.

Galbraith, H., & Miller, T. B. (1973). Effect of long chain fatty acids on bacterial respiration and amino acid uptake. *Journal of Applied Microbiology, 36*(4), 659-675.

Gänzle, M. G. (2012). Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *International Dairy Journal, 22*(2), 116-122.

Ghasemi, E., Mazaheri, R., & Tahmourespour, A. (2017). Effect of probiotic yogurt and xylitol-containing chewing gums on salivary s mutans count. *The Journal of Clinical Pediatrict Dentisrty, 41*(4), 257-263.

Gibson, G. R., Probert, H. M., Loo, J. V., & Rastall, R. A. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutritional Reseach Reviews, 17*, 259-275.

GmbH, B. D. (2013). *Bd™ brain heart infusion (bhi) agar* (pp. 1-3). N.P.: n.p.

GmbH, B. D. (2014). *Instructions for use-ready-to-usebottled media: Bd tryptic soy broth(tsb)* (pp. 1-3). N.P.: n.p.

Gong, Y., Tian, X. L., Sutherland, T., Sisson, G., Mai, J., Ling, J., & Li, Y. H. (2009). Global transcriptional analysis of acid-inducible genes in *Streptococcus mutans*: Multiple two-component systems involved in acid adaptation. *Microbiology Society Journal, 155*(Pt 10), 3322-3332.

Greene, J. D., & Klaenhammer, T. R. (1994). Factors involved in adherence of lactobacilli to human caco-2 cells. *Applied and Environmental Microbiology, 60*(12), 4487-4494.

Greenway, D. L., & Dyke, K. G. (1979). Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *Journal of General Microbiology, 115*(1), 233-245.

Grizard, D., & Barthomeuf, C. (1999). Non-digestible oligosaccharides used as prebiotic agents: Mode of production and beneficial effects on animal and human health. *Reproduction Nutrition Development, 39*(5-6), 563-588.

Guerzoni, M. E., Lanciotti, R., & Cocconcelli, P. S. (2001). Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*. *Microbiology Society Journal, 147*(Pt 8), 2255-2264.

Guo, L., McLean, J. S., Lux, R., He, X., & Shi, W. (2015). The well-coordinated linkage between acidogenicity and aciduricity via insoluble glucans on the surface of *Streptococcus mutans*. *Scientific Reports, 5*, 18015.

Halver, J. E. (1980). *Lipids and fatty acid.* N.P.: n.p.

Hamilton, I. R., & Ellwood, D. C. (1978). Effects of fluoride on carbohydrate metabolism by washed cells of *Streptococcus mutans* grown at various ph values in a chemostat. *Infection and Immunity, 19*(2), 434-442.

Hartemink, R., Quataert, M. C., van Laere, K. M., Nout, M. J., & Rombouts, F. M. (1995). Degradation and fermentation of fructo-oligosaccharides by oral streptococci. *J Appl Bacteriol, 79*(5), 551-557.

Hasslöf, P., Hedberg, M., Twetman, S., & Stecksén-Blicks, C. (2010). Growth inhibition of oral mutans streptococci and candida by commercial probiotic lactobacilli - an in vitro study. *BMC Oral Health, 10*, 18-18.

Haukioja, A. (2010). Probiotics and oral health. *European Journal of Dentistry, 4*(3), 348-355.

Hayek, S. A., & Ibrahim, S. A. (2013). Current limitations and challenges with lactic acid bacteria: A review. *Food and Nutrition Sciences, 04*(11), 15.

Hazan, R., Que, Y.-A., Maura, D., & Rahme, L. G. (2012). A method for high throughput determination of viable bacteria cell counts in 96-well plates. *BMC Microbiology, 12*, 259-259.

Hébert, E. M., Raya, R. R., & Giori, G. S. d. (2004). Nutritional requirements of *Lactobacillus delbrueckii* subsp. Lactis in a chemically defined medium. *Current Microbiology, 49*(5), 341-345.

Hébert, E. M., Raya, R. R., & Savoy de Giori, G. (2004). Evaluation of minimal nutritional requirements of lactic acid bacteria used in functional foods. In J. M. Walker, J. F. T. Spencer, & A. L. Ragout de Spencer (Eds.), *Environmental microbiology: Methods and protocols* (pp. 139-148). Totowa, NJ: Humana Press.

Hernandez-Hernandez, O., Muthaiyan, A., Moreno, F. J., Montilla, A., Sanz, M. L., & Ricke, S. C. (2012). Effect of prebiotic carbohydrates on the growth and tolerance of *Lactobacillus. Food Microbiology, 30*(2), 355-361.

HiMedia-Laboratories. (2015). *Tecnical data: Lactobaciluss mrs agar* (pp. 1-2). N.P.: n.p.

Hosoya, Norimasa, Dhorranintra, B., & Hidaka, H. (1988). *Utilization of [u-14c] fructooligosaccharides in man as energy resources, 5*(1), 67-74.

Jenkins, J. K., & Courtney, P. D. (2003). Lactobacillus growth and membrane composition in the presence of linoleic or conjugated linoleic acid. *Canadian Jornal of Microbioogyl, 49*(1), 51-57.

Jia, W., Li, H., Zhao, L., & Nicholson, J. K. (2008). Gut microbiota: A potential new territory for drug targeting. *Nature Reviews Drug Discovery, 7*, 123-129.

Kharat, A. S., & Tomasz, A. (2003). Inactivation of the srta gene affects localization of surface proteins and decreases adhesion of *Streptococcus pneumoniae* to human pharyngeal cells *in vitro*. *Infection and Immunity, 71*(5), 2758-2765.

Knight, G. M., McIntyre, J. M., Craig, G. G., Mulyani, & Zilm, P. S. (2008). The inability of *Streptococcus mutans* and *Lactobacillus acidophilus* to form a biofilm *in vitro* on dentine pretreated with ozone. *Australian Dental Journal, 53*(4), 349-353.

Koch, A. L. (1970). Turbidity measurements of bacteria cultures in some available commercial instruments. *Analytical Biochemistry, 38*(1), 252-259.

Kojima, Y., Ohshima, T., Seneviratne, C. J., & Maeda, N. (2015). Combining prebiotic and probiotics to develop novel synbiotic that supress oral pathogen. *Journal of oral bioscience, 58*, 27-32.

Koll-Klais, P., Mandar, R., Leibur, E., & Kjaeldgaard, M. (2004). High levels of salivary Lactobacilli in estonian schoolchildren. *European Journal of Paediatrict Dentisrty, 5*(2), 107-109.

Kondepudi, K. K., Ambalam, P., Nilsson, I., Wadström, T., & Ljungh, A. s. (1012). Prebiotic-non-digestible oligosaccharides preference of probiotic bifidobacteria and antimicrobial activity against clostridium difficile. *Clinical microbiology, 18*(5), 489-497.

Kuhnert, W. L., Zheng, G., Faustoferri, R. C., & Quivey, R. G., Jr. (2004). The f-atpase operon promoter of *Streptococcus mutans* is transcriptionally regulated in response to external pH. *Journal of Bacteriology, 186*(24), 8524-8528.

Lamont, R. J., & Jenkinson, H. F. (2010). Microbilogy of caries *Oral microbilogy at glance* (1st ed., pp. 34-35). Singapore: Blackwell.

Lamsal, B. P. (2012). Production, health aspects and potential food uses of dietary prebiotic gaactooligosaccharides. *Journal of the Science of Food and Agriculture, 92*(10), 2020-2028.

Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, D. (1970). *Lactobacillus acidophilus (moro 1900) hansen and mocquot.* N.P.: n.p.

Li, J. Y., Zhang, L. W., Du, M., Han, X., Yi, H. X., Guo, C. F., . . . Hou, A. J. (2011). Effect of tween series on growth and cis-9, trans-11 conjugated linoleic acid production of *Lactobacillus acidophilus* f0221 in the presence of bile salts. *International Journal of Molecular Sciences, 12*(12), 9138-9154.

Lin, X., Chen, X., Tu, Y., Wang, S., & Chen, H. (2017). Effect of probiotic lactobacilli on the growth of *Streptococcus mutans* and multispecies biofilms isolated from children with active caries. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research, 23*, 4175-4181.

Liu, M., Bayjanov, J. R., Renckens, B., Nauta, A., & Siezen, R. J. (2010). The proteolytic system of lactic acid bacteria revisited: A genomic comparison. *BMC Genomics, 11*, 36.

Loesche, W. J. (1986). Role of *Streptococcus mutans* in human dental decay. *Microbiology Reviews, 50*(4), 353-380.

Loesche, W. J., Stephen, E., Earnest, R., & Burt, B. (1984). Longitudinal investigation of bacteriology of human fissure decay: Epidemiological studies in molars shortly after eruption. *Infection and Immunity,* 46(3), 765-772.

Macfarlane, G. T., Steed, H., & Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology, 104*(2), 305-344.

Mahajan, A., Singh, B., Kashyap, D., Kumar, A., & Mahajan, P. (2013). Interspecies communication and periodontal disease. *Scientific World Journal, 2*(7) 65-68.

Malanovic, N., & Lohner, K. (2016). Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochimica et Biophysica Acta (BBA)-Biomembranes,1858*(5), 936-946.

Mathews, C. K., hold, K. E. v., & Ahern, K. G. (2000). *Biochemistry*. N.P.: Robin Heyden.

McGhie, D., Hutchison, J. G., Nye, F., & Ball, A. P. (1977). Infective endocarditis caused by streptococcus mutans. *British Heart Journal, 39*(4), 456-458.

MIDI, I. (2012). *Sherlock microbial identification system version 6.2 (MIS operating manual)*. N.P.: n.p.

Moynihan, P. J. (1998). Update on the nomenclature of carbohydrates and their dental effects. *Journal of Dentistry, 26*(3), 209-218.

Mussatto, S. I., & Mancilha, I. M. (2007). Non-digestable oligosacharides: A review. *Carbohydrate polymer, 68*, 587-597.

Nair, G. B., & Takeda, Y. (2011). Probiotic foods in health and disease (1st ed., pp. 87- 109). India: Science.

Nase, L., Hatakka, K., Savilahti, E., Saxelin, M., Ponka, A., Poussa, T., . . . Meurman, J. H. (2001). Effect of long-term consumption of a probiotic bacterium, *Lactobacillus rhamnosus* gg, in milk on dental caries and caries risk in children. *Caries reseach, 35*(6), 412- 420.

Naumoff, D. G. (2001). Beta-fructosidase superfamily: Homology with some alpha-l-arabinases and beta-d-xylosidases. *Proteins, 42*(1), 66-76.

Nikawa, H., Makihira, S., Fukushima, H., Nishimura, H., Ozaki, Y., Ishida, K., . . . Aimi, R. (2004). *Lactobacillus reuteri* in bovine milk fermented decreases the oral carriage of mutans streptococci. *International journal of Food Microbial, 95*(2), 219-223.

Nyvad, B., & Kilian, M. (1990). Comparison of the initial streptococcal microflora on dental enamel in caries-active and caries-inactve individuals. *caries reseach, 24*, 267 - 272.

Obst, M., Hehn, R., Vogel, R. F., & Hammes, W. P. (1992). Lactose metabolism in *Lactobacillus curvatus* and *Lactobacillus sake*. *FEMS Microbiology Letters, 97*(3), 209-214.

Ocaña, V., & Nader-Macías, M. E. (2001). Adhesion of *Lactobacillus vaginal* strains with probiotic properties to vaginal epithelial cells. *Biocell: official jurnal of the Sociedades Latinoamericans de Microscopia Electronica, 25*(3), 265-273.

Oh, S., Rheem, S., Sim, J., Kim, S., & Baek, Y. (1995). Optimizing conditions for the growth of *Lactobacillus casei* yit 9018 in tryptone-yeast extract-glucose medium by using response surface methodology. *Applied and Environmenntal Microbiology, 61*(11), 3809-3814.

Oliveira, A. J. B. d., Goncalves, R. A. C., Chierrito, T. P. C., Santos, M. M. l. d., Souza, L. M. d., Gorin, P. A. J., . . . Iacomini, M. (2011). Structure and degree of polymerisation of fructo-oligosaccharides present in roots and leaves of stevia rebaudiana (bert.) bertoni. *Food Chemistry, 129*(2), 305-311.

Ortakci, F., & Sert, S. (2012). Stability of free and encapsulated *Lactobacillus acidophilus* ATCC4356 in yogurt and in an artificial human gastric digestion system. *Journal of Dairy Science, 95*(12), 6918-6925.

Pan, X.-d., Chen, F.-q., Wu, T.-x., Tang, H.-g., & Zhao, Z.-y. (2009). Prebiotic oligosaccharides change the concentrations of short-chain fatty acids and the microbial population of mouse bowel. *Journal of Zhejiang University. Science. B, 10*(4), 258-263.

Pandey, N., Malik, R. K., Kaushik, J. K., & Singroha, G. (2013). Gassericin a: A circular bacteriocin produced by lactic acid bacteria *Lactobacillus gasseri*. *World Journal of Microbioogy and Biotechnology, 29*(11), 1977-1987.

Park, S.-N., Ahn, S.-J., & Kook, J.-K. (2015). Oleanolic acid and ursolic acid inhibit peptidoglycan biosynthesis in *Streptococcus mutans* UA159. *Brazilian Journal of Microbiology, 46*(2), 613-617.

Perrone, M., Gfell, L. E., Fontana, M., & Gregory, R. L. (1997). Antigenic characterization of fimbria preparations from *Streptococcus mutans* isolates from caries-free and caries-susceptible subjects. *Clinical Diagnostic Laboratory Immunology, 4*(3), 291-296.

Peters, Jeanne S., & Chin, C.-K. (2003). Inhibition of photosynthetic electron transport by palmitoleic acid is partially correlated to loss of thylakoid membrane proteins. *Plant Physiology and Biochemistry, 41*(2), 117-124.

Petersen, F. C., Assev, S., van der Mei, H. C., Busscher, H. J., & Scheie, A. A. (2002). Functional variation of the antigen i/ii surface protein in *Streptococcus mutans* and *Streptococcus intermedius*. *Infection and Immunity, 70*(1), 249-256.

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., . . . Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology, 1*, 57-63.

Quigley, M. E., Hudson, G. J., & Englyst, H. N. (1999). Determination of resistant short-chain carbohydrates (non-digestible oligosaccharides) using gas-liquid chromatography. *Food Chemistry, 66*(3), 381-390.

Quivey, R. G., Kuhnert, W. L., & Hahn, K. (2000). Adaptation of oral streptococci to low pH *Advances in microbial physiology* (Vol. 42, pp. 239-274). N.P.: Academic Press.

Raj, A., Bhati, P., & Bhadekar, R. (2015). Effect of lactic acid bacteria on biofilm formation by *Streptococcus mutans*: An *in vitro* study. *Internatioal journal of phamaceutical sciences and research*, 2533-2538.

Ray, C. A., Gfell, L. E., Buller, T. L., & Gregory, R. L. (1999). Interactions of *Streptococcus mutans* fimbria-associated surface proteins with salivary components. *Clinical Diagnostic Laboratory Immunology, 6*(3), 400-404.

Resta-Lenert, S., & Barrett, K. E. (2003). Live probiotics protect intestinal epithelial cells from the effects of infection with enteroinvasive *Escherichia coli* (EIEC). *Gut, 52*(7), 988-997.

Roberfroid, M. (2007). Introducing inulin-type fructans. *British Journal of Nutrition, 93*(S1), S13-S25.

Roberfroid, M. (2007). Prebiotics: The concept revisited. *The Journal of Nutrition, 137*, 830S-837S.

Robinson, R. K., Carl A, B., & Patel, P. D. (2000). Encyclopedia of food microbiology. *Streptococcus* (Vol. 2, pp. 2117-2127). Great Britain: The Bath Press.

Robinson, R. K., Carl A, B., & Patel, P. D. (2000). Encyclopedia of food microbiology. *Lactobacillus* (Vol. 2, pp. 1134 - 1157). Great Britain: The Bath Press.

Rogers, H. J., Perkins, H. R., & Ward, J. B. (1980). Membrane structure and composition in micro-organisms *Microbial cell walls and membranes* (pp. 72-104). Dordrecht: Springer Netherlands.

Rose, R. K., Hogg, S. D., & Shellis, R. P. (1994). A quantitative study of calcium binding by isolated streptococcal cell walls and lipoteichoic acid: Comparison with whole cells. *Journal of Dental Research, 73*(11), 1742-1747.

Russell, R. R., Aduse-Opoku, J., Sutcliffe, I. C., Tao, L., & Ferretti, J. J. (1992). A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. *Journal of Biology Chemistry, 267*(7), 4631-4637.

Rustan, A. C., & Drevon, C. A. (2001). Fatty acids: Structures and properties Encyclopedia of life sciences (pp. 1-7). New York: John Wiley & Sons.

Ryan, V., Hart, T. R., & Schiller, R. (1980). Size determination of *Streptococcus mutans* 10499 by laser light scattering. *Biophysical Journal, 31*(3), 313-324.

Saad, N., Delattre, C., Urdaci, M., Schmitter, J. M., & Bressollier, P. (2013). An overview of the last advances in probiotic and prebiotics field. *LWT- Food Science and Technology, 50*, 1-16.

Saavedra, J. M., & Tschernia, A. (2007). Human studies with probiotics and prebiotics: Clinical implications. *British Journal of Nutrition, 87*(S2), S241-S246.

Sabater-Molina, M., Larque, E., Toorella, F., & Zamora, S. (2009). Dietary fructo-oligosaccharides and potential benefits on health. *The Journal of physiology and Biochemistry, 65*(3), 315-328.

Saini, R., Saini, S., & Sharma, S. (2011). Biofilm: A dental microbial infection. *Journal of Natural Science, Biology, and Medicine, 2*(1), 71-75.

Sako, T., Mastumoto, K., & Tanaka, R. (1999). Recent progress on research and applications of non-digestable galacto-oliogosacchrides. *International Diary Journal, 9*, 69-80.

Scientific Committee on Food, SCF. (2001). *Statement on the use of resistant short chain carbohydrates (oligofructose and oligogalactose) in infant formulae and in follow-on formulae*. Retrieved November 22, 2015, from https://ec.europa.eu/food/sites/food/files/.../sci-com_scf_out103_en.pdf

Scientific Committee on Food, SCF. (2003). Report of the scientific committee on food on the revision of essential requirements of infant formulae and follow-on formulae. Retrieved November 22, 2015, from https://ec.europa.eu/food/sites/food/files/safety/docs/sci-com_scf_out199_en.pdf

Shigwedha, N., Hal, P. H.-V., Jia, L., Sichel, L., & Zhang, S. (2016). Prebiotics: Metabolism and symbiotic synergy with probiotics in promoting health. In V. Rao & L. G. Rao (Eds.), *Probiotics and prebiotics in human nutrition and health* (pp. 41-57). Rijeka: InTech.

Silza, M., Jacobus, N. V., Deneke, C., & Gorbachi, S. L. (1987). Antimicrobial substance from a human Lactobacillus strain. *Antimicrobial Agents and Chemotherpy, 31*(8), 1231-1233.

Singh, R. P., Damle, S. G., & Chawla, A. (2011). Salivary mutans streptococci and lactobacilli modulations in young children on consumption of probiotic ice-cream containing bifidobacterium lactis Bb12 *and Lactobacillus acidophilus* La5. *Acta Odontologica Scandinavica, 69*(6), 389-394.

Speert, D. P., Wannamaker, L. W., Gray, E. D., & Clawson, C. C. (1979). Bactericidal effect of oleic acid on group a streptococci: Mechanism of action. *Infection and Immunity, 26*(3), 1202-1210.

Spiegel, J. E., Rose, R., Karabell, P., Frankos, V. H., & Schmitt, D. F. (1994). Safety and benefits of fructo-oligosaccharides as food ingredients. *Food Technology, 48*(1), 389-394.

Stamatova, I., & Meurman, J. H. (2009). Probiotics: Health benefits in the mouth. *American Journal of dentistry, 22*, 329 - 338.

Streit, F., Delettre, J., Corrieu, G., & Beal, C. (2008). Acid adaptation of *Lactobacillus delbrueckii* subsp. *Bulgaricus* induces physiological responses at membrane and cytosolic levels that improves cryotolerance. *Journal of Applied Microbiology, 105*(4), 1071-1080.

Tahmourespour, A., & Kermanshahi, R. K. (2011). The effect of a probiotic strain (lactobacillus acidophilus) on the plaque formation of oral streptococci. *Bosnian Journal of Basic Medical Sciences, 11*(1), 37-40.

Teanpaisan, R., & Dahlen, G. (2006). Use of polymerase chain reaction techniques and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for differentiation of oral Lactobacillus species. *Oral Microbiology and Immunology, 21*(2), 79-83.

Tester, R. F., & Al-Ghazzewi, F. H. (2011). A preliminary study of the synbiotic effects of conjac glucomannan hydrolysates(gmh) and lactobacilli on the the growth of the oral bacterium *Streptococcus mutans*. *Nutrition & Food Science, 41*(4), 234 - 237.

Teughels, W., Van Essche, M., Sliepen, I., & Quirynen, M. (2008). Probiotics and oral healthcare. *Periodontology 2000, 48*, 111-147.

Teuri, U., Korpela, R., Saxelin, M., Montonen, L., & Salminen, S. (1998). Increased fecal frequency and gastrointestinal symptoms following ingestion of galacto-oligosaccharide-containing yogurt. *Journal of Nutritional Science and Vitaminology (Tokyo), 44*(3), 465-471.

Todar, K. (2008). Todar's online textbook of bacteria (pp. unpage). Retrieved from http://www.textbookofbacteriology.net/structure_5.html.

Torres, D. P. M., Gonçalves, M. d. P. F., Teixeira, J. A., & Rodrigues, L. R. (2010). Galacto-oligosaccharides: Production, properties, applications, and significance as prebiotics. *Comprehensive review in Food Science and Food safety, 9*(5), 438-454.

Vollmer, W. (2008). Structural variation in the glycan strands of bacterial peptidoglycan. *FEMS Microbiology Reviews, 32*(2), 287-306.

Vrese, M. d., Stegelmann, A., Bernd Richter, S. F., Laue, C., & Schrezenmeir, J. (2001). Probiotics—compensation for lactase insufficiency. *The American Journal of Clinical Nutrition, 73*(2), 421S-429S.

Wallock-Richards, D. J., Marles-Wright, J., Clarke, D. J., Maitra, A., Dodds, M., Hanley, B., & Campopiano, D. J. (2015). Molecular basis of *Streptococcus mutans* sortase a inhibition by the flavonoid natural product trans-chalcone. *Chemical Communications, 51*(52), 10483-10485.

Wang, L. L., & Johnson, E. A. (1992). Inhibition of *Listeria monocytogenes* by fatty acids and monoglycerides. *Applied Environmental Microbiology, 58*(2), 624-629.

Wasfi, R., Abd El-Rahman, O., Zafer, M., & M. Ashour, H. (2018). Probiotic lactobacillus sp. Inhibit growth, biofilm formation and gene expression of caries-inducing *Streptococcus mutans*. *Journal of medicine and biochemistry.*

Watanuki, M., Y. Wada, and K. Matsumoto. (1996). Digestibility and physiological heat of combustion of β-1-6 galacto-oligosaccharides *in vitro*. *Annu. Rep. Yakult Central Inst. Microbiol. Res., 16,* 1-12.

Watson, D., O'Connell Motherway, M., Schoterman, M. H., van Neerven, R. J., Nauta, A., & van Sinderen, D. (2013). Selective carbohydrate utilization by Lactobacilli and Bifidobacteria. *Journal of Applied Microbiology, 114*(4), 1132-1146.

Wecke, J., Madela, K., & Fischer, W. (1997). The absence of d-alanine from lipoteichoic acid and wall teichoic acid alters surface charge, enhances autolysis and increases susceptibility to methicillin in *Bacillus subtilis*. *Microbiology Society Journal, 143*(9), 2953-2960.

Wei, L., Keqi, W., Yi, S., Hong, Y., Bing, H., & Xiaoxing, Z. (2015). Influences of structures of galactooligosaccharides and fructooligosacharides on the fermentation in vitro by human intestinal microbiota. *Journal of functional food, 13*, 158-168.

Widdel, F. (2007). Theory and measurement of bacterial growth. *Di dalam Grundpraktikum Mikrobiologie, 4*(11), 1-11.

Wu, C. C., Lin, C. T., Wu, C. Y., Peng, W. S., Lee, M. J., & Tsai, Y. C. (2015). Inhibitory effect of *Lactobacillus salivarius* on *Streptococcus mutans* biofilm formation. *Molecular Oral Microbiology, 30*(1), 16-26.

Yli-Knuuttila, H., Snall, J., Kari, K., & Meurman, J. H. (2006). Colonization of *Lactobacillus rhamnosus* GG in the oral cavity. *Oral Microbiol Immunol, 21*(2), 129-131. doi:10.1111/j.1399-302X.2006.00258.x

Yu, L., Lei, T., Ren, X., Pei, X., & Feng, Y. (2008). Response surface optimization of l-(+)-lactic acid production using corn steep liquor as an alternative nitrogen source by *Lactobacillus rhamnosus* CGMCC 1466. *Biochemical Engineering Journal, 39*(3), 496-502.

Zeng, L., & Burne, R. A. (2013). Comprehensive mutational analysis of sucrose-metabolizing pathways in *Streptococcus mutans* reveals novel roles for the sucrose phosphotransferase system permease. *Journal of Bacteriology, 195*(4), 833-843.

APPENDIX

ใบรับรองด้านความปลอดภัยทางชีวภาพ

ชื่อโครงการ (ภาษาไทย)	**การส่งเสริมการทำงานของโปรไบโอติกด้วยพรีไบโอติกในการยับยั้งการเจริญเติบโตของจุลินทรีย์ก่อโรคฟันผุ**
ชื่อโครงการ (ภาษาอังกฤษ)	The enhancement of prebiotics on probiotics of inhibiting dental caries pathogen
ชื่อผู้วิจัย	นายสันติชัย นุ่นปาน
ชื่ออาจารย์ที่ปรึกษา	ทญ.ดร.กรชนก วชัคผาบนท์
สังกัดหน่วยงาน/คณะ	คณะทันตแพทยศาสตร์
เลขสำคัญโครงการ	NUIBC MI 59-09-56
เลขที่รับรองโครงการ	60-06
ประเภทการรับรอง	งานประเภทที่ 2
การรับรองครั้งที่ 1	ข้อเสนอการวิจัยนี้ได้ผ่านการพิจารณาและรับรองจากคณะกรรมการเพื่อความปลอดภัยทางชีวภาพ มหาวิทยาลัยนเรศวร ครั้งที่ 1/2560 วันที่ 20 ตุลาคม 2559 เห็นว่ามีความสอดคล้องกับแนวทางปฏิบัติเพื่อความปลอดภัยทางชีวภาพ จึงเห็นควรให้ดำเนินการวิจัยด้านความปลอดภัยทางชีวภาพ ตามข้อเสนอการวิจัยนี้ได้
วันหมดอายุครั้งที่ 1	วันที่ 20 ตุลาคม 2560

ลงนาม

(ดร.วิสาข์ สุพรรณไพบูลย์)
ประธานคณะกรรมการเพื่อความปลอดภัยทางชีวภาพ
มหาวิทยาลัยนเรศวร

หมายเหตุ ใบรับรองฉบับนี้ใช้ควบคู่กับหนังสือราชการ เลขที่ ศธ 0527.01.33(1)/ว 2645 ลงวันที่ 10 พฤศจิกายน 2559

ศูนย์จุลินทรีย์
สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย
35 หมู่ 3 เทคโนธานี ต.คลองห้า อ.คลองหลวง จ.ปทุมธานี 12120
โทร: 0 2577 9034 โทรสาร: 0 2577 9031, 0 2577 9009

ข้อตกลงในการให้บริการสายพันธุ์จุลินทรีย์ วว.

คลังเก็บรักษาสายพันธุ์จุลินทรีย์ วว. (TISTR Culture Collection) ฝ่ายวิทยาศาสตร์ชีวภาพ (ฝวช.) สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย (วว.) ให้บริการสายพันธุ์จุลินทรีย์ตามคำขอบริการเฉพาะผู้ใช้บริการที่ตกลงปฏิบัติตามเงื่อนไขดังต่อไปนี้

1. สายพันธุ์จุลินทรีย์ ชิ้นส่วนสารพันธุกรรม (DNA, RNA replicates) และอนุพันธ์ต่าง ๆ (derivertives) ที่สร้างโดยสายพันธุ์จุลินทรีย์ที่ให้บริการโดย วว. เพื่อการศึกษา ค้นคว้า วิจัย ทดสอบ และวัตถุประสงค์อื่นที่ได้รับอนุญาตจาก วว. โดยไม่รวมวัตถุประสงค์เชิงพาณิชย์ ทั้งนี้ผู้ใช้บริการสายพันธุ์จุลินทรีย์ต้องคำนึงถึงความปลอดภัยต่อสาธารณะ (ensured public safety) หากเป็นสายพันธุ์ที่เป็นพิษ ผู้ใช้บริการจะต้องทราบแม้ไม่มีการบ่งบอก และยอมรับความเสี่ยงที่เกิดขึ้นโดยปฏิบัติตามกฎหมาย (Law) กฎเกณฑ์ (Regulation) และข้อกำหนด (Guideline) ของประเทศไทย และระหว่างประเทศ
2. ผู้ใช้บริการต้องทำการเก็บรักษา และทำลายสายพันธุ์จุลินทรีย์ภายหลังการใช้อย่างเหมาะสมและถูกต้องตามหลักวิชาการ
3. ผู้ใช้บริการไม่สามารถจดทะเบียนหรือมีสิทธิ์ในทรัพย์สินทางปัญญาทุกประเภทจากสายพันธุ์จุลินทรีย์
4. หากผู้ใช้บริการมีการเปลี่ยนแปลงวัตถุประสงค์ในการใช้สายพันธุ์จุลินทรีย์จะต้องแจ้งให้ วว. ทราบเป็นลายลักษณ์อักษรและต้องได้รับอนุญาตจาก วว. ก่อนจึงจะดำเนินการตามวัตถุประสงค์ใหม่ได้
5. ผู้ใช้บริการไม่สามารถส่งมอบสายพันธุ์จุลินทรีย์ ชิ้นส่วนสารพันธุกรรม และอนุพันธ์ต่าง ๆ แก่บุคคลที่สาม เว้นแต่เป็นผู้ร่วมงาน/โครงการที่เกี่ยวข้องในหน่วยงานเดียวกัน หากผู้ใช้บริการต้องการส่งมอบสายพันธุ์จุลินทรีย์ให้แก่ผู้ร่วมงาน/โครงการที่อยู่ต่างหน่วยงาน ผู้ใช้บริการจะต้องแจ้งให้ วว. ทราบเป็นลายลักษณ์อักษรและต้องได้รับอนุญาตจาก วว. ก่อนจึงจะดำเนินการได้
6. ผู้ใช้ต้องแสดงรหัสประจำสายพันธุ์ (TISTR number เช่น TISTR 006) ในรายงานและสิ่งตีพิมพ์ทุกประเภท พร้อมทั้งต้องจัดส่งสิ่งตีพิมพ์ดังกล่าวแก่ วว. จำนวน 2 ชุด โดยอาจอยู่ในรูปของเอกสาร (reprint) สำเนา (photocopy) หรือแผ่น CD
7. หากการใช้สายพันธุ์จุลินทรีย์ของผู้ใช้บริการกุดคามค่อสิทธิหรือสร้างความเดือดร้อนแก่บุคคลอื่น ผู้ใช้บริการต้องรับผิดชอบและดำเนินการแก้ไขปัญหาด้วยตนเอง โดยไม่สามารถเรียกร้องค่าเสียหายใด ๆ จาก วว.
8. ข้อตกลงนี้มีอายุสามปี (3) ปี นับแต่วันลงนาม หากครบตามกำหนดแล้ว ผู้ใช้บริการต้องการใช้สายพันธุ์จุลินทรีย์นั้นต่อ ผู้ใช้บริการต้องแจ้งให้วว. ทราบเป็นลายลักษณ์อักษรและต้องได้รับอนุญาตจากวว.ก่อนจึงจะดำเนินการได้
9. สายพันธุ์จุลินทรีย์ภายใต้ข้อตกลงนี้ ได้แก่ TISTR 1365T

ข้าพเจ้าได้รับทราบและยินดีปฏิบัติตามข้อตกลงดังกล่าวข้างต้นทุกประการ

"ผู้ใช้บริการ"

(ลงชื่อ) .....
(.....)
หน่วยงาน .....
.....
วันที่ 25 ก.ค. 59

วว.TISTR

Table 17 The percentages of cellular fatty acids from *S. mutans* cultured in prebiotics-supplemented media

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
Saturated fatty acids											
Capric acid ($C_{10:0}$)	-	-	0.22	-	0.25	-	0.29	-	0.21	0.16	0.23
9-methyl undecanoic acid ($C_{12:0\ anteiso}$)	-	0.22	-	-	-	0.20	0.52	0.33	-	-	-
Lauric acid ($C_{12:0}$)	0.78	0.36	0.57	-	0.53	0.55	0.81	0.33	0.47	0.66	0.64
Myristic acid ($C_{14:0}$)	2.61	1.50	1.90	1.75	1.91	2.36	1.79	2.09	1.90	2.68	2.31
13-Methyltetradecanoic acid ($C_{15:0\ iso}$)	-	0.11	0.08	1.24	0.15	0.32	0.18	0.25	0.12	0.18	0.15
12-Methyl myristic acid ($C_{15:0\ anteios}$)	-	0.21	0.21	-	0.32	0.42	0.42	0.41	0.35	0.35	0.48
14-Methylpentadecanoic acid ($C_{16:0\ iso}$)	-	-	-	4.57	-	0.42	-	1.46	-	0.20	0.11
Palmitic acid ($C_{16:0}$)	36.37	31.50	31.68	28.14	30.92	36.32	28.61	-	32.25	42.22	33.44

Table 17 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
10-Methylhexadecanoic acid ($C_{16:0\ 10\text{-methyl}}$)	-	-	-	-	-	-	-	0.63	-	-	-
15-Methylhexadecanoic acid ($C_{17:0\ iso}$)	-	-	-	1.37	-	0.20	-	0.19	-	0.05	-
14-Methylhexadecanoic acid ($C_{17:0\ anteiso}$)	-	-	0.09	3.84	0.11	0.19	0.29	0.44	-	0.10	0.26
Heptadecanoic acid ($C_{17:0}$)	-	0.26	0.22	1.38	0.20	0.23		0.39	0.23	0.21	0.20
10-Methylheptadecanoic acid ($C_{17:0\ 10\text{-methyl}}$)	-	-	-	-	-	-	-	1.60	-	-	-
Steric acid ($C_{18:0}$)	5.43	6.63	5.71	5.23	5.33	5.27	5.19	5.24	5.19	7.93	5.80
$C_{17:0\ 2OH}$	-	0.40	0.56	-	0.62	0.40	0.75	0.42	0.56	0.47	0.58

Table 17 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
10-Methyloctadecanoic acid ($C_{18:0\ 10\text{-methyl, TBSA}}$)	-	0.08	-	-	-	-	-	0.16	-	-	-
17-Methyloctadecanoic acid ($C_{19:0\ iso}$)	-	0.56	0.50	2.27		0.38	0.80	0.51	0.49	0.49	-
Arachidic acid ($C_{20:0}$)	-	0.74	0.62	0.27	0.63	0.36	0.78	0.39	0.62	0.75	0.55
Unsaturated fatty acids											
(10Z)-10-Pentadecenoic acid ($C_{15:1\ \omega5c}$)	-	-	-	-	0.16	-	-	-	-	-	-
cis-7-Palmitoleic acid ($C_{16:1\ \omega9c}$)	3.94	2.11	2.58	2.09	2.65	3.06	2.34	2.68	2.49	3.24	2.82
cis-11-Palmitoleic acid ($C_{16:1\ \omega5c}$)	0.59	0.32	0.54	-	0.53	0.59	0.46	-	0.52	0.59	0.54
$C_{17:1\ iso\ \omega5c}$	-	-	-	-	-	-	0.21	-	-	-	-

Table 17 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
(7Z)-13-Methyl-7-Hexadecenoic acid ($C_{17:1\ anteiso\ \omega9c}$)	-	0.10	0.11	-	0.13	-	0.22	0.15	0.10	0.07	0.08
(11Z)-11-Icosenoic acid ($C_{20:1\ \omega9c}$)	16.41	21.09	18.87	-	17.60	14.98	17.59	14.54	18.63	-	16.35
Oleic acids ($C_{18:1\ \omega9c}$)	19.80	20.79	22.79	24.35	24.73	19.33	27.93	19.73	24.59	21.76	21.31
$C_{18:1\ \omega7c}$	14.07	12.71	12.50	10.76	12.21	14.14	10.36	12.85	11.14	17.40	13.08
$C_{19:1\ iso\ I}$	-	0.18	0.16	-	0.15	0.14	0.18	0.15	0.49	0.26	0.25
Gondoic acid ($C_{20:1\ \omega9c}$)	16.41	21.09	18.87	15.25	17.60	14.98	17.59	14.54	18.63	-	16.35
Unsaturated fatty acids	54.81	57.43	57.66	52.47	58.41	52.38	59.29	51.15	57.61	43.54	54.60
Saturated fatty acids	45.19	42.57	42.36	47.52	41.57	47.62	40.43	48.85	42.39	56.45	44.75

Note: * Fatty acids name (amount of carbon atom: amount of double bond in structure)

Table 18 The percentages of secreted fatty acids from *S. mutans* cultured in prebiotics-supplemented media

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
Saturated fatty acids											
Capric acid ($_{C10:0}$)	0.32	-	0.32	-	-	-	0.31	0.99	0.26	1.28	0.35
9-Methylundecanoic acid (12:0 anteiso)	0.41	-	0.45	-	-	0.74	0.34	0.38	0.43	0.37	0.37
Lauric acid (12:0)	0.23	-	0.38	-	-	-	0.28	0.14	0.19	0.17	0.26
Myristic acid (14:0)	0.38	0.32	0.35	0.28	-	0.63	0.29	0.38	0.27	0.37	0.27
13-Methyltetradecanoic acid (15:0 iso)	-	-	-	0.14	-	0.23	-	0.75	-	-	-
12-Methylmyristic acid (15:0 anteiso)	0.13	-	-	0.22	-	0.63	-	0.38	-	-	-
14-Methylpentadecanoic acid (16:0 iso)	-	-	-	0.26	-	0.36	-	1.94	-	-	-
Palmitic acid (16:0)	13.55	15.15	13.92	16.06	14.34	15.69	13.77	14.39	13.56	13.66	13.24
15-Methylhexadecanoic acid (17:0 iso)	-	-	-	0.15	-	-	-	0.76	-	-	-

Table 18 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
14-Methylhexadecanoic acid ($C_{17:0\ anteiso}$)	-	-	-	0.30	-	-	-	1.66	-	-	-
Margaric acid ($C_{17:0}$)	0.22	0.31	0.28	0.18	-	-	0.19	0.50	0.33	0.16	0.21
Stearic acid ($C_{18:0}$)	3.23	3.46	3.25	2.49	2.86	2.38	2.96	3.77	3.29	3.01	3.81
$C_{17:0\ 2OH}$	3.66	2.90	3.30	3.24	3.44	3.33	3.61	2.66	3.27	3.19	3.57
17-Methylstearic acid ($C_{19:0\ iso}$)	1.86	2.17	1.75	2.27	1.62	2.57	1.76	1.99	1.76	2.10	1.75
16-Methyloctadecanoic acid ($C_{19:0\ anteiso}$)	0.18	-	-	-	-	-	-	-	-	-	-
Arachidic acid ($C_{20:0}$)	0.31	0.44	0.42	0.27	-	-	0.30	0.29	0.35	0.31	0.32
Unsaturated fatty acids											
(10Z)-10-Pentadecenoic acid ($C_{15:1\ \omega5c}$)	-	-	-	-	-	-	-	0.23	-	-	0.63

Table 18 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
(12Z)-12-Heptadecenoic acid ($C_{17:1iso\ \omega5c}$)	-	-	-	-	-	-	-	-	-	-	0.77
(7Z)-13-Methyl-7-Hexadecenoic acid ($C_{17:1\ anteiso\ \omega9c}$)	0.18	0.17	0.19	-	-	0.40	0.13	0.17	0.17	0.15	0.13
cis-Margarolcic acid ($C_{17:1\ \omega8c}$)	0.21	0.19	0.20	0.18	-	-	0.19	0.19	0.18	0.20	0.21
Oleic acid ($C_{18:1\ \omega9c}$)	74.18	74.05	74.25	72.70	76.66	71.41	74.93	67.42	74.76	73.63	73.20
(12Z)-12-Nonadecenoic acid ($C_{19:1\ \omega7c}$)	-	-	-	-	-	0.38	-	-	-	-	-
Gondoic acid ($C_{20:1\ \omega9c}$)	-	-	-	-	-	-	-	-	-	0.51	-
Unsaturated fatty acids	75.52	75.25	75.60	73.95	77.74	73.43	76.19	69.02	76.30	75.38	75.84
Saturated fatty acids	24.48	24.75	24.42	25.86	22.26	26.56	23.81	30.98	23.71	24.62	24.15

Note: * Fatty acids name (amount of carbon atom: amount of double bond in structure)

Table 19 The percentages of cellular fatty acids from *L. acidophilus* cultured in prebiotics-supplemented media

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
Saturated fatty acids											
Capric acid ($C_{10:0}$)	-	-	-	0.18	-	-	-	-	-	-	-
9-Methylundecanoic acid ($C_{12:0\ anteiso}$)	0.68	0.68	0.57	-	0.64	0.69	-	0.78	-	0.51	-
Lauric acid ($C_{12:0}$)	0.57	0.36	0.92	0.63	-	0.37	-	0.99	-	0.32	
Myristic acid ($C_{14:0}$)	0.97	0.64	-	2.32	0.98	0.91	0.91	0.84	0.91	0.65	0.9
12-Methylmyristic acid ($C_{15:0\ anteios}$)	-	-	-	0.12	-	-	-	-	-	-	-
Palmitic acid ($C_{16:0}$)	8.3	7.95	7.5	44.83	7.88	7.81	7.63	8.78	7.78	7.15	8.56
Margaric acid ($C_{17:0}$)	-	-	-	0.2	-	-	-	-	-	-	-
Stearic acid ($C_{18:0}$)	1.79	2.11	2.12	10.27	2.14	1.74	1.94	2.10	1.57	1.62	2.38

Table 19 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
C $_{17:0\ 2OH}$	1.31	1.16	1.02	0.52	1.17	1.23	1.28	1.28	1.35	1.06	1.14
17-Methylstearic acid (C $_{19:0\ iso}$)	1.19	1.30	1.2	0.48	1.32	1.13	1.29	1.33	1.29	0.98	1.37
Arachidic acid (C $_{20:0}$)	12.74	7.44	10.63	1.01	9.96	8.67	10.15	7.29	8.17	8.38	7.12
Unsaturated fatty acids											
(10Z)-10-Pentadecenoic acid (C $_{15:1\ \omega5c}$)	-	0.35	-	-	-	-	-	-	-	-	
Palmitoleic acid (C $_{16:1\ \omega7c}$)	1.31	1.26	1.12		1.14	1.63	1.1	1.15	1.24	1.32	1.87
cis-7-Palmitoleic acid (C $_{16:1\ \omega}$)	-	-	-	2.16	-	-	-	-	-	-	-
cis-11-Palmitoleic acid (C $_{16:1\ \omega5c}$)	-	-	-	0.38	-	-	-	-	-	-	-
C $_{17:1\ iso\ \omega5c}$	-	0.44	-	-	0.3	-	-	-	-	-	0.28

Table 19 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
cis-Margarolcic acid ($C_{17:1\ \omega8c}$)	-	-	-	0.28	-	-	-	-	-	0.24	-
Olcic acid ($C_{18:1\ \omega9c}$)	66.63	68.28	67.43	18.01	67.67	67.74	68.13	67.89	69.48	67.30	68.31
cis-Vaccenic acid ($C_{18:1\ \omega7c}$)	-	-	-	18.48	-	-	-	-	-	-	-
(12Z)-12-Nonadecenoic acid ($C_{19:1\omega7c}$)	4.24	7.75	7.48	-	6.55	7.8	7.31	7.25	7.94	10.28	8.05
Arachidic acid ($C_{20:0}$)	12.74	7.44	10.63	1.01	9.96	8.67	10.15	7.29	8.17	8.38	7.12
Unsaturated fatty acids	**72.45**	**79.0**	**76.04**	**39.37**	**75.91**	**77.45**	**76.8**	**76.61**	**78.93**	**79.33**	**78.53**
Saturated fatty acid	**27.55**	**21.0**	**23.96**	**60.63**	**24.09**	**22.55**	**23.2**	**23.39**	**21.07**	**20.67**	**21.47**

Table 19 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
Saturated fatty acids											
Capric acid ($C_{10:0}$)	-	0.31	0.27	0.87	0.28	1.03	0.27	1.22	0.29	1.40	-
9-Methylundecanoic acid ($C_{12:0\ anteiso}$)	0.47	0.32	0.55	0.42	0.41	0.45	0.40	0.45	0.54	0.36	0.50
Lauric acid ($C_{12:0}$)	0.53	0.26	0.23	0.55	0.21	0.27	0.19	0.3	0.23	0.39	0.22
Myristic acid ($C_{14:0}$)	-	0.21	0.31	0.30	0.30	0.39	0.29	0.37	0.32	0.42	0.3
Palmitic acid ($C_{16:0}$)	14.75	15.9	14.93	14.8	14.06	14.45	14.26	14.93	14.25	14.53	14.31
Margaric acid ($C_{17:0}$)	-	0.16	0.30	0.18	0.22	0.19	0.33	0.21	-	0.19	-
Stearic acid ($C_{18:0}$)	2.66	2.88	3.40	3.20	3.39	2.94	2.91	2.93	3.13	2.87	3.82
$C_{17:0\ 2OH}$	4.01	3.48	3.07	3.56	3.66	4.04	3.77	4.03	3.97	4.26	3.30

Table 19 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
17-Methylstearic acid ($C_{19:0\ iso}$)	1.66	2.04	2.01	2.31	1.67	1.97	1.76	2.07	1.89	2.02	1.71
Arachidic acid ($C_{20:0}$)	0.70	0.42	0.50	0.27	0.43	0.56	0.43	0.54	0.48	0.78	0.57
Unsaturated fatty acids											
(10Z)-10-Pentadecenoic acid ($C_{15:1\ w5c}$)	-	-	-	-	1.20	-	-	-	-	-	0.75
Palmitoleic acid ($C_{16:1\ \omega7c}$)	1.11	1.07	0.89	1.05	1.08	1.15	0.99	1.11	1.00	1.07	1.07
$C_{17:1\ iso\ \omega5c}$	-	-	-	-	0.82	-	-	-	-	-	0.83
(9Z)-9-Heptadecenoic acid ($C_{17:1\ \omega8c}$)	-	-	-	-	-	-	-	-	-	0.19	-
cis-Margaroleic acid ($C_{17:1\ \omega8c}$)	-	0.19	-	0.21	0.16	-	-	-	-	-	-
Oleic acid ($C_{18:1\ \omega9c}$)	74.13	72.62	73.26	71.54	71.94	72.41	74.24	71.65	73.68	71.36	72.62
Gondoic acid ($C_{20:1\ \omega9c}$)	-	-	-	0.56	-	-	-	-	-	-	-
Unsaturated fatty acids	75.22	74.03	74.43	73.60	75.37	73.71	75.39	72.95	74.90	72.78	75.27
Saturated fatty acids	24.78	25.97	25.57	26.40	24.63	26.29	24.61	27.05	25.10	27.22	24.73

Note: * Fatty acids name (amount of carbon atom: amount of double bond in structure)

Table 20 The percentages of cellular fatty acid from the co-cultured between *S. mutans* and *L. acidophilus* in prebiotics-supplemented media

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
Saturated fatty acids											
Lauric acid ($C_{12:0}$)	0.49	0.40	0.54	-	0.33	0.30	-	0.32	0.35	0.38	0.38
Myristic acid ($C_{14:0}$)	0.90	0.76	0.92	0.59	0.67	0.70	0.49	0.56	0.69	0.69	0.66
12-Methylmyristic acid ($C_{15:0\ anteiso}$)	-	-	-	-	-	-	-	-	-	0.13	-
Palmitic acid ($C_{16:0}$)	10.61	10.85	11.09	11.66	10.72	10.43	11.64	8.63	13.16	8.31	8.43
Stearic acid ($C_{18:0}$)	3.00	2.56	3.09	1.97	2.64	2.50	1.93	2.16	3.33	2.13	2.38
$C_{17:0\ 2OH}$	0.81	0.83	0.71	0.83	0.77	0.78	0.88	0.85	0.84	0.68	0.64
17-Methylstearic acid ($C_{19:0\ iso}$)	0.85	0.83	0.91	0.51	0.72	0.68	0.63	0.87	0.80	0.74	0.73
Arachidic acid ($C_{20:0}$)	3.92	2.18	1.94	2.08	3.19	1.94	1.81	2.00	1.46	1.95	1.53

Table 20 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
Unsaturated fatty acids											
cis-11-Palmitoleic acid ($C_{16:1\ \omega5c}$)	-	-	-	-	-	-	-	-	0.09	-	-
Palmitoleic acid ($C_{16:1\ \omega7c}$)	1.34	1.27	1.29	1.51	1.16	1.08	1.57	1.22	1.26	1.24	1.19
cis-Margaroleic acid ($C_{17:1\ \omega8c}$)	0.25	0.23	0.28	0.23	0.23	0.25	-	0.20	0.20	0.20	0.22
$C_{17:1\ iso\ \omega5c}$	-	-	-	0.33	-	-	-	-	-	-	-
Oleic acid ($C_{18:1\ \omega9c}$)	60.29	64.14	63.91	62.43	60.44	60.74	61.28	65.38	57.57	65.25	62.68
$C_{19:1\ iso\ I}$	-	-	-	-	0.22	0.18	-	0.20	0.26	-	-
(12Z)-12-Nonadecenoic acid ($C_{19:1\omega7c}$)	15.70	13.30	12.80	15.35	16.03	17.73	17.58	16.59	15.68	16.83	19.72

Table 20 (cont.)

Fatty acids*	Percentage of cellular fatty acids										
		1%		2%		3%		4%		5%	
	MRS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS	GOS	FOS
$C_{19:1\ \omega 11c}$	-	-	-	-	-	-	-	-	0.12	-	-
Gondoic acid ($C_{20:1\ \omega 9c}$)	2.47	2.66	2.53	2.51	2.88	2.69	2.21	1.02	4.18	1.46	1.44
Unsaturated fatty acids	**79.42**	**81.59**	**80.80**	**82.36**	**80.96**	**82.67**	**82.62**	**84.61**	**79.37**	**84.99**	**85.25**
Saturated fatty acids	**20.58**	**18.41**	**19.20**	**17.64**	**19.04**	**17.33**	**17.38**	**15.39**	**20.63**	**15.01**	**14.75**

Note: * Fatty acids name (amount of carbon atom: amount of double bond in structure)

Table 21 The percentages of secreted fatty acids in *S. mutans* and *L. acidophilus* co-cultured in galacto-oligosaccharides

Fatty acids*	MRS		GOS									
			1%		2%		3%		4%		5%	
	Mc	St	Mc	St	Mc	St	Mc	St	Mc	St	Mc	St
Saturated fatty acids												
Capric acid ($C_{10:0}$)	0.34	0.30	-	0.42	-	-	-	-	-	-	-	-
Lauric acid ($C_{12:0}$)	0.14	0.12	-	-	-	0.16	-	0.41	-	-	-	0.13
Myristic acid ($C_{14:0}$)	0.25	0.19	0.19	0.20	0.23	0.29	0.25	0.64	0.30	0.34	0.31	0.34
Palmitic acid ($C_{16:0}$)	14.83	14.48	15.06	13.96	14.92	14.90	15.12	17.02	15.21	15.09	15.04	14.19
Margaric acid ($C_{17:0}$)	0.11	0.11	0.12	-	-	0.12	0.11	0.14	0.11	-	0.10	0.12
Stearic acid ($C_{18:0}$)	2.90	3.17	3.46	3.78	3.62	3.74	3.67	3.15	3.33	4.47	3.32	3.70
$C_{17:0\ 2OH}$	3.29	3.94	3.29	3.55	3.23	3.87	3.26	3.37	3.57	3.66	3.31	3.54

Table 21 (cont.)

Fatty acids*	MRS		GOS									
			1%		2%		3%		4%		5%	
	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T
17-Methylstearic acid ($C_{19:0\ iso}$)	1.56	1.81	1.96	2.16	1.97	2.08	1.88	1.68	1.94	2.11	1.98	2.14
Arachidic acid ($C_{20:0}$)	0.21	0.82	0.32	0.89	0.31	0.90	0.34	0.80	0.30	1.08	0.31	1.15
Unsaturated fatty acids												
Palmitoleic acid ($C_{16:1\ \omega7c}$)	1.07	0.96	0.86	0.87	0.84	0.89	0.88	1.35	1.10	1.11	0.92	0.86
cis-Margaroleic acid ($C_{17:1\ \omega8c}$)	0.16	0.17	0.17	-	-	0.15	0.16	0.19	0.15	-	0.15	0.14
$C_{18:3\ \omega6c\ (6,9,12)}$	-	-	-	-	-	-	-	0.15	-	-	-	-
Oleic acid ($C_{18:1\ \omega9c}$)	75.15	73.92	74.57	74.16	74.88	72.91	74.33	71.10	73.99	72.13	74.55	73.27

Note: *M_C indicated culture-based medium without bacteria, *S_T indicated supernatant from the co-culture.

Table 22 The percentages of secreted fatty acids in *S. mutans* and *L. acidophilus* co-cultured in fructo-oligosaccharides

Fatty acids*	MRS		FOS									
			1%		2%		3%		4%		5%	
	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T
Saturated fatty acids												
Capric acid ($C_{10:0}$)	0.34	0.30	-	0.42	-	-	-	-	-	-	-	-
Lauric acid ($C_{12:0}$)	0.14	0.12	-	-	-	0.16	-	0.41	-	-	-	0.13
Myristic acid ($C_{14:0}$)	0.25	0.19	0.19	0.20	0.23	0.29	0.25	0.64	0.30	0.34	0.31	0.34
Palmitic acid ($C_{16:0}$)	14.83	14.48	15.06	13.96	14.92	14.90	15.12	17.02	15.21	15.09	15.04	14.19
Margaric acid ($C_{17:0}$)	0.11	0.11	0.12	-	-	0.12	0.11	0.14	0.11	-	0.10	0.12
Stearic acid ($C_{18:0}$)	2.90	3.17	3.46	3.78	3.62	3.74	3.67	3.15	3.33	4.47	3.32	3.70

Table 22 (cont.)

Fatty acids*	MRS		FOS									
			1%		2%		3%		4%		5%	
	Mc	St	Mc	St	Mc	St	Mc	St	Mc	St	Mc	St
C $_{17:0\ 2OH}$	3.29	3.94	3.29	3.55	3.23	3.87	3.26	3.37	3.57	3.66	3.31	3.54
17-Methylstearic acid (C $_{19:0\ iso}$)	1.56	1.81	1.96	2.16	1.97	2.08	1.88	1.68	1.94	2.11	1.98	2.14
Arachidic acid (C $_{20:0}$)	0.21	0.82	0.32	0.89	0.31	0.90	0.34	0.80	0.30	1.08	0.31	1.15
Unsaturated fatty acids												
Palmitoleic acid (C $_{16:1\ \omega7c}$)	1.07	0.96	0.86	0.87	0.84	0.89	0.88	1.35	1.10	1.11	0.92	0.86
cis-Margaroleic acid (C $_{17:1\ \omega8c}$)	0.16	0.17	0.17	-	-	0.15	0.16	0.19	0.15	-	0.15	0.14
C $_{18:3\ \omega6c\ (6,9,12)}$	-	-	-	-	-	-	-	0.15	-	-	-	-

Table 22 (cont.)

Fatty acids*	MRS		FOS									
			1%		2%		3%		4%		5%	
	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T	M_C	S_T
Oleic acid ($C_{18:1\ \omega9c}$)	75.15	73.92	74.57	74.16	74.88	72.91	74.33	71.10	73.99	72.13	74.55	73.27
$C_{18:3\ \omega6c\ (6,9,12)}$	-	-	-	-	-	-	-	0.15	-	-	-	-

Note: *M_C indicated culture-based medium without bacteria, *S_T indicated supernatant from the co-cultured.