EFFECT OF NICOTINE ON *STREPTOCOCCUS MUTANS*

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the School of Dentistry,
Indiana University

November 2014

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

I would like to dedicate this work to my grandfather, Guigen Huang, who has given me numerous support and guidance in the past three decades. You are the light tower of my life. Also, I would like to dedicate this work to my husband, Yue Lu, thank you for your love and your patience for five years of waiting for my graduation. I would like to thank my parents, Yu Huang and Ke Tang, for your emotional and financial support.

ACKNOWLEDGEMENTS

This project could not have been done alone. I sincerely thank the people, both in United Stated and in China, who have helped me along my Ph.D. journey in Indiana University School of Dentistry. I am so overwhelmed by the thankful feelings because I have received help hundreds and thousands of times in the last five years. I can't list all of your names here, but I do remember you and your dedicated support in my heart. And I will carry this thankful heart wherever I travel to.

There is a special thanks to my mentor, Dr. Richard L. Gregory. Thank you for offering me a precious chance to study at IUSD. It's really a very nice school with a great Ph.D. program. Thank you for being my mentor and giving me tons of support from daily life to research. And, I especially appreciate the flexibility you give to us.

To my committee members and other senior researchers, Dr. Fengyu Song, Dr. L. Jack Windsor, Dr. Wanzhu Tu, Dr. Christine Wu, Dr. William J. Babler, Dr. Frank X. Yang, Dr. Dong Xie, Youyun Yang, Junjie Zhang, Chung-Ping Liao, Clif W. Duhn, Malgorzata M. Kamocha (Gosia) and Zizheng Dong. Thank you for your insightful suggestions and advanced technical support.

To the members (past and present) of Dr. Gregory's lab: Zhiyun (Cloud) Chen, Cunge (Ellen) Zheng, Wallace V. McCarlie, Jennifer Foltz, Mingyun Li, Grace Gomez, Ghada Batarseh, Alaa Sabrah, and other undergraduate, dental and graduate students, thank you for making my lab life easier and colorful.

To the dental school, thank you for giving me a fellowship and a chance for a better education. Special thanks to our Dean John N. Williams, you are the most easy-going and charming Dean I have ever met. I also thank other Ph.D. students in our program and secretaries Barbara Lerner, Julia M. Daley-Backus and Kathy Sterrett.

To the Oral Health Research Institute personnel, thank you for the many happy moments you have given me. Special thanks to Sharon Gwinn, you are always very helpful in every aspect. Thanks to Beverly J. Spriggs and Dr. Ana for your everyday warm welcomes.

To all my friends, roommates and relatives, including but not limited to Xia Xu, Shujie Cheng, Jieru Bai, Meiping Ye, Liangping Li, Subei Li, Shuangshuang Xie, Yumin Wang, Fei Huang, Xueting Zhou, Weiping Zhang, Si Wang, Ming Sun, Yang Liu, Xia Guo, Nan Ru, Jun Sun, Nida Tanataweethum, Josh Evans, Li Yang, Xuelian Huang and Quan Chen. Thank you for your support throughout this wonderful journey in the United States.

Ruijie Huang

EFFECT OF NICOTINE ON *STREPTOCOCCUS MUTANS*

Streptococcus mutans is a key contributor to dental caries. Smokers have increased caries, but the association between tobacco, nicotine, caries and *S. mutans* growth is little investigated. In the first section, seven *S. mutans* strains were used for screening. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) were 16 mg/ml (0.1 M), 32 mg/ml (0.2 M), and 16 mg/ml (0.1 M), respectively, for most of the *S. mutans* strains. Growth of planktonic *S. mutans* cells was significantly repressed by 2.0-8.0 mg/ml nicotine concentrations. Biofilm formation and metabolic activity of *S. mutans* was increased in a nicotine-dependent manner up to 16.0 mg/ml. Scanning electron microscopy (SEM) revealed higher nicotine-treated *S. mutans* had thicker biofilm and more spherical bacterial cells than lower concentrations of nicotine. In the second section, confocal laser scanning microscopy (CLSM) results demonstrated that both biofilm bacterial cell numbers and extracellular polysaccharide (EPS) synthesis were increased by nicotine. Glucosyltransferase (Gtf) and glucan binding protein A (GbpA) protein expression of *S. mutans* planktonic cells were upregulated, while GbpB protein expression of biofilm cells were downregulated by nicotine. The mRNA expression of those genes were mostly consistent with their protein results. Nicotine was not directly involved in *S. mutans* LDH activity. However, since it increased the total number of bacterial cells in biofilm; total LDH activity

of *S. mutans* biofilm was increased. In the third section, a PCR-based multiple species cell counting (PCR-MSCC) method was designed to investigate the effect of nicotine on *S. mutans* in a ten mixed species culture. The absolute *S. mutans* number in mixed biofilm culture was increased but the percentage of *S. mutans* in the total number of bacterial cells was not changed.

In conclusion, nicotine enhanced biofilm formation and biofilm metabolism of *S. mutans*, through stimulating *S. mutans* planktonic cell Gtfs and Gbps expression. This leads to more planktonic cells attaching to dental biofilm. Increased *S. mutans* cell numbers, in biofilms of single species or ten mixed species, resulted in higher overall LDH activity. More lactic acid may be generated and contribute to caries development in smokers.

Richard L. Gregory, Ph.D., Chair

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BHI+YE	Brain Heart Infusion + 5% Yeast Extract
CFU_R	Colony forming unit of reference species
CLSM	Confocal laser scanning microscopy
CSC	Cigarette smoke condensate
C_T	q-PCR cycle number when fluorescence reaches the threshold value
$C_{T,R}$	C_T value of reference mixed culture
$C_{T,X}$	C_T value of unknown mixed culture
DMFT	Decayed/missing/filled teeth
E	Efficiency of q-CPR amplification
E. faecalis	*Enterococcus faecalis*
E_R	E of reference mixed culture
E_X	E of unknown mixed culture
Gbp	Glucan-binding Protein
Gtf	Glucosyltransferase
I_R	PCR band intensities of the reference mixed cultures
I_X	PCR band intensities of the unknown mixed cultures
L. casei	*Lactobacillus casei*
LDH	Lactate Dehydrogenase
MBC	Minimum Bactericidal Concentration

MBIC	Minimum Biofilm Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MMP	Matrix metalloproteinase
MRBM	Metabolism Relative to Biofilm Mass
nAChRs	Nicotinic acetylcholine receptors
N_R	Reference cell number processed for DNA extraction
N_X	Unknown cell number in mixed culture processed for DNA extraction
$N_{X,O}$	Original unknown cell number in mixed culture
p	Dilution of unknown mixed culture before DNA extraction
PBS	Phosphate Buffered Saline
PVDF	Polyvinylidene Fluoride
P. gingivalis	*Porphyromonas gingivalis*
q	Number of different species in mixed culture
q-PCR	Quantitative Polymerase Chain Reaction
S. aureus	*Staphylococcus aureus*
SDS	Sodium Dodecyl Sulfate
SEM	Scanning electron microscopy
S. epidermidis	*Staphylococcus epidermidis*
S. gordonii	*Streptococcus gordonii*
S. mitis	*Streptococcus mitis*
S. mutans	*Streptococcus mutans*
S. oralis	*Streptococcus oralis*

SPSS	Statistical Package for Social Science
S. salivarius	*Streptococcus salivarius*
S. sanguinis	*Streptococcus sanguinis*
TIMP	Tissue Inbibitors of Metalloproteinases
TSB	Tryptic Soy Broth
TSBS	Tryptic Soy Broth + 1% Sucrose
V_R	Volume of reference species processed for DNA extraction

CHAPTER ONE

Introduction

1.1 Dental Caries

Dental caries is a bacterial infectious disease. It impairs the physiological balance of tooth demineralization and remineralization, causing tooth minerals to be lost and caries formation. With the absence of proper clinical treatment, severe pain and tooth loss may occur. Dental caries is one of the world's major diseases presenting in 60-90% of the school age pediatric population and most of the adult population (Petersen and Lennon, 2004). The three essential factors for dental caries formation are exposed teeth, dietary carbohydrates and bacteria (Chankanka, 2010). The tooth surface (tooth enamel) has 96% mineral salts. The primary mineral is a crystalline calcium phosphate named hydroxyapatite, which makes teeth the hardest tissue in the human body (Mihu et al., 2008). Demineralization is the process of removing minerals in the form of ions due to the acid metabolized from food by resident bacteria. Remineralization is the process of gaining minerals in the form of ions due to carbonic acid precipitating mineral ions. The process of demineralization and remineralization maintains the mineral balance of tooth. If the balance is impaired, caries will occur. The capability of dental plaque to produce lactic acid and to dissolve tooth minerals is associated with caries development (Kleinberg et al., 1982). Furthermore, *Streptococcus mutans*, lactobacilli and other bacteria facilitate the procedure by utilizing lactic acid to create a low pH environment (Hu and Sandham, 1972).

1.2 Streptococcus mutans

S. mutans is a gram-positive, facultatively anaerobic coccus bacterium that is considered one of the primary cariogenic bacterial species. Two distinguished characteristics make *S. mutans* the primary pathogen: presence in dental biofilm and lactic acid generation. Three groups of enzymes contribute to *S. mutans* cariogenicity; glucosyltransferase (Gtf), glucan binding protein (Gbp) and lactate dehydrogenase (LDH). Gtf metabolizes sucrose to fructose and glucose and synthesizes glucose into glucan, which is a major source of a sticky extracellular polysaccharide (EPS) of dental biofilm. EPS facilitates *S. mutans* to attach to the tooth surface, aggregate in biofilm and attack the host immune system (Koo et al., 2010). Gbp is related to *S. mutans* cell surface binding (Hazlett et al., 1999). LDH metabolizes glucose, fructose, lactose and other forms of sugar into lactic acid and lowers environmental pH (Sommer et al., 1985).

1.2.1 Glucosyltransferase

Gtfs are a family of enzymes that catalyze the transformation of glucosyl groups from one chemical component to another and contributes to *S. mutans* cariogenicity (Vasilev et al., 2010). *S. mutans* expresses three distinct Gtfs. GtfB, GtfC and GtfD are encoded by *gtfB*, *gtfC* and *gtfD*, respectively. GtfB and GtfC are encoded by highly homologous genes and they primarily synthesize α-1,3-rich water-insoluble glucan polysaccharide (Aoki et al., 1986; Hanada and Kuramitsu, 1988), while GtfD is responsible for the synthesis of α-1,6-rich water-

soluble polysaccharides (Hanada and Kuramitsu, 1989). Gtfs also enhance bacterial cohesion in biofilm (Bowen and Koo, 2011).

1.2.2 Glucan Binding Protein

Gbps are defined as non-Gtf glucan-binding proteins that serve as cell-surface receptors for glucan (Banas and Vickerman, 2003). There are four major Gbps, GbpA, GbpB, GbpC and GbpD. GbpA and GbpD share homology of glucan-binding domains with Gtfs and their functions are to serve as a glucan-binding enzyme (Banas and Vickerman, 2003). GbpA is related to *S. mutans* biofilm smoothness (Hazlett et al., 1999). GbpB is involved in *S. mutans* bacterial cell-wall synthesis and cycling, and the GbpB expression of clinical strains is correlated to biofilm formation ability (Mattos-Graner et al., 2001). GbpC is responsible for dextran-dependent aggregation (Sato et al., 1997) and related to bacterial biofilm structure (Banas and Vickerman, 2003). GbpA and GbpC have been shown to optimize bacterial aggregation and plaque architecture (Banas et al., 2001).

1.2.3 Lactate Dehydrogenase

LDH is the enzyme that catalyzes the conversion of pyruvate to lactate with concomitant interconversion of NADH and NAD^+ and produces lactic acid as the final product (Sommer et al., 1985). *S. mutans* produces LDH constitutively if the level of fructose-1,6-diphosphate is adequate (Brown and Wittenberger, 1972) as

the catalytic activity of LDH requires the presence of fructose-1,6-diphosphate with a pH range of 5 to 6.2 (Sommer et al., 1985). LDH deficiency is lethal to *S. mutans* due to the level of accumulated toxic intermediate products such as pyruvate (Chen et al., 1994; Hillman et al., 1996). Since LDH activity is directly related to *S. mutans* lactic acid production, LDH is considered a *S. mutans* virulence factor. LDH facilitates *S. mutans* to dissolve tooth minerals and lead to dental caries.

1.3 Ecosystem and Commensal Bacteria

There are over 700 bacterial species in dental biofilm. Most of them are usually harmless commensal bacteria. Commensal bacteria compete with virulent bacteria and protect human health. *Streptococcus sanguinis* is considered an oral commensal bacterium that antagonizes *S. mutans* (Kreth et al., 2005). The ratio of *S. mutans* to *S. sanguinis* has been used as a caries risk factor in clinical caries risk assessments (Alaki et al., 2002; Loesche et al., 1984). A larger ratio indicates a higher caries risk. *S. sanguinis* is a Gram positive facultatively anaerobic coccus-shaped bacterium and is considered a commensal bacteria in dental biofilm. *S. sanguinis* competes with *S. mutans* by producing a bacteriocin (sanguicin) or excreting H_2O_2 (Merritt et al., 2005). H_2O_2 can inhibit the glycolysis and protein synthesis of target bacteria. Both biofilm and planktonic *S. mutans* cells are sensitive to H_2O_2 (Baldeck and Marquis, 2008). Low concentrations (16.3 mM) of H_2O_2 can strongly inhibit glycolysis and are primarily bacteriostatic.

Streptococcus gordonii possesses a similar mechanism to compete with *S. mutans*, but the potency is lower than *S. sanguinis* (Kreth et al., 2008).

Virulent bacteria have methods to compete with commensal bacteria. *S. mutans* produces bacteriocins, which are proteins with an inhibitory function to commensal bacterial growth of its own strain or strains similar to it (Kuramitsu et al., 2007), to inhibit commensal bacterial growth. Bacteriocins produced by *S. mutans* can be differentiated into two types of mutacins, lantibiotics and non-lantibiotics. The inhibition spectrum of lantibiotics on Gram-positive bacteria is relatively wide, while the non-lantibiotics have a narrow antimicrobial spectrum that only act on *S. sanguinis* (Senadheera and Cvitkovitch, 2008). Another mechanism of *S. mutans* inhibition on *S. sanguinis* is the large amount of organic acid produced by *S. mutans*. Although both *S. mutans* and *S. sanguinis* can metabolize glucose and produce lactate when incubated with excess glucose, *S. mutans* produces more acid than *S. sanguinis* due to the higher ATP-glucose phosphotransferase activity of *S. mutans* than *S. sanguinis*. Therefore, the lactic acid produced by *S. mutans* glucose metabolism is excreted into the environment and inhibits the growth of *S. sanguinis*, resulting in an increased ratio of *S. mutans* over *S. sanguinis* (Iwami and Yamada, 1980).

1.4 Smoking and Caries

Studies have shown a positive relationship between smoking and dental caries. Higher decayed/missing/filled teeth (DMFT) scores of permanent teeth were

detected in a Swedish smoking population than a non-smoking population (Axelsson et al., 1998). A study in Mexico found the same conclusion among cigarette smoking truck drivers (Aguilar-Zinser et al., 2008). In U.S, chewing tobacco was associated with root surface caries in a dose-dependent manner (Tomar and Winn, 1999). In Turkey, *S. mutans* colonization was positively associated with dmft scores of primary teeth in children exposed to passive smoke (Avsar et al., 2008). In Japan, pregnant mothers who are exposed to tobacco had children with a higher prevalence of caries than children with unexposed mothers (Tanaka et al., 2009). In Italy, tobacco usage increased the prevalence of caries in cadets at a military academy (Campus et al., 2011). Both behavioral and biological factors contribute to smokers' caries development. The association between smoking and *S. mutans* growth is still controversial. One study (Zonuz et al., 2008) reported that smoking upregulated the growth of oral *S. mutans*. However, another study (Sakki and Knuuttila, 1996) reported that smoking was strongly associated with lactobacilli and yeast but weakly associated with *S. mutans*, although the incidence of *S. mutans* was 69% in smokers and 57% in non-smokers. A recent study (Baboni et al., 2010) found that cigarette smoke condensate (CSC) enhanced *S. mutans* adhesion to acquired pellicle forming on orthodontic materials such as bands and acrylic resin. CSC stimulates the activity of host matrix metalloproteinase (MMP), which degrades collagens (Zhang et al., 2011). Since dentin contains type-I collagen, smoking contributes to caries development. Various tobacco extracts have different impacts on *S. mutans*. The effect of pouch and plug tobacco on *S.*

mutans growth is positive while snuff is neutral (Lindemeyer et al., 1981). The effects of nicotine on *S. mutans* planktonic growth was reported as biphasic, as 10^{-3}-10^{-4} M nicotine upregulated *S. mutans* growth, while 10^{-1}-10^{-2} and 10^{-6}-10^{-7}M nicotine inhibited its growth (Keene and Johnson, 1999). However, our preliminary study has not confirmed the biphasic pattern, which might be explained by different methodologies used.

Nicotine (*Nicotiana tabacum*) is a plant alkaloid in tobacco, accounting for about 0.6-3.0% of the dry tobacco weight. It inhibits fibroblasts (Biondo-Simoes Mde et al., 2009) via nicotinic acetylcholine receptors (nAChRs) (Wang et al., 2010) and facilitates *Porphyromonas gingivalis* invasion of epithelial cells (Cogo et al., 2009) and human gingival fibroblasts (Schena et al., 1995; Tipton and Dabbous, 1995). Nicotine has been shown to cause oxidative stress in human cells (Kang et al., 2011). Transcriptional repressor Rex is responsible for the regulation of *S. mutans* oxidative stress and biofilm formation (Bitoun et al., 2011). Previous studies (Falkler et al., 1987; Keene and Johnson, 1999) suggested that nicotine affected *S. mutans* gene expression. However, the detailed mechanisms are unknown.

1.5 Specific Aims and Null Hypothesis

The overall goal of this study was to investigate if and how nicotine facilitated *S. mutans* growth, metabolism and competition with other dental commensal

bacteria and thus contributes to smokers' dental caries development. To accomplish the overall goal, the following three specific aims were accomplished.

Specific aim 1: To assess the general effects of nicotine on *S. mutans* overall planktonic cell growth, biofilm formation and metabolism.

Very few studies have investigated how nicotine affects *S. mutans*. It is necessary to have some fundamental growth data that would serve as the guidance for further studies. Those fundamental growth data includes (1) the minimum inhibitory concentration, minimum bactericidal concentration and minimum biofilm inhibitory concentration of nicotine on *S. mutans*, and (2) the effect of nicotine on *S. mutans* planktonic cell growth, biofilm formation and general metabolism.

Specific aim 2: To assess the effects of nicotine on *S. mutans* LDH activities, and Gtf and Gbp expression at the mRNA and protein levels.

Bacterial attachment to the tooth surface is critical for cell multiplication. Gtfs and Gbps are dynamically involved in *S. mutans* surface attachment. EPS synthesis is critical in maintaining bacterial cells in biofilm, and it is regulated by Gtfs. Acid metabolism is critical for caries generation. LDH is the enzyme that catalyzes the conversion of pyruvate to lactate and then to lactic acid. CSC stimulated *S. mutans* gbpA, gbpB and Gtfs (Zheng and Gregory, unpublished data). However,

very few studies have reported the effects of nicotine on the expression of Gtfs, Gbps and LDH in *S. mutans*.

Specific aim 3: To assess the effects of nicotine on *S. mutans* growth competition with other commensal bacteria.

Commensal bacteria are considered the normal microflora in humans and co-evolved with their hosts (Tlaskalova-Hogenova et al., 2004). Single species investigations go deep to explore the detailed regulation pathways, while multiple species investigations broadly explore bacterial interactions. Multiple species research is closer to the clinical situation than single species research. There are some clinical reports of bacterial profiles of smokers, but no *in vitro* studies have investigated the effects of nicotine on multiple bacterial competitions.

The null hypothesis was the *S. mutans* growth, including planktonic cell growth, biofilm formation, metabolism and binding protein expression, is independent from the presence of nicotine.

CHAPTER TWO

Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Seven *S. mutans* strains UA159 (ATCC 700610), UA130 (ATCC 700611), 10449 (ATCC 25175), A32-2 (isolated from a severe caries patient by our lab) (Gregory et al., 1998), NG8 (Dr. Song Lee, Dalhousie University, Canada), LM7 (Dr. Suzanne M. Michalek, University of Alabama-Birmingham, USA) and OMZ175 (Dr. Suzanne M. Michalek), and nine other bacterial strains: *S. gordonii* (ATCC 35105), *Streptococcus mitis* (ATCC 49456), *Streptococcus oralis* (ATCC 35037), *Streptococcus salivarius* (ATCC 27975), *S. sanguinis* (SK36, Dr. Todd O. Kitten, Virginia Commonwealth University Philips Institute), *Staphylococcus aureus* (COL, Dr. Steven R. Gill and Ann Gill, University of Rochester Medical Center), *Enterococcus faecalis* (ATCC 29212), *Lactobacillus casei* (ATCC 393) and *Staphylococcus epidermidis* (PR62A, Dr. Steven R. Gill and Ann Gill) were used . In Project I, all of the seven S. mutans strains were used; in Project II, S. mutans UA159 was used; in Project III, S. mutans UA159 and other species were used(Table 2.1.1). The strains were stored at -80°C in tryptic soy broth (TSB) or Brain Heart Infusion with 5% Yeast Extract (BHI+YE) with 20% glycerol before use. The growth media, growth duration and nicotine concentrations are summarized in Tables 2.1.2, 2.1.3 and 2.1.4, respectively. Unless otherwise stated, the growth condition was 5% CO_2 at 37°C. Nicotine (1 g/ml, $\geq$99%, liquid) was purchased from Sigma-Aldrich Chemical Co., St. Louis, MO.

2.2 Project I: Effect of Nicotine on *S. mutans* Growth and Metabolism

2.2.1 MIC/MBC and Planktonic Cell Growth

Minimum inhibitory concentration (MIC) is the lowest concentration of an agent that inhibits the visible growth of a microorganism, and the minimum bactericidal concentration (MBC) is the lowest concentration of an agent that kills the microorganism. The MIC and MBC of nicotine against *S. mutans* were determined by a two-fold dilution method (Song et al., 2006). Briefly, an overnight *S. mutans* culture (10^6 CFU/ml, final concentration unless stated otherwise, determined by spiral plating) was treated with 0, 4.0, 8.0, 16.0, and 32.0 mg/ml nicotine for 24 hours in sterile 96-well flat bottom microtiter plates (Fisher Scientific, Co., Newark, DE). The turbidities of the bacterial cultures were measured by optical density at 595 nm (OD_{595nm}) using a spectrophotometer (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA). The MIC was defined as the lowest concentration of nicotine that yielded a turbidity change equal or less than 0.050 (Song et al., 2006). To determine the MBC, bacterial cultures (10 µl) from the wells with nicotine concentrations equal to and higher than the MIC were transferred onto Tryptic Soy Agar (TSA, Difco, Detroit, MI) plates and incubated for 48 hours. The MBC was defined as the lowest concentration of nicotine that had no visible bacterial colonies on the agar plates after 48 hours incubation.

Planktonic cell growth assays are similar to MIC assays. Briefly, an overnight bacterial culture (10^6 CFU/ml) was grown in the presence of 0, 0.25, 0.5, 1, 2, 4

and 8 mg/ml nicotine for 24 hours. After the incubation, the planktonic unbound cells were gently aspirated and transferred to a new 96-well plate followed by reading at OD_{595nm}.

2.2.2 MBIC and Crystal Violet Assay (Biofilm Formation)

The minimum biofilm inhibitory concentration (MBIC) is the lowest concentration of an agent that inhibits the visible biofilm formation of a microorganism (Xu et al., 2010). To determine the MBIC, an overnight *S. mutans* culture (10^6 CFU/ml) in TSB was grown in the presence of 0, 4.0, 8.0, 16.0 and 32.0 mg/ml nicotine and TSBS for 24 hours in 96-well microtiter plates. After the planktonic cells were gently removed, biofilm was fixed with 10% formaldehyde (Vasilev et al., 2010) for 30 minutes and stained with 0.5% crystal violet for another 30 minutes. After washing biofilm three times with saline, crystal violet was extracted from the biofilm cells by 200 µl of 2-propanol for 1 hour. The extract was diluted 1:5 with fresh 2-propanol and read at OD_{490nm}.

The crystal violet assay used for biofilm formation estimation was similar to the method of MBIC, but the nicotine concentrations used in biofilm formation were 0, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/ml (sub-MIC concentrations).

2.2.3 Biofilm Metabolic Activity

S. mutans biofilm metabolic activity was measured by a method described by Pierce et al. (Pierce et al., 2008), which was based on biofilm cells reducing 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to a water-soluble orange compound (Pierce et al., 2008). *S. mutans* biofilm was grown in TSBS without nicotine in 96-well-plates for 24 hours, followed by another 24 hours growth in TSBS supplemented with 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mg/ml nicotine. Biofilm was gently washed twice with saline, XTT reagent added and the plate kept in the dark in 5% CO_2 at 37°C for 2 hours. After incubation, the XTT reagent was transferred to another 96-well-plate to detect the color change by reading at OD_{490nm}. In the meantime, the relative biofilm mass of identically treated wells was also detected by the crystal violet assay described above. The ratio of specific biofilm metabolic activity/mass, which illustrated single cell metabolism relative to biofilm mass (MRBM), was calculated.

2.2.4 Scanning Electron Microscopy (SEM)

TSBS containing 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml nicotine was inoculated with an overnight *S. mutans* UA159 culture (10^6 CFU/ml) and incubated for 24 hours in 2-well Lab-Tek Chamber slides (Thermo Fisher Scientific, Rochester, NY). Biofilm was washed twice with saline, fixed with 1% glutaraldehyde overnight, washed twice with distilled water, dehydrated by a series of ethanol rinses (70%, 85%, 95%, and 100%), immersed in Hexamethyldisilazane (HMDS,

Electron Microscopy Sciences, Hatfield, PA) for 10 minutes, and dried in a desiccator. After sputter coating with gold-palladium, samples were analyzed by SEM (JEOL JSM-5310LV; JEOL Techniques, Ltd., Tokyo, Japan) at 500X, 1,500X and 20,000X magnifications in the Electron Microscopy Laboratory, School of Dentistry, Indiana University.

2.2.5 Kinetic Growth

For planktonic cell kinetic growth, TSB containing 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml nicotine was inoculated with an overnight *S. mutans* UA159 culture (10^6 CFU/ml) in microtiter plate. The plate was sealed by SealPlate® adhesive sealing film (EXCEL Scientific, Inc., Victorville, CA, USA) and incubated in the spectrophotometer at 37°C without CO_2 supply. The turbidity of each well was read every 20 minutes in a 24 hours period at OD_{595nm}. The turbidity results at 2, 4, 8, 12 and 24 hours were extracted from the kinetic data.

For biofilm kinetic growth, TSBS containing 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml nicotine was inoculated with an overnight *S. mutans* UA159 culture (10^6 CFU/ml) in five identical microtiter plates at 37°C with 5% CO_2 supply. At 2, 4, 8, 12 and 24 hour points, one of the microtiter plates was removed from the incubator and processed for the crystal violet assay described before.

2.3 Project II: Effect of Nicotine on *S. mutans* UA159 Cell Binding, EPS Synthesis and LDH Activity

2.3.1 Confocal Laser Scanning Microscopy (CLSM)

S. mutans (UA159) was grown in TSBS with 0, 1, 2 and 4 mg/ml nicotine and 1 μM Alexa Fluor 647® red fluorescent dye (Dextran conjugates, 10,000 MW, Molecular Probes Inc., OR, USA) in four-well Lab-Tek Chamber slides (Thermo Fisher Scientific, Rochester, NY, USA) for 24 hours (Xiao and Koo, 2010). The dextran conjugates were incorporated into EPS during biofilm matrix synthesis, and therefore it represented the amount of EPS. Biofilm was washed three times with deionized water (diH_2O) and incubated at room temperature with 1 μM SYTO® 9 green fluorescent dye (Nucleic acid stains, Molecular Probes Inc., OR, USA), which binds to bacterial nucleic acid and represents bacterial cell numbers in biofilm. Biofilm was then washed three times with diH_2O. After biofilm was air dried, ProLong Gold Antifade Reagents (Molecular Probes Inc., OR, USA) was added to the biofilm to enhance its resistance to photobleaching. Fluorescent images were obtained by an Olympus FV1000-MPE Confocal/Multiphotonmicroscope (Olympus Corp., USA) with Olympus FV10-ASW software (Olympus Corp., USA) in the Division of Nephrology, Indiana Center for Biological Microscopy, Indiana University School of Medicine. The Alexa Fluor 488 and Alexa Fluor 647 fluorescent channels were selected to detect green and red fluorescence, respectively. The vertical distance between the highest and lowest scanning layers where biofilm presented was defined as biofilm thickness.

The image processing and analysis program was written according to Heydorn's principles (Heydorn et al., 2000) by MATLAB® R2012a (The MathWorks, Inc.) via the codes:

(1) Fluorescence intensity

im = imread('image name');

im_g = im(:,:,N$^{\Psi}$);

ans = sum(sum(im_g));

ans/(K*K$^{\delta}$)

(2) Fluorescence coverage

im = imread('image name');

im_g = im(:,:,N$^{\Psi}$);

result = im_g>M$^{\kappa}$;

ans = sum(sum(result));

ans/(K*K$^{\delta}$)

Footnotes:

$^{\Psi}$N = 1 for red fluorescence (EPS), N = 2 for green fluorescence (bacteria)

$^{\kappa}$M is the background color intensity, which varies in different experiments. In the present study, M = 50.

$^{\delta}$K is the pixel value in each dimension of an image.

2.3.2 Western Blotting.

Western blotting was used to investigate *S. mutans* Gtfs, GbpA and GbpB protein expression. The primary antibodies were kindly provided by Dr. Daniel J.

Smith (Forsyth Institute, USA). *S. mutans* was grown in TSBS with 0, 1, 2 and 4 mg/ml nicotine for 12 hours. Both planktonic and biofilm cells were harvested separately, washed three times with saline (0.9% sodium chloride) and suspended in lysis buffer [9 M urea, 2% nonidet-P40 and 1% dithiothereitol (DTT)]. Samples were sonicated [Branson Sonifier 450 (Branson Ultrasonics Corp., CO, USA) with duty cycle 50% and output control equal to 7] on ice with 0.2 mm microglass beads (B. Braun Melsungen AG., Melsungen, Germany) for 5 minutes in each cycle and for three cycles in total (Sanui and Gregory, 2009). Cellular proteins were harvested by centrifugation at 2,000×g. The RC PC Protein Assay (BIO-RAD, Bio-Rad Laboratories, Inc., CA, USA) was used to determine protein concentrations. The samples were mixed with an equal volume of sample buffer [64 mM Tris-HCl, pH 6.8, 25% glycerol, 0.15 mM bromophenol blue, 69 mM sodium dodecyl sulfate (SDS)] and incubated at 95°C for 10 minutes. Three μg samples from each treatment were loaded onto Tris-HCl electrophoresis gels (Ready Gel Tris-HCl Gel, Bio-Rad Laboratories, Inc., CA, USA), electrophoresed at 200 mA for 75 minutes and transferred to a polyvinylidene fluoride (PVDF) membrane at 30 V overnight. The PVDF membrane was then blocked in PBS + 0.1% Tween 20 + 5% non-fat milk (PBS-TM) for 2 hours, incubated with primary antibody dissolved in PBS-TM at appropriate concentrations (Gtfs 1:750, GbpA 1:2000, GbpB 1:5000) for 2 hours, washed three times with PBS-TM, incubated with anti-rat horseradish peroxidase secondary antibody dissolved in PBS-TM (Gtfs 1:1000, GbpA 1:5000, GbpB 1:5000), washed three times with PBS-TM, rinsed with PBS, incubated with

enhanced chemiluminescence substrate (ECL, Pierce ECL Western Blotting Substrate, Thermo Scientific, IL, USA) and exposed to classic blue autoradiography film (Molecular Technologies, MO, USA)(Li et al., 2010). ImageJ (Version 1.8, NIH) was used to quantitative the intensity of bands.

2.3.3 Quantitative-PCR (q-PCR) of *gtfs* and *gbps*

The q-PCR protocol described previously was followed with some modifications (Xu et al., 2012). Briefly, *S. mutans* was grown in TSBS with 0 and 2 mg/ml nicotine for 12 hours. Both planktonic and biofilm cells were harvested separately and washed three times with saline. Approximately 6×10^7 cells were stabilized by RNAprotect™ Bacteria Reagent (QIAGEN, MD, USA). Each *S. mutans* cell sample was suspended in 100 µl of lysis buffer (30 mM Tris-HCl pH 8.0, 1 mM EDTA and 15 mg/ml lysozyme) with 10 µl of 10 KU/ml mutanolysin (Sigma-Aldrich, MO, USA) and 15 µl of proteinase K (QIAGEN), incubated at 37°C with agitation for 90 minutes, and sonicated for 5 cycles of 10 seconds/cycle (52% amplitude, Sonic Dismembrator, Model 500, Fisher Scientific). The RNA was isolated and purified by RNeasy Mini Kit (QIAGEN). RNA concentration was determined by NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). SuperScript III First-Strand Synthesis System (Invitrogen™, Life Technologies Corp., CA, USA) was used to synthesize cDNA from 10 µg RNA. Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies Corp., CA, USA), *S. mutans* gtfB, gtfC, gtfD, gbpA, gbpB, gbpC and gbpD primers (0.375 µM, Table 2.3) and cDNA template (2 µl) were processed for q-PCR replication (ABI PRISM® 7000

Sequence Detection System, Applied Biosystems, Life Technologies Corp.). q-PCR data was generated and analyzed by 7000 System SDS software (Applied Biosystems, Life Technologies Corp.) with all default settings. The original purified RNA samples were loaded with all the primers and Fast SYBR Green Master Mix for q-PCR as control to exclude genomic DNA contamination. The gene expression fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method described by Livak (Livak and Schmittgen, 2001).

2.3.4 LDH Assay

LDH assays were used to determine cellular LDH activity of *S. mutans* treated by nicotine. Briefly, *S. mutans* was treated with 0, 0.5, 1, 2 and 4 mg/ml nicotine in TSBS for 24 hours, 45 µl of resuspended *S. mutans* biofilm cells was mixed with 5 µl of LDH Assay Lysis Solution and incubated in a microtiter plate for 45 minutes. Fresh 100 µl of LDH Assay Mixture (LDH Assay Cofactor Preparation : LDH Assay Substrate : LDH Dye Solution = 1:1:1) was added to 50 µl of cell lysate and the microtiter plate was incubated in the dark at room temperature for 30 minutes. The reaction was terminated by the addition of 15 µl of 1N HCl. The absorbance was read at OD_{490nm}. The background absorbance at OD_{690nm} was measured and subtracted from the primary measurement, according to the manufacturer's manual. When the biofilms for the LDH assay were prepared, another set of biofilms for the crystal violet assay was prepared as well. It was necessary to adjust the LDH data by the amount of formed biofilm in order to

estimate a unit cell of LDH activity. The crystal violet assay was processed as described before.

2.4 Project III: Effect of Nicotine on *S. mutans* UA159 Proliferation in A Ten Species Mixed Culture

2.4.1 Primer Design

To differentiate the ten species within a mixed culture and to count the cell number of each species, a PCR-based multiple species cell counting (PCR-MSCC) method was designed. The primers used bacterial non-repeated chromosome sections as the template. Since each bacterial cell has only one chromosome, the DNA template copy number represents the bacterial cell number. The primers were designed based on the chromosome section sequence of one species that was different from the other. Therefore, the species-specific primers would only amplify the species-specific DNA template.

The BLAST® tool on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare genomes and to find the DNA sequence for species-specific primer design. A random 10,000 bp gene sequence of one strain was compared with the genome of a second strain. From the Graphic Summary of the BLAST results, a 1000 bp sequence of the first strain without similarity to the second strain was identified. This sequence was then compared with the genome of the rest of the eight strains. If no similarity was found in any of the rest of the strains, this sequence was defined as the species-specific sequence. If similarity

was found in one of the other strains, we repeated the process from the beginning with a second sequence or a third one until this sequence was proved to be different from all of the other genomes. In the present study, the species-specific chromosome section of each strain was blasted with all of the rest of the strains, totaling 45 comparisons, to make sure all of the primers were unique to a certain species within the 10 species mixed culture. The primer-BLAST tool on the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design the species-specific primers based on the species-specific sequence.

Since four of the strains used did not have their genome information available on the NCBI website, the genomes of *S. mitis* B6, *S. oralis* Uo5, *S. salivarius* CCHSS3 and *E. faecalis* V583 were used to surmise the genomes of *S. mitis* ATCC 49456, *S. oralis* ATCC 35037, *S. salivarius* ATCC 27975 and *E. faecalis* ATCC 29212, respectively. The final primer sequences are listed in Table 2.4.

2.4.2 Primer Specificity Test

To test the validation of primers that were designed based on surmised gene sequences and to further confirm the specificity of the primers used, polymerase chain reaction (PCR) was used to test the amplification product of each species whole DNA with all of the species-specific primers. Briefly, overnight bacterial cultures of each strain were individually grown in BHI+YE broth for 10 hours. Cells were harvested at their log phase and washed three times by PBS. Cells were suspended in 100 µl lysis buffer (30 mM Tris-HCl, 1 mM EDTA and 15

mg/ml lysozyme, pH = 8.0) with 10 µl mutanolysin (1000 U/ml) and 15 µl proteinase K (72 AU/ml) at 37^{o}C for 90 minutes with agitation (Xu et al., 2012). Buffer AL of DNeasy® 96 Blood & Tissue Kit (QIAGEN, Valencia, CA) was added to each sample, which was then sonicated for 10 seconds (52% amplitude, Sonic Dismembrator, Model 500, Fisher Scientific) and repeated 5 times on ice. The sonicator tip was rinsed with 10% bleach followed by distilled water between different samples. The remaining DNA extraction steps were followed using the manufacturer's protocol. The quantity and quality of the extracted DNA were determined by NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). The total DNA (100 ng) of each strain was amplified by all of the species-specific primers (0.25 µM) and TaKaRaTaq™ Polymerase (Chemicon International, Temecula, CA). The initialization step was 94^{o}C 5 minutes, the amplification step had 30 cycles of 94^{o}C 30 seconds, 55^{o}C 30 seconds and 72^{o}C 30 seconds, and the final elongation step was 72^{o}C 10 minutes. The amplification product was confirmed by agarose gel electrophoresis.

2.4.3 Validation Range Test of the Method

Each test has a validation range, if one result is beyond this range it becomes invalid and should be excluded. Quantitative-PCR (q-PCR) was used to estimate the system error, which represents the maximum acceptable tolerance of cell number variations between samples in the present study. The DNA samples (200 ng) of each species were loaded in a MicroAmp Optical 96-well Reaction plate (Invitrogen, Grand Island, NY) with the primers (0.25 µM) of each species and

Fast SYBR Green Master Mix (Applied Biosystems, Grand Island, NY). q-PCR amplification was performed on an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). All of the default settings were used. The raw data (i.e., the data generated by *S. mutans* DNA versus all the primers from ten species) was extracted and normalized by the same species-primers paired data (i.e., the data generated by *S. mutans* DNA versus *S. mutans* species-specific primers). The amount of q-PCR product amplified by its own species-specific primers was defined as 1 (control), and the amount of products amplified by other primers were calculated based on their fold changes compared to control.

2.4.4 q-PCR Efficiency Test

Since absolute PCR product quantification would be used to estimate bacterial cell numbers, the q-PCR efficiency *E* of each paired strain-primers used in the present study need to be estimated. Briefly, the DNA sample of each strain was serially 1:2 diluted four times, and 2 µl of the undiluted and diluted samples were loaded with the species-specific primers for q-PCR as described before. Linear regression lines were used to estimate *E*. The x-value presented 1:2^n dilutions of DNA and the y-value presented Ct values. The slope ($\Delta y / \Delta x$), which represents *E,* was estimated by linear regression (Microsoft Office 2011, Version 14.1.0, Seattle, WA).

2.4.5 Sample Preparation and q-PCR

There were two different mixed samples; one was the reference mixed sample and the other one was the unknown mixed sample. The former one would be used as the reference and the latter would be the tested sample that we were interested in.

To prepare the reference mixed sample, overnight cultures of the ten species were diluted 1:100 in BHI and inoculated individually for 10 hours (BHI instead of TSB was used because for some strains their growth is poor in TSB). After incubation, their individual colony forming units per ml (CFU/ml) were determined by spiral plating on blood agar plates. The DNA of those samples was extracted as described before, and the same amount (by volume) of samples from each DNA extraction were mixed. Since the ten species were mixed together, the original extracted DNA from each species was diluted 1:10.

To prepare the unknown mixed samples, according to microbiome data (Peterson et al., 2013), overnight *S. mutans* UA159, *S. gordonii*, *S. mitis*, *S. oralis*, *S. salivarius*, *S. sanguinis*, *S. aureus*, *E. faecalis*, *L. casei* and *S. epidermis* cultures were mixed in a 1:3:26:6:1:9:1:1:1:1 ratio (the percentages of *S. salivarius*, *S. aureus*, *E. faecalis*, *L. casei* and *S. epidermis* were not reported in Peterson et al., 2013, they were treated as 1 in the present study). The bacterial number of each strain in overnight broth was pre-calculated based on CFU/ml on agar plates, they were *S. mutans* 1.0×10^8 CFU/ml, *S. gordonii* 5×10^7

CFU/ml, *S. mitis* 4.0×10^8 CFU/ml, *S. oralis* 1.3×10^8 CFU/ml, *S. salivarius* 2.0×10^7 CFU/ml, *S. sanguinis* 1.5×10^7 CFU/ml, *S. aureus* 1.2×10^8 CFU/ml, *E. faecalis* 1.1×10^8 CFU/ml, *L. casei* 3.6×10^8 CFU/ml and *S. epidermis* 4.0×10^8 CFU/ml, respectively. To achieve the final 1:3:26:6:1:9:1:1:1:1 cell ratio, 61.5 µl of *S. mutans*, 369 µl of *S. gordonii*, 400 µl of *S. mitis*, 284 µl of *S. oralis*, 308 µl of *S. salivarius*, 3.69 ml of *S. sanguinis*, 51 µl of *S. aureus*, 56 µl of *E. faecalis*, 17.1 µl of *L. casei* and 15.4 µl of *S. epidermis* cultures were mixed together (Fig. 1-B). The mixed culture (1.0×10^6 total CFU/ml, 5 ml/well) was grown in triplicate in TSB or TSBS for 48 hours in six-well-plates. In TSB broth, planktonic cells were harvested. In TSBS broth, planktonic cells were discarded and the biofilm cells were harvested. The saved cells were washed three times with PBS. Half of the harvested cells were discarded to limit the bacterial number within the DNA extraction kit capacity. Cell total DNA was extracted and processed for q-PCR as described before. The C_T value for the reference sample (control, $C_{T,R}$) and the unknown sample ($C_{T,X}$) of each species were recorded.

2.4.6 Cell Quantification Algorithm

According to Livak and Schmittgen(Livak and Schmittgen, 2001), the equation for PCR exponential amplification is:

$$X_n = X_0 \times (1+E_x)^n \quad [1]$$

Where X_n is the molecule number amplified by q-PCR at cycle n, X_0 is the initial molecule number, E_x is the amplification efficiency of the reaction, and n is the

cycle number. For reference sample (R) and unknown sample (X), they reached threshold at different threshold cycles (C_T).

$$X_T = X_0 \times (1+E_X)^{C_{T,X}} \quad [2]$$

$$R_T = R_0 \times (1+E_R)^{C_{T,R}} \quad [3]$$

$C_{T,R}$ and $C_{T,X}$ are the threshold cycles for reference and unknown samples, respectively. Since the reference and unknown samples belong to the same strain and the same primers are used, their thresholds and efficiencies are considered the same. Thus,

$$X_T = R_T \quad [4]$$

$$E_X = E_R = E \quad [5]$$

dividing [2] by [3] and bringing equation [4] and [5] gives

$$\frac{X_T}{R_T} = \frac{X_0 \times (1+E_X)^{C_{T,X}}}{R_0 \times (1+E_R)^{C_{T,R}}} = \frac{X_0 \times (1+E)^{C_{T,X}}}{R_0 \times (1+E)^{C_{T,R}}} = 1 \quad [6]$$

rearranging provides the expression

$$\frac{X_0}{R_0} = (1+E)^{C_{T,R}-C_{T,X}} \quad [7]$$

The reference microbial cell number (N_R) processed for DNA extraction is estimated as

$$N_R = CFU_R \times V_R \quad [8]$$

where CFU_R is the cell concentration and V_R is the volume of processed cells. The percentage of sample lost during DNA extraction is assumed to be k. Moreover, in preparing the reference mixed culture of q different species, the DNA concentration of each species is 1:q diluted. Thus,

$$R_0 = N_R(1-k)/q \quad [9]$$

and for the unknown mixed sample, without further dilution,

$$X_0 = N_X(1-k) \quad [10]$$

where N_x is the specific microbial number in the mixed culture.

Bringing [9] and [10] into [7] results in

$$\frac{N_X(1-k)}{N_R(1-k)/q} = (1+E)^{C_{T,R}-C_{T,X}} \quad [11]$$

rearranging gives the expression

$$N_X = (1+E)^{C_{T,R}-C_{T,X}} \times \frac{N_R}{q} \quad [12]$$

bringing [8] into [12] gives

$$N_X = (1+E)^{C_{T,R}-C_{T,X}} \times \frac{CFU_R \times V_R}{q} \quad [13]$$

if the unknown mixed culture sample is 1:p diluted before DNA extraction to ensure appropriate cell numbers are processed for DNA extraction (≤2×10^9), the original bacterial number $N_{X,O}$ in the mixed culture is

$$N_{X,O} = N_X \times p \quad [14]$$

bringing [14] into [13] gives

$$N_{X,O} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{p}{q} \quad [15]$$

This is the final equation to calculate the microorganism number in the mixed culture. If the reference single culture instead of reference mixed culture is used and the unknown mixed culture sample is not diluted before DNA extraction, then q = 1 and p = 1, and the equation is

$$N_{X,O} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \quad [16]$$

2.5 Statistical Analysis

Each experiment was independently repeated at least three times. The statistical analysis for each assay were listed in Table 2.5. Briefly, for all the experiments, except the q-PCR for *gtfs* and *gbps* expression and kinetic growth, One-way Analysis of Variance (ANOVA) and the Tukey's multiple-comparisons test were used for statistical analysis by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Student t-test was used for q-PCR data analysis. Two-way ANOVA and the Tukey's multiple-comparisons test were used for kinetic growth (SigmaPlot 12.0, Systat Software, Inc., San Jose, CA, USA). The level of significance was set at $P < 0.05$.

Project	Bacterial Strain
Project I	*S. mutans* (UA159/ATCC 700610) *S. mutans* (UA130/ATCC 700611) *S. mutans* (10449/ATCC 25175) *S. mutans* (A32-2) *S. mutans* (NG8) *S. mutans* (LM7) *S. mutans* (OMZ175)
Project II	*S. mutans* (UA159/ATCC 700610)
Project III	*S. mutans* (UA159/ATCC 700610) *S. gordonii* (ATCC 35105) *S. mitis* (ATCC 49456) *S. oralis* (ATCC 35037) *S. salivarius* (ATCC 27975) *S. sanguinis* (SK36) *S. aureus* (COL) *E. faecalis* (ATCC 29212) *L. casei* (ATCC 393) *S. epidermidis* (PR62A)

Table 2.1.1 Bacterial strains used in each project

Project	Inoculation	Bacterial Growth
Project I	TSB	TSB (Planktonic cells) TSBS (Biofilm cells)
Project II	TSB	TSBS (Planktonic cells) TSBS (Biofilm cells)
Project III	BHI+YE	TSB (Planktonic cells) TSBS (Biofilm cells)

Table 2.1.2 Growth media for each project

Project	Assay	Duration (hours)
Project I	MIC/MBC	24+48
	Planktonic	24
	Biofilm	24
	XTT	24+24
	SEM	24
	Kinetic growth	24
Project II	CLSM	24
	Western blot	12
	q-PCR	12
	LDH	24
Project III	PCR-MSCC	48

Table 2.1.3 Growth duration of each project

Project	Assay	Nicotine Concentrations (mg/ml)
Project I	MIC/MBIC	0, 4, 8, 16 and 32
	Planktonic	0, 0.25, 0.5, 1, 2, 4 and 8
	Biofilm	0, 0.25, 0.5, 1, 2, 4 and 8
	XTT	0, 0.25, 0.5, 1, 2, 4 and 8
	SEM	0, 0.25, 0.5, 1, 2, 4 and 8
	Kinetic growth	0, 0.25, 0.5, 1, 2, 4 and 8
Project II	CLSM	0, 1, 2 and 4
	Western blot	0, 1, 2 and 4
	q-PCR	0 and 2
	LDH	0, 0.25, 0.5, 1, 2 and 4
Project III	PCR-MSCC	0, 2 and 4

Table 2.1.4 Nicotine concentrations used in each project

Primers		Sequences
16S rRNA	F	5’-AGCGTTGTCCGGATTTATTG-3’
	R	5’-CTACGCATTTCACCGCTACA-3’
gtfB	F	5’-TACACTTTCGGGTGGCTTGG-3’
	R	5’-AGAAGCTGTTTCCCCAACAGT-3’
gtfC	F	5’-AGCAGATTCAACTGACGACCG-3’
	R	5’-TCAGTAACAGTGGCGGTTGG-3’
gtfD	F	5’-TGCAAGCGACGGAAAACAAG-3’
	R	5’-GCCTGTCAGAGCTTCACCAT-3’
gbpA	F	5’-TCATCAGGCACAGAACCACC-3’
	R	5’-CAGTTGAGGCTCGTTTCCCT-3’
gbpB	F	5’-AGGGCAATGTACTTGGGGTG-3’
	R	5’-TTTGGCCACCTTGAACACCT-3’
gbpC	F	5’-TCTGGTTTTTCTGGCGGTGT-3’
	R	5’-GTCAATGCTGATGGAACGCC-3’
gbpD	F	5’-TTGACTCAGCAGCCTTTCGT-3’
	R	5’-CTTCTGGTTGATAGGCGGCA-3’

Table 2.3 Specific primers for *gtfs* and *gbps* q-PCR

F: forward

R: reverse

Strains		Sequences
S. mutans	F	5’-AGTCGTGTTGGTTCAACGGA-3’
	R	5’-TAAACCGGGAGCTTGATCGG-3’
S. gordonii	F	5’-GCCTTAATAGCACCGCCACT-3’
	R	5’-CCATCTCTGTTGTTAGGGCGT-3’
S. mitis	F	5’-CATCTCACGGGTTGAAGCCT-3’
	R	5’-CCTCGCAGACTAAATTCGCC-3’
S. oralis	F	5’-GGCCGTGAGAATGTGATTGC-3’
	R	5’-TGTTACAGCCTGACCACCAC-3’
S. salivarius	F	5’-CTGCTCTTGTGACAGCCCAT-3’
	R	5’-ACGGGAAGCTGATCTTTCGTA-3’
S. sanguinis	F	5’-TCAGCAAATCCCCCAGGTTC-3’
	R	5’-AACGGAGTGTCAGCGAAGTT-3’
S. aureus	F	5’-TCAGATGAGCAAGCTTCACCAA-3’
	R	5’-TGGCTGTACTGCTGCTATACG-3’
E. faecalis	F	5’-CGCGAACATTTGATGTGGCT-3’
	R	5’-GTTGATCCGTCCGCTTGGTA-3’
L. casei	F	5’-AAGAAAGGCTCACTGGTCGG-3’
	R	5’-TTTTGGCCCGGATTCGATGA-3’
S. epidermidis	F	5’-CATATGGACCTGCACCCCAA-3’
	R	5’-GCAACTGCTCAACCGAGAAC-3’

Table 2.4 Specific primers for PCR-MSCC

F: forward

R: reverse

Project	Assay	Statistics
Project I	MIC/MBIC	N/A
	Planktonic	One-Way ANOVA + Tukey
	Biofilm	One-Way ANOVA + Tukey
	XTT	One-Way ANOVA + Tukey
	SEM	N/A
	Kinetic growth	Two-Way ANOVA + Tukey
Project II	CLSM	One-Way ANOVA + Tukey
	Western blot	One-Way ANOVA + Tukey
	q-PCR	Student t-test
	LDH	One-Way ANOVA + Tukey
Project III	PCR-MSCC	One-Way ANOVA + Tukey

Table 2.5 Statistical Analysis

CHAPTER THREE

Results

3.1 Project I: Effect of Nicotine on *S. mutans* Growth and Metabolism

3.1.1 MIC/MBC/MBIC

The MIC/MBC results of nicotine were consistent among all the *S. mutans* strains (Table 3.1). The MIC was 16 mg/ml (0.1 M), and the MBC was 32 mg/ml (0.2 M). The MBIC of nicotine on *S. mutans* was 16 mg/ml (0.1 M), except strains 10449 and OMZ175 which were both 8 mg/ml (0.05 M).

3.1.2 Planktonic Culture Growth

Overall, the planktonic growth of *S. mutans* was repressed by 4 and 8 mg/ml nicotine concentrations (Fig. 3.1.1). The growth of UA159 and UA130 was not significantly affected by nicotine. The growth of 10449, NG8, A32-2, LM7, and OMZ175 was repressed by 2.0-8.0 mg/ml nicotine, and the growth of A32-2 was significantly increased by 0.25 and 0.5 mg/ml nicotine.

3.1.3 Biofilm Formation

S. mutans biofilm formation was increased in a nicotine-dependent manner, although the increase still varied from one strain to another (Fig. 3.1.2). *S. mutans* UA159, UA130, 10449, A32-2, LM7, and OMZ175 had increased biofilm mass in some nicotine concentrations between 0.5 to 4 mg/ml. At 8.0 mg/ml of

nicotine, biofilm formation was increased in UA159, A32-2, and NG8 cultures, while decreased in 10449. The nicotine concentrations inducing maximum biofilm formation were 4.0-8.0 mg/ml, except strain LM7 (1.0 mg/ml).

3.1.4 Biofilm Metabolic Activity

The overall biofilm metabolic activity of all seven *S. mutans* strains significantly increased in a nicotine dose dependent manner (Fig. 3.1.3). The biofilm metabolic activity started to increase primarily in nicotine concentrations of 0.50-2.0 mg/ml, and the maximum metabolic activity of most strains was observed at a nicotine concentration of 8.0 mg/ml. However, *S. mutans* UA159, 10449, NG8, and OMZ175 still maintained higher biofilm metabolic activity at 16 mg/ml than the control (0 mg/ml nicotine) cultures, while *S. mutans* UA130 had lower biofilm metabolic activity than the control. The maximum metabolic activity of A32-2 was observed at a nicotine concentration of 16.0 mg/ml. The lowest metabolic activity for all *S. mutans* strains was observed at 32.0 mg/ml nicotine.

The MRBM of most *S. mutans* strains (UA159, 10449, A32-2, NG8 and OMZ 175) was significantly increased in most strains (UA130, 10449, NG8 and OMZ175) in a nicotine dose dependent manner up to 8.0 mg/ml and in others (UA159 and A32-2) up to 16.0 mg/ml (Fig. 3.1.4).

3.1.5 Scanning Electron Microscopy

The *S. mutans* UA159 biofilm mass was obviously increased in 2.0-8.0 mg/ml nicotine-treated groups (Fig. 3.1.5, A1-G1). Without nicotine, *S. mutans* cells were more randomly distributed with shorter chain length than their counterparts treated with 0.5-2.0 mg/ml nicotine (Fig. 3.1.5, A2-E2). Fewer *S. mutans* cells were observed in the gaps between the biofilm accumulations among the 4.0 and 8.0 mg/ml nicotine groups than lower nicotine groups (Fig. 3.1.5, F2-G2), nevertheless, more *S. mutans* cells were aggregated in those nicotine groups. The morphological changes of the *S. mutans* cells were also notable. In the 8.0 mg/ml nicotine-treated group, the cells developed a spherical appearance instead of the usual ellipsoid shape (Fig. 3.1.5, G3).

3.1.6 Kinetic Growth

In the *S. mutans* UA159 planktonic cell growth curve, *S. mutans* growth was stimulated by 0.25 and 0.5 mg/ml nicotine. Time to log phase of *S. mutans* growth at 1 and 2 mg/ml nicotine was longer than 0 nicotine control. The growth of *S. mutans* at 4 and 8 mg/ml nicotine was inhibited (Fig. 3.1.6, Panel A). *S. mutans* growth in 2, 4 and 8 mg/ml nicotine was statistically significantly decreased at 12 and 24 hours from 0 nicotine control. *S. mutans* growth in 1 mg/ml nicotine was significantly decreased at 8 mg/ml (Fig. 3.1.6, Panel B).

In the *S. mutans* UA159 kinetic biofilm formation, *S. mutans* biofilm was significantly increased in 1, 2 and 4 mg/ml nicotine at 8, 12 and 24 hours than 0

nicotine control. *S. mutans* biofilm in 8 mg/ml nicotine was significantly decreased at 8 hours but significantly increased at 24 hours (Fig. 3.1.7).

Table 3.1 MIC, MBC, and MBIC of nicotine on *S. mutans* strains

***S. mutans* Strains**	**MIC (mg/ml)**	**MBC (mg/ml)**	**MIBC (mg/ml)**
UA159 (ATCC700610)	16	32	16
UA130 (ATCC 700611)	16	32	16
10449 (ATCC 25175)	16	32	8
A32-2	16	32	16
NG8	16	32	16
LM7	16	32	16
OMZ175	16	32	8

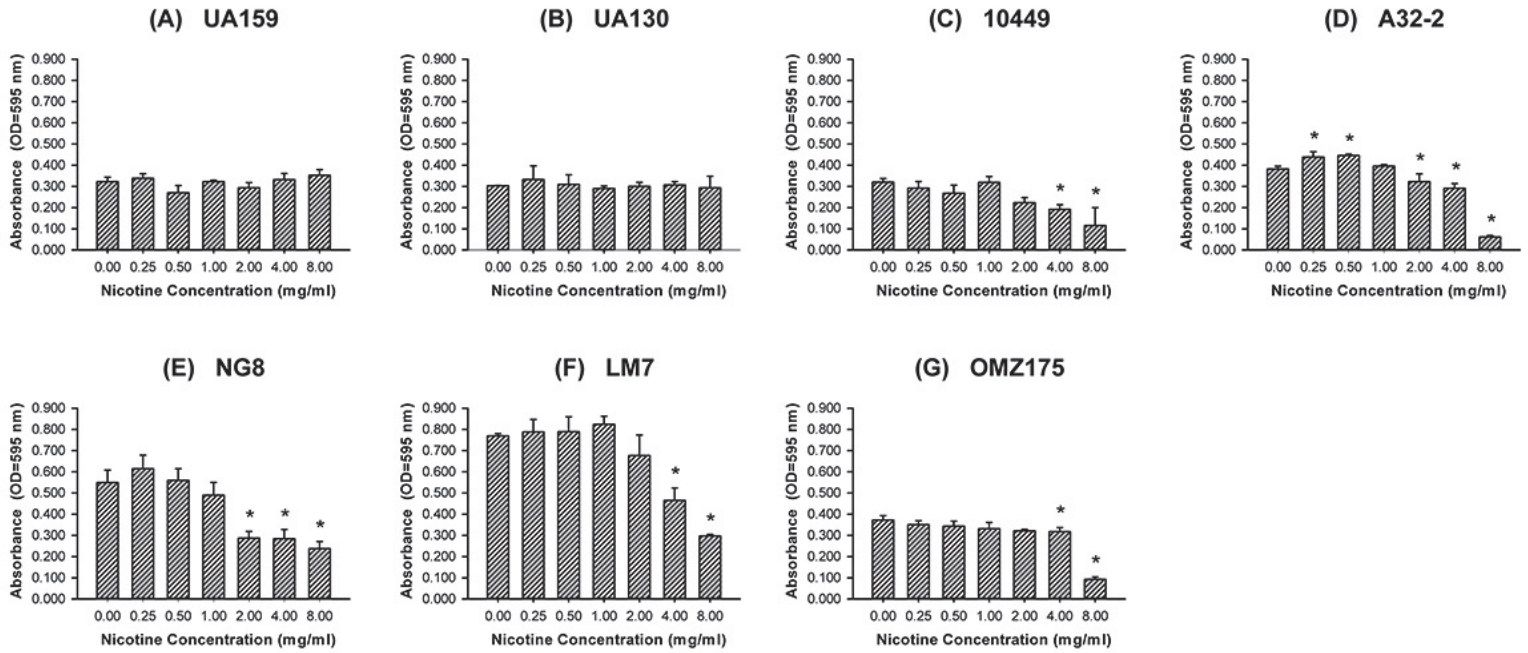


Fig. 3.1.1 **Planktonic culture growth of seven strains of *S. mutans* (A-G) in the presence of nicotine after 24 hours.**

The absorbance (OD_{595nm}) of *S. mutans* planktonic cells in different nicotine concentrations is shown with means and standard deviations. The experiment was repeated three times and asterisks indicate significant differences compared with the 0 mg/ml nicotine control.

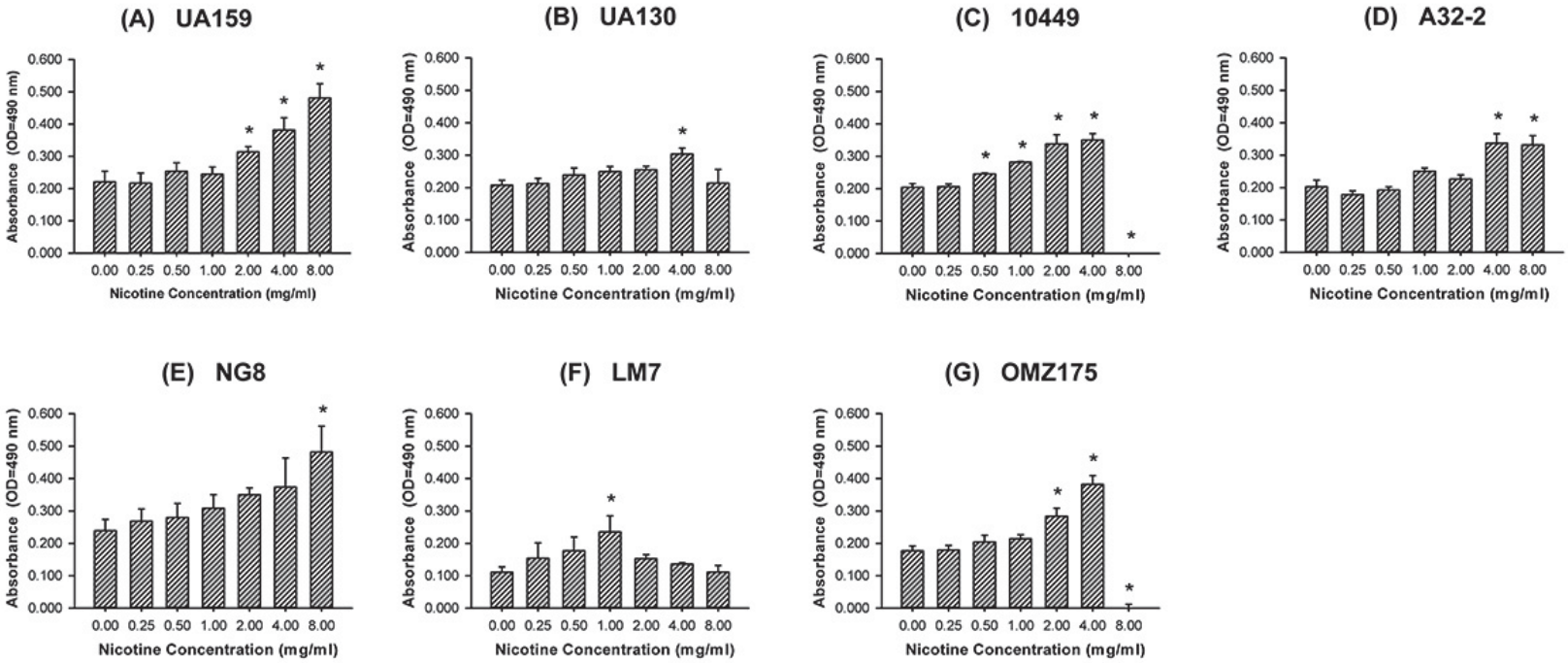


Fig. 3.1.2 **Biofilm formation of seven strains of *S. mutans* (A-G) in the presence of nicotine after 24 hours.**

The absorbance (OD_{490nm}) of crystal violet stained *S. mutans* biofilm in different nicotine concentrations is shown with means and standard deviations. The experiment was repeated three times and asterisks indicate significant differences compared with the 0 mg/ml nicotine control.

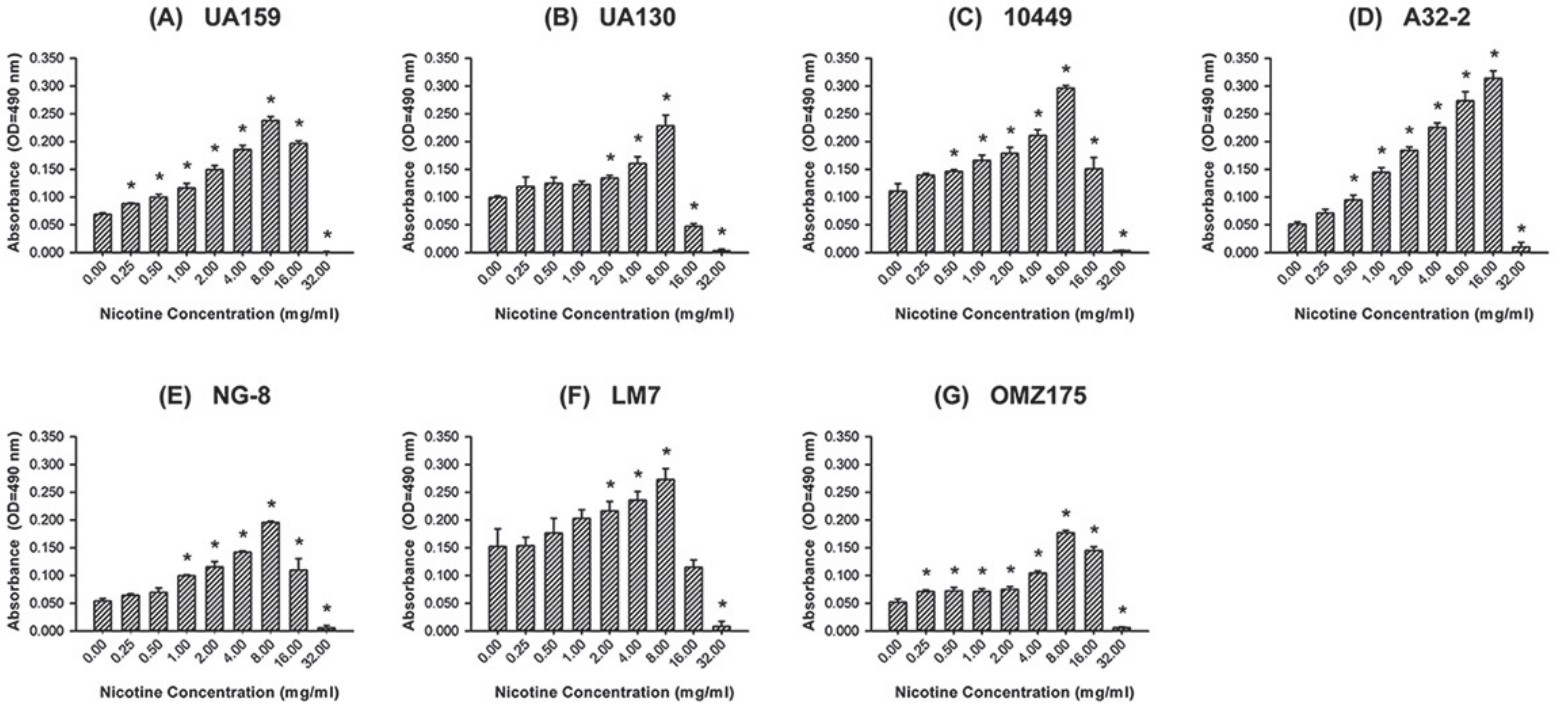


Fig. 3.1.3 **Biofilm metabolic activity of seven strains of *S. mutans* (A-G) in the presence of nicotine after 24 hours using an XTT/menadione assay.**

The absorbance (OD_{490nm}) reflecting the metabolic activity of *S. mutans* biofilm cells in different nicotine concentrations is shown with means and standard deviations. The experiment was repeated three times and asterisks indicate significant differences compared with the 0 mg/ml nicotine control.

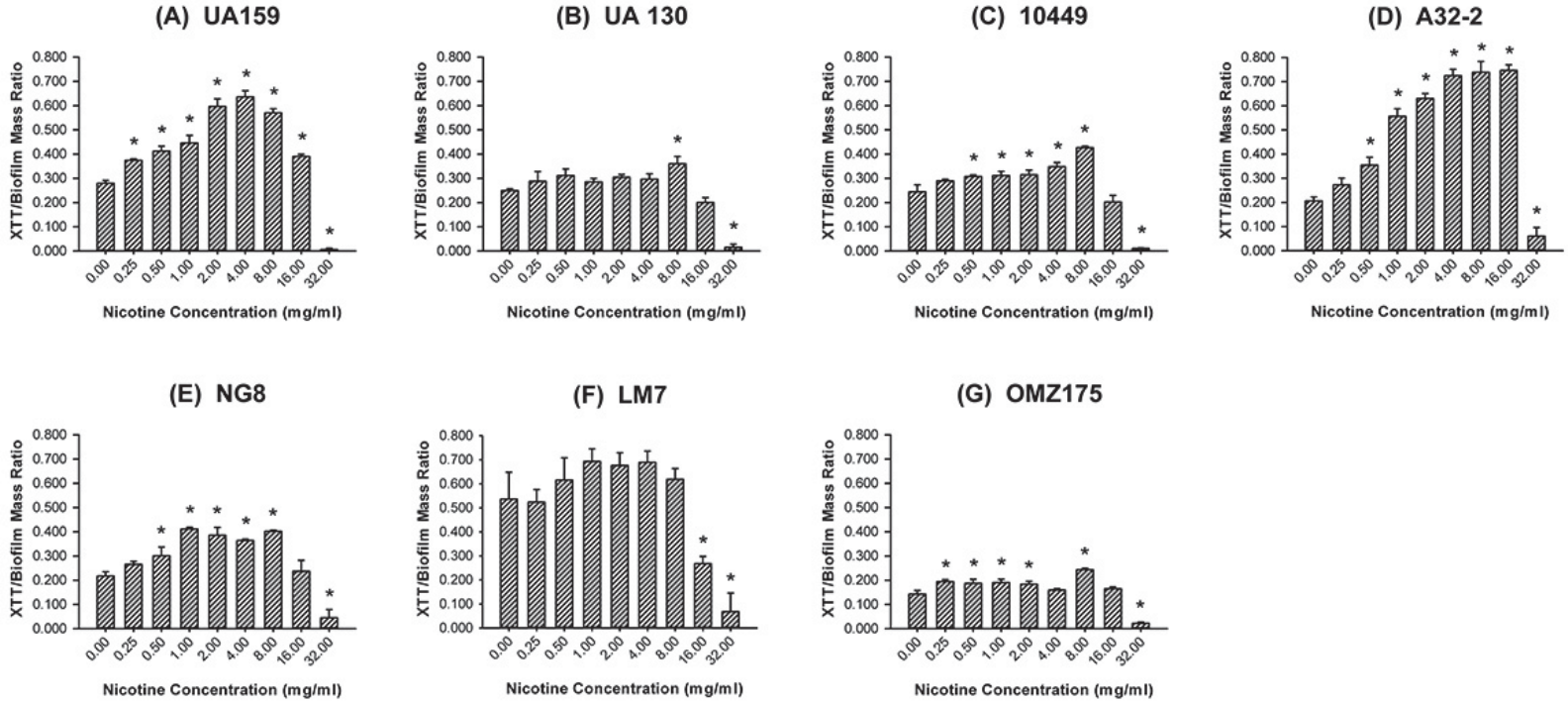


Fig. 3.1.4 **Metabolism relative to biofilm mass (MRBM) of seven strains of *S. mutans*.**

The biofilm metabolic activity of each sample was devided by its own biofilm formation result. The experiment was repeated three times and asterisks indicate significant differences compared with the 0 mg/ml nicotine control.

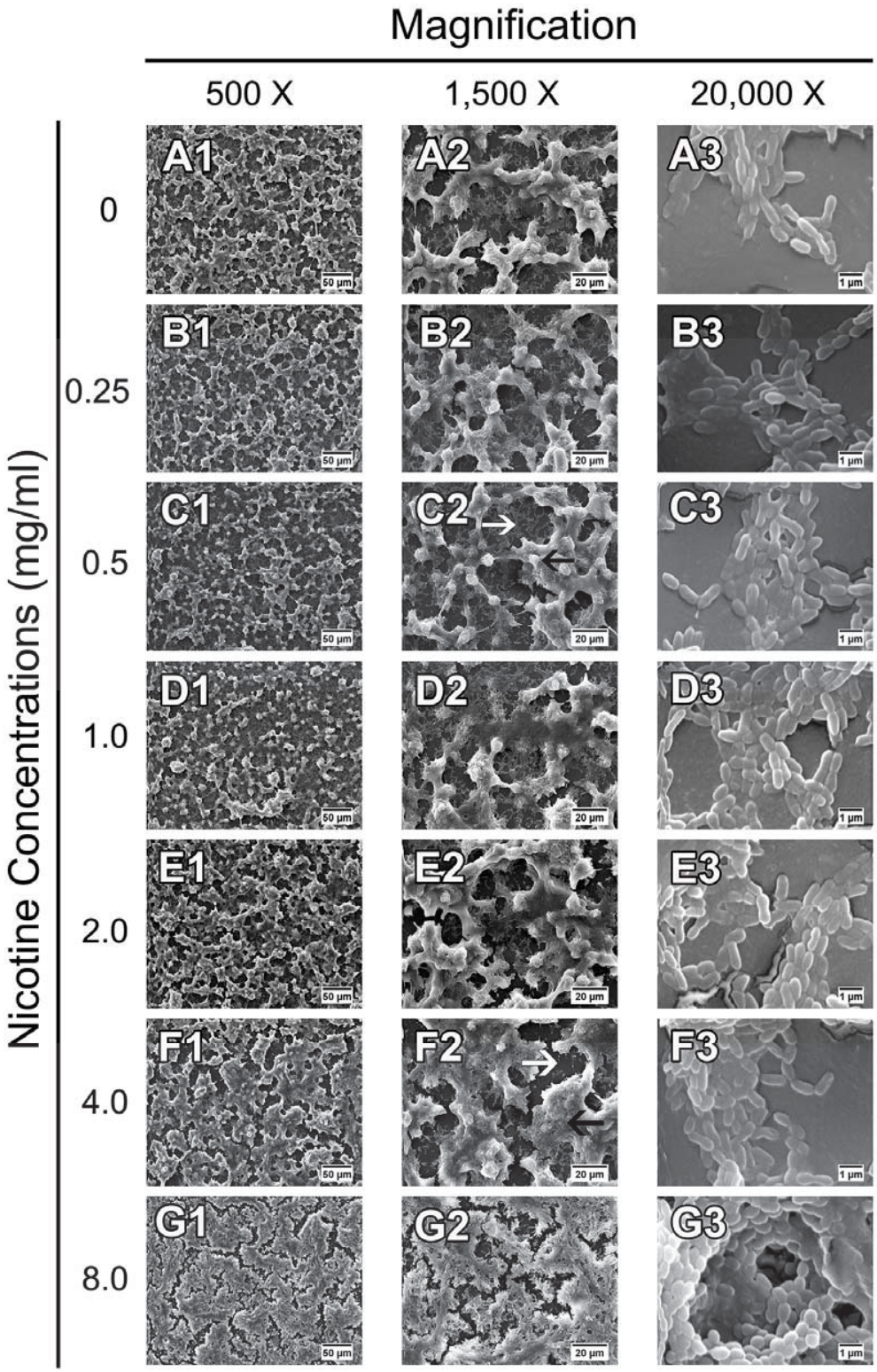

Magnification
500 X
1,500 X
20,000 X
Nicotine Concentrations (mg/ml)
0
0.25
0.5
1.0
2.0
4.0
8.0
A1
A2
A3
B1
B2
B3
C1
C2
C3
D1
D2
D3
E1
E2
E3
F1
F2
F3
G1
G2
G3
50 µm
20 µm
1 µm

Fig. 3.1.5 **Scanning electron microscopy of *S. mutans* UA159 treated with 0, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/ml nicotine for 24 hours.**

Images were obtained at 500X (A1-G1), 1,500X (A2-G2) and 20,000X (A3-G3) magnifications. Compared with low nicotine treated groups, high nicotine treated ones indicated thicker biofilm with more spherical bacterial cells. More bacterial cells were present in the gaps (white arrows) between the biofilm accumulations (black arrows) in 0-1.0 mg/ml nicotine than in 2.0-8.0 mg/ml nicotine.

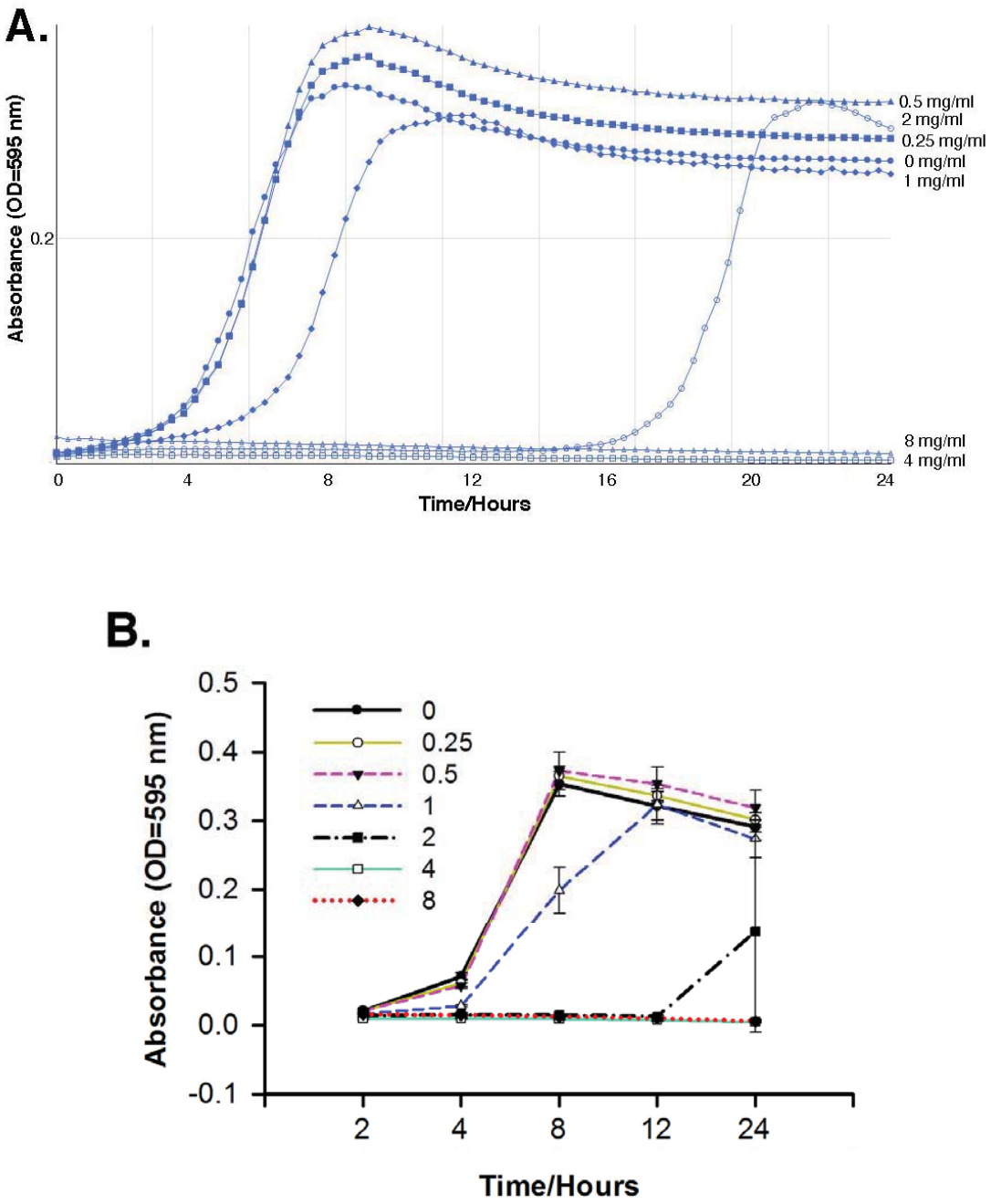


Fig. 3.1.6 ***S. mutans* Growth Curve with Nicotine.**

S. mutans UA159 was grown in TSB with 0-8 mg/ml nicotine for 24 hours in spectrophotometer at 37°C without CO_2 supply. **Panel A** demonstrated the

growth curve of *S. mutans*. In **Panel B**, the absorbance data was extracted at 2, 4, 8, 12 and 24 hour points. Each experiment was independently repeated three times.

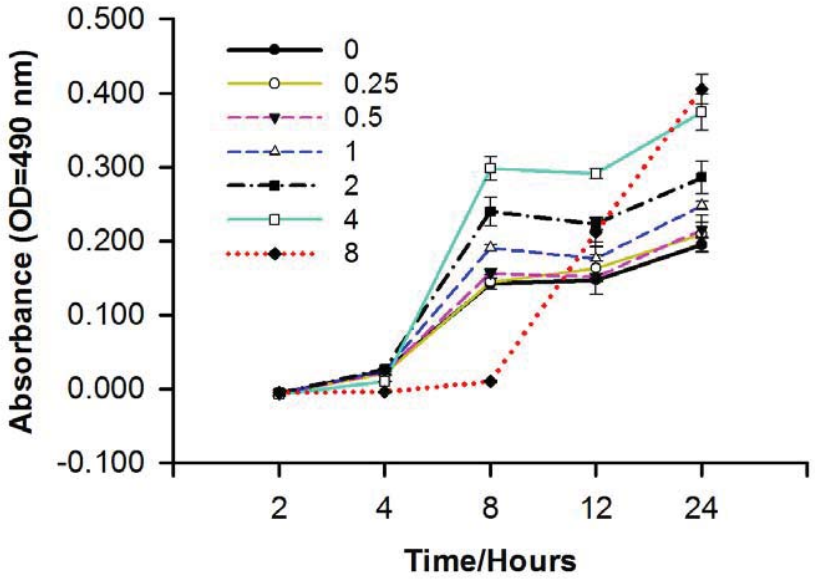


Fig. 3.1.7 ***S. mutans*** **Kinetic Biofilm Formation with Nicotine.**

S. mutans UA159 was grown in TSBS with 0-8 mg/ml nicotine. At 2, 4, 8, 12 and 24 hours, biofilm formation was quantified by crystal violet assay. Each experiment was independently repeated three times.

3.2 Project II: Effect of Nicotine on *S. mutans* UA159 Cell Binding, EPS Synthesis and LDH Activity

3.2.1 Confocal Laser Scanning Microscopy (CLSM)

Both *S. mutans* UA159 bacterial cell numbers and EPS synthesis were increased by nicotine treatment. Biofilm cells in the 0 nicotine controls were spread out with several small aggregates, while the cells in the nicotine treated samples were more aggregated and contained larger aggregates (Fig. 3.2.1). There were less blank areas, which are indicative of biofilm channels, in the nicotine-treated groups than the non-treated groups. These results are consistent with previous SEM data. Bacterial cell and EPS volumes were significantly increased in the 2 and 4 mg/ml nicotine groups. Bacteria and EPS biofilm coverage demonstrated a trend to be increased by nicotine treatment, but only bacterial cell coverage at 4 mg/ml was significant. In addition, biofilm thickness was significantly increased at 4 mg/ml (Table 3.2).

3.2.2 Western Blotting

The GbpA and GbpB expression of planktonic cells was 8.1 and 2.5 fold higher, respectively, than that of biofilm cells (Fig. 3.2.2.1). Expression of Gtfs from biofilm cells was difficult to detect with 3 μg of the samples and its data was not included. Planktonic cell GbpA expression was significantly increased up to 4.0 fold by nicotine, Gtfs expression was increased up to 2.2 fold but this was not statistically significant, and GbpB was unchanged (Fig. 3.2.2.2). Biofilm cell

GbpA was unchanged while GbpB was decreased 75% by nicotine treatment (Fig. 3.2.2.3).

3.2.3 q-PCR of *gtfs* and *gbps*

The mRNA levels of planktonic cell *gtfC, gbpA* and *gbpB* were increased 1.2 to 2 fold by nicotine, but none of the augmentation was statistically significant due to the large standard deviations (Fig. 3.2.3). The mRNA level of biofilm cell *gtfB, gtfC, gtfD, gbpB* and *gbpD* was 0.46 to 0.88 fold significantly decreased by nicotine. No significant genomic DNA was detected in the original RNA samples.

3.2.4 LDH Assay

Overall, *S. mutans* biofilm LDH activity was significantly increased at 1, 2 and 4 mg/ml nicotine (Fig. 3.2.4). However, after adjustment by the biofilm volume, which was determined by crystal violet assay (data not shown), there was no difference among those groups. This indicates nicotine does not directly affect LDH activity of *S. mutans*, but with the increased bacterial cell number induced by nicotine treatment, the overall biofilm LDH activity was increased.

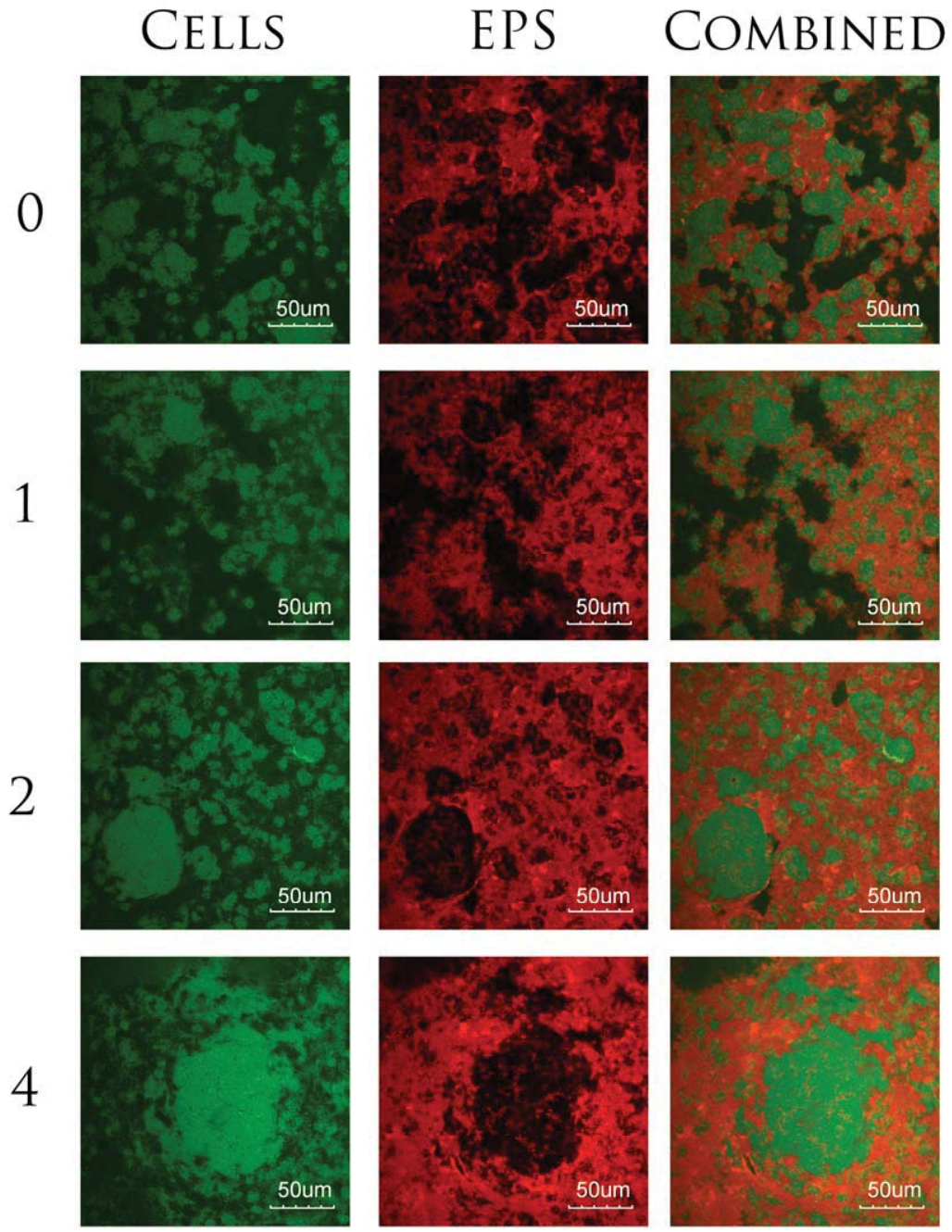


Fig. 3.2.1 **Multiplication of *S. mutans* UA159 biofilm bacterial cells and synthesis of EPS with nicotine.**

S. mutans was treated with 0-4 mg/ml nicotine (left axis) for 24 hours in TSBS. Bacterial cells were labeled with SYTO® 9 green fluorescence and EPS was

labeled with Alexa Fluor 647® red fluorescence. Results indicated bacterial cell multiplication and EPS synthesis were increased by nicotine. Bacterial cells were more aggregated at higher nicotine concentrations than lower concentrations.

Table 3.2 Biofilm coverage, volume and thickness of *S. mutans* UA159 treated with nicotine

Nicotine (mg/ml)	Bacteria		EPS		Thickness (μm)
	Volume ($\mu m^2/\mu m$)	Coverage (%)	Volume ($\mu m^2/\mu m$)	Coverage (%)	
0	47.67±6.45	35.33±3.84	69.33±13.08	55.38±12.24	38.67±3.42
1	50.80±4.47	40.32±10.09	71.36±11.25	60.41±10.01	41.06±0.69
2	60.27±4.82*	45.87±3.57	99.13±16.25*	72.75±13.26	42.95±2.81
4	77.60±6.47**	69.27±4.28*	96.90±4.75*	73.63±1.68	52.41±4.01*

Asterisks indicate significant difference from the 0 nicotine control sample. * denotes $P<0.05$, and ** denotes $P<0.01$.

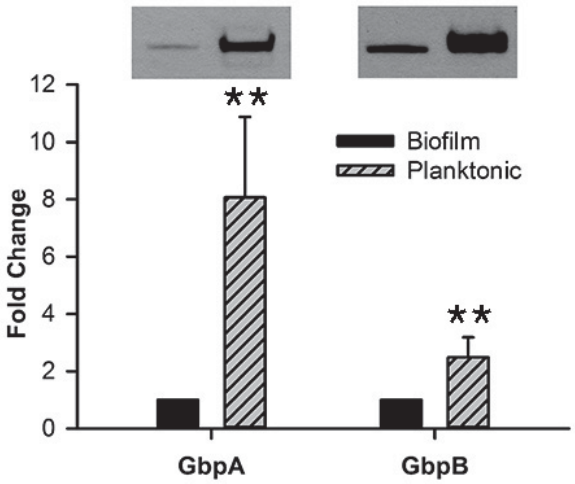


Fig. 3.2.2.1 **Western blot of *S. mutans* UA159 Gbps expression without nicotine.**

S. mutans was grown in TSBS without nicotine for 12 hours. Both planktonic and biofilm cells were harvested. The intensity of biofilm cells was set at 1 and the fold change of the planktonic cells was calculated. The GbpA and GbpB expression of biofilm cells compared to planktonic cells were demonstrated. Asterisks indicate significant differences from the biofilm. ** denotes P<0.01.

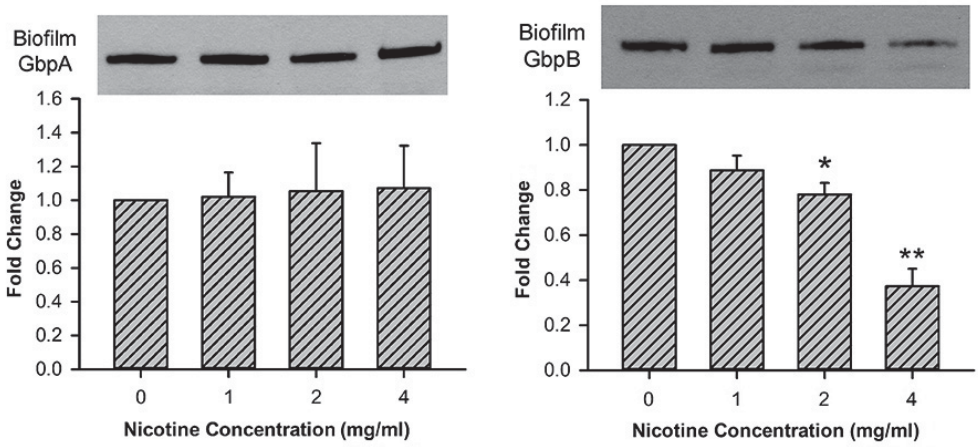


Fig. 3.2.2.2 **Western blot of *S. mutans* UA159 biofilm cell Gbps expression with nicotine.**

S. mutans was grown in the presence of 0-4 mg/ml nicotine for 12 hours in TSBS. Biofilm cells were harvested. The 0 nicotine control is set at 1 while the fold increase from the 0 control was calculated. GbpA and GbpB expression of biofilm cells treated with different nicotine concentrations were demonstrated. Asterisks indicate significant differences from the 0 nicotine control sample. * denotes $P<0.05$, and ** denotes $P<0.01$.

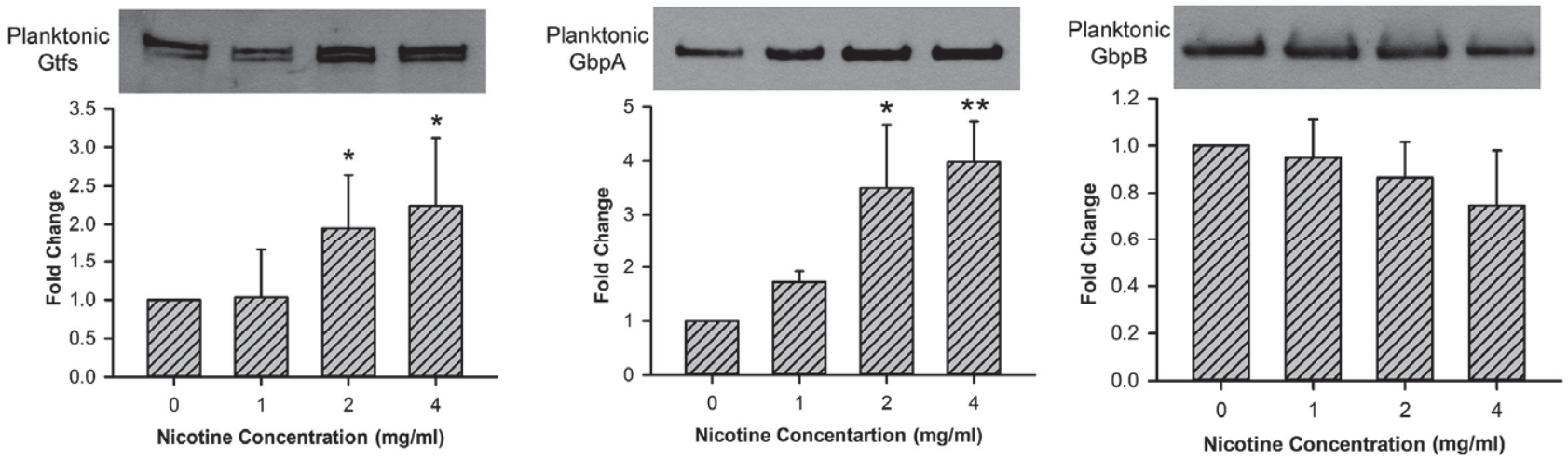


Fig. 3.2.2.3 **Western blot of *S. mutans* UA159 planktonic cell Gtfs and Gbps expression with nicotine.**

S. mutans was grown in the presence of 0-4 mg/ml nicotine for 12 hours in TSBS. Planktonic cells were harvested. The 0 nicotine control was set at 1 while the fold increase from the 0 control was calculated. Gtfs, GbpA and GbpB expression of planktonic cells treated with different nicotine concentrations were demonstrated. Asterisks indicate significant differences from the 0 nicotine control sample. * denotes $P<0.05$, and ** denotes $P<0.01$.

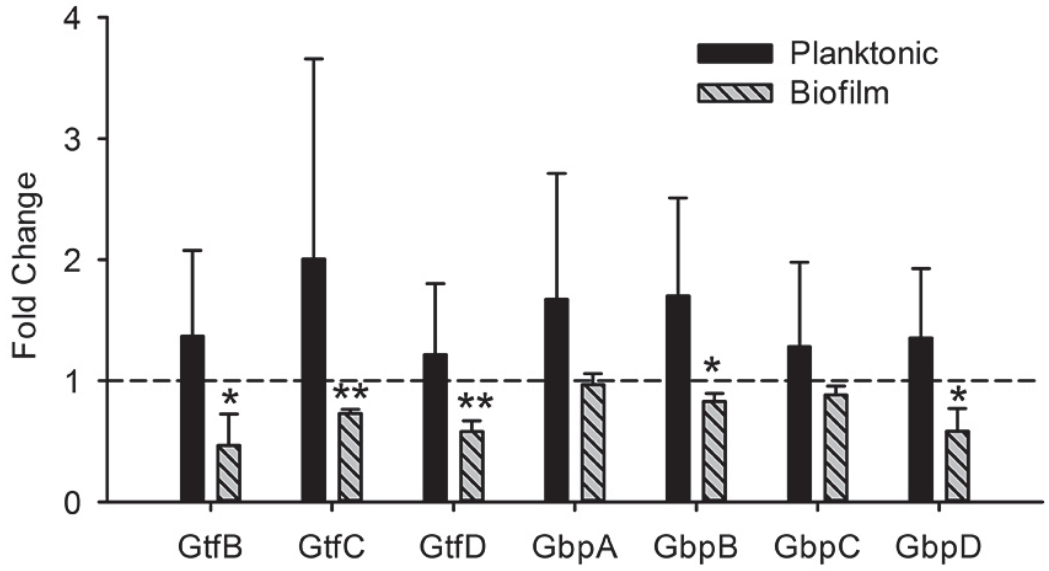


Fig. 3.2.3 ***S. mutans* UA159 *gtfs* and *gbps* gene expression.**

S. mutans was grown in the presence of 0 and 2 mg/ml nicotine for 12 hours in TSBS. Both planktonic and biofilm cells were harvested for q- PCR. The 2 mg/ml nicotine-treated q-PCR is shown. Black solid bars represent planktonic cell gene expression while gray streaked bars represent biofilm cell gene expression. The reference line of 1 indicated gene expression of the 0 nicotine group for each gene. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes: $P<0.01$.

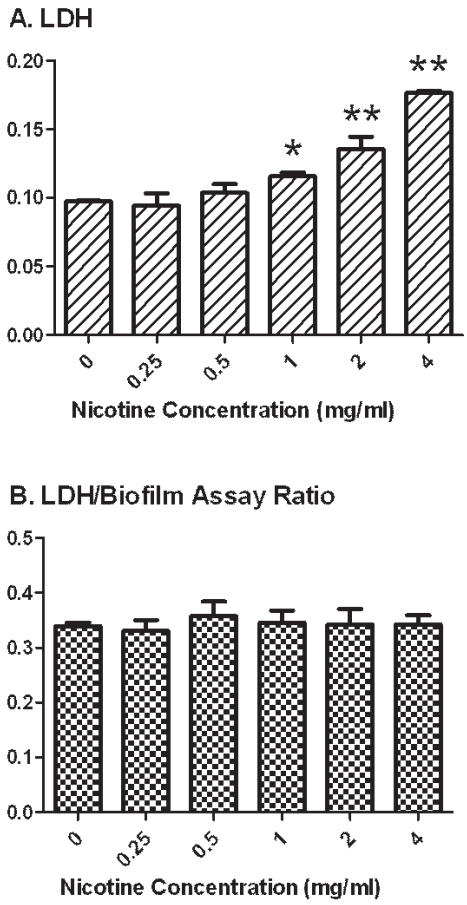


Fig. 3.2.4 **LDH activity of *S. mutans* UA159 with nicotine.**

S. mutans was treated with 0-4 mg/ml nicotine for 24 hours in TSBS. Biofilm was washed and processed for LDH assay. The manufacturer's protocol was followed. **Panel A** demonstrates the overall biofilm LDH activity. Since nicotine stimulates *S. mutans* biofilm formation, crystal violet assay was used to estimate the amount of biofilm in each treatment (data not shown). **Panel B** demonstrates the ratio of LDH assay over the crystal violet assay data. This represents unit cell LDH activity. Asterisks indicate significant differences between nicotine treated and non-treated. * denotes $P<0.05$, and ** denotes $P<0.01$.

3.3 Project III: Effect of Nicotine on *S. mutans* UA159 Proliferation in A Ten Species Mixed Culture

3.3.1 Primer specificity test

The primer specificity test results demonstrated that the DNA of each species was amplified only by its own species-specific primers (Fig. 3.3.1), although the intensity of each band varied.

3.3.2 PCR-MSCC validation range test

The validation range test results indicated most of the errors happened at the 10^{-6} range, which means for PCR-MSCC any two species cell numbers should vary no more than 10^5 if a 10% error is acceptable or no more than 10^4 if a 1% error is acceptable (Table 3.3.1). Specifically, 4.4% errors occured at the 10^{-4} range, 25.6% errors occured at the 10^{-5} range, 56.7% errors occured at the 10^{-6} range and 13.3% errors occured at the 10^{-7} range.

3.3.3 q-PCR efficiency test

The q-PCR efficiency test implied the median *E* is 82.7% with range from 70.0% to 98.5%. The coefficient of determination (R^2) of every regression line was equal to or larger than 0.95 (Fig. 3.3.2).

3.3.4 Cell proliferation of 10 mixed species

The parameters for cell number calculation were listed in Table 3.3.2. The cell quantification results demonstrated the cell number of the ten species varied from 2.51×10^3 to 8.87×10^8 cells. Since the most significant difference was at the 10^5 range, the data of *S. aureus* and *S. epidermidis* were removed to prevent bias. The next lowest colony number was observed in *L. casei* with 3.45×10^5 cells, which was different from the highest cell number at an acceptable range of 10^3. Planktonic cell growth of *S. mutans* in the mixed culture was not significantly changed by nicotine treatment (Fig. 3.3.4.1), which was consistent to its individual planktonic cell growth (Fig. 3.1.1). Biofilm formation of *S. mutans* in the mixed culture was significantly increased at 2 and 4 mg/ml nicotine (Fig. 3.3.4.1), which was consistent to its individual biofilm formation (Fig. 3.1.2). Planktonic cell growth and biofilm formation of *S. gordonii* were increased at 4 mg/ml (Fig. 3.3.4.2). Planktonic cell growth of *S. mitis* was increased at 2 mg/ml but not 4 mg/ml, and biofilm formation of *S. mitis* indicated a decrease trend (Fig. 3.3.4.3). Planktonic cell growth of *S. oralis* was not significantly changed by nicotine, while biofilm formation of *S. oralis* was significantly increased at 4 mg/ml (Fig. 3.3.4.4). Planktonic cell growth of *S. salivarius* was significantly increased at 4 mg/ml nicotine and its biofilm formation was significantly increased at 2 and 4 mg/ml nicotine (Fig. 3.3.4.5). Planktonic cell growth of *S. sanguinis* was significantly increased at 4 mg/ml nicotine, and its biofilm formation was significantly increased at 2 and 4 mg/ml nicotine (Fig. 3.3.4.6). Planktonic cell growth of *E. faecalis* was not significantly changed by nicotine, but its biofilm formation was

significantly increased at 2 and 4 mg/ml nicotine (Fig. 3.3.4.7). Planktonic cell growth of *L. casei* was significantly decreased at 4 mg/ml nicotine, and its biofilm formation was decreased at 2 and 4 mg/ml nicotine (Fig. 3.3.4.8).

The percentage of each bacterial species cell number within the mixed culture was calculated by the cell number of a certain species divided by the sum of the ten species cell number. In planktonic mixed cultures, *E. faecalis* was increased in 2 mg/ml but decreased in 4 mg/ml, while *S. oralis* was decreased in 2 mg/ml but increased in 4 mg/ml. However, if *E. faecalis* and *S. oralis* were considered as a single component, their proportions were consistently decreased by nicotine. The proportions of *S. salivarius* and *S. gordonii* were consistently increased by nicotine. The proportions of *S. sanguinis* and *S. mutans* were not significantly changed by nicotine. The proportion of *L. casei* was decreased by nicotine (Fig. 3.3.4.9; Table 3.3.3).

In the biofilm mixed cultures, the proportions of *S. oralis*, *S. mitis*, *L. casei* were decreased by nicotine. The proportion of *E. faecalis* was increased by nicotine. The proportions of *S. gordonii*, *S. sanguinis* and *S. mutans* were not changed. Notably, the proportion of *L. casei* in the biofilm mixed cultures was higher that it in planktonic cell mixed cultures (Fig. 3.3.4.10; Table 3.3.3).

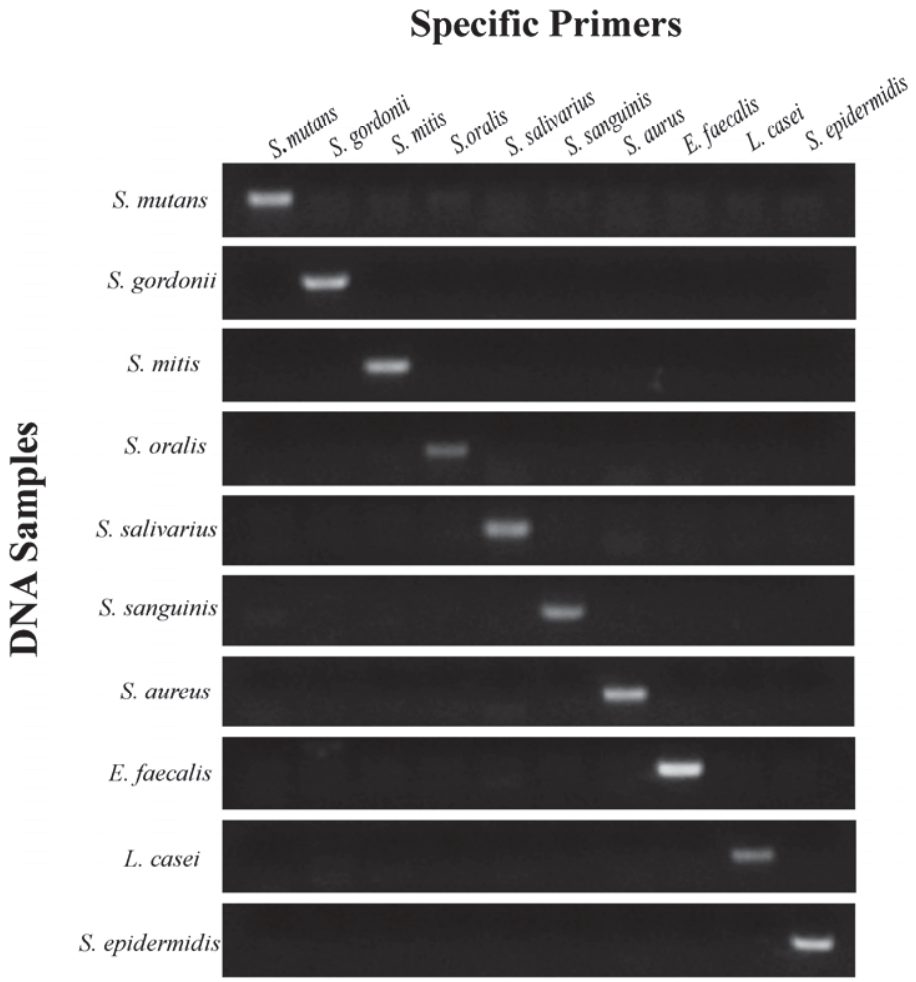


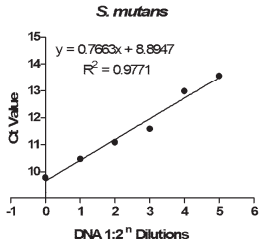
Fig. 3.3.1 **Primers specificity.**

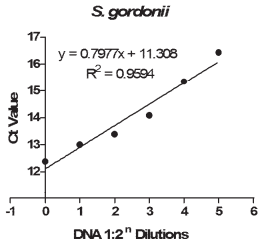
The total DNA of each species was loaded with every pair of the species-specific primers for PCR. Each horizontal row represents the DNA extracted from one strain, and each vertical column represents the strain specific-primers of each strain.

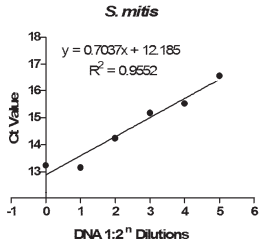
		Primers									
		S. mutans	*S. gordonii*	*S. mitis*	*S. oralis*	*S. salivarius*	*S. sanguinis*	*S. aureus*	*E. faecalis*	*L. casei*	*S. epidermidis*
DNA	*S. mutans*	1	3.01×10^{-6}	1.71×10^{-6}	1.03×10^{-6}	3.51×10^{-6}	6.25×10^{-7}	3.53×10^{-6}	4.57×10^{-6}	7.03×10^{-7}	1.97×10^{-6}
	S. gordonii	1.45×10^{-5}	1	1.44×10^{-5}	2.03×10^{-6}	6.78×10^{-6}	1.15×10^{-6}	4.47×10^{-6}	2.73×10^{-5}	2.35×10^{-5}	4.86×10^{-6}
	S. mitis	2.92×10^{-4}	1.67×10^{-5}	1	2.38×10^{-6}	3.92×10^{-5}	4.57×10^{-6}	8.05×10^{-5}	1.68×10^{-4}	1.58×10^{-5}	8.06×10^{-6}
	S. oralis	2.75×10^{-5}	7.12×10^{-6}	1.93×10^{-5}	1	1.69×10^{-5}	2.83×10^{-5}	4.32×10^{-5}	7.94×10^{-5}	2.01×10^{-5}	1.27×10^{-5}
	S. salivarius	9.14×10^{-6}	8.76×10^{-6}	1.95×10^{-6}	5.18×10^{-7}	1	6.42×10^{-7}	2.79×10^{-6}	3.79×10^{-6}	1.22×10^{-6}	4.72×10^{-5}
	S. sanguinis	4.03×10^{-5}	3.53×10^{-4}	2.91×10^{-6}	1.37×10^{-6}	2.28×10^{-5}	1	2.16×10^{-6}	9.07×10^{-6}	1.65×10^{-5}	1.92×10^{-6}
	S. aureus	2.27×10^{-6}	3.39×10^{-5}	2.73×10^{-5}	1.10×10^{-6}	4.51×10^{-6}	1.02×10^{-6}	1	2.43×10^{-6}	3.66×10^{-6}	2.12×10^{-6}
	E. faecalis	3.66×10^{-6}	1.98×10^{-4}	2.53×10^{-6}	3.00×10^{-7}	3.87×10^{-6}	3.02×10^{-7}	2.62×10^{-6}	1	7.53×10^{-7}	4.51×10^{-6}
	L. casei	7.90×10^{-6}	4.29×10^{-6}	1.25×10^{-5}	2.59×10^{-7}	1.04×10^{-6}	2.61×10^{-7}	7.69×10^{-7}	7.28×10^{-7}	1	2.81×10^{-6}
	S. epidermidis	7.63×10^{-6}	6.33×10^{-6}	3.30×10^{-6}	8.19×10^{-7}	4.32×10^{-6}	2.59×10^{-6}	9.39×10^{-6}	7.74×10^{-6}	2.27×10^{-6}	1

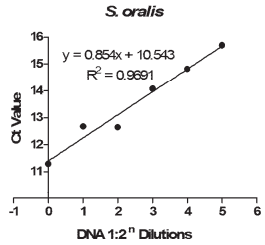
Table 3.3.1 Validation range test of the method

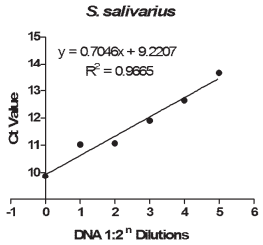
The total DNA of each species was loaded with every pair of the species-specific primers for q-PCR. The amount of q-PCR product amplified by its own species-specific primers was defined as 1 (control), and the amount of products amplified by other primers were calculated based on their fold changes compared to control. Each horizontal row represents DNA extracted from one strain, and each vertical column represents the species-specific primers of each strain.

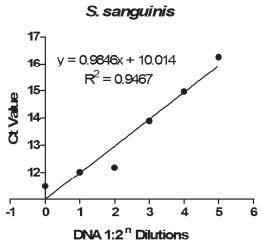

S. mutans
y = 0.7663x + 8.8947
R2 = 0.9771
Ct Value
DNA 1:2n Dilutions

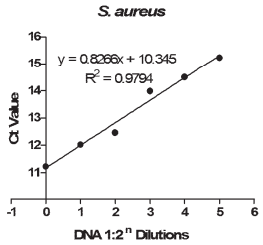

S. gordonii
y = 0.7977x + 11.308
R2 = 0.9594
Ct Value
DNA 1:2n Dilutions

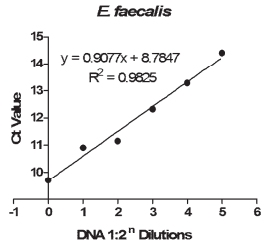

S. mitis
y = 0.7037x + 12.185
R2 = 0.9552
Ct Value
DNA 1:2n Dilutions

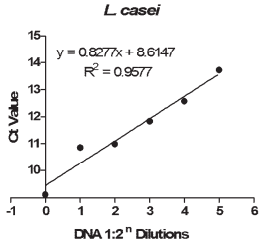

S. oralis
y = 0.854x + 10.543
R2 = 0.9691
Ct Value
DNA 1:2n Dilutions


S. salivarius
y = 0.7046x + 9.2207
R2 = 0.9665
Ct Value
DNA 1:2n Dilutions


S. sanguinis
y = 0.9846x + 10.014
R2 = 0.9467
Ct Value
DNA 1:2n Dilutions


S. aureus
y = 0.8266x + 10.345
R2 = 0.9794
Ct Value
DNA 1:2n Dilutions


E. faecalis
y = 0.9077x + 8.7847
R2 = 0.9825
Ct Value
DNA 1:2n Dilutions


L. casei
y = 0.8277x + 8.6147
R2 = 0.9577
Ct Value
DNA 1:2n Dilutions

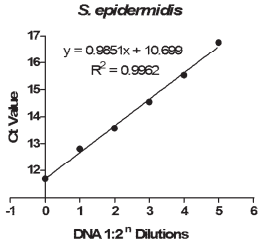

S. epidermidis
y = 0.9851x + 10.699
R2 = 0.9962
Ct Value
DNA 1:2n Dilutions

Fig. 3.3.2 **q-PCR efficiency test.**

The x-axis represents DNA 1:2^n dilutions and the y-axis represents Ct values. Ideally, if one sample is diluted 1:2 ($\Delta x = 1$), it will take one more cycle ($\Delta y = 1$) to reach the same threshold. Ideally, the slope ($\Delta y / \Delta x$), which represents the amplifying efficiency *E*, is equal to 1. For actual samples the slope is less than 1.

	E	$C_{T,R}$	$C_{T,X}$*	CFU_R	V_R	t	q
S. mutans	0.7663	13.00	16.49	3.28×10^{8}	5	2	10
S. gordonii	0.7977	14.34	15.24	4.79×10^{7}	5	2	10
S. mitis	0.7037	15.86	19.95	5.41×10^{8}	5	2	10
S. oralis	0.8540	14.75	13.80	4.40×10^{8}	5	2	10
S. salivarius	0.7046	12.79	11.46	1.41×10^{8}	5	2	10
S. sanguinis	0.9846	13.55	13.07	2.14×10^{7}	5	2	10
S. aureus	0.8266	13.38	20.28	2.63×10^{8}	5	2	10
E. faecalis	0.9077	11.51	13.15	4.48×10^{8}	5	2	10
L. casei	0.8277	12.19	16.80	1.29×10^{8}	5	2	10
S. epidermidis	0.9862	14.84	20.30	1.91×10^{7}	5	2	10

Table 3.3.2 Parameters for each species cell number calculation

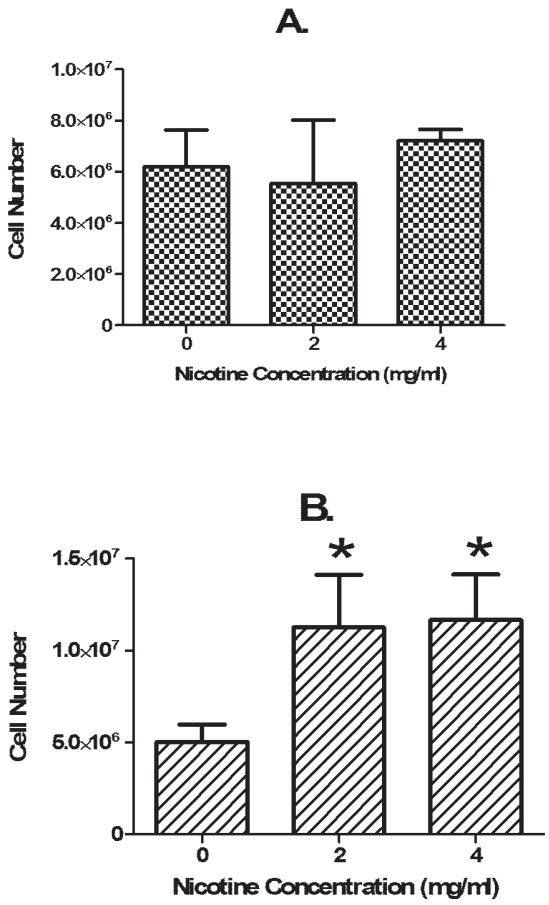


Fig. 3.3.4.1 ***S. mutans*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

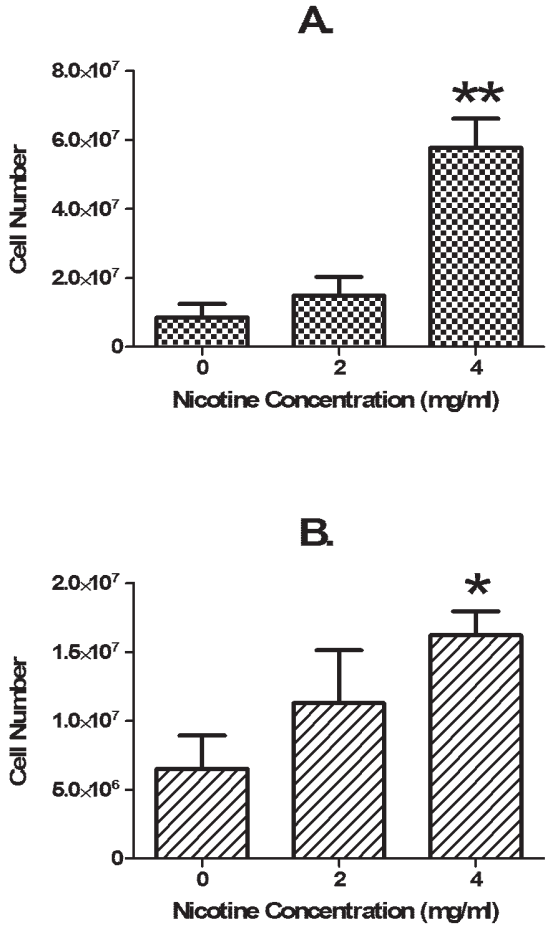


Fig. 3.3.4.2 ***S. gordonii*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

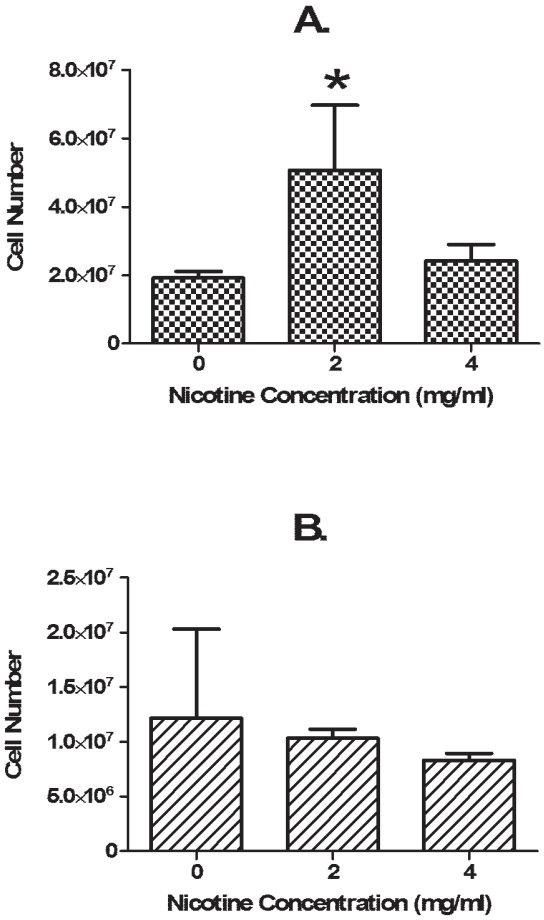


Fig. 3.3.4.3 ***S. mitis*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

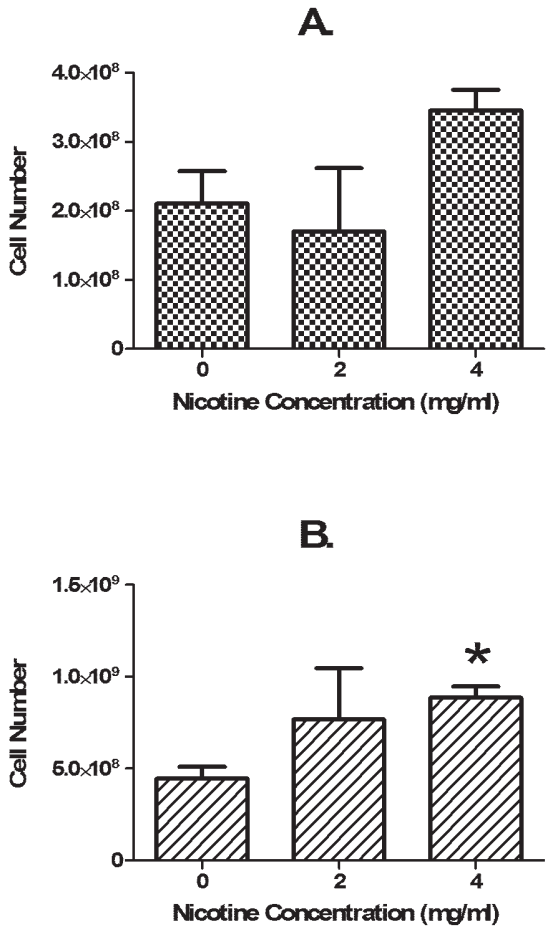


Fig. 3.3.4.4 ***S. oralis*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

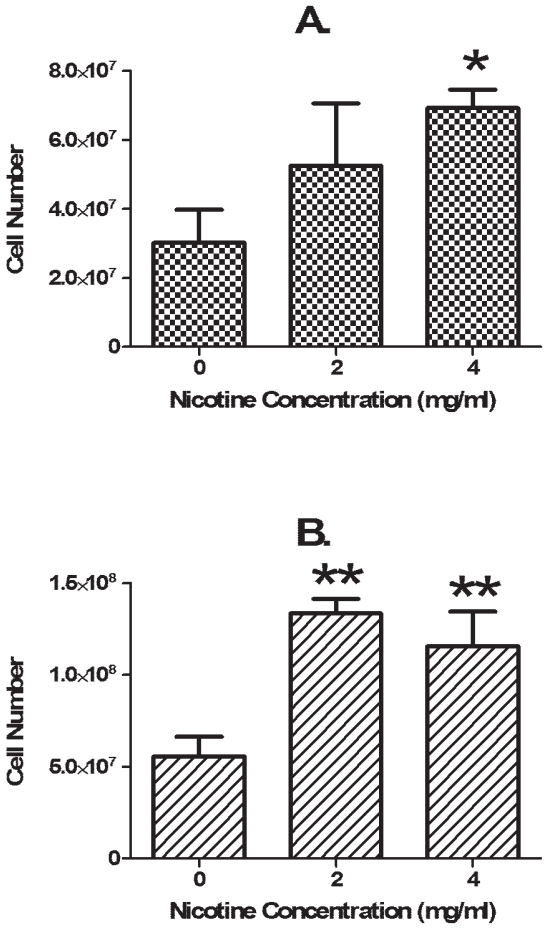


Fig. 3.3.4.5 ***S. salivarius*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

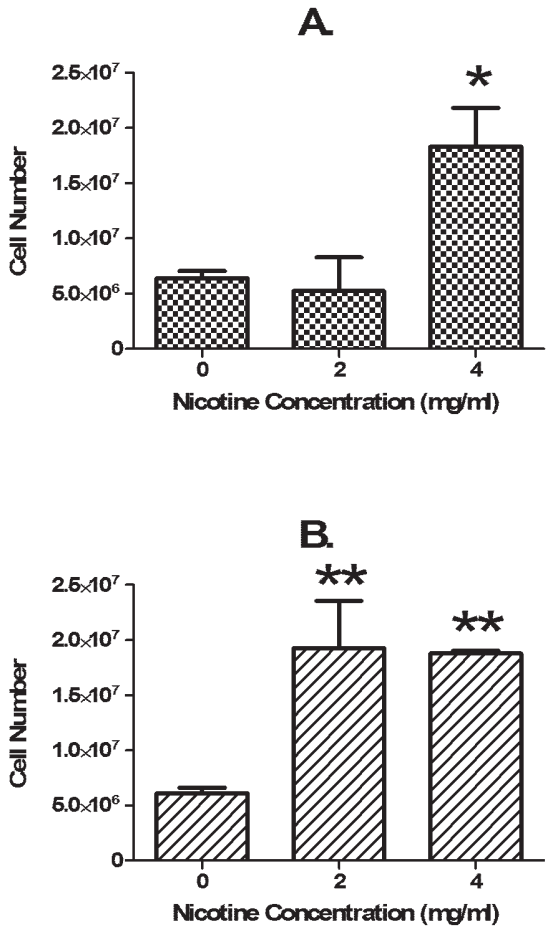


Fig. 3.3.4.6 ***S. sanguinis*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and **: denotes $P<0.01$.

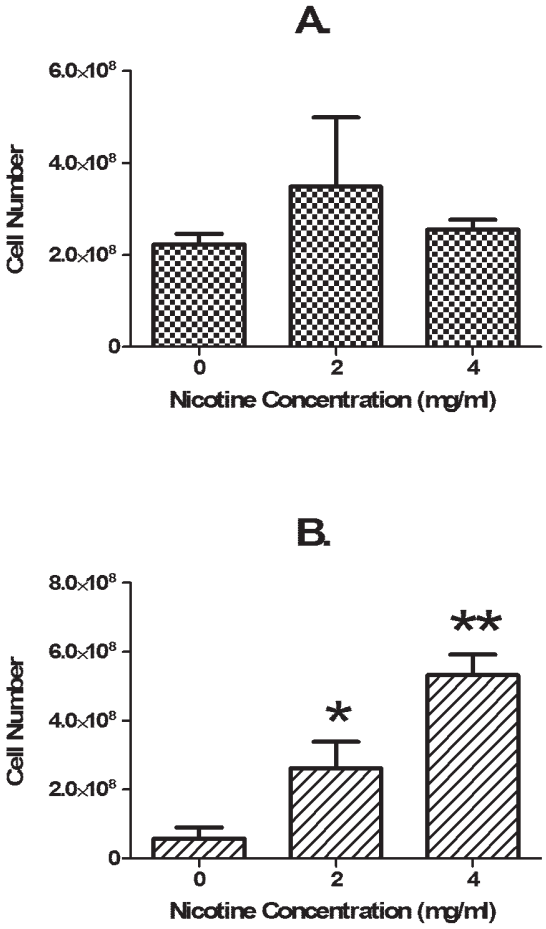


Fig. 3.3.4.7 ***E. faecalis*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

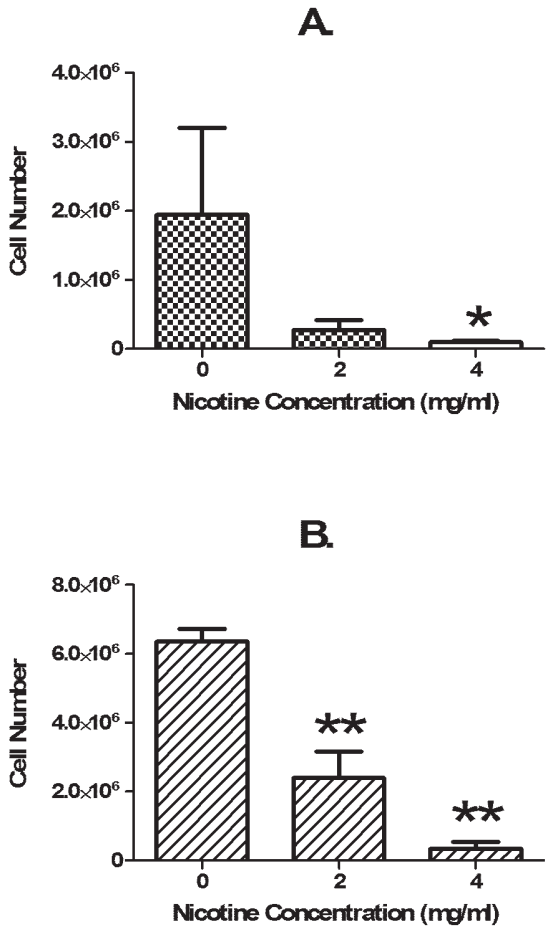


Fig. 3.3.4.8 ***L. casei*** **cell number in 10 species planktonic or biofilm mixed culture.**

Panels A and B demonstrate cell numbers in planktonic and biofilm cultures (5 ml), respectively. Cell number was calculated based on equation [15; see above] as follows:

$$N_{X,F} = (1+E)^{C_{T,R}-C_{T,X}} \times CFU_R \times V_R \times \frac{t}{q}$$

where $N_{X,F}$ is the final bacterial number of one species in mixed culture, E is the q-PCR amplification efficiency, C_T is the cycle number when fluorescence

reaches the threshold value, CFU_R is the colony forming unit of the reference species determined by plating on appropriate bacterial media, V_R is the volume of the reference species processed for DNA extraction, t is the dilution of the unknown mixed culture before DNA extraction, and q is the number of different species in the mixed culture. Asterisks indicate significant differences between nicotine treated and non-treated samples. * denotes $P<0.05$, and ** denotes $P<0.01$.

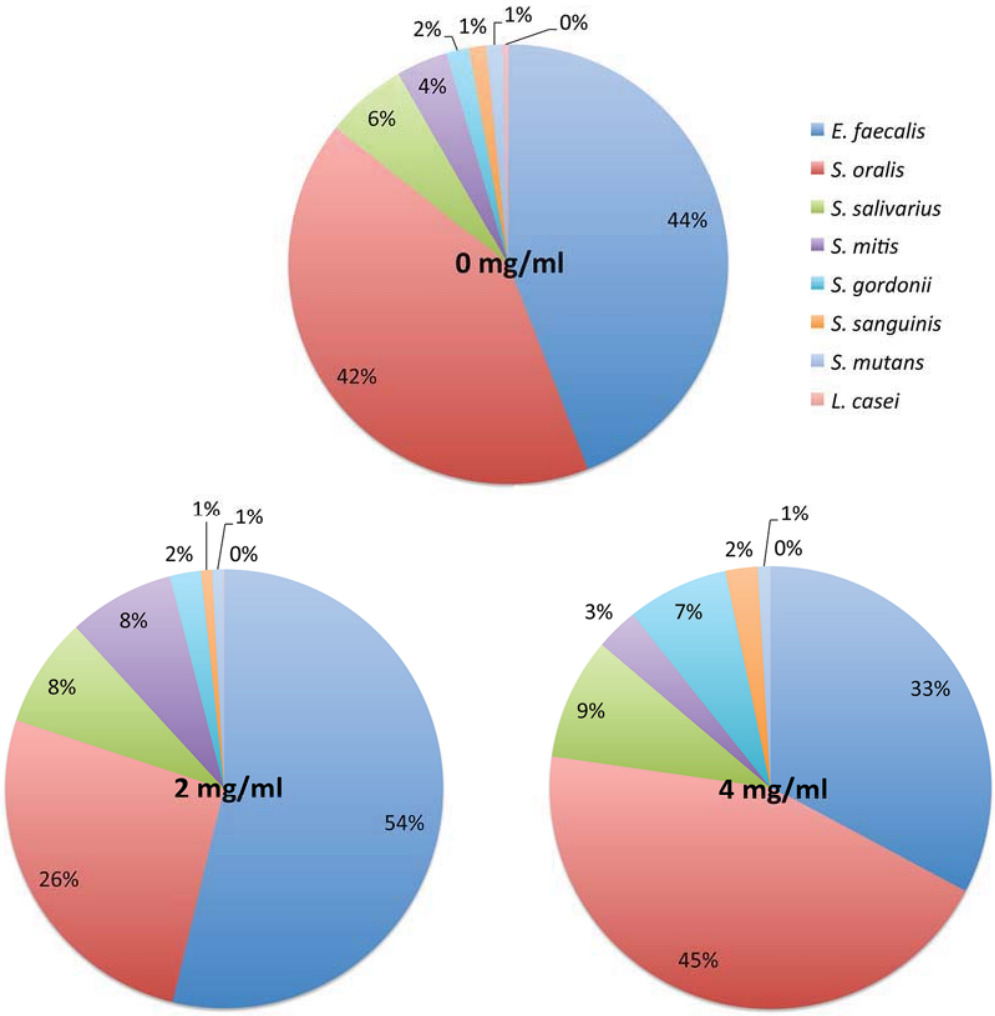


Fig. 3.3.4.9 **Proportion of each species in mixed planktonic culture.** Upper panel, lower left panel and lower right panel represent 0, 2 and 4 mg/ml nicotine treated mixed planktonic cultures, respectively. Different colors refer to different species, and the proportion value of each species is labeled on the pie graphs.

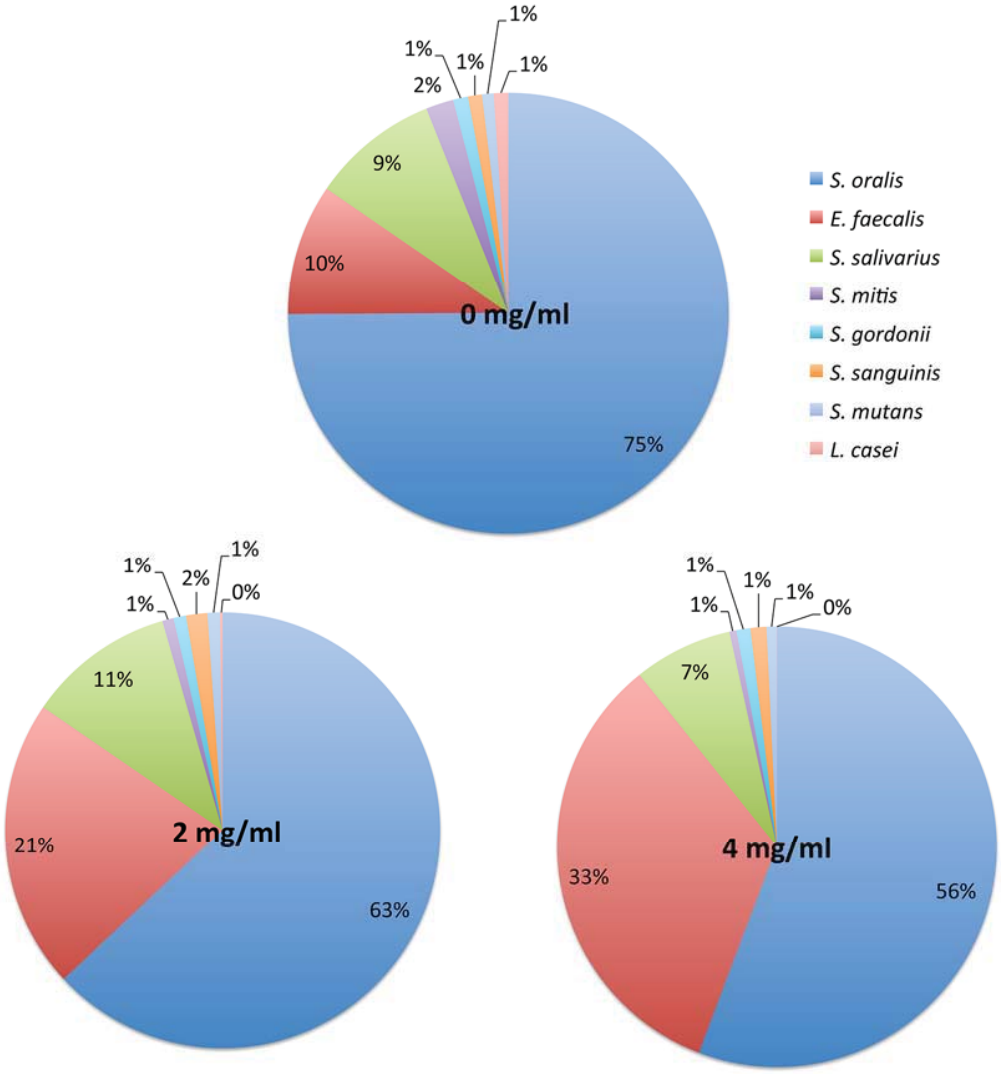


Fig. 3.3.4.10 **Proportion of each species in mixed biofilm culture.**

Upper panel, lower left panel and lower right panel represent 0, 2 and 4 mg/ml nicotine treated mixed biofilm cultures, respectively. Different colors refer to different species, and the proportion value of each species is labeled on the pie graphs

	Planktonic Cells			Biofilm Cells		
	0	2	4	0	2	4
E. faecalis	100.0%	122.0%	74.3%	100.0%	84.1%	74.5%
S. oralis	100.0%	63.2%	107.0%	100.0%	223.0%	346.8%
S. salivarius	100.0%	135.7%	149.3%	100.0%	117.1%	77.7%
S. mitis	100.0%	206.2%	81.8%	100.0%	41.6%	25.7%
S. gordonii	100.0%	137.5%	443.2%	100.0%	84.8%	93.4%
S. sanguinis	100.0%	63.9%	185.9%	100.0%	154.4%	115.1%
S. mutans	100.0%	69.7%	75.6%	100.0%	109.3%	86.6%
L. casei	100.0%	10.9%	3.2%	100.0%	18.4%	2.0%

Table 3.3.3 Cell number changes by percentage. The percentage changes of cell number by its percentage in the mixed culture (Fig. 3.3.4.9 and Fig. 3.3.4.10) were calculated.

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CHAPTER FOUR

Discussion

4.1 Effect of Nicotine on *S. mutans* Growth and Metabolism

To our knowledge, this is the first report analyzing the MIC/MBC/MBIC of nicotine on *S. mutans*. The MIC and MBC of nicotine on *S. mutans* reported in the present study were 16 and 32 mg/ml, respectively. We previously reported the MIC and MBC of CSC were 4 ± 0 and 7 ± 2 mg/ml, respectively (Zheng C. and Gregory R.L., unpublished data), which is lower than nicotine. This difference can be explained by the thousands of other complex components in CSC (Siegmund et al., 1999) such as tar that also inhibits *S. mutans* growth (Zonuz et al., 2008).

It is well known that planktonic bacteria are more sensitive to chemicals and antibiotics than biofilm cells (Sedlacek and Walker, 2007). For planktonic cells, growth of most *S. mutans* strains was inhibited at a nicotine concentration of 4.0 mg/ml; while for biofilm cells, growth of most *S. mutans* strains was enhanced at a nicotine concentration of 4.0 mg/ml. Keene et al. reported 16.2 mg/ml and 1.62 mg/ml nicotine inhibited *S. mutans* strain 10449 planktonic cell growth (Keene and Johnson, 1999), which was partly confirmed by our present study. The difference results for the 1.62 mg/ml nicotine treated *S. mutans* 10449 strain could be explained by the different culture starting concentrations, which were approximately 10^3-10^4 CFU/ml and 10^6 CFU/ml for their and our studies, respectively.

Biofilms are the preferred form of growth for most bacteria (Huang et al., 2011) and protein expression in biofilm is different from protein expression in planktonic cells (Sanui and Gregory, 2009). The present study revealed that the enhancement by nicotine on planktonic cells is minor, because only A32-2 demonstrated increased growth at nicotine concentrations of 0.25 and 0.5 mg/ml. The growth curve data indicated significant inhibition on *S. mutans* growth at 4 and 8 mg/ml might be explained by the lack of extra 5% CO_2 supply. Conversely, the enhancement of nicotine on biofilm cells appears to be significant and consistent, as all seven of the strains demonstrated increased biofilm. Furthermore, the overall biofilm metabolism of all seven *S. mutans* strains was enhanced by nicotine treatment, even at the MBIC nicotine concentration (16.0 mg/ml). However, the highest MRBM of most strains (UA130, 10449, NG8 and OMZ175) was observed at 8.0 mg/ml. The overall metabolic activity is equivalent to single cell activity multiplied by the number of biofilm cells, therefore, cell number increase was the chief contributor to the high *S. mutans* overall metabolism at 16.0 mg/ml of nicotine. The explanation for the strong cell proliferation less than the MBIC was that in the MBIC assay, overnight planktonic cells were treated with TSBS and nicotine at the beginning, therefore nicotine affected the inoculated cells. In the metabolism activity assay, biofilm was formed before nicotine was applied, therefore, nicotine affected the whole biofilm structure instead of single planktonic cells. This difference again confirmed that biofilm is more resistant than planktonic cells (Sedlacek and Walker, 2007).

From the SEM results (Fig. 3.1.5), it is clear that nicotine enhanced *S. mutans* biofilm mass. The bacterial cells present in gaps outside the thick biofilm accumulations were organized in networks with longer cell chains in 0.5 and 1.0 mg/ml nicotine than in the control samples. In 2.0-8.0 mg/ml nicotine, the number of cells in the gaps was reduced and the accumulated biofilm was augmented (1,500X). Cell morphology was also changed by nicotine treatment (20,000X) resulting in more spherical-shaped cells (Fig. 3.1.5). From this data, it is clear that nicotine causes effects on *S. mutans* biofilm.

One homogeneous gene sequence of β2 nAChRs was found in *S. mutans* and its expression was upregulated by 1.0 mg/ml nicotine (DelaCruz K., Jouravlev A. and Gregory R.L., unpublished data). Nicotine has been shown to cause oxidative stress in human cells (Kang et al., 2011), and transcriptional repressor Rex has been reported to regulate *S. mutans* oxidative stress response and biofilm formation (Bitoun et al., 2011). Both Falkler et al. (Falkler et al., 1987) and Keene et al. (Keene and Johnson, 1999) have suggested that nicotine affects *S. mutans* gene expression, unfortunately, there are still questions about how metabolic processes are involved, whether the processes are related to lactic acid production or not, as well as which mRNAs are related to biofilm formation. Further research should focus on those aspects.

The nicotine concentration in the saliva of tobacco users varies due to different individual saliva secreting volumes, time of consuming tobacco, amount of

tobacco consumed, measuring time, and measuring methods (Curvall et al., 1990; Robson et al., 2010; Schneider et al., 1997). One study (Feyerabend et al., 1982) reported a salivary nicotine range of 0-0.31 mg/ml for non-smokers, 0-1.33 mg/ml for slight or medium smokers, and 0-2.27 mg/ml for heavy smokers. Other studies indicated nicotine levels ranging from 70 to 1560 μg/ml (Hoffmann and Adams, 1981), 367-2496 ng/ml in stimulated saliva and 900-4612 ng/ml in unstimulated saliva (Robson et al., 2010), or 115 ng/ml in smokers (Dhar, 2004). However, no studies have reported dental biofilm levels of nicotine making it difficult to know what the actual concentration *S. mutans* residing in the dental biofilm is exposed to.

4.2 Effect of Nicotine on *S. mutans* Cell Binding, EPS Synthesis and LDH Activity

From the CLSM results (Fig. 3.2.1), both bacterial cells and EPS were significantly increased by nicotine treatment. To better understand biofilm structure, two parameters were used to interpret the figures. The first parameter is volume, which is calculated by adding the color intensity of each pixel on the image. This represents total cell numbers. The second parameter is coverage, which is calculated by counting the presence of color at each pixel site. For the image analysis, we used Heydorn's principles (Heydorn et al., 2000). But since theirs were 3-dimensional images and ours are 2-dimensional images, we deleted the z-axis factor from our algorithm. For bacterial cells, both volume and coverage were increased in a nicotine dependent manner (Fig. 3.2.1 and Table

3.2). This demonstrates that as more bacterial cells attach to the biofilm they occupy more space. For EPS, unlike bacterial cells, only EPS volume was increased with nicotine treatment. The coverage data indicated an increased trend but this was not statistically significant. EPS is essential for bacterial adhesion-cohesion, and it plays a critical role in creating low pH microenvironments and providing binding sites for bacterial attachment (Koo et al., 2013). EPS also serves as a local sugar reservoir that can be fermented to acids, which contributes to caries development (Koo et al., 2010; Koo et al., 2013). Therefore, the increased EPS synthesis by nicotine may increase smokers' caries risk.

Gtfs are a family of enzymes that catalyze the transformation of glucosyl groups and contributes to *S. mutans* cariogenicity (Vasilev et al., 2010). *S. mutans* expresses three distinct Gtfs, GtfB, GtfC and GtfD. GtfB and GtfC are encoded by highly homologous genes and they primarily synthesize α-1,3-rich water-insoluble polysaccharide (Aoki et al., 1986; Hanada and Kuramitsu, 1988), while GtfD is responsible for the synthesis of α-1,6-rich water-soluble polysaccharides (Hanada and Kuramitsu, 1989). Although the roles of Gtfs are different from each other, their functions actually overlap as the water-soluble polysaccharide synthesized by GtfD is the primer for GtfB (Bowen and Koo, 2011). The Gtfs (GtfB and GtfC) protein expression was very low in biofilm cells such that it could hardly be detected when similar amounts of biofilm cell total protein were loaded with planktonic cell total protein (data not shown). Planktonic cell Gtfs proteins

were significantly increased at 2 and 4 mg/ml nicotine (Fig. 3.2.2.3). In particular, the GtfC band (lower band in Western blot results) indicated more significant changes than the GtfB band (upper band). This is consistent with *gtfB* and *gtfC* gene expression (Fig. 3.2.3). The *gtfD* expression of planktonic cells was also demonstrated by an upregulation trend with nicotine treatment, but none of the *gtfs* upregulation was significant due to the large standard deviations. Typically, RNA extraction from planktonic cells is easier than that from biofilm cells (Cury and Koo, 2007). But with the presence of sucrose, planktonic cell RNA extraction becomes more difficult than biofilm cell RNA extraction. Several solutions were tried, such as increasing lysing time, increasing sonication time, adjusting cell numbers, or extraction with chloroform, but none of them worked. It was interesting that nicotine stimulated planktonic cell *gtfs* but inhibited biofilm cell *gtfs* (Fig. 3.2.3). *S. mutans* biofilm cell protein synthesis has been reported to be different from planktonic cell protein synthesis, such as glycolytic enzymes related to acid formation were inhibited in biofilm cells (Svensater et al., 2001). *gtfs* expression was 2-3 fold lower in non-nicotine treated biofilm cells than planktonic cells (data not shown). One possible explanation for this phenomenon is the regulation effect of the *S. mutans* quorum sensing (QS) system, which senses microbial cell numbers, environmental stresses, carbohydrate types and its quantity (Smith and Spatafora, 2012). Since bacterial density is much higher in biofilm than planktonic cells, the QS system may inhibit *gtfs* expression to reduce cell density. So far, QS systems have only been found in biofilm cells, not in planktonic cells (Huang et al., 2011). Therefore, the *gtfs* and Gtfs of *S. mutans*

planktonic cells were not interfered by the QS system and were upregulated by nicotine. However, since planktonic cells gained more binding ability with the presence of nicotine, more cells attached to biofilm. The increased cell accumulation in biofilm would be sensed by the QS system, which then turned on the *gtfs* downregulation pathway to limit more biofilm cell accumulation.

Theoretically, Gtfs belong to the Gbps family because they are related to cell binding. But in most literature, Gbps are defined as non-Gtf glucan-binding proteins that serve as cell-surface receptors for glucan (Banas and Vickerman, 2003). GbpA and GbpD share homology of glucan-binding domains with Gtfs. The *gbpA* knockout mutant strain forms a smoother biofilm than the wild type (Hazlett et al., 1999). That means *gbpA* makes biofilm coarse. Planktonic GbpA was increased four fold by nicotine (Fig. 3.2.2.3) and the *gbpA* also demonstrated a nicotine upregulation trend (Fig. 3.2.3). Those upregulations were correlated to the more aggregated and coarse biofilm surface observed in the CLSM (Fig. 3.2.1) and SEM (Fig. 3.1.5) results. GbpB is involved in bacterial cell-wall synthesis (Mattos-Graner et al., 2001). The GbpB and *gbpB* expression in biofilm cells were significantly decreased with nicotine treatment. As mentioned above, autoinducer-2 (AI-2), a signaling molecule of QS system, has been speculated to regulate *gtfs* and *gbps* expression (Schauder et al., 2001). Since there is increased EPS in the environment (Fig. 3.2.1) already with the presence of nicotine, the QS system downregulates cell-wall synthesis. GbpC, which is related to bacterial biofilm structure (Banas and Vickerman, 2003), is not

significantly affected by nicotine. The function of GbpD is similar to Gtfs and GbpA serving as a glucan-binding enzyme (Banas and Vickerman, 2003), and the effect of nicotine on *gbpD* is similar to *gtfs* and *gbpA* as well (Fig. 3.2.3). GbpA and GbpB expression in planktonic cells were eight and three fold higher than that in biofilm cells, respectively (Fig. 3.2.2.1), because of the absence of QS system in planktonic cells. Unfortunately GtfD, GbpC and GbpD protein data was not available due to the lack of primary antibodies.

LDH is the enzyme that catalyzes the conversion of pyruvate to lactate with concomitant interconversion of NADH and NAD^+ and produces lactic acid as the final product (Sommer et al., 1985). Since LDH activity is directly related to *S. mutans* lactic acid production, LDH is considered one of *S. mutans* major virulence factors. Although nicotine does not directly regulate *S. mutans* LDH activity (Fig. 3.2.4, panel B), nicotine increases total bacterial cells in the biofilm, the overall LDH activity is increased (Fig. 3.2.4, panel A). Therefore, nicotine indirectly enhanced lactic acid production in smokers. Type I collagen is the major organic component of dentin. Collagen degradation by endogenous collagenases, MMP-2, MMP-8 and MMP-9, is enhanced in the presence of lactic acid or low pH (Tjaderhane et al., 1998). CSC affects membrane-associated MMPs and tissue inbibitors of metalloproteinases (TIMPs) and stimulates human gingival fibroblasts–mediated collagen degradation (Zhang et al., 2011). Smokers with gingival recession would have their teeth roots exposed to bacteria. Similar

to crown dentin, root dentin is susceptible to collagenase degradation at low pH (Dung et al., 1995).

4.3 Effect of Nicotine on *S. mutans* Proliferation in A Ten Species Mixed Culture

One limitation with species-specific primer design is the limited bacterial genome information and the limited number of available bacterial strains. We had been trying to work with fully sequenced strains, but we failed because of at least one of the following reasons: no genome information was available for that species; the strain is available from ATCC, but its sequence was unknown; or the sequence of the strain was known but was not available from ATCC or could not be shipped to the US. But at least for the latter two situations, we could surmise the sequence and test it. To design primers based on a different strain of the same species is a gamble. In the present study, the species-specific primers of seven out of ten species were found in the first trial, two in the second trial, and the last one in the fifth trial. This problem should not be a permanent barrier because ATCC is frequently collecting newly sequenced strains and the genome information of many bacterial strains that are being under sequenced will be available in the future.

For all of the regular PCR amplified products, only species-specific bands were observed. Therefore, the species-specific primer design worked. Many factors affect band intensity of PCR products, such as Mg, Taq polymerase, DNA

template and primer concentrations, primer length, GC content, primer/template ratio, thermocycler settings, etc. (Tyler et al., 1997). In addition, PCR product length, ethidium bromide concentration and shutter speed were noted to significantly affect band intensity. In the present study, GC content varied from 59.07 to 60.32% and PCR product length varied from 169 to 361 bp, while all of the other factors were the same for all samples. This primer specificity test may be skipped if the validation range test is used. Those two tests provide the same experimental results from semi-quantitative or quantitative aspects. The primer specificity test was included in the present study because its expense was less than the validation range test and it was found to be adequate for screening.

The PCR-MSCC system error, which was measured by the validation range test, was relatively low (Table 2). It may further be minimized by optimizing the primer design, i.e.. changing primer length, altering terminal nucleotide, selecting a reasonable GC content and Tm, etc. (Dieffenbach et al., 1993). But because the system error was acceptable for the current study, there was no need to pursue minimum system errors.

In the q-PCR efficiency test, according to Ginzinger (Ginzinger, 2002), the efficiency needs to be at or above 90% for the relative quantitation method. The efficiencies of the present study varied from 70.0 to 98.5%, and the absolute quantitation method should be used. For most of the q-PCR efficiency studies, DNA template concentrations ($\log_{10}X$) were used as the X-axis, and the efficiency

calculation equation was $E = 10^{-1/slope} - 1$ with the ideal slope of -3.32 (Bustin et al., 2009; Ginzinger, 2002; Nigro et al., 2001). In the present study, we changed the X-axis to $1:2^n$ dilutions of DNA template, this made the efficiency much easier to predict because *E* = *slope*. However, if the absolute DNA template would like to be used, the X-axis could be stated as $(\log_2 X)^{-1}$. R^2 in the present study could approach 1 by adding five more DNA template dilution points for the linear regression (Bustin et al., 2009; Ginzinger, 2002).

In the cell quantification test, the maximum difference occurred between *S. oralis* (8.87×10^8) and *S. epidermidis* (6.97×10^3). The difference was 1.27×10^5 cells. If the *S. oralis* number was multiplied by the system error of the *S. oralis* DNA template with *S. epidermidis* primers then (8.87×10^8) × (1.27×10^{-5}) = 1.13×10^4 cells. This number is even larger than the estimated *S. epidermidis* number. Therefore, the *S. epidermidis* species was excluded because the error was not acceptable. The *S. aureus* species was excluded for the same reason. *S. epidermidis* is frequently found in the skin microflora and less commonly in the mucosal microflora (Fey and Olson, 2010), and *S. aureus* is frequently found in respiratory track and skin microflora. Possibly, their growth was inhibited by oral microflora. The reason for including those two species in the ten species mixed culture was that we would like to test the validation of PCR-MSCC in as many species as possible. Moreover, the colony numbers of *S. epidermidis* and *S. aureus* were not very low in the ten species culture grown in BHI+YE for 24 hours (around 10^5 cells per sample, data not shown). Possibly, BHI+YE is a preferred

media for *S. epidermidis* and *S. aureus*. TSB or TSBS instead of BHI or BHI+YE with 1% sucrose were used in the multiple mixed cultures because they need to be consistent with the first two section studies.

The cell number of each species in mixed cultures can be estimated through either regular PCR or q-PCR. The present study has demonstrated the q-PCR method. If the PCR method is used, the PCR band intensity should be semi-quantified by image software (i.e., ImageJ), and the final equation will be

$$N_{X,O} = \frac{I_R}{I_X} \times CFU_R \times V_R \times \frac{p}{q} \quad [17]$$

where I_R and I_X are the band intensities of the reference mixed and unknown mixed cultures, respectively.

There are several methods typically used for microbial quantification. Fluorescence *in situ* hybridization (FISH) is the best option for microbial quantitation *in situ*. The average laboratory is usually equipped to has access to a general confocal laser scanning microscope that can differentiate three to five different fluorescent wavelengths, but microscopes of ten different channels are rare and very expensive (Al-Ahmad et al., 2007; Zijnge et al., 2010). The antibiotic resistance gene labeling method costs time. Colony morphology recognition on selected agar plates is limited to the number of species. 16S rRNA sequencing is time efficient and works well with ten species, but the expense is high. If one species generates 40 full-length 16S rRNA clones (calculation based on Peterson et al. data) (Peterson et al., 2013) and the retail price for sequencing

one DNA clone is $5, triplicates of ten species multiplied by two groups (control and treated) will cost 40×5×10×3×2 = $12,000. But if the PCR-MSCC method is used, the expense for each species in a single sample is $2 (spent on the Fast SYBR Green Master Mix). The total PCR-MSCC price for 10 species will be 1×2×10×3×2 = $120, an approximate savings of 99% (the expense on other supplies have been excluded from the calculation because they are the same for both the PCR-MSCC and16S rRNA sequencing).

Theoretically, PCR-MSCC can be applied to over 100 species because the primers can target any region of the genome, and are not limited to the 16S rRNA region. For two species of 93% similarity, for instance, if the genome size of one is ~2,000,000 bp, there will be 2,000,000×(1-93%) = 140,000 bp region that can be used for species-specific primer design. If each DNA sequence cloned by the primers is 500 bp, the total available length is enough to detect 280 mixed species.

PCR-MSCC can also be used to compare different strains within the same species, such as *S. mutans* UA159 versus *S. mutans* GS-5. PCR-MSCC was defined as PCR-based multiple species cell counting. Actually, PCR-based multiple *strain* cell counting might be more appropriate because it is designed based on strain instead of species. Since in the present study, ten different species instead of strains were used, the name with *species* was used. PCR-MSCC is limited to *in vitro* studies to date. But it is still important because *in vitro*

studies are the foundation of *in vivo* studies and most of the mechanistic explorations are carried out in *in vitro* studies.

S. mutans, as mentioned before, is a Gram positive facultative anaerobic coccus. The presence of *S. mutans* in oral plaque microflora varies from 0 to 50% (Hamada and Slade, 1980). Generally, *S. mutans* is absent or the cell number is very low in caries-free subjects while the cell number is high in caries-affected subjects (Bowden, 1997; Sanchez-Perez and Acosta-Gio, 2001). For instance, a total of 8 phenotypes of *S. mutans* were detected in four caries-free subjects while 158 phenotypes were detected in the same number of caries-affected subjects (Peterson et al., 2013). The absolute count, which is the absolute bacterial cell number of *S. mutans* in biofilm, was significantly increased by nicotine. But the relative count, which is the percentage of *S. mutans* in mixed culture, was not changed. In the Peterson et al. study, both absolute and relative *S. mutans* counts were increased in caries-affected subjects. The increments were from 8 to 570 and 0.037% to 2.236%, respectively (Peterson et al., 2013). In the Sanchez-Perez and Acosta-Gio study, *S. mutans* absolute count per ml was reported. 31±34, 31±9 and 630±13 CFU/ml of *S. mutans* were observed in caries-free, low caries (less than 5 lesions) and high caries (more than 5 lesions) subjects, respectively (Sanchez-Perez and Acosta-Gio, 2001). In association with previous findings that nicotine does not affect *S. mutans* LDH activity, the higher absolute *S. mutans* count in smokers would yield more lactic acid and contribute to smokers caries development.

S. sanguinis is a Gram positive facultative aerobic coccus. It is the second most abundant species in dental biofilm after *S. mitis*, taking up 9% of the total biofilm cells (Peterson et al., 2013). *S. sanguinis* is believed to antagonize *S. mutans* growth in dental microflora. *S. sanguinis* produces hydrogen peroxide to inhibit *S. mutans* growth, and on the other hand, *S. mutans* produces acid and bacteriocins to inhibit *S. sanguinis* growth (Huang et al., 2011). The higher ratio of *S. mutans* over *S. sanguinis* usually indicates a higher carious risk (Preza et al., 2008), for instance the ratio is 187.69×10^{-3} (570/3037) and 6.21×10^{-3} (8/1288) in caries affected and caries-free subjects, respectively (Peterson et al., 2013). Noticeably, the absolute count of *S. sanguinis* is increased in caries affected subjects than caries-free subjects with crown caries (Peterson et al., 2013), but the count is decreased in subjects with root caries (Preza et al., 2008).The difference between crown lesions and root lesions is the oxygen content, which is the substrate of hydrogen peroxide produced by *S. sanguinis*. In an oxygen deprived environment, although *S. sanguinis* can survive, its competition ability with other strains is compromised (Huang et al., 2011). In the present study, the absolute numbers of *S. sanguinis* were increased at 4 mg/ml nicotine in planktonic mixed culture and increased at 2 and 4 mg/ml nicotine in biofilm mixed culture. This finding was consistent to the microbial profile of crown lesions (Peterson et al., 2013), in which higher oxygen content is present. Since aerobic instead of anaerobic incubation environment was used in the present study, it makes sense that the data was closer to what was observed with crown lesions

than root lesions. However, in nutrient-rich growth conditions, such as with sucrose, no inhibition between *S. sanguinis* and *S. mutans* was observed (Kreth et al., 2005). Sucrose had been added in the mixed biofilm culture, and the extra sugar provided a more friendly growth condition that both strains could grow well in without competition for nutrients.

S. gordonii is a Gram positive coccus. It is one of the pioneer bacteria known to attach to tooth surfaces after tooth brushing. Its major role in dental microflora is to provide binding sites for later colonizers such as *S. mutans*. Like *S. sanguinis*, *S. gordonii* has been demonstrated to inhibit *S. mutans* growth *in vitro* through producing hydrogen peroxide (Kreth et al., 2008). The synthesis of hydrogen peroxide is regulated by *S. gordonii* pyruvate oxidase, which is encoded by *spxB* (Zheng et al., 2011). *S. gordonii* has been detected in both root caries-free and caries affected subjects, but more predominant in caries-free subjects (Preza et al., 2008). However, another primary tooth caries microbial profile indicates *S. gordonii* is mainly detected on the intact tooth surfaces of caries affected subjects (Becker et al., 2002). A third study investigating bacterial microbiomes of tooth crown surfaces demonstrated higher *S. gordonii* counts in caries affected subjects than caries-free subjects (Peterson et al., 2013). Since the mechanism *S. gordonii* uses to compete with *S. mutans* is similar to *S. sanguinis*, it is likely that *S. gordonii* would have a higher count in an aerobic environment. The absolute cell numbers of *S. gordonii* in planktonic and biofilm mixed cultures were increased by nicotine. Those increased *S. gordonii* cells might either

produce more hydrogen peroxide to inhibit *S. mutans* growth or provide more binding sites for *S. mutans* attachment. Further studies need to be conducted before making a comprehensive conclusion.

L. casei is considered the second most important carious pathogen due to its ability to produce lactic acid. *L. casei* itself is pH sensitive (Russell, 1992), with an optimal pH of 5.5 (Zacharof, 2009). The presence of *L. casei* is higher in root caries and primary tooth caries subjects than caries-free subjects (Becker et al., 2002; Preza et al., 2008). In the present study, *L. casei* was significantly decreased in both planktonic and biofilm mixed culture in the presence of nicotine. This means the presence of nicotine is not a favorable factor for *L. casei* growth.

S. salivarius is a Gram positive coccus. It accounts for less than 1% of the total cells in dental biofilm (Peterson et al., 2013). In the presence of sucrose, *S. salivarius* produces a thick polysaccharide capsule (Scheie and Rolla, 1984). Although it could serve as a probiotic for *Streptococcus pyogenes* infection (Burton et al., 2006), *S. salivarius* is strongly associated with crown caries of primary and permanent teeth (Becker et al., 2002; Peterson et al., 2013). Possibly, the increased polysaccharide contributes to biofilm formation and the increased biofilm results in local higher *S. mutans* count and lower pH. Nicotine has significantly increased both planktonic and biofilm *S. salivarius* absolute numbers, which may be correlated to the increased caries incidence in smokers.

S. mitis is a Gram positive facultative anaerobic coccus. It is the most abundant microbial species in dental biofilm. The abundance of *S. mitis* is approximately 25% (Peterson et al., 2013). In both primary tooth and root caries, *S. mitis* is primarily present in caries-free subjects or intact enamel of caries affected subjects, and the presence of *S. mitis* is low in white spots and dentin of caries affected subjects (Becker et al., 2002; Preza et al., 2008). In the present study, *S. mitis* in mixed biofilm cultures exhibited a decreased trend in both absolute cell numbers and percentages, although the decrease was not significant. To some extent, this *S. mitis* data with nicotine treatment is consistent to the *S. mitis* data in caries affected subjects.

S. oralis is a Gram positive coccus. It is ranked as the fourth most abundant species in dental biofilm, accounting for up to 6% of the total cell numbers (Peterson et al., 2013). Its presence is superficially associated, if not independent, to caries status (Becker et al., 2002; Peterson et al., 2013; Preza et al., 2008). The present study indicated significantly increased numbers of *S. oralis* at 4 mg/ml nicotine.

E. faecalis is a Gram positive facultative anaerobic microbe. In the oral cavity, it is usually found in root canal treated teeth (Gomes et al., 2006). Its relationship with dental caries is not very clear. In the present study, the absolute number of

E. faecalis was increased while the relative percentage of *E. faecalis* was decreased with nicotine treatment.

The literature results and current study results of bacterial species cell number changes were summarized and compared (Table 4). Overall, nicotine has a generally stimulatory effect on streptococcal species growth. In comparison with human microbiome data, the relative count of each species in the present study is far from reality. Several factors including available bacterial species, growth media and growth environmental conditions influence the growth of each species. The current study was just a pilot study in multiple species research. Further studies need to include more bacterial species and optimize the growth conditions.

4.4 Conclusion

In conclusion, nicotine enhanced biofilm formation and biofilm metabolism of *S. mutans*, through stimulating *S. mutans* planktonic cell Gtfs and Gbps expression. This leads to more planktonic cells attaching to dental biofilm. Increased *S. mutans* biofilm cell numbers, in a 10 mixed species model, resulted in higher overall LDH activity. More lactic acid may be generated and contribute to caries development in smokers.

	Literature	Current study	
	Relationship with caries	Planktonic	Biofilm
S. mutans	+	no change	+
S. gordonii	+/-	+	+
S. mitis	-	+/no change	No change
S. oralis	+ (minor)	no change	+
S. salivarius	+	+	+
S. sanguinis	+/-	+	+
E. faecalis	+ with endodontic failure	no change	+
L. casei	+	-	-

Table 4 Comparison of literature results versus current study results.

The literature results are based on the relationship between bacteria counts and caries, the status of smoking was unknown. The current study results are based on the relationship between bacteria counts and nicotine treatment.

ACKNOWLEDGEMENTS

The final, definitive versions of some of the contents of this dissertation have been published and used by permission.

European Journal of Oral Science, 2012 Jun; 120(4):319-325. All rights reserved.

http://onlinelibrary.wiley.com/doi/10.1111/j.1600-0722.2012.00971.x/full

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Education

2009-2014 Ph.D., Indiana University, USA
2004-2009 D.D.S., West China School of Stomatology, Sichuan University, China

Honors, Awards, Fellowships

2014 IN-AADR/IUSD Research Day Shofu PhD Student Oral Presentation Award
2014 IUPUI Graduate School Travel Grant
2012 IBASM poster presentation in graduate level second place winner
2012-2013 IBASM Graduate Student Research Fund
2012 IUPUI GPSG Education Enhancement Travel Grant
2010-2014 Graduate/Dentistry Fellowship

Conferences Attended

2014 AADR, Charlotte, NC (oral presentation)
2014 IBASM, Turkey Run State Park, IN (oral & poster presentation)
2013 IADR/AADR, Seattle, WA (oral presentation)
2013 IBASM, McCormick's Creek State Park, IN (poster presentation)
2012 AADR, Tampa, FL (poster presentation)
2012 IBASM, Wabash College, IN (poster presentation)
2011 IADR/AADR, San Diego, CA (poster presentation)
2011 IBASM, Brown County, IN (poster presentation)

Service and Memberships

2012-2014 IBASM student representative
2012 IUPUI international graduate students' mentorship
2010-2014 IBASM student membership
2010-2014 American Association for Dental Research (AADR) student membership
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Publications

1. **Huang R**, Li M, Gregory RL. Nicotine promotes *Streptococcus mutans* extracellular polysaccharide synthesis, cell binding and lactate dehydrogenase activity. (in progress)
2. **Huang R**, Zhang J, Yang XF, Gregory RL. PCR-based multiple species cell counting *in vitro*. (in progress)

3. **Huang R**, Li M, Ye M, Yang K, Xu X, Gregory RL. Effects of nicotine on *Streptococcus gordonii* growth, biofilm formation and cell attachment. Appl Environ Microbiol. 80(23):7212-7218. (2014)
4. Whatley JD, Spolnik KJ, Vail MM, Adams BH, **Huang R**, Gregory RL, Ehrlich Y. Susceptibility of a methacrylate based root canal filling to degradation by bacteria found in endodontic infections. Quintessence Int. 45(8): 647-652. (2014)
5. Li MY, **Huang RJ**, Zhou XD, Gregory RL. Role of sortase in *Streptococcus mutans* under the effect of nicotine. Int J Oral Sci 5(4):206-11. (2013)
6. Li M, **Huang R**, Zhou X, Zhang K, Zheng X, Gregory RL. Effect of nicotine on dual-species biofilms of *Streptococcus mutans* and *Streptococcus sanguinis*. FEMS Microbiol Lett 350:125-132. (2013)
7. Weng Y, Chong VJ, Howard L, **Huang R**, Gregory RL, Xie D. A novel antibacterial dental resin composite. J Biomaterials Nanobiotechnol. 3:130-135. (2012)
8. **Huang R**, Li M, Gregory RL. Effect of nicotine on growth and metabolism of *Streptococcus mutans*. Eur J Oral Sci 120(4):319-325 (2012)
9. **Huang R**, Li M, Gregory RL. Bacterial interactions in dental biofilm. Virulence 2(5):435-444. (2011)
10. **Huang R**. Tooth surface recording. Br Dent J. 204:5. (2008)