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Los Angeles

Development of *In Vitro* Denture Biofilm Models for Studying

Denture-related Halitosis and Stomatitis

A thesis submitted in partial satisfaction

of the requirements of the degree Master of Science

in Oral Biology

by

Tingxi Wu

2012

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Tingxi Wu

2012

ABSTRACT OF THE THESIS

Development of *In Vitro* Denture Biofilm Models for Studying

Denture-related Halitosis and Stomatitis

by

Tingxi Wu

Master of Science in Oral Biology

University of California, Los Angeles, 2012

Professor Wenyuan Shi, Chair

Denture stomatitis and denture-related halitosis are two of the most prevalent denture related infectious diseases. The establishment of denture biofilm models for disease-associated pathogens is essential in further investigating the pathogenesis of these diseases. Chapter I and II of this thesis reported the successful development of denture

biofilm model for *Candida albican*, the main pathogen of denterun stomatitis; as well as the denture models for halitosis-related bacteria, including *Klebsiella pneumonia*, *Fusobacterium nucleatum*, *Tannerella forsythia*, *Veillonella atypica*. We further demonstrated that the established denture models can be used for evaluating efficacy of denture-cleansers. Chapter III is focused on further exploring the association between denture stomatitis and denture-related halitosis. We revealed the interaction between *C. albican* and halitosis related pathogens using *in vitro* co-aggregation assay. A further investigation of the possible association between stomatitis-associated *C. albicans* and halitosis-associated bacteria *in vivo* is necessary.

The thesis of Tingxi Wu is approved.

Renate Lux

Xuesong He

Shen Hu

Wenyuan Shi, Committee Chair

University of California, Los Angeles

2012

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## INTRODUCTION OF THE THESIS

Dentures are prosthetic devices fabricated to replace missing teeth. By restoring their chewing ability, dentures can significantly improve patients' state of health and quality of life. Conventional dentures are removable which supported by soft and hard tissues of oral cavity. They are mostly made of Polymethylmethacrylate (PMMA). The denture surfaces provide colonization niches for oral microbes, resulting in the formation of denture plaque, a biofilm-like structure containing a variety of microbial species of oral origin (Paranhos Hde F 2007). Microbes within denture plaques are protected from hosts' immunological response and often exhibit high resistance to antimicrobial agents (Mah TF 2001). Accumulating evidence indicates that denture plaque could serve as reservoirs of potential pathogens, and the adherence and accumulation of denture plaque on denture surface play an important role in oral infectious diseases, such as denture stomatitis, halitosis (or bad breath), and angular cheilitis; as well as systemic diseases including chronic respiratory pulmonary disease, cardiovascular diseases, and arthritic disorders (MacEntee MI 1985, Budtz-Jørgensen E 1981).

Denture stomatitis and halitosis are two of the most prevalent denture-related infectious diseases (MacEntee MI 1985, Budtz-Jørgensen E 1981, Coulthwaite L 2007). Denture stomatitis is often characterized by inflammation of the oral mucosa in direct contact with the dentures and a positive correlation between *Candida albicans* and clinical symptoms has been verified. Denture-related halitosis is often associated with unclean or badly

adjusted dentures and a variety of malodor-producing oral bacterial species have been implicated in causing the related symptoms (Gendreau L 2011, Nalcaci R 2007). A recent study further indicated a possible positive association between these two clinical conditions (Baran I 2009).

The high occurrence of stomatitis and halitosis in denture-wearer subjects calls for better understanding of the pathogenesis of *C. albicans* and odor-producing bacterial species, particularly their denture-surface associated pathogenesis. One important step towards achieving these goals is to develop biofilm models of the implicated microbes on denture surfaces. Meanwhile, simple and reproducible biofilm models are also needed for screening antimicrobial agents and testing their efficacy in eliminating the associated pathogens.

The first chapter of this dissertation focuses on the establishment of denture biofilm models of halitosis-related bacteria and the application of these biofilm models in evaluating denture-cleansers. Our data indicated that all the selected halitosis-associated oral bacteria were capable of forming biofilm on denture surface and these established biofilm models can be successfully used to evaluate the efficacy of anti-microbial denture cleansers. The second chapter reported the development of a *C.albians* biofilm model system on real denture surfaces. Coupled with MTT colorimetric assay, this model was proven to be effective in evaluating anti-microbial denture cleansers. The last chapter of this dissertation investigated the possible association between denture stomatitis and denture-related halitosis. We demonstrated the interaction between *C. albians* and

halitosis-related oral bacterial species under *in vitro* conditions. Our data also revealed the distinct microbial profiles associated with denture stomatitis diseased sites compared to that of the healthy sites even within the same subjects. However, no clear concurrence was observed between *C. albicans* and selected halitosis related bacterial species among limited clinical samples tested. A larger number of clinical samples and high resolution 454 sequencing are being used to further explore the possible association between stomatitis-associated *C. albicans* and halitosis-associated bacteria *in vivo*.

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## **Chapter I**

**Development of *in vitro* denture biofilm models of halitosis related  
bacteria and its application in testing the efficacy of  
antimicrobial agents**

## ABSTRACT

Halitosis or bad breath is a common oral disease that often interferes with an individual's social life. Increasing evidence suggests possible association between wearing denture and increased risk of developing halitosis. It has been proposed that, by providing extra surface area for microbial colonization and biofilm formation, denture could serve as a reservoir for halitosis-related oral bacteria. However, the biofilm-forming ability of halitosis-related oral species on the denture surface is still poorly investigated. In this study, four halitosis-related strains (*Klebsiella pneumonia*, *Fusobacterium nucleatum*, *Tannerella forsythia*, and *Veillonella atypica*) were chosen and their biofilm formation on denture surface was evaluated by crystal violet staining and confocal laser scanning microscopy (CLSM). We demonstrated that, under proper environmental conditions, all four tested strains were able to form biofilm on rough polymethylmethacrylate (PMMA) denture surface, although to various degrees. Furthermore, the denture biofilms of *K. pneumonia* and *F. nucleatum* were successfully used as model systems to evaluate the efficacy of denture cleansers. In conclusion, we established mono-species denture biofilms of various halitosis-associated oral bacteria, which can be used for dental

product evaluation and serve as potential model systems for studying pathogenesis of denture-related halitosis.

## INTRODUCTION

Halitosis, also known as oral malodor or bad breath, is foul-smelling breath exhaled from the oral cavity. It affects one-third of the population and has profound physical, social and psychological impact on affected individuals (Rösing CK 2011). Halitosis is mainly due to the metabolic products produced by oral bacteria using proteinaceous compounds from saliva, gingival crevicular fluid, and epithelial cells as substrates (Loesche WJ 2000, Rosing CK 2011). The volatile sulfur compounds (VSCs), especially hydrogen sulfide (H<sub>2</sub>S), methyl mercaptan (CH<sub>3</sub>SH), and dimethyl sulfide [(CH<sub>3</sub>)<sub>2</sub>S] are the major molecules that cause oral halitosis (Persson S 1990). While other malodorous molecules including short-chain fatty acids as well as polyamines produced by anaerobic microbes residing on the surface of the tongue and in the periodontal pockets, also contribute to halitosis (Loesche WJ 2000).

A variety of oral bacteria have been shown to be able to produce malodorous compounds (Persson S 1990, Goldberg S 1997, Rogosa M 1964). Among them, *Tannerella forsythia* (formerly *Bacteroides forsythia*), *Fusobacterium nucleatum*, *Klebsiella pneumonia*,

*Veillonella atypica*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Treponema denticola*, and *Eubacterium sulci* are the most common species isolated from halitosis patients (Persson S 1989; Persson S 1990, Goldberg S 1997, Haraszthy VI 2007, Riggio MP 2008, Rogosa M 1964). These malodorous compound-producing bacteria are present at various sites in the oral cavity, particularly on the dorsum of the tongue, saliva, periodontal pockets, dentures, and dental restoration sites.

Recent studies have showed that the degree of denture hygiene is significantly associated with the halitosis symptom, and higher levels of VSC have been detected among denture-wearing subjects, particularly those elderly individuals wearing dentures overnight (Goldberg S 1997, Verran J 2005, Nalcaci R 2007). All these findings suggested that halitosis-related oral bacteria might be able to colonize denture surfaces, become residents of the denture plaque microbial communities and contribute to the denture-associated halitosis symptom. However, very few studies have been undertaken to investigate the colonization and biofilm formation of halitosis-related oral strains on denture surfaces. Meanwhile, with the increased demand for dental hygiene products, simple and reproducible denture biofilm models of halitosis-associated oral species are needed to test the effectiveness of these products, particularly denture cleansers, against denture-associated odor-causing bacteria.

In this study, four odor-producing bacterial species including *V. atypica*, *K. pneumonia*, *F. nucleatum* and *T. forsythia*, which could be recovered from denture plaque of halitosis patients, were chosen to develop *in vitro* mono-species denture biofilm models. Crystal

violet staining and confocal laser scanning microscopy (CLSM) were employed to analyze their biofilm formation and structures on denture surfaces. Furthermore, we assessed the application of these biofilm models to test the efficacy of antimicrobial agents.

## MATERIALS AND METHODS

### *Strains and growth conditions*

*Klebsiella pneumonia* IA 565 is a clinical isolate originally obtained from University of Iowa Hospitals and Clinics Special Microbiology Laboratory. The cells were inoculated in LB medium (Tryptone 10g/L Yeast Extract 5g/L NaCl 10g/L) and incubated in an aerobic chamber with 200 rpm shaking speed at 37 °C overnight. Cultures were diluted to OD = 0.1 in LB medium for biofilm growth. *Tannerella forsythia* ATCC 43037 were inoculated in TF medium described in reference, and incubated in an anaerobic chamber (85% N<sub>2</sub>, 10% H<sub>2</sub>, and 5% CO<sub>2</sub>) at 37 °C overnight (Sharma A, 1998). Cultures were diluted to OD = 0.1 in TF medium for biofilm growth. *Veillonella atypica* PK1910 were grown in TH with 0.06% lactic acid anaerobically (85% N<sub>2</sub>, 10% H<sub>2</sub>, and 5% CO<sub>2</sub>) at 37 °C overnight. Cell culture was diluted to OD=0.1 in 50% BHI (Difco, Detroit, MI) supplemented with 50 mM lactic acid and 50 mM PIPES for biofilm growth. *Fusobacterium nucleatum* ATCC 23726 were used to grow in Columbia broth (Difco,

Detroit, MI) under anaerobic conditions (85% N<sub>2</sub>, 10% H<sub>2</sub>, 5% CO<sub>2</sub>,) at 37 °C overnight. Cultures were diluted to OD = 0.1 in Columbia broth for biofilm growth.

### ***Denture discs fabrication***

The acrylic denture discs were manufactured by the same procedure as previously described (Li L 2010). All denture samples were disinfected with 2% (v/v) sodium hypochlorite solution (sigma-aldrich) for 10 minutes and immersed in sterilized ddH<sub>2</sub>O overnight prior to microbial inoculation.

### ***Growth of biofilms on denture disc surface***

2 ml of the diluted cultures of different species was respectively added into 12-well plates (Thermo Fisher Scientific Inc.) with a rough surface of denture discs. A control well was added the biofilm growth medium without bacteria. *T. forsythia*, *F. nucleatum*, *V. atypica* were inoculated in anaerobic chamber at 37 °C for 24 hours. *K. pneumonia* was inoculated in aerobic chamber at 37 °C for 24 hours.

### ***Crystal violet staining of biofilms on denture disc surface***

Biofilms formed on denture discs were stained with crystal violet following the procedure described in chapter two: “Samples were washed twice with phosphate-buffered saline

(PBS), air dried for 30 min, and stained with 0.4% (w/v) crystal violet in distilled water (at 0.22  $\mu\text{m}$  filtered before use) for 20 min. The stained samples were gently washed three times with sterilized distilled water and air dried for 30 min before being photographed. Images were taken with a D50 digital camera (Nikon, Japan).”

### ***Biofilm observation on denture surfaces with confocal laser scanning microscopy***

Biofilms grown on rough surface of denture disc with or without treatment were stained with 10  $\mu\text{M}$  SYTO 59 and 10  $\mu\text{M}$  SYTOX green (Invitrogen, US) in PBS buffer for 20 minutes at room temperature in the dark (Li L, 2010). A PASCAL 5 confocal laser scanning microscope (Zeiss, Germany) equipped with a 20 $\times$  and 63 x objectives (Plan-Neofluar/NA 0.5) was used to observe samples. Excitation at 633 nm with an argon laser in combination with a 650 nm band-pass emission filter was used for SYTO 59 fluorescence imaging. SYTOX green signals were visualized using 488 nm excitation with a helium-neon laser and a 503-530 nm band-pass emission filter.

### ***Evaluation of the treatment efficacy of denture cleansers***

Biofilms of *K. pneumonia* and *F. nucleatum* grown on denture discs were washed three times with PBS buffer before treatment. Two Polident® denture cleansing tablets 50767 and M138-12 (GlaxoSmithKline, UK) were separately dissolved in 150 ml 37 °C distilled water according to the manufacturer’s instructions, treatment with PBS as negative

control. Each sample was treated with 50 ml of the treatment solution for 5 min respectively, sequentially washed three times with PBS buffer. After treatment, samples were tackled with mechanical disruption, following dilution and plating. Treatment efficacy was evaluated by surviving colony-forming units on LB agar plates (for *K. pneumonia*) and Columbia agar supplemented with 5% sheep blood (Colorado Serum company) (for *F. nucleatum*) compared with PBS treated group.

### ***Statistical analysis***

Significance of differences between average values was analyzed by Student's t tests.

## **RESULTS**

### **Biofilm formation of halitosis-related oral strains on rough polymethylmethacrylate (PMMA) denture discs**

In order to establish denture biofilm models of halitosis-associated oral bacteria, four odor-producing strains were tested for their ability to form biofilm on PMMA denture discs. Crystal violet staining revealed that under the conditions described in Materials and Methods, all four species were capable of forming biofilms on denture surface, although to different extents, as manifested by the different intensity of the purple staining on the denture discs (Figure 1.1).

### **CSLM analysis of denture biofilms of halitosis-related oral strains**

Crystal violet staining data suggested that all four tested halitosis-related bacteria are capable of forming mono-biofilm on the surface of denture discs. In an effort to confirm their biofilm formation ability and to further study their biofilm structures, denture biofilm of each bacterial species was stained with Live/Dead stain and examined using a confocal microscope.

CSLM analyses revealed distinct architecture for each of the halitosis-related strains. Among the 4 tested species, *F. nucleatum* formed biofilm with the most smooth and homogeneous structure and could be seen covering the irregular denture surface (Fig.1.2(A)). The similar surface coverage could be observed for *T. forthysia*, however, its biofilm thickness distribution is uneven with some “ripple-like” structures extending from the base (Fig.1.2(B)). For *V. atypical*, although it can cover most of the denture surface, its biofilm is less dense and compact compared with *F. nucleatum* and *T. forthysia* (Fig.1.2(C)). The biofilm formed by *K. pneumonia* greatly differed in appearance from the rest of the tested strains. It formed thin, “patch-like” structure without covering the entire surface, leaving some of the denture surface un-occupied (Fig 1.2(D)). The majority of the bacteria cells within all the tested mono-species biofilms showed intense green fluorescence, indicating they were alive.

### **Evaluating the antimicrobial efficacy of denture cleansers against denture biofilms of halitosis-related strains**

The successful establishment of halitosis-related bacteria biofilms on the denture surfaces makes them ideal model systems for testing dental products. In this study, *K. pneumonia* and *F. nucleatum* denture biofilms were employed for evaluating the efficacy of antimicrobial denture cleanser—Polident®. Two Polident® formulas, Polident 50767 and Polident M138-12, were included in this study. The Polident tablets were dissolved in water as described in Material and Methods section, and used to treat *F. nucleatum* and *K.*

*pneumonia* denture biofilms. After removing the treatment by several washes with PBS, the colony-forming unit count, which reflects cell viability was used as a final read-out. Results showed that *F. nucleatum* single species biofilm grown on denture disc was sensitive to Polident M138-12 treatment with no viable cells being detected after 5 min treatment; while  $10^2$ - $10^3$  surviving colony-forming units could still be recovered from denture biofilm after treatment with Polident 50767 (Fig.1.3(A)). Meanwhile, both formulas demonstrated similar killing efficacy toward *K. pneumonia* denture biofilm, resulting in about 4 orders of magnitude reduction (from  $\sim 10^8$  to  $\sim 10^4$ ) in cell viability ( $P < 0.01$ ) after 5 min treatment (Fig.1.3(B)).

Furthermore, denture biofilms were stained with Live/Dead stain and examined by CSLM to reveal the live (green fluorescence) and dead (red fluorescence) cell population following different treatments. Confocal laser imaging showed that, compared to the PBS-treated mono-species denture biofilms where the majority of the cells displayed green fluorescence, treatment of *K. pneumonia* biofilm with Polident M138-12 resulted in a biofilm with high percentage of red-fluorescence labeled single cells as well as microcolonies; while the same treatment of *F. nucleatum* denture biofilm induced an almost complete shift of fluorescence signal from green to red (Fig 1.4. (A, B)). These data suggested more effective killing of Plodent M138-12 against *F. nucleatum*, which is consistent with cfu plating data.

## DISCUSSION

Denture-related halitosis imposes a serious problem, particularly for elderly denture-wearers. The colonization of odor-producing oral bacteria on denture surface or/and their integration into denture plaque has been suspected to play an important role in causing halitosis. However, the colonization and biofilm formation ability of halitosis-related bacteria on denture surfaces has remained poorly understood. In this study, four oral bacteria species, including *F. nucleatum*, *T. forsythia*, *V. atypica* and *K. pneumonia*, were chosen to investigate their biofilm forming ability on the surface of PMMA, the most commonly used denture material. All these oral species could be frequently isolated from denture plaque of halitosis patients, and have been shown to be able to produce malodorous compounds and implicated in halitosis (Persson S 1989, Persson S 1990, Goldberg S 1997, Haraszthy VI 2007, Riggio MP 2008, Rogosa M 1964).

Our study demonstrated that all four tested halitosis-related strains were capable of forming biofilm on PMMA surface. The ability to colonize and develop biofilm on denture surface not only enables these bacteria to explore extra niche for growth, but also protects themselves against host immune defense as well as external stress such as antimicrobial agents. In case of the poor oral hygiene, dentures could potentially serve as a reservoir for these odor-producing bacteria, resulting in persistent halitosis symptom. Our result also revealed very different biofilm formation abilities and structures among

the tested species, with *F. nucleatum* and *T. forthysia* being the better biofilm former on PMMA surface. This is consistent with previous studies showing different bacteria exhibited differential colonization abilities on the same surfaces; while surfaces of different physical properties could also greatly affect the biofilm formation ability of the same bacterial strains (Li L 2010). It would be interesting to know if the ability to form biofilm on denture surface would correlate with the frequency of these halitosis-related strains being isolated from denture plaque.

A variety of dental products, including denture cleanser which often contains antimicrobial compounds, have been developed for treating denture-related halitosis by reducing or eliminating odor-producing bacteria. Our study demonstrated the successful application of the newly developed denture biofilms in evaluating the efficacy of two antimicrobial denture cleanser—Polident® 50767 and Polident® M138-12. Differential killing of *F. nucleatum* and *K. pneumonia* biofilm was monitored for the same denture cleanser formula; meanwhile, different susceptibilities were observed for the same strain when treated with two different cleanser formulas. These data indicated that, the established denture biofilm models could serve as useful models in the screening and evaluation of dental products, particularly products for treating denture-related halitosis.

In conclusion, mono-species denture biofilms of various halitosis-associated oral bacteria were established in this study and used for testing antibacterial effectiveness of dental products. These mono-species biofilm could potentially be expanded into dual or

multispecies denture biofilms and serve as model systems for dental product evaluation, as well as studying pathogenesis of denture-related halitosis.

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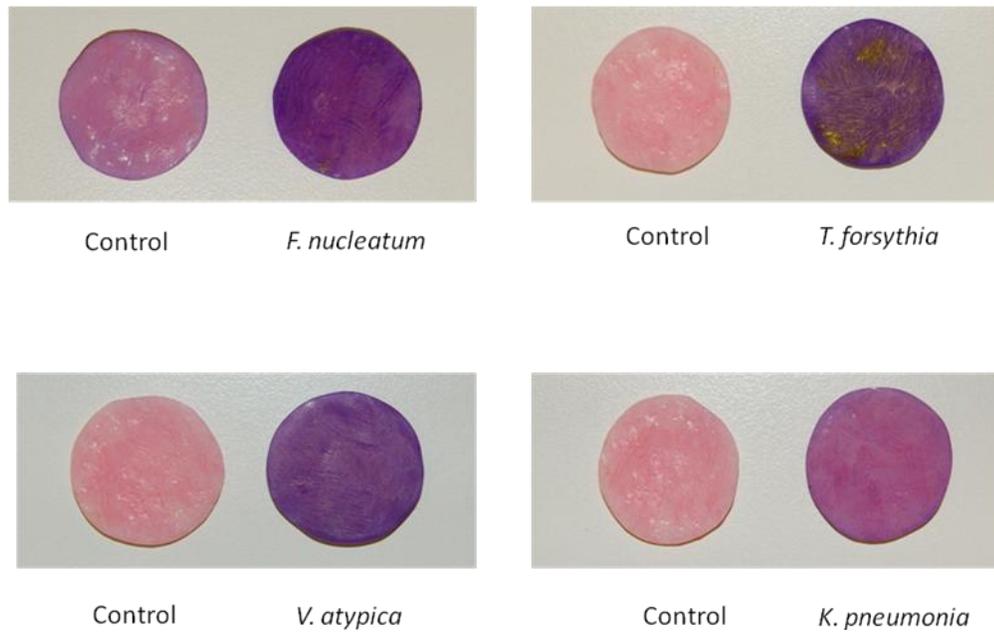
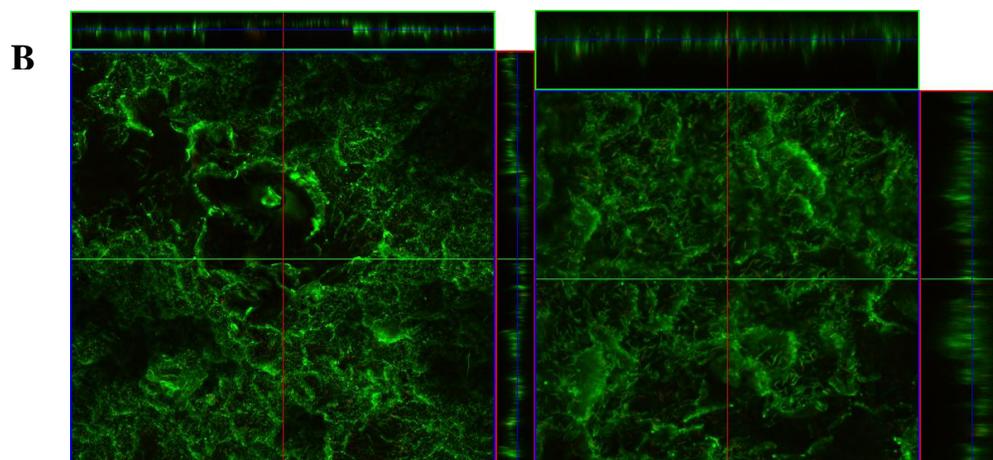
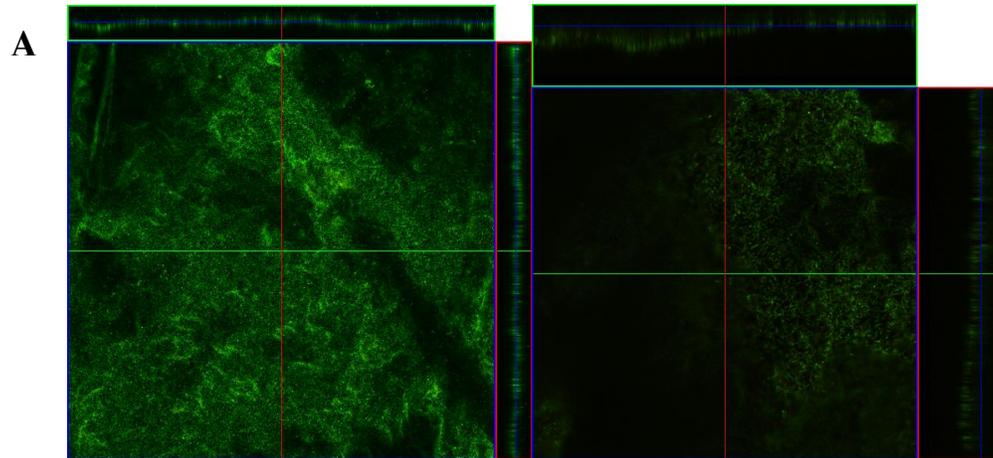


Fig. 1.1 Biofilm formation on denture surface. Representative images of *F. nucleatum*, *T. forsythia*, *V. atypica*, *K. pneumonia* formed on denture discs. These biofilms are indicated by crystal violet staining. This experiment was triplicate.



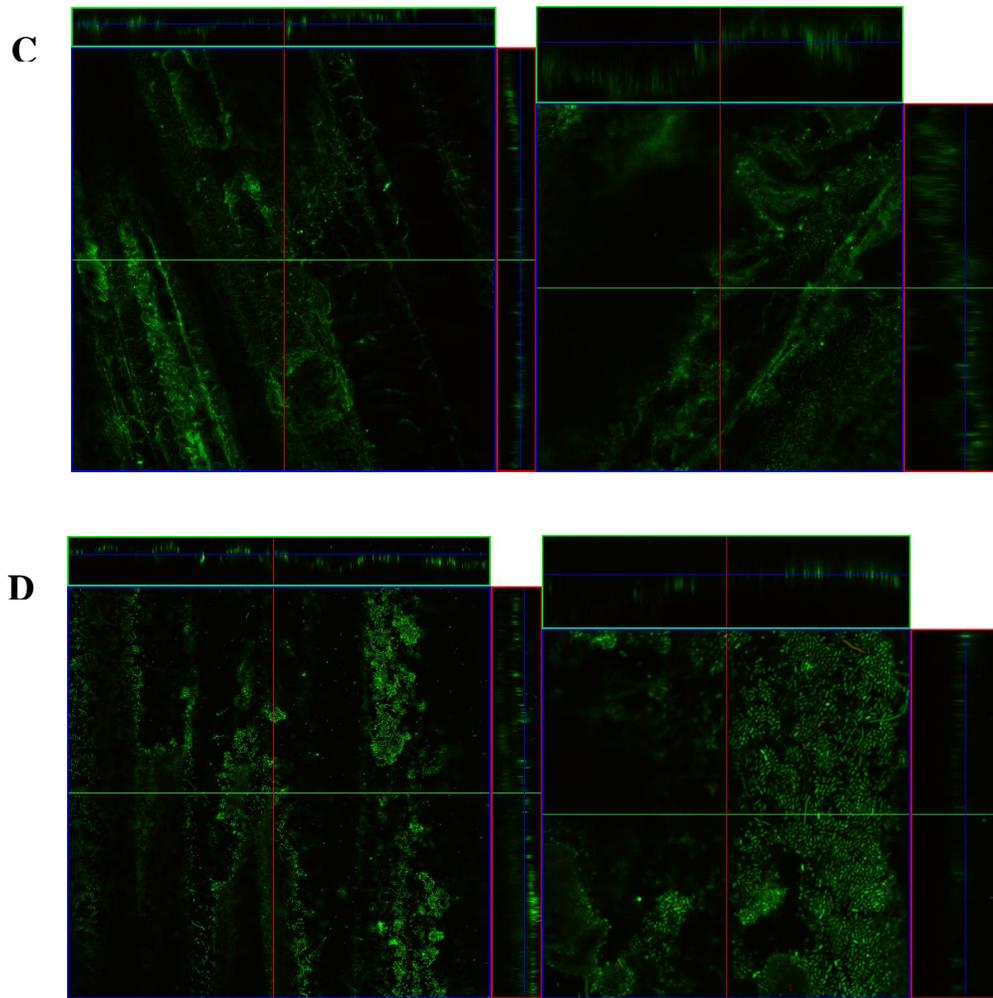


Fig. 1.2 CLSM images of biofilm on denture surface. Representative images of *F. nucleatum* (A), *T. forsythia* (B), *V. atypical* (C), *K. pneumonia* (D) formed on denture discs with the left side of images are 20 x, the right side of images are 63x. Four random fields of view were examined for each sample.

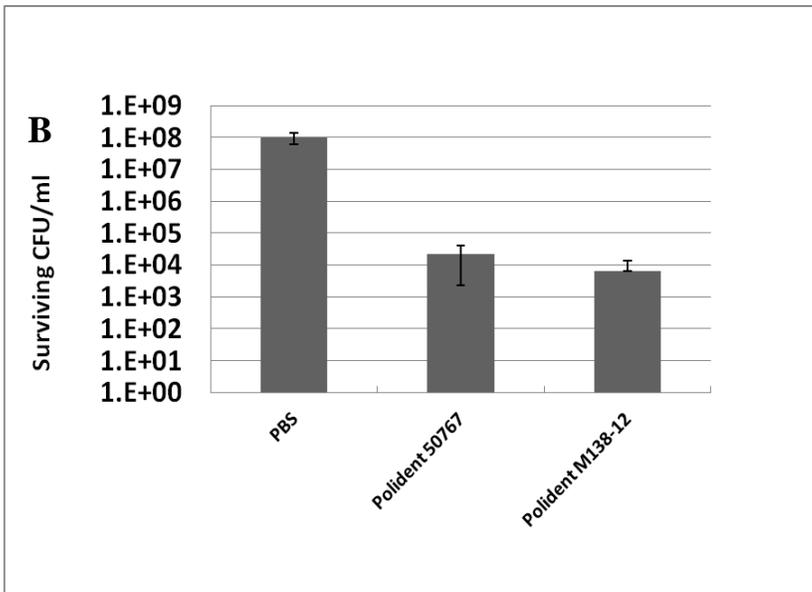
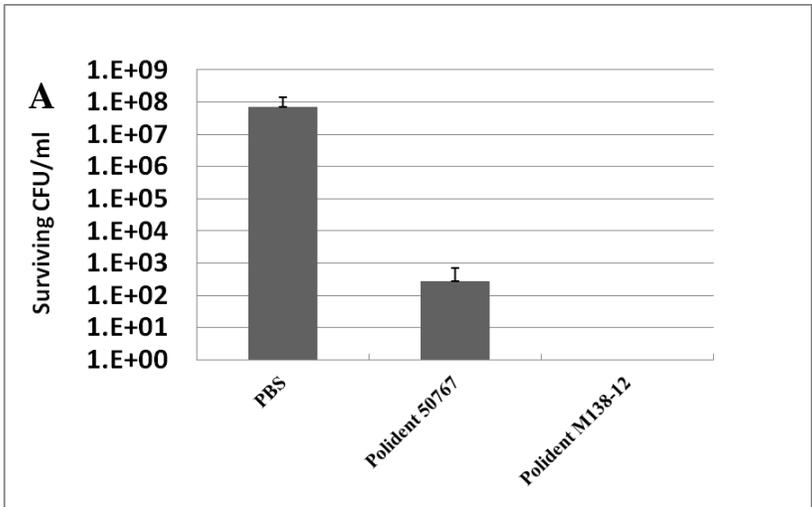


Fig. 1.3 Antimicrobial treatment efficacy against biofilms formed on denture surface. The biofilms of *F. nucleatum* (A) and *K. pneumoniae* (B) were treated with denture cleanser for 5 min followed by mechanical disruption and plating ( $P < 0.05$ ). Standard error of three replicates is presented.

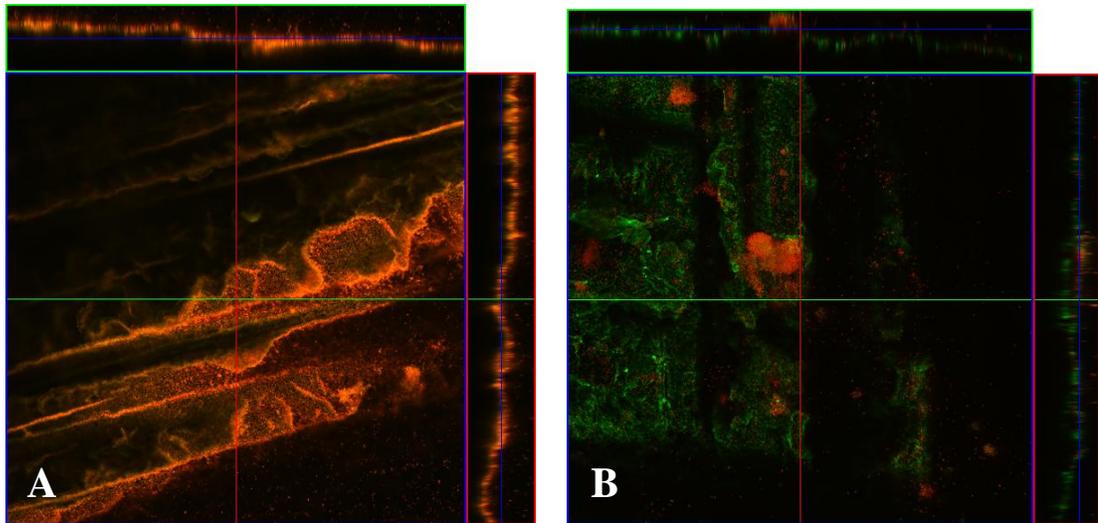


Fig. 1.4 CLSM images of *F. nucleatum* (A), *K. pneumoniae* (B) biofilms formed on denture surface against antimicrobial treatment. Four random fields of view were examined for each sample.

## **Chapter II**

### **Development of a new model system to study *Candida albicans* colonization on denture surfaces**

The following manuscript was published in Journal of Prosthodontics, in press

## ABSTRACT

Dentures are often colonized with a variety of microorganisms including *Candida albicans* which contributes to denture stomatitis. Several *in vitro* models have been previously established to study denture related microbial colonization and evaluate treatment efficacy of denture cleansers. However, those models typically fail to appreciate the complex topology and heterogeneity of denture surfaces and lack effective ways to accurately measure microbial colonization. The purpose of this study is to study microbial colonization with a new model system which is based on real dentures to more realistically mimic *in vivo* conditions. Scanning electron microscopy was used to observe topological structures among surfaces from different parts of the denture. Employing *Candida albicans* as a model microorganism, we established microbial colonization on different denture surfaces. Moreover, we applied a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) colorimetric assay to quantify without the necessity of biofilm removal *C. albicans* colonization on dentures and evaluate treatment efficacy of denture cleansers. There were significant variations in topological structures among surfaces from different parts of the denture, with the unpolished side having the highest amounts of indentations and pores. The distinct denture surfaces support microbial colonization differently, with the unpolished side containing the highest level of microbial colonization and biofilm formation. Furthermore,

the modified MTT colorimetric assay proved to be an accurate assay to measure biofilm formation on dentures and evaluate treatment efficacy of denture cleansers. This new denture model system in conjunction with the MTT colorimetric assay is a valuable tool to study denture related microbiology and treatment approaches.

## INTRODUCTION

Dentures are prosthetic devices commonly used in adults which are designed to replace missing teeth (Dolan TA 2001, Seman K 2007, Henriksen BM 2003, MacEntee MI 1985). Although dentures enhance the patient's ability to chew, their utilization is strongly associated with several diseases, including local as well as systemic infections. Local infections include denture stomatitis, angular cheilitis, and contact allergy to denture materials; while systemic diseases associated with dentures include chronic respiratory disease, cardiovascular diseases, diabetes, and arthritic disorders (MacEntee MI, 1985). Denture stomatitis is the most common disease which manifests as inflammation of oral mucosa that contacts dentures (Gendreau L 2011). *Candida albicans* is known to be the main contributor to this disease as it colonizes and subsequently forms a biofilm on the denture surface (Salerno C, 2011).

In order to study denture related microbial colonization and determine the efficacy of different denture cleansers, a variety of *in vitro* bacterial or fungal biofilm models have been established using microtiter plates, acrylic strips and discs (Nett J 2006, Nikawa H 1999, Chandra J 2001, Harrison Z 2004, Thein ZM 2007, Li L 2010, Jahn B 1995). While these are simple, convenient, economic models to study microbial colonization, and test the antimicrobial efficacy of denture cleansers, they have intrinsic limitations. The model systems using acrylic strips and discs do not account for the complex topology

of the denture structures. In addition, treatment efficacies are mainly determined by scraping, vortexing, or sonicating microbes off the strips or discs, which is ineffective and often leads to inaccuracies in CFU counting. To address this problem, investigators have applied 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-based colorimetric assays to measure the metabolic activities of total microbial flora, and demonstrated that this assay was a more accurate and convenient way to evaluate microbial viability than scraping and CFU counting (Jahn B 1995, Levitz SM 1985, Hawser SP 1995). This approach, however, has not been used so far to study microbial colonization on real dentures. Furthermore, all dentures have a polished side (called smooth surface in this study) and an unpolished side (called rough surface in this study). In clinical applications, the smooth surfaces are typically exposed to the oral cavity, while the rough surfaces are in contact with the oral mucosa. Most previously described model systems do not consider the possible differences in microbial colonization between these two different sides (Chandra J 2001, Harrizon Z 2004, Thein ZM 2007, Li L 2010, Jahn B 1995).

In this study, we aimed to develop a new model system in which microbial biofilms were grown on real dentures taking into consideration their complex topological structures and surface features. The MTT colorimetric assay was employed to validate its utility to measure microbial colonization on real dentures compared to the traditional methods of scraping, vortexing or sonication and to evaluate treatment efficacies of denture cleansers. *C. albicans* was chosen as the model microorganism for this study.

## MATERIALS AND METHODS

### *Strains and growth conditions*

*C. albicans* strain 5296 is a clinical isolate obtained from the University of California Los Angeles hospital. The cells were inoculated in YDP medium (10 mg/ml yeast extract, 20 mg/ml dextrose, 20 mg/ml peptone) and incubated in a 5% (v/v) CO<sub>2</sub> chamber at 30°C (Murrilo LA 2005).

### *Denture materials*

The acrylic denture discs used in this study were manufactured as previously described (Li L 2010). The partial dentures were provided by GlaxoSmithKline and were manufactured by commercial vendors following standard procedures. All denture samples were disinfected with 75% (v/v) ethanol treatment prior to microbial inoculation.

### *Growth of C. albicans biofilms on various surfaces*

A previously described protocol was applied with modifications (Li L 2010). *C. albicans* biofilms were grown on dentures, denture discs, and plastic wells. For these experiments, the overnight cultured *C. albicans* cells were diluted in Ham's F-12 medium (Fisher, US) to approximately 10<sup>4</sup> cells/ml as inoculum.

*C. albicans* biofilms formed in plastic wells were used to generate standard curves for the MTT assay. In these experiments, 2 ml of serial diluted inoculum (10<sup>3</sup>-10<sup>8</sup> cells/ml) was added into each well of 12-well plates (Fisher, US) and incubated aerobically for 3 h at 37°C to form the biofilm on polystyrene surfaces.

*C. albicans* biofilms formed on acrylic denture discs were used in some of the experiments for comparison and analysis. 2 ml of diluted inoculum (10<sup>4</sup> cells/ml) and a sterile acrylic denture disc with a rough or smooth surface were added into each well of 12-well plates and cultured aerobically on a shaker at 140 rpm for 24 h at 37°C to form the biofilms on denture discs.

For our new model system, sterilized acrylic dentures were submerged in 80 ml of inoculum (10<sup>4</sup> cells/ml) in a 250 ml conical flask, and aerobically cultured on a shaker at 140 rpm for different time periods at 37°C to form biofilms.

#### ***Crystal violet staining of biofilms on denture surfaces***

*C. albicans* biofilms formed on dentures and denture discs were stained with crystal violet according to a published protocol with some modifications (Saotome K 1989). Samples were washed twice with phosphate-buffered saline (PBS), air dried for 30 min, and stained with 0.4% (w/v) crystal violet in distilled water (at 0.22 µm filtered before use) for 20 min. The stained samples were gently washed three times with sterilized distilled water and air dried for 30 min before being photographed. Images were taken with a D50 digital camera (Nikon, Japan).

### ***Examination of biofilms on denture surfaces with confocal laser scanning microscopy***

*C. albicans* biofilms grown on both rough and smooth surfaces of dentures were stained with 10  $\mu$ M SYTO 9 and 10  $\mu$ M SYTOX orange (Invitrogen, US) in PBS buffer for 30 min at room temperature in the dark.<sup>13</sup> The specimens were observed through a PASCAL 5 confocal laser scanning microscope (Zeiss, Germany) equipped with a 20 $\times$  objective (Plan-Neofluar/NA 0.5). Excitation at 488 nm with an argon laser in combination with a 505-530 nm band-pass emission filter was used for SYTO 9 fluorescence imaging. SYTOX orange signals were visualized using 543 nm excitation with a helium-neon laser and a 560-615 nm band-pass emission filter.

### ***Analysis of denture surfaces with SEM***

Specimens were coated with a 10 nm thick film of platinum in a sputter coater (EMS 575 high resolution sputter coater with thin film monitor, EMS Inc, Hatfield, PA) and images were obtained using an XL30 S FEG scanning electron microscope (FEI Inc, Hillsboro, Oregon) operating at 5kVa.

### ***Mechanical disruption of *C. albicans* biofilm on partial denture***

*C. albicans* biofilms grown on denture surfaces were disrupted and removed by scraping, vortexing or sonication, respectively. For scraping, each biofilm on a denture was scraped

thoroughly with a scraper for 3 min. For vortexing, dentures with biofilms were vortexed in 20 ml PBS buffer containing 3 mm glass beads (Fisher) for 3 min. For sonication, dentures with biofilms were placed in a 20 ml PBS solution and sonicated at 7W energy level for 3 min using a Sonic Dismembrator (Fisher). The residual biofilms remaining on treated dentures were visualized with crystal violet staining (as described above).

### ***MTT colorimetric assay***

The MTT colorimetric assay is based on the cleavage of MTT into a blue-colored formazan by living cell enzymes and the amount of formazan formed is correlated to the number of viable cells. The MTT assay was performed as previously described with modifications (Sylvester PW 2011, Garn H 1994, Pires RH 2011). The MTT assay solution was prepared by dissolving 1 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Biosynth, US) 1% (w/v) glucose and 10  $\mu$ M menadione (Acros, US) in PBS buffer. The solution was filtered at 0.22  $\mu$ m and pre-warmed to 37°C prior to use. The MTT solution was added to *C. albicans* biofilm samples, incubated at 37°C for 3 h, and then carefully removed. The formazan crystals formed in viable cells were dissolved by acid-isopropanol (5 ml 3N HCl in 95 ml isopropanol). The optical density of this solution was determined at 540 nm using a microplate reader (Bio-Rad, US). Solutions without biofilms were used as blank controls.

The MTT standard curve was generated using *C. albicans* biofilms grown on polystyrene surfaces (as described above). The biofilms formed by different numbers of viable cells

(103-108 cells/ml) were obtained by inoculating serially diluted *C. albicans* cells in Ham's F-12 medium prior to cultivation. After 3 h of incubation, medium was removed from each well and the biofilms were washed three times with PBS buffer, and then incubated with 2 ml MTT solution for 3 h. The formation of formazan crystals by viable cells was determined as described above. The viable cell numbers in the respective biofilm samples formed by different initial inocula were also assessed by colony-forming units (CFU) on BHI agar plates supplemented with 800 mg/ml spectinomycin and 100 mg/ml kanamycin as previously described (Li L 2010). The MTT standard curve was determined by plotting the number of viable cells (CFU counts obtained from different inocula) on the x-axis against the optical density on the y-axis (MTT assay read out of different inocula) using log/log power regression analysis. Viable cell numbers were evaluated through the formula obtained from the standard curve. Individual standard curves were generated for each experiment.

In order to assay the viable cells in *C. albicans* biofilms formed on denture surfaces, the medium was removed and each denture was incubated with 18 ml MTT solution for 3 h. The biofilms containing formazan crystal were submerged in 5 ml acid-isopropanol and vortexed with glass beads for 10 min. 200 ml of this acid-isopropanol solution was removed and transferred into the wells of a 96-well plate to measure the optical density. The corresponding viable cell numbers were calculated using the formula obtained from the MTT standard curve (see above). The growth curve of a biofilm on a denture was generated by graphing the dependent variable number of cells on the x-axis versus optical density on the y-axis.

### ***Evaluation of the treatment efficacy of an antifungal cleanser***

*C. albicans* 24 h biofilms grown on both denture discs and dentures were washed three times with PBS buffer to remove non-adherent cells. A Polident® denture cleansing tablet (GlaxoSmithKline, UK) was dissolved in 150 ml distilled water according to the manufacturer's instructions. 75% (v/v) ethanol was used as a positive treatment control and PBS was used as the corresponding negative control. Each sample was treated with 50 ml of the treatment solution for 3 min, 5 min and 10 min, respectively. After treatments, solutions were removed and dentures were washed three times with PBS buffer. The treatment efficacy was evaluated by the MTT assay described above.

## RESULTS

### **Analysis of topological structures of different denture surfaces and their influence on biofilm formation of *C. albicans***

Our goal is to create a more realistic denture biofilm model that can mimic *in vivo* conditions. In this study, we used real dentures to support microbial biofilm formation to better appreciate the complex topology and heterogeneity of denture surfaces derived by standard manufacturing processes. As the first step, we decided to better understand their physical structures. As shown in Fig. 2.1, different parts of the denture were viewed by SEM. The polished side (Fig. 2.1A) showed an extremely smooth surface devoid of pores and indentations (Fig. 2.1C), while the unpolished side (Fig. 2.1B) displayed a rough surface with many irregularities and pores (Fig. 2.1D).

The variations in physical structures present in smooth and rough denture surfaces shown in Fig. 1 may lead to differences in microbial colonization. To test this hypothesis, we used *C. albicans* as the model microbe to grow biofilms on manufactured dentures as described in Materials and Methods. Assayed with crystal violet staining, we found that different surfaces had different abilities to support biofilm formation under these growth conditions. Biofilms on both sides of the dentures were unevenly distributed and much more biomass accumulated on rough surfaces (Fig. 2.2B), compared to smooth surfaces (Fig. 2.2A). To further examine the differences in biofilms grown on both rough and

smooth surfaces, we also grew biofilms on both surfaces of the denture discs similar to the procedures applied to real dentures. As shown in Figs. 2.2C and D, A dense, tightly attached biofilm was formed on the rough surface of the denture disc (Fig. 2.2D); whereas a less dense, loosely attached biofilm was found on the smooth surface of the denture disc (Fig. 2.2C), similar to our observations on real dentures (Fig. 2.2A and B). We then used laser scanning confocal microscopy to examine the architectural differences between the biofilms formed on both rough and smooth surfaces. A thicker biofilm with tight aggregates was observed on rough surfaces (Fig. 2.2F), while only thin biofilms with loose aggregates were observed on smooth surface (Fig. 2.2E). Most interestingly, using SEM, we found that microbes on the rough surfaces often colonized within the cracks and pores (Fig. 2.2G).

### **Evaluation of standard procedures for measuring microbial colonization on real dentures**

Based on the above data, the new model system using real dentures showed different microbial colonization patterns on different sides of a denture, similar to what had been observed with dentures obtained from patients. Despite this advantage, the use of real dentures also created new problems, especially the difficulty to accurately measure the number of bacteria colonizing the denture surfaces. As shown in Figure 2.3, standard approaches used to count microbial CFUs on denture strips or discs, such as scraping, vortexing or sonication, proved to be ineffective for this denture model since substantial amounts of biofilms remained on the dentures after the treatments, especially on the rough sides (Figs. 2.3B, D, and F).

### **Adaptation of the MTT assay to determine microbial colonization of the new denture model system**

Recognizing the deficiency of the standard procedures used above, we decided to adapt the MTT assay for this new model system for the reasons described in Materials and Methods.

As the first step, a standard curve was constructed to evaluate the correlation between viable cell numbers and optical readings from the MTT assay (Fig. 2.4A). After establishing the good power relationship ( $R^2 > 0.9$ ) between viable cell numbers and optical readings, we applied the MTT assay to measure *C. albicans* 24 hr-biofilm developed on dentures. As expected, the viable cell numbers determined by the MTT assay were significantly higher than the inaccurate CFU counts produced by standard procedures ( $P < 0.01$ ), as shown in Fig. 2.4B.

Furthermore, we also demonstrated that the MTT assay was suitable for monitoring the dynamic process of biofilm formation on dentures. As shown in Fig. 2.4C, the accumulation of viable cells on dentures was consistent with previous findings, and exhibited logarithmic growth in the first 24 hr, followed by a stationary phase from 24 hours to 72 hr, and a decrease in cell viability after 72 hr.

### **Evaluation of antifungal efficacy with the new denture model system in conjunction with the MTT colorimetric assay**

In this section, we further validated the utility of this new denture model system and determined its ability to evaluate the treatment efficacy of a denture cleanser in comparison with the denture disc assay. The *C. albicans* biofilms formed on denture discs and dentures were treated with Polident® according to the manufacturer's instructions. The Polident solution was able to completely eliminate viable *C. albicans* biofilms on denture discs after 5 min treatment, while it only reduced *C. albicans* viability by three to four orders of magnitude on the dentures ( $P < 0.01$ ) (Fig. 2.5). The elimination of viable *C. albicans* biofilms on dentures was achieved with a 10 min-treatment of Polident solution ( $P < 0.01$ ).

## DISCUSSION

In this study, we established a new *in vitro* denture biofilm model system using real dentures to form microbial biofilms under shaking conditions. Our studies showed that the polished (smooth) and unpolished (rough) surfaces of dentures had great difference in their topological structures, which led to different outcomes in biofilm formation. In particular, the rough surfaces were found to contain extensive indentations and pores that provided niches for increased *C. albicans* colonization. It should be noted that, since in clinical applications the rough side of a denture is in direct contact with the oral mucosa, this enhanced ability to support *C. albicans* colonization on a rough surface could thus contribute to denture associated stomatitis. The finding also suggested that this new model overcomes many limitations of previous model systems, and more realistically mimick *in vivo* conditions (Nett J 2006, Nikawa H 1999, Chandra J 2001, Harrison Z, 2004, Thein ZM 2007, Li L 2010, Jahn B 1995).

Since this new model use real dentures containing complex topological structures, standard biofilm quantification methods are not suitable due to the difficulties of biofilm removal from the surface. Our data shows that the MTT assay is a good alternative and is suitable for this model system. In addition to its ability to measure biofilms on denture surfaces, the MTT assay is also useful in monitoring the dynamic process of biofilm

formation, creating the possibility for many future applications to use this model system (in conjunction with MTT) to study denture biofilm related biological questions.

The data reported in this paper also demonstrated that this model system is a useful tool to evaluate the treatment efficacy of antimicrobial cleansers for dentures. It is particularly interesting to note the variability in treatment efficacy when we tested the same denture cleanser with a denture disc based *in vitro* model as compared to the real denture based new model system. We found that it took 10 min to kill all *Candida* cells on the denture, while 5 min treatment was sufficient to kill all *Candida* cells on the denture discs. This difference could be due to difference in the properties of the biofilms formed on actual dentures, further validating the positive features of this new model system for future applications in screening antimicrobial products since it may more realistically reflect *in vivo* conditions.

In conclusion, the present study describes a more realistic denture biofilm model system which reflects the real topology and surface properties of dentures and their associated abilities for microbial colonization and biofilm formation. In conjunction with the MTT colorimetric assay, it is a convenient, cost-effective and high-throughput tool to study denture related microbiological and treatment questions. Furthermore, this denture biofilm model is not limited to *C. albicans* related stomatitis; it could easily be modified and adapted to various other biofilms that are associated with denture-related local or systemic diseases, including respiratory pathogens such as *Streptococcus pneumoniae* and *Enterobacter cloacae*. This model system could also be modified and adapted to other denture materials such as porcelain and composite resins for additional studies.

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**Figure 1**

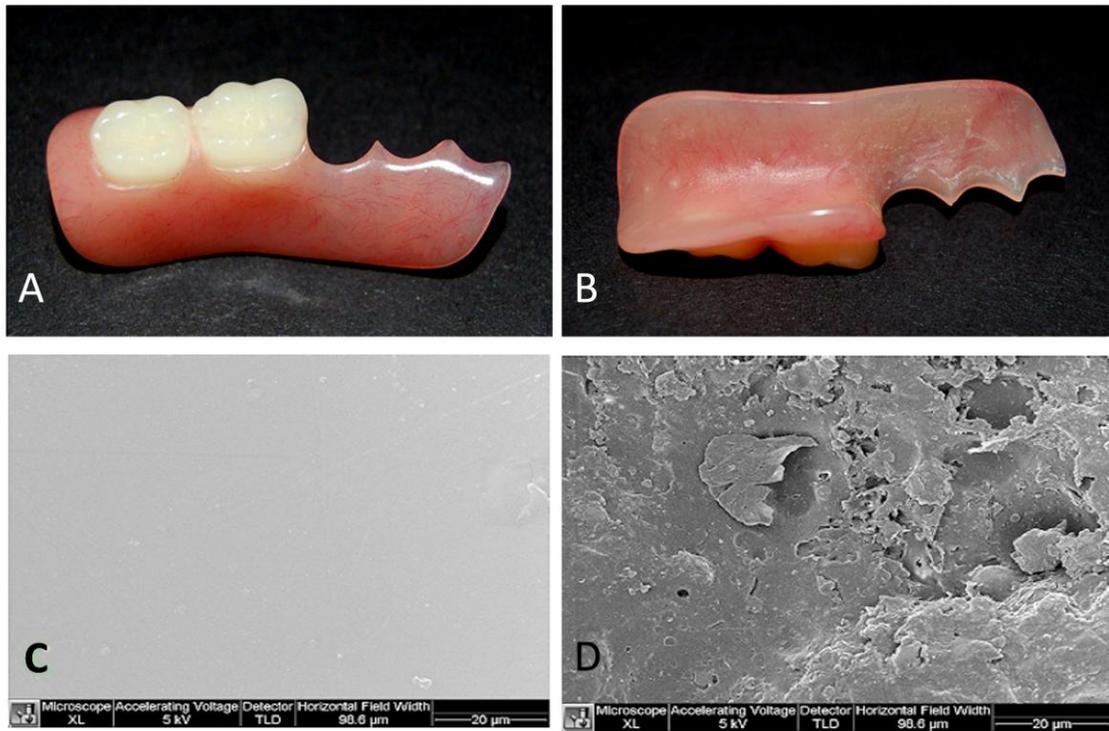


Figure 2.1 Representative images of denture surfaces. Shown are the (A) polished (smooth) and (B) unpolished (rough) surfaces of the partial dentures. SEM images (5000x magnification) of the (C) smooth and (D) rough surfaces of the partial dentures. Four random fields of view were examined for each sample.

Figure 2

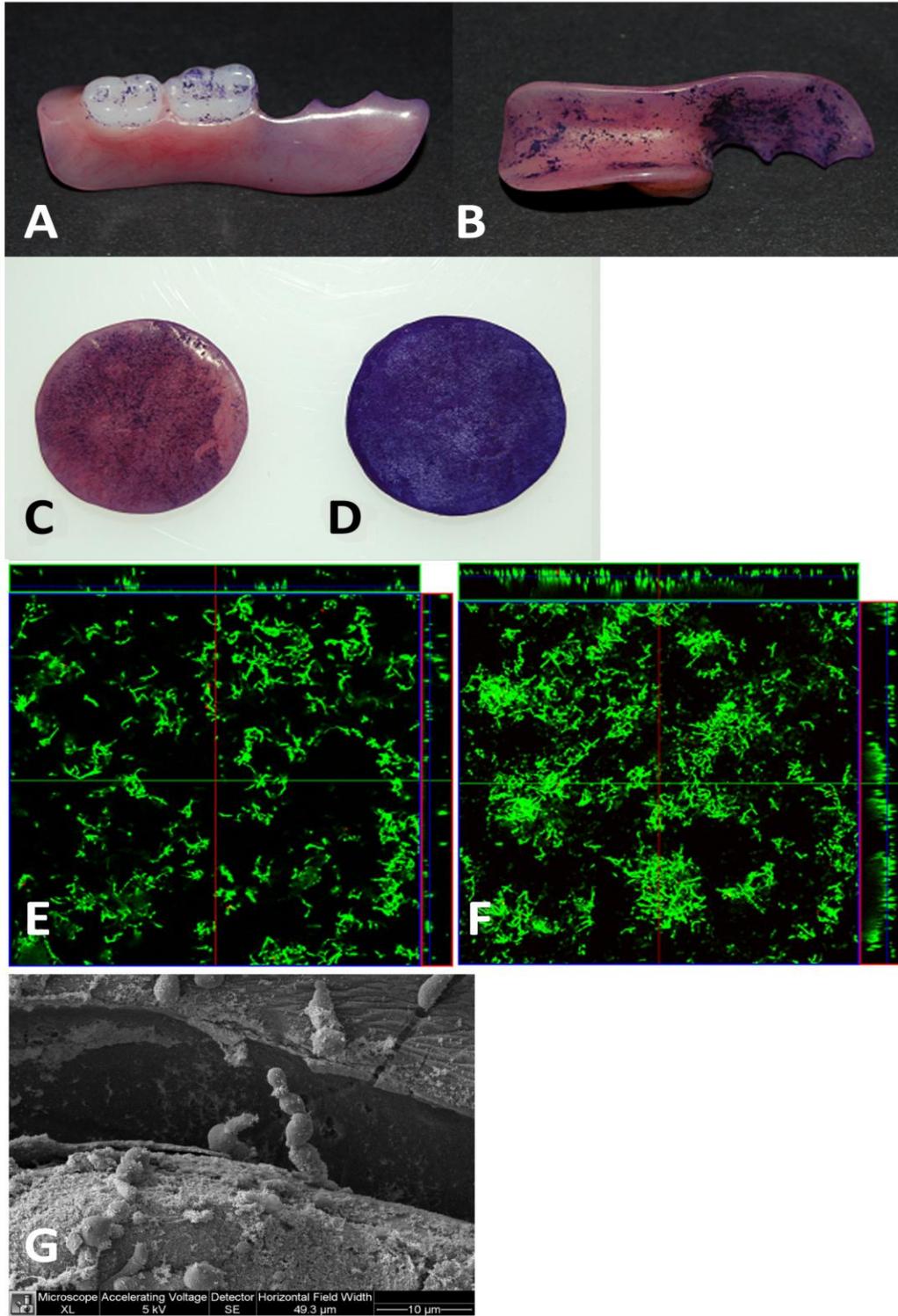


Figure 2.2 *C. albicans* biofilm formation on dentures and denture discs. (A and B) *C. albicans* biofilms formed on the smooth (A) and rough (B) denture surfaces were stained by crystal violet shown in blue. (C and D) *C. albicans* biofilms formed on the smooth (C) and rough (D) surfaces of denture discs that were stained by crystal violet. (E and F) *C. albicans* biofilms formed on the smooth (E) and rough (F) surfaces of denture discs that were examined with CLSM after staining with SYTO 9 and SYTOX Orange. (G) *C. albicans* cells colonizing the surface imperfections of the rough denture surface. Four random fields of view were examined by SEM and CLSM for each sample.

**Figure 3**

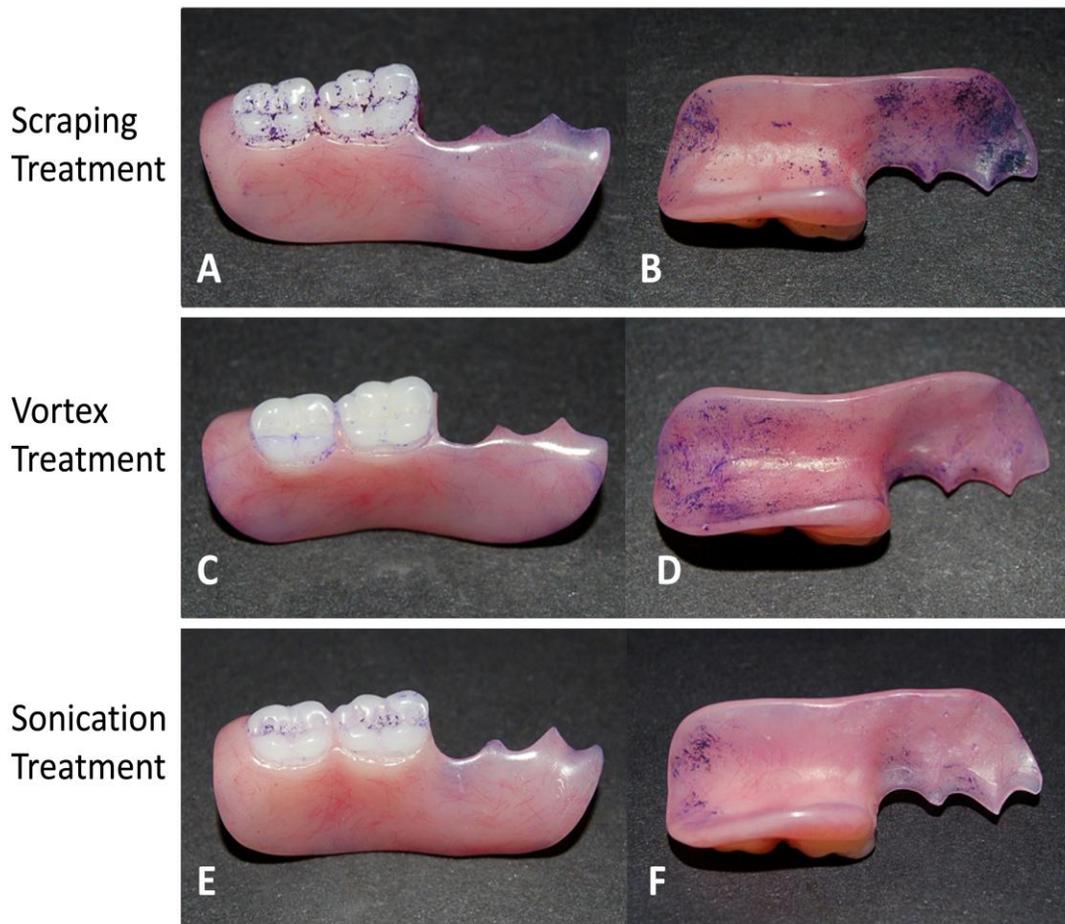


Figure 2.3 Biofilms remaining on dentures after removing *C. albicans* with scraping (A and B), vortexing (C and D) or sonication (E and F), respectively. The remaining biofilms on denture were visualized by crystal violet (shown in blue). Panels A, C and E are smooth surfaces while panels B, D and F are rough surfaces. The experiments were repeated three times.

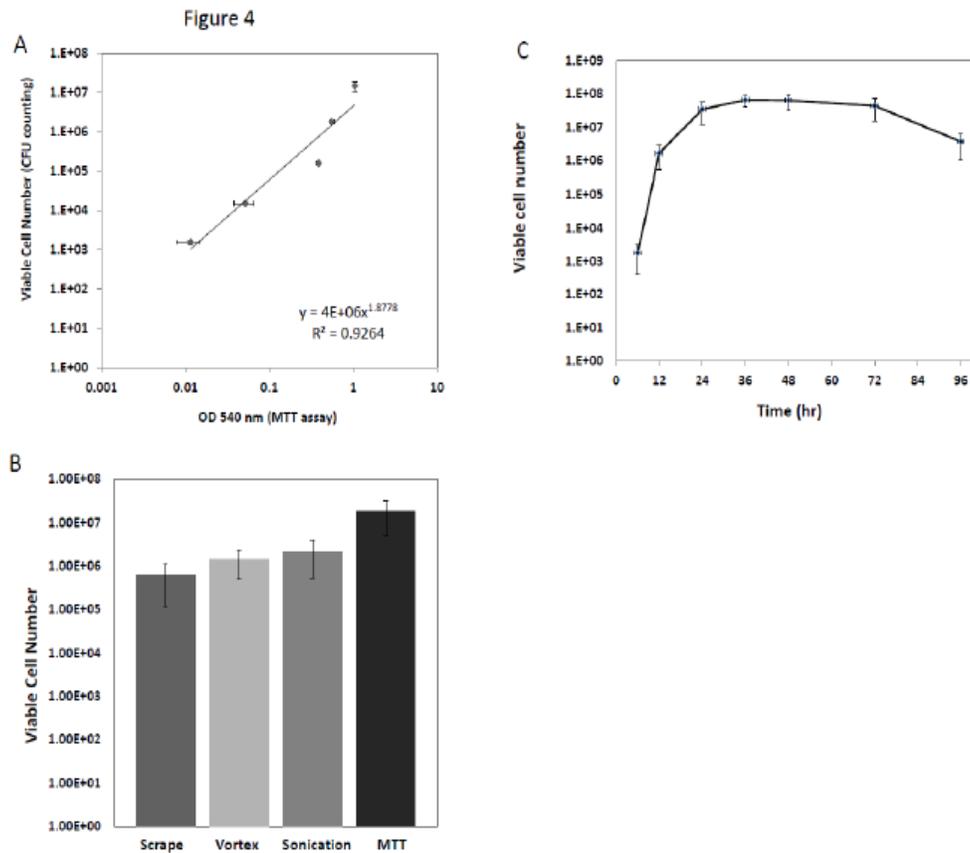


Figure 2.4 Quantification of viable cell number of *C. albicans* biofilms on denture surfaces. (A) Standard curve of the MTT assay was obtained for quantification of viable cell numbers of *C. albicans* biofilms. (B) Determination of viable cell number in *C. albicans* biofilm on dentures by CFU count through scraping, vortexing, sonication (three columns on the left) and MTT assay (the right column). The difference between scraping/vortexing/sonication and MTT is significant ( $P < 0.01$ ). (C) The growth curve of *C. albicans* biofilm grown on dentures was determined by MTT assay. All errors are within the 10 to 15 % range and experiments were repeated at least three times.

Figure 5

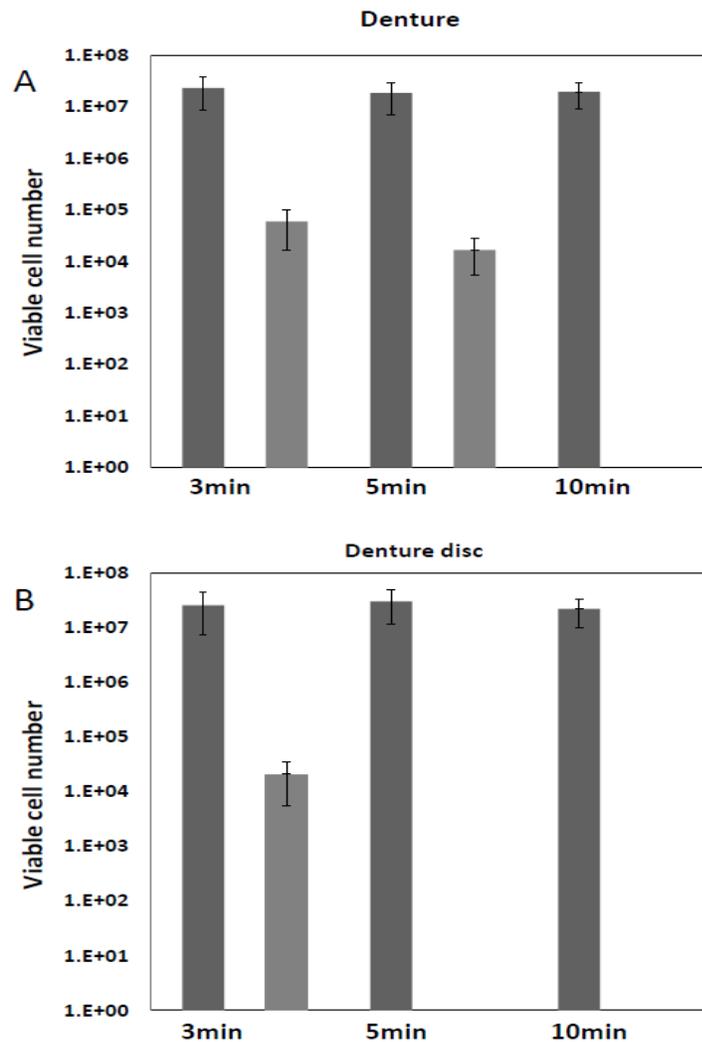


Figure 2.5 Antifungal treatment efficacy against *C. albicans* biofilms on denture discs and dentures. Biofilms on (A) dentures and (B) denture discs were treated with denture cleanser for 3 min, 5 min, 10 min. The treatment efficacy was determined by the MTT assay. The errors are within 15%. and the differences between control groups and treated groups are significant ( $P < 0.01$ ). All experiments were repeated three times.

■ Negative control (PBS), □ positive control (75% ethanol), ■ experimental group.

## **CHAPTER III**

***In vitro* and *in vivo* studies of the possible association between *Candida albicans* and  
halitosis-related bacteria**

## ABSTRACT

Denture stomatitis and halitosis are two of the most prevalent denture-related infectious diseases. Although clinic evidence indicates that these two oral health conditions are often concurrent, the association between the causative agents of denture stomatitis and halitosis-related oral bacterial species are still poorly defined. In this study, *Candida albicans*, the main pathogen of denture stomatitis and four halitosis-related species (*Klebsiella pneumoniae*, *Fusobacterium nucleatum*, *Tannerella forsythia*, and *Veillonella atypica*) were chosen to investigate their potential inter-kingdom interactions under planktonic as well as biofilm conditions. Fluorescence-based co-aggregation assay and confocal laser scanning microscopy (CLSM) analysis revealed significant coadherence between *C. albicans* and halitosis-related bacterial strains. Furthermore, clinical denture and tooth plaque samples from healthy as well as denture stomatitis patients were screened for the presence of *C. albicans* and aforementioned four halitosis strains. Result showed that high frequency of *C. albicans* detection was associated with stomatitis patients, although no significant concurrence of *Candida* and halitosis strains was observed. Denaturing gradient gel electrophoresis (DGGE) analysis further revealed distinct microbial profiles between denture plaques collected from healthy and diseased sites among the subjects and within the same subjects. Our study demonstrated interaction between *C. albicans* and halitosis-related species under *in vitro* conditions, which could potentially facilitate the co-existence of these clinically relevant microbes

within denture plaques, resulting in the concurrence of denture stomatitis and halitosis.

## INTRODUCTION

The human oral cavity harbors a wide range of microbial species, including bacteria, fungi, protozoa, mycoplasmas, and virus, with bacteria being the most dominant residents. These microorganisms form highly structured, multispecies communities (Coulthwaite L 2007). The resident microbes within the communities often display intricate physical and chemical interactions, perform physiological functions and induce microbial pathogenesis, which could potentially result in a variety of oral diseases, including dental caries, periodontitis, stomatitis and halitosis (Coulthwaite L 2007).

*Candida albicans* is an opportunistic fungal pathogen often found in human oral cavity. It becomes a resident of the commensal flora by competing and cooperating with many oral bacteria species and its growth is often kept in check by host defense mechanisms as well as antagonistic effects exerted by other commensal bacteria (Odds FC 1994). However, under certain conditions, the change of microenvironments or host immune response could transform *C. albicans* from commensal microbe to pathogen capable of causing infections, such as denture-related stomatitis (Odds FC 1994).

Clinical study revealed in denture wearers a possible association between denture stomatitis and halitosis, another health condition caused by the malodorous compounds produced by certain oral bacterial species (Baran I 2008). Examination of the infection sites of denture stomatitis often revealed the presence of significant bacterial growth on both mucosa and fitting surface of the denture (Ramage G 2004). There is increasing

evidence that *C. albicans* forms direct physical associations with certain oral bacterial species, and this type of inter-kingdom interaction could facilitate the colonization and integration of *C. albicans* into the multispecies microbial communities. The high frequency of concurrence of denture stomatitis and halitosis in denture wearing patients suggested a possible physical association between *C. albicans* and halitosis-related oral bacterial species. In this study, 4 halitosis-related oral strains, including *K. pneumonia*, *F. nucleatum*, *T. forsythia*, and *V. atypica* were tested for their physical interaction with *C. albicans* under planktonic and biofilm conditions. Meanwhile, clinical denture plaque samples from denture stomatitis patients were analyzed for their associated microbial profiles, as well as the concurrence of *C. albicans* and these odor-producing strains.

## Materials and methods

### *Strains and growth conditions*

*K. pneumonia* IA 565, *T. forsythia* ATCC 43037, *V. atypica* PK1910 and *F. nucleatum* ATCC 23726 were used in this study and their growth conditions were described in Chapter I. *C. albicans* 5296 was cultivated as described in Chapter II. *Streptococcus gordonii* DL1 was cultivated in Brain Heart Infusion broth (BD, USA) at 37 °C under anaerobic condition (85% N<sub>2</sub>, 10% H<sub>2</sub>, and 5% CO<sub>2</sub>).

### *Fluorescence-based assay*

Bacterial cells were collected from the culture, washed and re-suspended in phosphate-buffered saline (PBS) to final OD<sub>600</sub> of 10. 200 µl of cell suspension was transferred to two 1.5 ml microfuge tubes. The cells in one of the tubes were stained with 1.5 µl 10 µM Syto 9 green fluorescent nucleic acid stain for 20 minutes at room temperature as fluorescence group. Cells were washed 10 times with coaggregation buffer (150 mM NaCl, 1 mM Tris, 0.1 mM CaCl<sub>2</sub>, 0.1 mM MgCl<sub>2</sub>) and resuspended in 200 µl coaggregation buffer before use; while the other tube with un-stained cells served as blank. *C. albicans* cells were collected by centrifugation, washed and re-suspended in phosphate-buffered saline (PBS) and adjusted to OD<sub>600</sub> of 10.

For coaggregation assay, bacteria (Cyto9-labeled or un-labeled) and *C. albicans* were diluted with co-aggregation buffer to a final concentration of OD600 of 0.5 and 1, respectively. Testing bacteria with *C.albicans* were mixed in a volume of 250 ul reaction tube. The tubes were vigorously vortexed for 10 s and allowed to stand at room temperature. At 0 m, 10 m and 30 m after vortexing, 100 µl of upper layer supernatant was taken and 10 x diluted in coaggregation buffer. 250 µl of diluted supernatant was measured for its fluorescence intensity using fluorometer (Bio-Rad, US). The pair with un-stained bacteria cells was used as blank.

The control group with bacteria (Cyto9-labeled or un-labeled) only were diluted in co-aggregation buffer to a final concentration of OD600 of 0.5 in a volume of 250 ul reaction tubes. The measurement procedure was the same as the described above. The group with un-stained bacteria cells was used as blank in the control group.

The result was recorded as the percentage of fluorescence signal remaining in the supernatant after incubation relative to the initial signal intensity.

#### ***Growth of dual species biofilms of C. albicans and halitosis-associated bacteria***

For dual species biofilm, 2 ml of Ham F-12 medium (Hyclone, US) containing *C. albicans* and bacterium with starting inoculum of OD600 of 0.1 for each was added into 6-well plates (Thermo Fisher Scientific Inc.) with 2cm x 2cm plastic coverslips (Fisher US). For single species biofilm, 2 ml of Ham F-12 diluted cell suspension of different species (OD=0.1) was added into 6-well plates (Thermo Fisher Scientific Inc.) with 2cm

x 2cm plastic coverslips. *T. forsythia*, *F. nucleatum*, *V. atypica* were incubated in anaerobic chamber at 37 °C for 8 hours; while *K. pneumonia* was grown aerobically at 37 °C for 8 hours.

***Observation of biofilms on plastic coverslips with confocal laser scanning microscopy (CLSM)***

Plastic coverslips were taken out from culture wells and washed three times with phosphate buffered saline (PBS). Biofilms grown on plastic surfaces were stained with 10 µM SYTO 59 and 10 µM SYTOX green (Invitrogen, US) in PBS buffer and kept in dark for 20 minutes at room temperature. A PASCAL 5 confocal laser scanning microscope (Zeiss, Germany) equipped with a 40x objectives (Plan-Neofluar/NA 0.5) was used to observe samples. Excitation at 633 nm with an argon laser in combination with a 650 nm band-pass emission filter was used for SYTO 59 fluorescence imaging. SYTOX green signals were visualized using 488 nm excitation with a helium-neon laser and a 503-530 nm band-pass emission filter as describe in Chapter I.

***Subjects and sampling***

Three groups of subjects in total were included in this study. Group 1 contained ten healthy denture wearers (five women and five men; mean age  $69.8 \pm 4.73$  years); group 2 had 10 denture wearers diagnosed as denture stomatitis (five women and five men; mean age  $61.6 \pm 12$  years) based on the guideline for denture stomatitis diagnosis; group 3

included four subjects (two women and two men; mean age  $65 \pm 6.32$  years) who wear two sets of dentures at the same time, one was diagnosed as exhibiting denture stomatitis symptom, while the other stayed healthy (Budtz-Jorgensen & Bertram, 1970). These individuals had not used biocide-containing dentifrice for at least 6 months before sample collection. Subjects had not been treated for any systemic disease nor were they taking any prescription or nonprescription medications. All subjects provided written informed consent to participate in the current study.

For sample collection, sterile toothpicks (Fisher US) were used to scrape off plaque from the denture surface contacting with the oral mucosa. Plaque was also collected from the tooth on the opposite side from the missing tooth. QIAamp DNA Micro Kit (Qiagen, US) was used to extract DNA from collected plaque samples according to the manufacturer's instructions. DNA quality and quantity were checked by NanoDrop 2000 spectrophotometer (Thermo, US).

#### ***Detection of fungi and halitosis-related species by polymerase chain reaction (PCR)***

PCR was employed to detect the presence of fungi as well as four of the selected halitosis-related bacterial strains within the plaque samples. Fungi and bacterial species specific primers were designed based on previous studies (Liu Y 2008, Igarashi E 2009, Kirakodu S 2008, Ghannoum MA 2010).

For PCR reaction, 10 ng DNA was used as template to set up a reaction mixture of 25 µl containing 1x PCR buffer, 1.5mM MgCl<sub>2</sub>, 0.1mM each of the four dNTPs, 1 unit Taq DNA polymerase (Invitrogen, USA) and 1 µM of each primer. The PCR reaction conditions were as follows: for *K. pneumonia*, one pair of specific primers was used Pf (5'-ATT TGA AGA GGT TGC AAA CGA T-3') and Pr2 (5'-CCG AAG ATG TTT CAC TTC TGA TT-3'); the reaction condition was 94 °C for 10 min; 35 cycles of 94°C for 30s, 57 °C for 20 s, and 72 °C for 20 s, and a final extension at 72 °C for 10 min. For *V. atypica*, one pair of specific primers was used ATYF (5'- TCT CTT GTT GAA GAA TTA GAA CGC-3') and VR (5'- GTGTAACAAGGGAGTACGGACC-3'); the PCR condition was 15 94 °C for 15 min, 20 cycles of 92 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min, followed by 5 min at 72 °C. For *T. forsythia*, one pair of specific primers was used 5'- GCGTATGTAACCTGCCCGCA-3' and 5'- TGCTTCAGTGTCAGTTATACCT-3'; the reaction condition was 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 5 min. For *F. nucleatum*, one pair of specific primers was used 5'- AGAGTTTGATCCTGGCTCAG-3' and 5'- GTCATCGTGCACACAGAATTGCTG -3'; the PCR condition was 94 °C for 3 min; 23 cycles of 94 °C for 1 min, 61.5 °C for 1 min, and 72 °C for 30s, and a final extension at 72°C for 5 min. For Fungi, one pair of universal primers was used ITS1F (5'- CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'); the cycling condition was 94 °C for 11 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30s, and 72 °C for 2 mins , and a final extension of 30min at 72 °C.

PCR products were analyzed by agarose gel electrophoresis (1% w/v) in 1×TAE buffer (40 mM Tris–HCl, 1.18 ml acetic acid, 2 mM EDTA, pH 8.0) and a constant voltage of 100V was applied. Images were digitally recorded using the Molecular Imager Gel Documentation system (Bio-Rad) to examine the presence of the amplified DNA product.

### *Denaturing Gradient Gel Electrophoresis (DGGE) analysis*

DGGE analysis was performed as previously described (Wang RK, 2011). Briefly, V3 region of bacterial 16S ribosomal RNA (rRNA) genes were PCR amplified with Bac1 (5'-

CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGACTACGTGCCAG

CAGCC-3') and Bac2 (5'-GGACTACCAGGGTATCTAATCC-3'). 8% polyacrylamide

gels with a denaturing urea/formamide gradient between 40% and 60% were used. PCR products were separated by electrophoresis for 17 h at 58 °C using a fixed voltage of 60 V

in a Bio-Rad DCode System (Bio-Rad Laboratories, US). The gels were then stained with

0.5 µg/mL ethidium bromide in 1× TAE buffer for 15 min, and destained for 10 min in

1× TAE buffer. DGGE profile images were digitally captured using the Molecular

Imager Gel Documentation system (Bio-Rad).

## RESULTS

### **Coaggregation of selected halitosis related bacteria with *C. albicans***

Four selected halitosis related strains (*K. pneumonia*, *V. atypica*, *F. nucleatum*, *T. forsythia*) were tested for their binding ability with *C. albicans*. *S. gordonii* was used as the control strain due to its co-aggregation phenotype with *C. albicans* in previous studies (Jenkinson HF 1990). To more quantitatively measure the co-adherence between *C. albicans* and bacterial strains, we developed fluorescence-based co-aggregation assay, in which bacteria were labeled with fluorescence before being mixed with un-labeled *C. albicans*. Since co-aggregated bacteria cells often precipitate at the bottom together with *C. albicans*, the remaining fluorescence signal in the supernatant would reflect the number of un-bound bacteria. Our result showed that, while there was only about 20% decrease in fluorescence intensity for the control species, a range of 60% to 80% reduction in signal intensity was observed for the tested halitosis-related strains, suggesting positive co-aggregation phenotypes between *C. albicans* and four halitosis related strains (Fig.3.1).

### **CLSM analysis of early-stage dual species biofilm of *C. albicans* and halitosis-related bacteria**

Co-aggregation data showed that all the tested halitosis-related strains were able to adhere to *C. albicans*. In an effort to further investigate the direct physical association between candida and halitosis strains, early duo-species biofilm of *Candida* and halitosis-related bacteria were allowed to develop for 8 hours, biofilms were stained with Live/Dead staining and examined using a confocal microscope. Observation of single species biofilms showed that *C. albicans* formed a heterogeneous network structure composed of hyphae, while for the mono-species bacterial biofilms, other than *K. pneumonia* which formed thin, “mesh-like” structure with many un-occupied space, the rest of the bacterial strains were able to develop confluent single layer biofilm structures (Fig.3.2). *C. albicans*/bacteria dual species biofilms all exhibited heterogeneous structures including sparsely occupied areas with few bacteria cells and dense colonized region containing highly structured microcolonies. The microcolonies were composed of intertwined candida hyphae, forming scaffold-like structure; while the bacterial cells can be seen either decorated along the candida hyphae or forming small aggregates and tightly associated with fungal cells (Fig 3.2).

### **The prevalence of fungi and four halitosis related strains in denture plaque**

*C. albicans* and aforementioned four halitosis-related bacterial species were chosen as targets for PCR screening among the 20 clinical samples (10 each for healthy and stomatitis subjects). The prevalence of these species in the clinical denture plaque samples were shown in Table 3.1. Fungi were detected more frequently from denture

stomatitis patients (90%) than healthy subjects (50%). For those halitosis-related bacteria, other than *K. pneumonia* which can only be detected from stomatitis group, the rest three species could all be found in both groups. Their prevalence in healthy and denture stomatitis plaque was 60% and 40% for *F. nucleatum*, 30% and 40% for *T. forsythia*, 30% and 10% for *V. atypica*, 0% and 20% for *K. pneumonia*; respectively (Table 3.1). However, no significant association between fungi and halitosis-related bacteria can be established among these limited numbers of clinical samples.

### **PCR-DGGE analysis of microbial profiles in denture and tooth plaque from healthy and stomatitis subjects**

*C. albicans* has been shown to co-exist with other oral bacteria through physical association and chemical interaction, and denture stomatitis often involves mixed infections of *Candida* and a variety of bacteria. As an initial effort to characterize the denture stomatitis-associated microbial profiles, plaque samples from healthy and diseased subjects, as well as denture plaques from healthy and infection sites of the same patients were collected for PCR-DGGE profile analysis. Results showed that, regardless of whether they were from healthy or diseased subjects, there are consistently distinct microbial banding patterns between tooth and denture plaque from the same subjects (Fig.3.3(A,B)). More interestingly, when comparing the denture plaque samples collected from healthy and diseased subjects, a drastic difference in DGGE banding pattern could

be observed; this phenomenon also found in the healthy and diseased site within the same individuals (Fig.3.3(B,C)).

## DISCUSSION

The possible association between denture stomatitis (fungal infection) and denture-related halitosis (bacterial infection) has been suspected based on clinical study, however, the direct interaction or the co-existence between the principle causative agents of these two oral health conditions has not been well investigated.

In the current study, we developed a novel fluorescence-based co-aggregation assay, which can provides much increased sensitivity for more accurate quantification. Using this assay, we demonstrated that as the main etiological agent of denture stomatitis, *C. albicans* was able to co-aggregate with all the tested odor-producing, halitosis-related oral bacterial species under planktonic conditions. Furthermore, during the development of *in vitro* dual species biofilms, *C. albicans* and these bacterial strains co-adhered and formed highly structured mixed microcolonies occupying the abiotic surfaces. Co-aggregation/co-adherence between different microorganisms is a significant factor contributing to the development and maintenance of the microbial community. It also plays crucial role in modulating the gene expression and physiological functions of the microbes involved. Differential co-adherence among microbial could affect the structure as well as composition of the community and have great impact on the pathogenesis of polymicrobial infections under certain circumstances. The inter-generic co-aggregations have been demonstrated between candida and a variety of commensal oral bacteria

(Rickard AH, 2003). More importantly, the fungal infection and parallel or secondary bacterial infections have been documented in several clinical conditions, including a reported high risk of bacterial co-infection with yeast keratitis (Pate JC 2006). The observed direct physical association between *C. albican* hyphae, the most clinically relevant form of *Candida*, and odor-producing bacteria suggested a mutual selection and their preference of staying in close proximity of each other during community development. The physical association could potentially result in the concurrence of the denture stomatitis and denture-related halitosis, the two most common denture-associated health conditions.

A screen for the co-existence of candida and aforementioned 4 halitosis strains in the clinical denture plaque samples revealed significantly higher occurrence of *Candida* within the samples from denture stomatitis patients. However, PCR screening didn't reveal significant concurrence between *C. albicans* and tested halitosis strains in denture plaque collected from healthy or denture stomatitis patients. This could be due to the limited sample size. Meanwhile, a variety of odor-producing bacteria have been implicated in halitosis, while only 4 were chosen as targets in the current study, potential association between *Candida* and other odor-producing bacteria could be overlooked. Thus, a larger sample size as well as high resolution of the microbial profile analysis, such as 454 deep sequencing will provide more detailed information to further explore and test the proposed association between *C. albican* and halitosis-related bacteria.

DGGE analysis revealed distinct microbial profiles between tooth and denture plaque samples collected from the same subjects, this is consistent with previous studies showing different microbial flora associated with different oral tissues and dental appliance surfaces (Coulthwaite L 2007). More interestingly, a drastic difference in microbial profiles was observed among the subjects with and without denture stomatitis, and also from the healthy and diseased sites within the same individuals. This finding further supported the notion that oral diseases are often influenced by the whole microbial communities, not just single pathogens. Even though the main etiological agent for denture stomatitis is *C. albicans*, the clinical outcome and symptom can be affected by the structure as well as functions of the microbial communities it resides. Thus, a particular clinical condition could be associated with community with distinct microbial structure and composition. The observed inter-kingdom physical association between *C. albicans* and halitosis-related species under planktonic as well as biofilm growth conditions suggested potentially co-colonization of these microbes on the denture plaques *in vivo*, which could lead to the concurrence of denture stomatitis and halitosis. A larger number of clinical samples and high resolution 454 sequencing for analyzing microbial community as well as detecting other halitosis-associated bacteria species would provide more detailed information to further explore and test the proposed association (Pate JC 2006).

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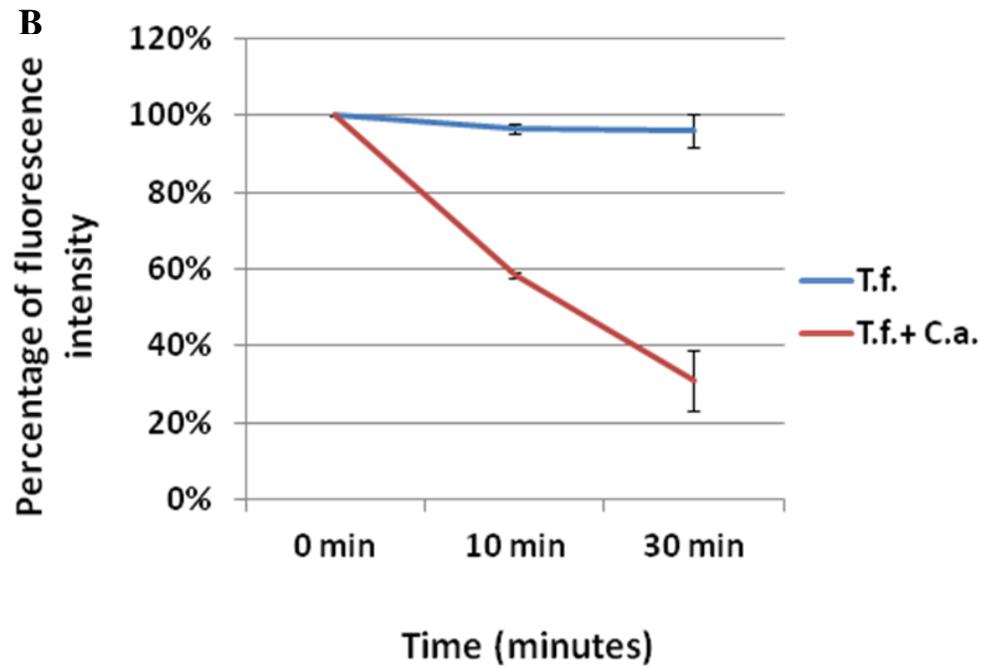
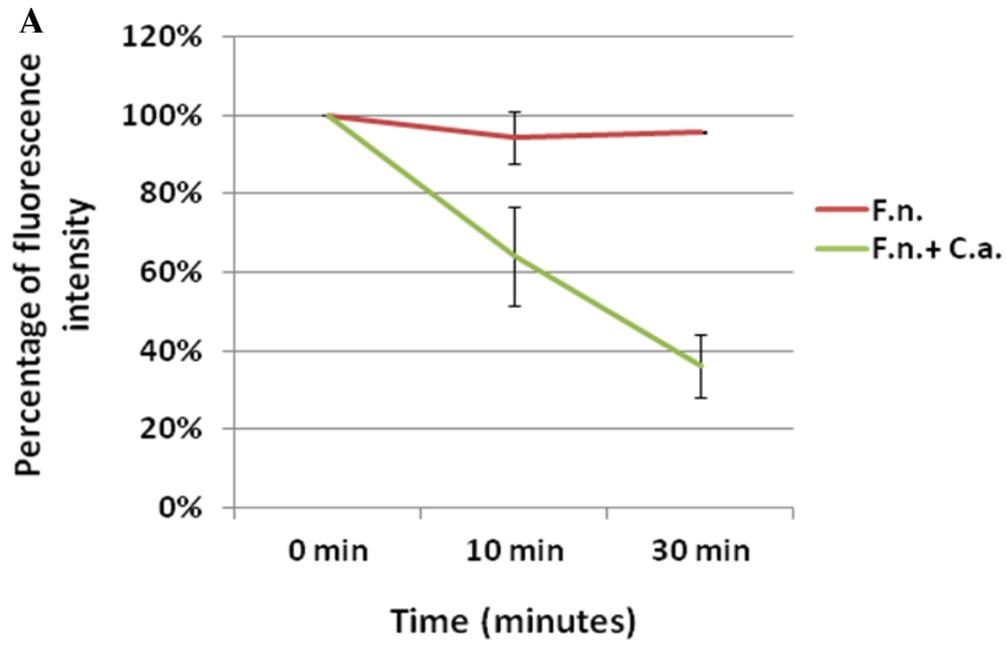
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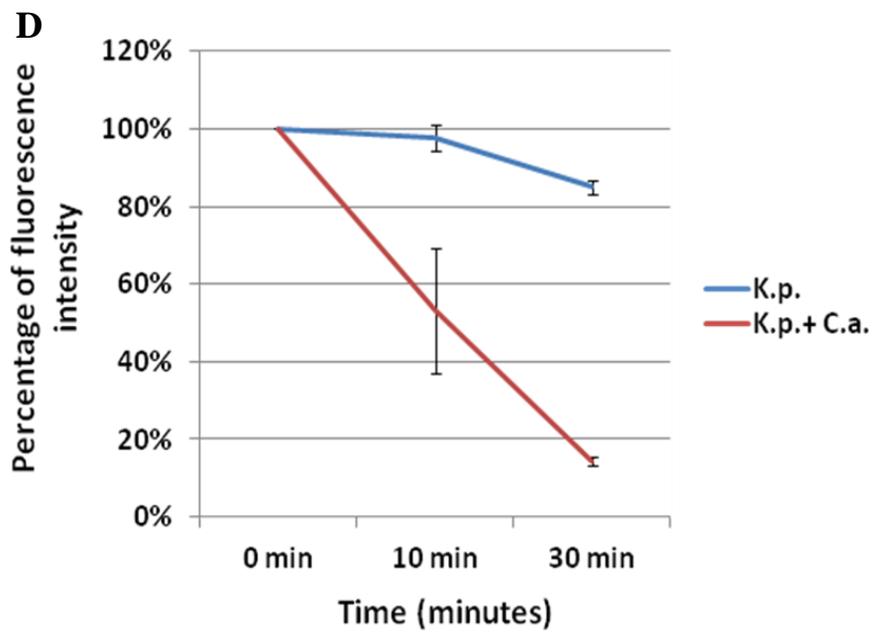
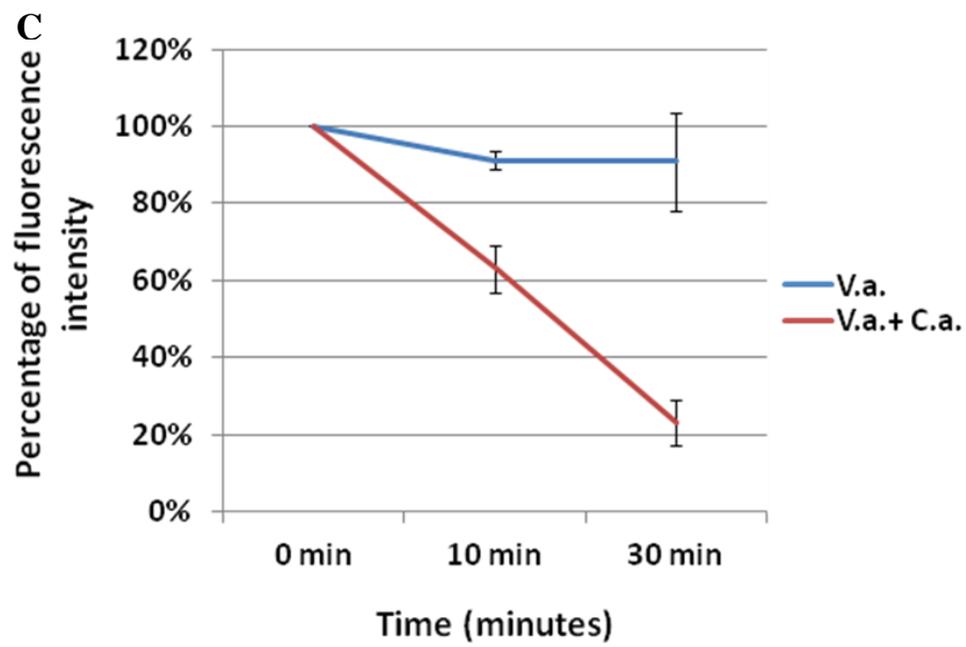
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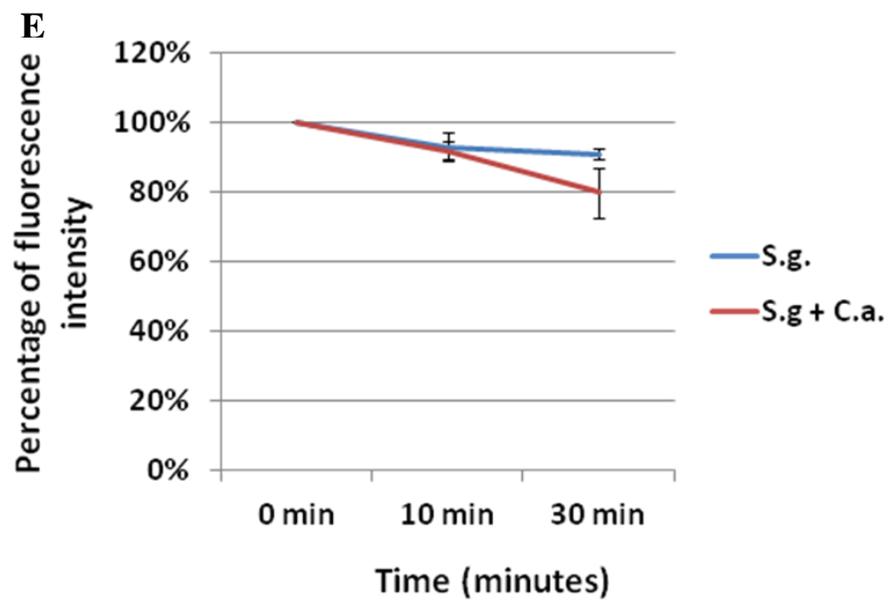
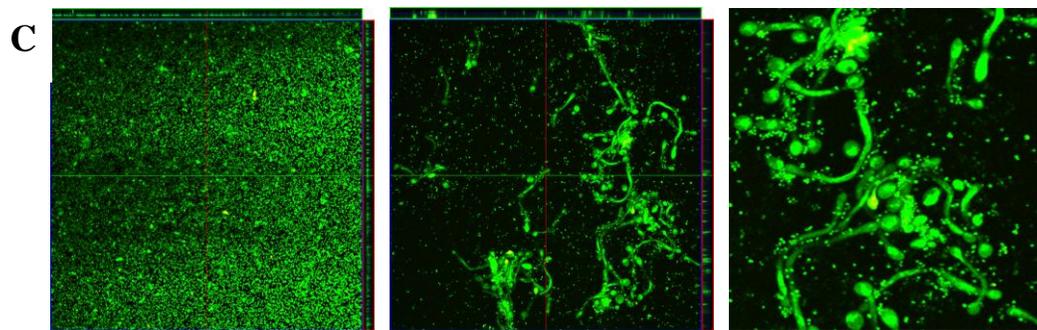
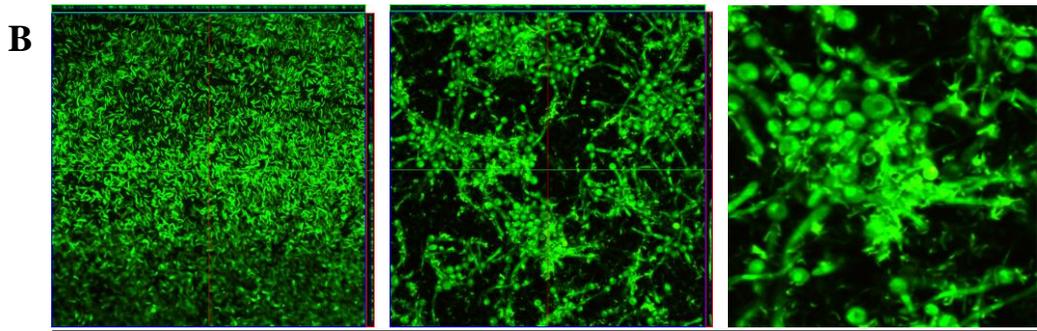
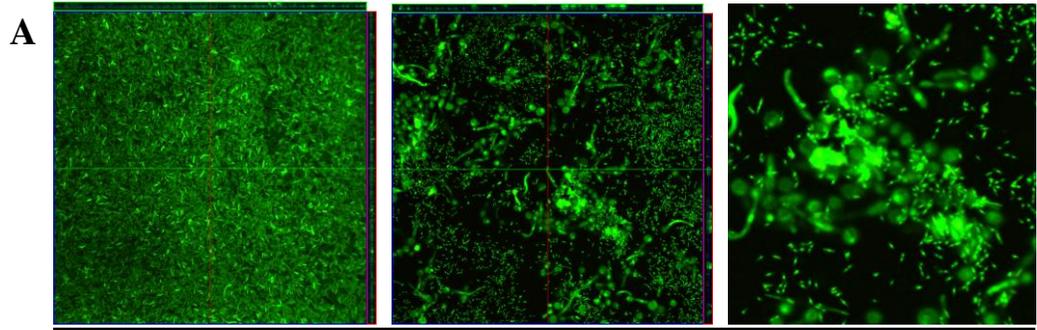


Fig. 3.1 Fluorescence-based coaggregation assay of *F. nucleatum* (A), *T. forsythia* (B), *V. atypica* (C), *K. pneumonia* (D), *S. gordonii* (E) with and without *C. albicans*. Standard error of three replicates is presented.



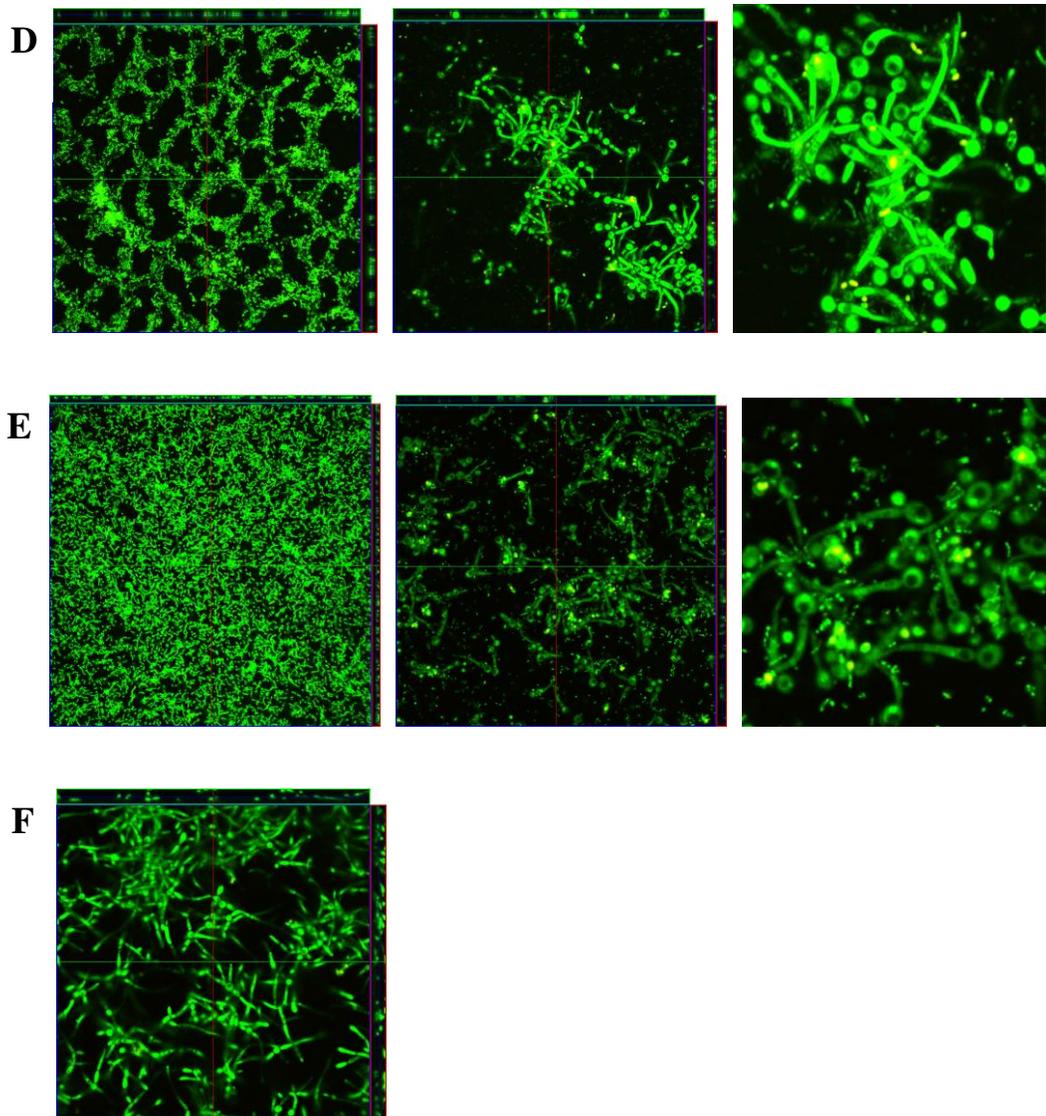
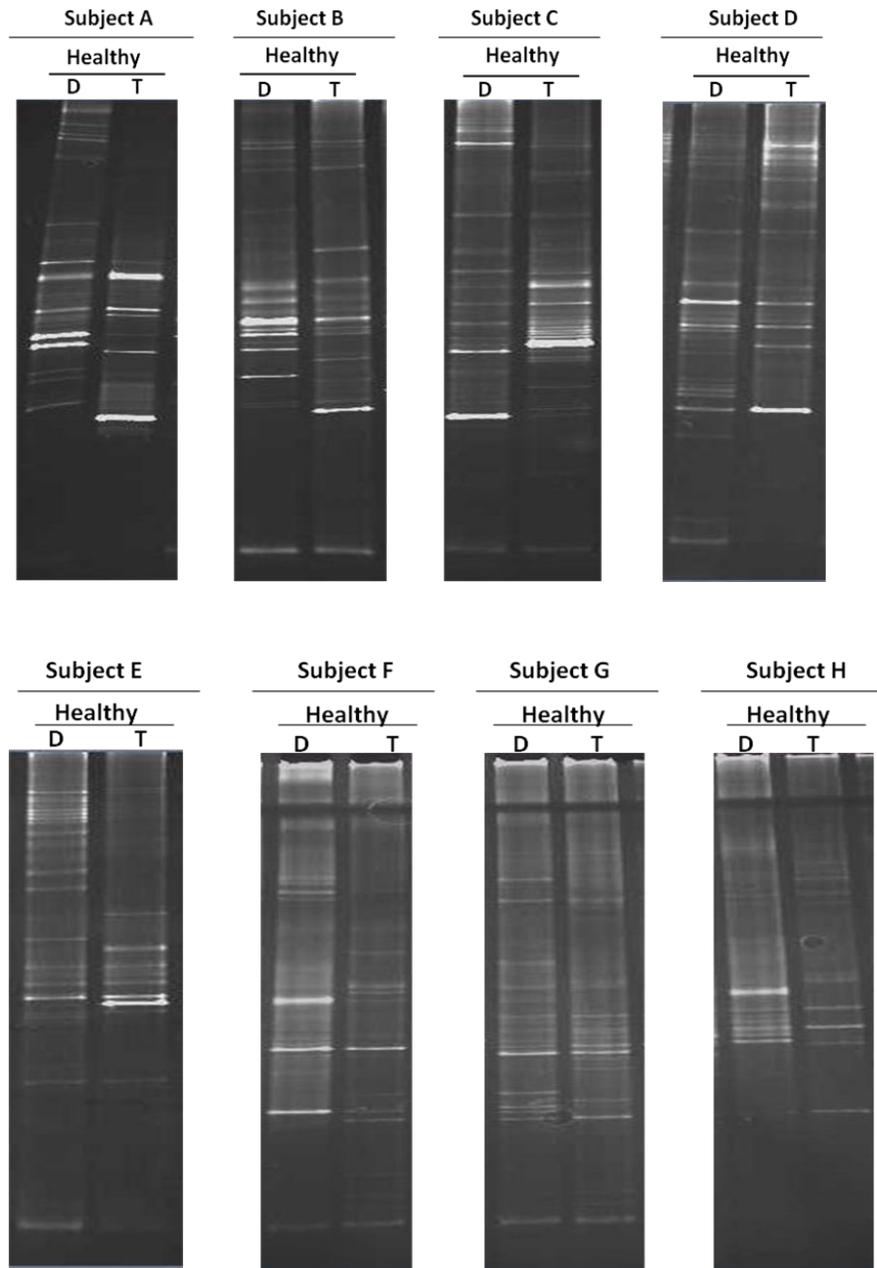
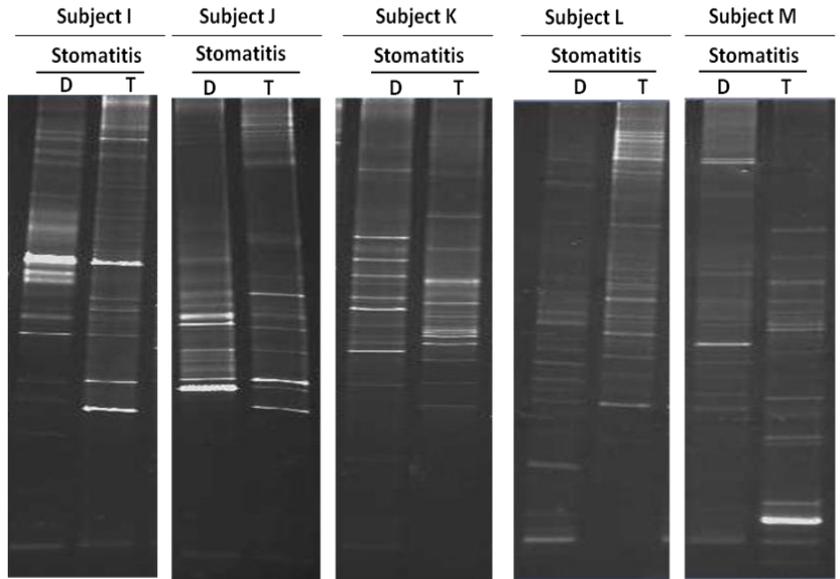


Fig 3.2 CLSM images of dual species biofilms consisting of a halitosis related bacterial species together with *C. albicans*. The left side is single species, the middle is dual species, and the right side is the magnified image from the picture in the middle. Four random fields of view were examined for each sample. (A: *F. nucleatum*, B: *T. forsythia*, C: *V. atypica*, D: *K. pneumonia*, E: *S. gordonii*, F: *C. albicans* )

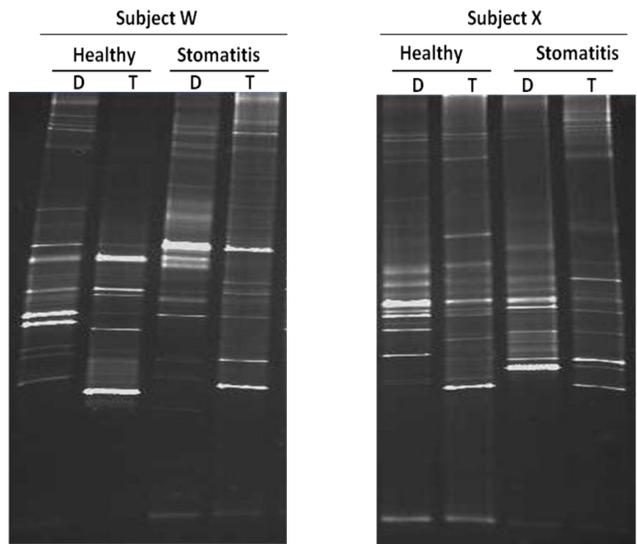
A



**B**



**C**



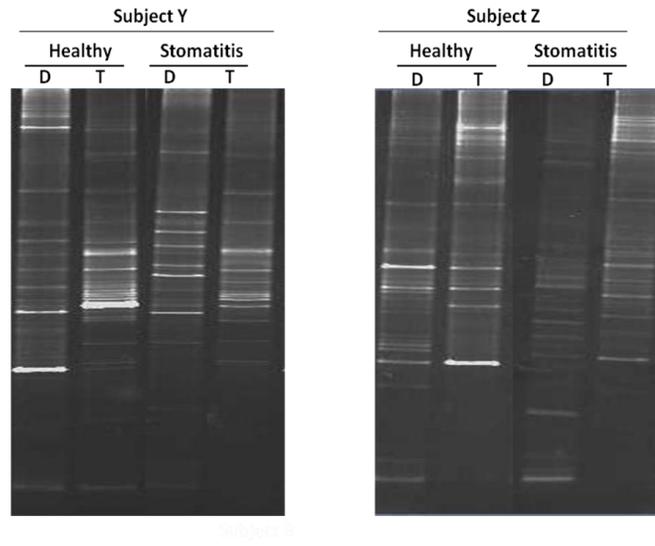


Fig 3.3 DGGE analysis of denture plaque and tooth plaque with and without denture stomatitis. Denture plaque (D) and tooth plaque (T) profile in healthy subjects (A), in denture stomatitis patients (B). Within the same subjects, denture plaque and tooth plaque profile in healthy sites and stomatitis sites (C).

Healthy subjects	1	2	3	4	5	6	7	8	9	10	Ratio
Fungi	1	0	0	1	0	0	1	1	0	1	50%
F.n.	1	0	1	1	0	1	0	1	1	0	60%
T.f.	0	0	1	0	0	1	0	0	1	0	30%
V.a.	0	0	1	1	0	1	0	0	0	0	30%
K.p.	0	0	0	0	0	0	0	0	0	0	0%

Denture stomatitis subjects	1	2	3	4	5	6	7	8	9	10	Ratio
Fungi	1	1	1	1	1	1	1	1	0	1	90%
F.n.	0	0	0	0	1	0	1	1	0	1	40%
T.f.	0	1	0	1	0	1	1	0	0	0	40%
V.a.	0	0	0	0	1	0	0	0	0	0	10%
K.p.	0	0	0	0	0	0	0	0	1	1	20%

Table. 3.1 The prevalence of fungi and halitosis-related species in denture plaque. Samples collected from 10 subjects with and without denture stomatitis were tested by PCR.

## **Chapter IV**

### **Summary and future directions**

The main accomplishment of this dissertation is the establishment of denture biofilm models of the etiological agents or implicated pathogens of both denture-related halitosis and stomatitis. These biofilm model systems were further proven to be useful in evaluating the efficacy of antimicrobial denture products. Furthermore, we demonstrated the direct cell-cell interaction between *C. albicans* and halitosis-related oral bacterial species under *in vitro* conditions, thus providing physical evidence for the possible association of denture-related halitosis and stomatitis. We can envision several future research directions based on the current study. 1) The established denture biofilm models could allow us to further investigate the pathogenesis of denture related stomatitis and halitosis. 2) The observed direct physical interaction between *C. albicans* and halitosis-related strains warrants further *in vivo* investigation using larger clinical samples size and 454 pyrosequencing to test the proposed association of these two denture-related clinical conditions. 3) Increasing evidence suggests that physical binding between two microbial species could result in different gene expression pattern which could affect their behavior within a multispecies community. It would be interesting to study the possible structural as well as functional changes following the co-adherence between *C. albicans* and halitosis-related species, and to investigate the potential effect of these inter-kingdom interactions on their pathogenesis.