Development of *In vitro* Multi-Species Biofilms to Explore Strategies to Prevent Biofilm-Associated Oral Diseases

by

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DEDICATION

I dedicate this work to my grandparents in Korea and
to my wife Danah

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Abstract

More than 80% of infectious diseases are caused by microbial biofilms. In the oral cavity, biofilms are composed of hundreds of distinct bacteria. The accumulation of oral biofilms can lead to caries and periodontal disease. From a public health standpoint, the cost of treatment for oral diseases remain high, at over $100 billion dollars per year in US alone. The primary aim of this project was to evaluate new anti-biofilm agents using novel *in vitro* biofilm model systems. In addition, the biology of oral biofilms and their relationship to oral diseases are discussed in the context of their development in laboratory biofilm model systems.

Nisin is a unique bacteriocin generated by a group of *Lactococcus* and *Streptococcus* species. The objective of the first part of the work was to determine if nisin exhibited broad-spectrum antimicrobial effects against oral bacteria. In addition, the presented work showed that nisin inhibited the growth and maintenance of saliva derived biofilms developed in static and controlled-flow biofilm model systems. Even at higher concentrations, nisin did not exhibit cytotoxic effects on human oral cells.

L-arginine is an amino acid that is present in low levels in the oral cavity. At lower concentrations, L-arginine is a nutrient source for arginolytic bacteria. The objective of the second part of the work was to explore the short-term effects of high concentrations of L-arginine on oral biofilms. A modified Swinnex model system was used to develop the multi-species oral biofilms and the FlowCam® was used to monitor the biofilm

dispersion. In a dose-dependent manner, L-arginine treatment resulted in biofilm destabilization through enhanced biofilm dispersion. Based on community analyses of the biofilms and the dispersed cells, L-arginine did not result a significant shift in the community compared to the water-treated controls.

In summary, the work presented in this thesis demonstrated that complex biofilms, that contain species present in the human oral cavity, can be developed using three different *in vitro* biofilm model systems. In addition, we showed that nisin and L-arginine has high potential to disrupt the *in vitro* developed multi-species biofilms and these compounds have potential as novel anti-biofilm agents.

Chapter I

Introduction

Oral Biofilm and Disease

The oral cavity is a nutrient rich habitat for complex community of microorganisms. Indeed, the mouth offers numerous sites for bacteria to adhere and prosper as oral biofilms, which is also known as the dental plaque (Marsh 2004). A biofilm is defined as a matrix enclosed bacterial population(s) existing together on a living and non-living surfaces (Donlan and Costerton, 2002; Flemming and Wingender, 2010). As a sessile structure, the microbial cells of the biofilm are irreversibly attached to each other and to the bound substratum. In addition, the biofilm structures self-produce exopolymeric substances (EPS), which have numerous functions to protect and maintain the biofilm architecture (Flemming and Wingender, 2010). Bacterial populations embedded in the matrix function and live together, and communicate with one another through a process known as quorum sensing (QS) (Zhang and Dong, 2004).

The Microbial Community of Oral Biofilm

Oral biofilms represent the second most diverse microbiome found in the human body next to the gut (Kilian *et al*., 2016). The oral biofilm community consists of more than 700 different bacterial species, where distinct groups of bacterial communities are present in different locations (ie. supragingival, subgingival, tongue; Fig.I.1) (Palmer 2014). The major phyla groups that represent the oral microbiome include: *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Spirochaetes*, *SR1*, *Synergistes*, *Tenericutes* and *TM7* (Bik *et al*., 2010; Dewhirst *et al*., 2010). The major genera include: *Streptococcus*, *Corynebacterium*, *Neisseria, Haemophilus, Actinomyces, Fusobacterium, Rothia, Veillonella, Granulicatella, Prevotella, Porphyromonas, Capnocytophaga, Leptotrichia, Selenomonas, Treponema and Gemella* (Keijser *et al*., 2008; Zaura *et al*., 2009). However, depending on the oral habitat, sequencing platforms (e.g., Illumina, PGM, 454) and the type of analysis performed (ie. 16S rRNA, whole genome sequencing), the presence and abundance of these bacterial groups can vary and are not limited to the phyla and genera mentioned above (Palmer 2014). Furthermore, based on findings from current sequencing technologies, there are still numerous unidentified bacterial taxa (Palmer 2014). However, based on the current knowledge of the oral biofilm ecology, it is clear that the biofilm communities associated with health and disease are significantly different from each other. For example, subgingival biofilms collected from subjects diagnosed with periodontal disease have higher biomass and diversity (Abusleme *et al*., 2013). In addition, periodontal diseased-associated biofilms are known to have higher proportions

of bacterial species such as *Porphyromonas gingivalis* and *Treponema denticola,* which are known periodontal pathogens (Dewhirst *et al*., 2010; Wade 2013).

Biofilm-Associated Oral Diseases

Two of the most well described oral biofilm-associated diseases are caries and periodontal disease (Marsh 2006). Dental caries is characterized by the local destruction of enamel and dentin surfaces by acidic products produced by bacterial fermentation of dietary carbohydrates (Matsui and Cvitkovitch, 2010). Periodontal disease is a complex inflammatory disorder of the periodontal tissue, where chronic persistence can lead to soft and hard tissue destruction (Philstrom *et al*., 2005). In both diseases, biofilms play a major role in initiation and progression of the disease processes. In the US alone, over $100 billion dollars are spent each year on dental services, where majority of these treatments are likely due to caries and periodontal disease (Chronic Disease Prevention and Health Promotion, 2017). Furthermore, oral diseases are a global health burden, where it has a major impact on people's daily lives and well-being (Petersen *et al*., 2005). Thus, to lower the substantial public health burden generated by oral biofilms, the development of novel strategies to prevent and better treat biofilm-associated oral diseases are in need of further research. In addition, because of the anatomic closeness of the oral biofilms to the blood stream, an oral infection can cause biofilm-associated systemic diseases (Li *et al*., 2000). The spread of pathogenic oral bacteria and their components can trigger the body's immune system

and have been linked to systemic diseases such as diabetes, pneumonia and cardiovascular diseases (Li *et al*., 2000).

Strategies of Targeting Biofilms

Infectious diseases impose a major health burden to our society. Millions of people are affected by bacterial-related infectious disease, where more than 80% of human infections are caused by biofilms (Costerton *et al*., 1999). In order to prevent and treat biofilm-associated oral diseases, several strategies can be implemented. From a clinician's perspective, diet (ie. reduction of frequent sugar and fermentable carbohydrate intake) and proper oral hygiene (ie. brushing and flossing) are critical for controlling oral biofilms. Adequate biofilm control through brushing and flossing is essential to avoid over-accumulation of biofilms, which can promote inflammation and calculus development. The public health burden of oral biofilms continues, as most individuals fail to practice proper oral hygiene techniques (Morris *et al*., 2001). The usual mechanical biofilm control procedures primarily focuses on the enamel surfaces. However, the enamel surfaces represent only about 25% of all of the biological surfaces present in the oral cavity for biofilm adhesion (Kerr *et al*., 1991). The most difficult areas in the oral cavity to keep biofilm-free are the interproximal areas between teeth and at the junction of the soft and hard tissues, and below the gums. Hence, effective chemical biofilm control strategies may help better control the biofilm accumulation on both hard and soft tissues to improve oral health.

In the following sub-sections of Chapter 1, the focus of discussion will be on novel strategies to eradicate oral biofilms. Specifically, the focus will be on the natural and synthetic antimicrobial/biofilm agents that have been reported to have therapeutic potential against oral biofilms.

Biofilm Inactivating Agents

In commercial oral health care products, such as mouthwash and toothpastes, a variety of biofilm inactivating agents can be present. For example, in mouthwashes, antimicrobial agents such as cetylpyridinium chloride (CPC) and chlorhexidine gluconate (CHX) are commonly found (Mandel 1994; Asadoorian and Williams, 2005). When considering these two agents, dental professionals prescribe CHX solution for control of pathogenic biofilm infection (Mandel 1994). CHX has potent antimicrobial activity and has good substantivity, where about 30% of the solution is retained in the oral cavity following usage (Cummins 1992). CPC is a quaternary ammonium compound, and is also widely formulated into anti-biofilm mouthrinses (Marsh 2010). CPC exhibits strong antimicrobial activity at low concentrations, since 0.075%-CPC containing mouthrinses were shown to be effective against multi-species oral biofilms (Rao *et al*., 2011). However, unlike CHX, CPC loses its activity when adsorbed to a surface and is less susbstantive compared to other molecules (Scheie and Petersen, 2003). Like many of the other antimicrobial agents and detergents, CPC and CHX are both cationic molecules, which have a membrane inactivating function by interacting with the negatively charged bacterial membrane (Smith *et al*., 1991; Rao *et al*., 2011).

Inhibiting Bacterial Adhesion: Cell to Cell and Cell to Surface

Bacterial adhesion to surfaces and/or to other microorganisms (autoaggregation and coaggregation) can be targeted to inhibit the formation of biofilms. Autoaggregation is when a single species aggregates on their own, whereas, coaggregation is defined as the highly specific aggregation of two or more distinct bacterial species (Katharios-Lanwermeyer *et al*., 2014). From early studies of oral biofilm development, it was demonstrated that the primary bacterial adhesion to a pellicle-coated enamel surface is a highly specific process (Liljemark and Bloomquist, 1996). Gram-positive oral streptococci such as *Streptococcus gordonii* initiate primary colonization through specific interactions mediated by bacterial adhesins (Jakubovics *et al.*, 2014). The successional colonization of oral bacteria require partner specific coaggregation interactions, which has been validated and recognized as a critical process for the development and maturation of oral biofilms (Kolenbrander and London, 1993; Kolenbrander *et al*., 2006).

Molecules extracted from the natural environment have gained considerable attention in dentistry as anti-biofilm agents. For example, a group of plant extracts were tested *in vitro*, *ex vivo* and *in situ* to demonstrate their effectiveness against oral biofilms (Karygianni *et al*., 2016). A high molecular weight material (NDM) from cranberry juice has been shown to inhibit intergeneric coaggregation of oral bacteria (Weiss *et al*., 2002). Furthermore, NDM was shown to reduce the adherence of oral *streptococci* strains to saliva-coated hydroxyapatite beads (Yamanaka *et al*., 2004). Lactose and related sugars were demonstrated to inhibit coaggregation between *Fusobacterium*

nucleatum and *P. gingivalis* (Kolenbrander *et al*., 1989). Dealcoholized red wine components were shown to have anti-adhesion and anti-biofilm properties against *Streptococcus mutans* (Daglia *et al*., 2010). Investigators have hypothesized that the anti-adhesive properties of natural molecules could be partly due to the action of organic acids (van Loveren *et al*., 2012). CPC and CHX can also directly inhibit cell-to-cell coaggregation in addition to the biofilm inactivating mechanisms (Smith *et al*., 1991). However, it is important to note that not all of the anti-biofilm agents can inhibit coaggregation, since coaggregation is a highly specific, and strong adhesin-receptor interaction (Rickard *et al*., 2003). Furthermore, the coaggregation interactions among oral bacteria are extremely broad and diverse, which may account for the success and failures of different types of anti-biofilm compounds (Rickard *et al*., 2003).

Surface anti-adhesion agents for the prevention of biofilm formation have numerous applications not limited to dentistry. Bacterial biofilms present major problems for implant-related medical devices, such as the hip/knee implants and dental implants (Donlan, 2001; Costerton *et al*., 2005). During the initial attachment and the primary colonization phase of biofilm formation, planktonic are generally more sensitive to antimicrobials and to immune cell responses than those present in biofilms (Salwiczek *et al*., 2013). Hence, antimicrobial coatings have shown promising results in reducing biofilm formation (Hasan *et al*., 2013; Salwiczek *et al*., 2013). Such anti-biofilm surface coating materials include natural and synthetic antimicrobial peptides (AMPs), polymers and polysaccharides, metal ions and other anti-adhesive molecules (Salwiczek *et al*., 2013). For example, coating the surface with a naturally occurring polysaccharide chitosan was shown to be effective in inhibiting the biofilm formation of bacteria and

fungi (Carlson *et al*., 2008). Nisin has been used to create a low-fouling antimicrobial coating to prevent biofilm adhesion and formation (Vreuls *et al*., 2010). For dental implants, others have shown that altering the surface roughness and coating the surface with antimicrobial agents promoted better osteointegration while limiting the biofilm formation (Abdulkareem *et al*., 2015). Thus, the modification of the biofilm-attaching surface is another strategy to prevent biofilm formation.

Targeting the Biofilm Glue – EPS

As previously mentioned, the biofilm matrix, which is a complex mixture of extracellular polymeric substances (EPS), plays critical roles for the maintenance of biofilm structure (Flemming and Wingender, 2010). The EPS function as a structural scaffold for the biofilm and is responsible for surface adhesion and for cohesion within the biofilm structure (Flemming and Wingender, 2010). Hence, an anti-biofilm strategy where the EPS is targeted can promote biofilm dispersion and eradication (Kaplan 2010). Some examples of the matrix degrading enzymes that promote biofilm dispersal include glycosidases, proteases, deoxyribonucleases and DNases (Kaplan 2010; Jakubovics *et al*., 2013). Particularly, dispersin B is a well-known matrix-degrading enzyme produced by a periodontal pathogen, *Aggregatibacter actinomycetemcomitans* (Kaplan *et al*., 2003). *A. actinomycetemcomitans* is not a motile species, but it can release itself from the intact biofilm through using dispersin B (Manuel *et al*., 2007). Other notable enzymes associated with biofilm matrix disruption include mutanase, glucanase and amylogucosidase-glucose oxidase (Marsh 2010). In a complex microbial

community like the oral biofilm, it is likely that species interactions such as competition, mutualism, predation or parasitism resulted in the evolution of certain species to possess innate dispersal mechanisms (Kaplan 2010).

Most, if not all, of the matrix-degrading enzymes mentioned above mediate biofilm dispersion. For biofilm dispersion, there are at least distinct mechanisms that have been reported, which are erosion, sloughing and seeding (Kaplan 2014). “Erosion” describes a process where slow continuous release of single cells or small clusters of cells from a biofilm occur during the early biofilm development period. “Sloughing” is the sudden detachment of large portions of the biofilm structure, which occur in the later stages of biofilm formation (Lappin-Scott and Bass, 2001; Stoodley et al., 2001). Both erosion and sloughing processes can occur in an active or passive manner, where the active dispersion is initiated by the bacteria themselves, and the passive dispersion is through the external forces (ie. shear force, chemical agents, human intervention) (Kaplan 2010). Lastly, “dispersion” through seeding refers to the central hollowing of the biofilm structure through the release of single cells or small clusters of cells (Ma *et al*., 2009). The seeding mechanism occurs only as an active dispersion process. For example, in this dissertation project, we hypothesized that L-arginine causes biofilm destabilization through the action of biofilm dispersion. The work presented here indicates that high concentrations of L-arginine promotes sloughing of the *in vitro* oral biofilms. In Chapter V, the potential role of high concentrations of L-arginine in enhancing biofilm destabilization will be discussed in detail.

Limitations of Current Strategies

Despite the tremendous advancements achieved in the development of anti-biofilm agents, limitations still exist within the current strategies. For example, in some of the most well-known antimicrobial agents for oral biofilm control, unwanted side effects have been reported. Prolonged usage of CHX causes browning of teeth and oral mucosa, promotes calculus formation, alter taste sensation and cause soft tissue ulcerations (Berchier *et al*., 2010). Mouthrinses containing strong antimicrobial agents such as CHX, CPC and alcohol can cause host cell damage in addition to their anti-biofilm effects (Carlin *et al*., 2012). Although *in vitro* studies have demonstrated great therapeutic potential for anti-adhesion coating materials and matrix degrading enzymes, the *in vivo* efficacy still remain elusive considering the complex interactions between the drug with the bacteria and the host cell-derived proteins (Chen *et al*., 2013). Furthermore, the translation of *in vitro* results to the *in vivo* conditions, and to clinical trials are lacking with anti-biofilm agents. This drawback is not limited to the field of biofilm research, but is also relevant to other scientific fields such as cancer biology (Mak *et al*., 2014). For example, a novel antimicrobial/biofilm agent may effectively inhibit biofilm formation and cause biofilm dispersion *in vitro*, but could generate a non-specific inflammatory and allergic response *in vivo* to limit their therapeutic potential. Thus, considering that there are still many limitations in the current biofilm control strategies, further research is needed to identify new anti-biofilm agents and model systems to exploit better approaches for biofilm control.

Overall Aims

The main objective of this dissertation work was to develop and apply novel *in vitro* biofilm model systems to study the oral biofilm physiology and strategies to inhibit biofilm formation and maintenance. Within this main objective, we utilized three different biofilm model systems, i) static biofilm model, ii) BioFlux microfluidic system, and iii) the modified Swinnex biofilm model system. Furthermore, we investigated the therapeutic potential of two novel anti-biofilm agents, nisin and L-arginine. Chapter I outlines the background information of oral biofilm and disease. In Chapter II, old and new *in vitro* biofilm model systems for oral biofilm studies are discussed. Current tools and technologies for biofilm analyses and limitations of model systems are further discussed. In Chapter III, the results obtained for the anti-biofilm activity of nisin on saliva derived oral biofilms are discussed. In Chapter IV, a comprehensive review of biomedical applications of nisin is discussed. In Chapter V, the effect of L-arginine on multi-species oral biofilms is discussed. Lastly, conclusions and future directions are discussed in the final chapter.

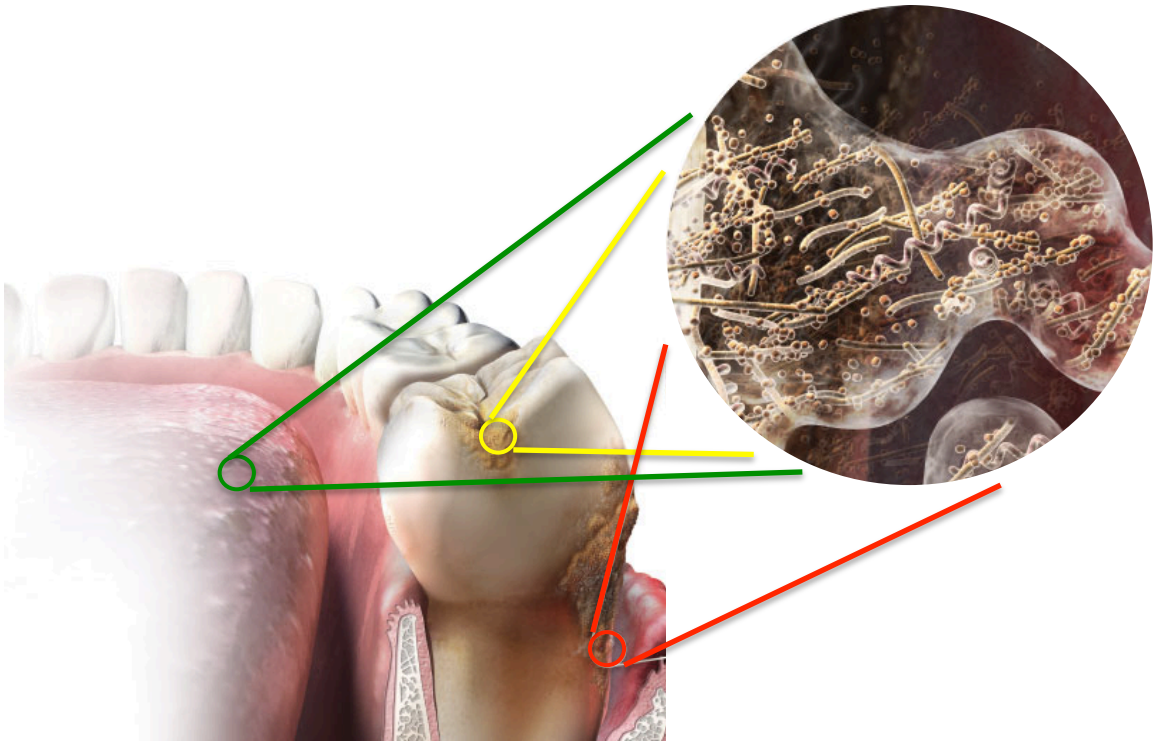


Figure I.1. Multi-Species Oral Biofilm. Dental plaque and biofilms that form on the tongue are examples of oral biofilms. Depending upon the age, an oral biofilm can be composed of hundreds of different bacterial species and forms on both soft and hard tissues around the oral cavity. In addition to biofilm age, biofilm community composition varies depending on the residing habitat. Three distinct locations for oral biofilm residence are shown in the figure as an example, i) the enamel surface (yellow), ii) the subgingival region (red), and iii) the tongue surface (green). Image adopted and modified from https://www.sciencenews.org/article/gum-disease-opens-body-host-infections.

Chapter II

In vitro Models to Study Oral Biofilms

The development and application of novel systems are critical for understanding biofilm biology (Hamilton *et al*., 2003; Coenye and Nelis 2010). The evolution of *in vitro* biofilm model systems has resulted in significant advancements in capabilities over the past few decades. For example, biofilms can be reconstructed in 3D using the images captured from the high-resolution microscopes with sophisticated imaging softwares. In Chapter II, the focus of discussions will be on *in vitro* biofilm model systems. The discussions will focus on the micro-scale models (systems that use small volume of media) that have been used to develop and evaluate multi-species biofilms. In addition, the application of both closed (ie. static) and open (ie. flowing, microfluidic) model systems will be discussed. Furthermore, the in vitro model biofilm systems used for the dissertation project will be discussed.

Static Microtiter Plate-based Biofilm Models

Arguably the most commonly used biofilm model system are is the Microtiter plate (MTP) -based system (Coenye and Nelis 2010; Azeredo *et al*., 2017). A MTP

biofilm model usually contains 6, 12, 24 or up to 96-wells per plate. The biofilms typically grow on the bottom of the plate well, or alternatively, a coupon (ie. hydroxyapatite discs) can be inserted into the well as the biofilm substratum. Usually, the biofilm substratum is plastic (ie. polystyrene) or glass (high quality optical glass) . However, the substratum can be modified as needed with different coating materials (Macia *et al*., 2014). The MTP biofilm model is a closed batch reactor-like system, meaning there is no exchange of media flowing in or out during the biofilm development period (Heersink and Goeres, 2003). Hence, the growth media must be frequently changed to avoid biofilm starvation and unwanted cell death.

Microtiter plates are available from numerous commercial vendors and have been widely accepted as a robust *in vitro* biofilm model system. The advantages include 1) simple and easy setup for experiments, 2) inexpensive cost relative to the experimental output, and 3) high throughput results for screening of anti-biofilm agents (Niu and Gilbert, 2004). An example of a classic MTP-based system is the Calgary biofilm device, which utilizes a 96-well plate design with pegs attached to the lid for biofilm formation (Fig. II.1) (Azeredo *et al*., 2017).

Glass Bottomed Static Biofilm Model

Both glass and plastic based microtiter plates are widely used for biofilm experiments (Merritt *et al*., 2005). Unlike the most assays using the continuous flow systems, the static MTP-based biofilm models do not require complex setups or specialized equipment. Single, dual, mixed and more complex multi-species oral biofilm

types have been studied using both glass and plastic based MTP-based models (Darrene and Cecile, 2016). The main advantage of using the glass based microtiter plates over the plastic is the ability to capture the high-resolution microscopy images directly from the biofilm substratum without altering the biofilm architecture. For the dissertation work outlined in Chapter III, twenty-four well glass bottomed sensoplates were used for screening and assessing short and long-term effects of nisin on oral biofilms (Greiner Bio-One, NC, USA; Fig. II.2).

Open Flow Microfluidic Biofilm Models

In contrast to a static, 'closed' biofilm model system, an 'open' system has continuous flowing of media during biofilm formation (Coenye and Nelis, 2010). A major advantage of an open system compared to a static system is the ability to precisely control the environmental parameters such as shear force (Lebeaux *et al*., 2013). Two well-known first generation of open flow biofilm model systems are the Modified Robbins device (MRD) and the Centers for Disease Control (CDC) biofilm reactor (Azeredo *et al*., 2016). Despite their proven reliability, these older models tend to be bulky, expensive and require a high volume of media per run. These features make them less *suitable* for conducting experiments with numerous strains and under different environmental conditions (Coenye and Nelis, 2010; Azeredo *et al*., 2016). In addition, the risk of contamination and introduction of artifacts are a major concern, since the inserted coupons must be removed for examination of developed biofilms (Azeredo *et al*., 2016). The MRD and the CDC biofilm reactors are still used today, but can be

considered outdated and cumbersome compared to the newer, more refined microfluidic devices (Nickel *et al*., 1985; Donlan *et al*., 2004) (Fig. II.3 A and B).

Swinnex Biofilm Model

The Swinnex biofilm model is a unique 'open' biofilm model system adapted from an earlier biofilm model called the 'baby machine' (Helmstetter and Cummings, 1963). The baby machine was developed to study the growth rate of single species, thin homogeneous biofilms and it is called the baby machine because it can be used to study the daughter cells released from the biofilms (Helmstetter and Cummings, 1963). However, due to the common technical difficulties associated with frequent leakage during the model run, contamination, and burst of filter membranes, a more practical the Swinnex biofilm model was developed (Gander and Gilbert, 1997). The Swinnex filter holder is commonly used for filtration of liquids for bacteria and liquid particles. Gander and Gilbert utilized a cellulose nitrate membrane as the biofilm substratum in the Swinnex filter holder to grow *Escherichia coli* biofilms and study their susceptibility to antibiotics (Gander and Gilbert, 1997). For the current project, the original Swinnex model was modified to study the biofilm biology of multi-species oral biofilms (Chapter V).

The Swinnex model is an ideal *in vitro* system if the biofilm dispersion is of interest (McBain, 2009). Similar to the baby machine model, the biofilm on the membrane can be assessed and in addition to the released daughter cells. The advantages of this model includes 1) the ability to monitor the different mechanisms of

biofilm dispersion processes (ie. sloughing) 2) all parts are reusable through sterilization, resulting in low cost for the setup, 3) the model has a micro-scale design, requiring small volume of media per sample, and 4) is versatile, since the biofilm and the detached daughter cells can be analyzed using microscopy and FlowCam® techniques. Some limitations are 1) short-term growth may not generate an adequate biomass for physical and molecular analyses and 2) a standardized protocol is absent since it is not a widely used model system.

BioFlux Microfluidic Biofilm Model

Microfluidic devices are modern 'open' systems that require only a small volume of media to conduct biofilm experiments. The material used to build a biofilm substratum for a microfluidic device includes glass, plastic and organic polymers (ie. poly-dimethylsiloxane) (McDonald *et al*., 2000; Becker and Gartner, 2008; Iliescu *et al*., 2012). An example of a microfluidic device applicable for studying multi-species oral biofilm is the BioFlux device (Benoit *et al*., 2010). The BioFlux system is a unique multi-channeled microfluidic device distributed by a company called Fluxion Biosciences (San Francisco, CA, USA) (Fig. II.4). Currently, Fluxion offers three different BioFlux systems (Bioflux 200, BioFlux 1000, BioFlux EZ) in combination with 6-, 24- and 48-well plate designs compatible with their machineries (https://bioflux.fluxionbio.com/). The cost of the entire setup can be high, however, if the plates are re-used and the system is frequently used; the cost in relation to the outcomes are considered reasonable.

For this dissertation project, the BioFlux 200 system with the 48-well BioFlux plate was used to evaluate the anti-biofilm properties of nisin on saliva-derived multi-species oral biofilms (Shin *et al*., 2015; Fig. II.4). The advantages of the BioFlux system includes 1) the high throughput capability, up to 24 samples per run using the 48-well plate design, 2) requiring less than 1 ml of media per sample for overnight biofilm development, 3) precise control of temperature and shear force in a confined microfluidic environment, and 4) the ability to directly evaluate spatial geometry of biofilms, down to a single cell level through high resolution microscopy (Benoit *et al*., 2010; Nance *et al*., 2013; Samarian *et al*,. 2014). Limitations of the BioFlux system includes 1) high initial cost for the system and tools, 2) the system itself is still new and needs further validation through cross-study comparisons, and 3) the achievement of a conventional anaerobic environment using mixed gases and nitrogen have not been validated to study oral anaerobic pathogens and subgingival biofilm communities.

Tools for Biofilm Analysis

Quantification of Biofilm Structure

The quantification of biofilm properties allows a deeper understanding of the spatial organization of the biofilm architecture, their heterogeneities and patterns of biofilm disruption as a result of an anti-biofilm or biofilm-modifying agent. There are number of different ways to quantify a biofilm structure for in depth analyses. Methods to assess the biofilm's physical properties include the traditional microbiological methods (ie. counting colony formation units), molecular methods (ie. quantitative polymerase chain reaction), physical methods (ie. by measuring the dry and wet weight), chemical methods (ie. dye staining; crystal violet) and microscopy methods (ie. confocal microscopy + fluorescent staining, electron and atomic force microscopy). In this sub-section of Chapter II, two of the most commonly used methods to quantify the biofilm structures will be discussed: 1) MTP-based methods (ie. Crystal violet staining method), and 2) Confocal microscopy method (ie. Fluorescent staining and 3D analysis). In addition, the newly developed FlowCam® approach will be discussed as a novel biofilm quantification method.

MTP-based Staining Method

For the biofilm staining methods, chemical dyes, fluorescent antibodies and nucleic acid stains are commonly used to quantify the biofilm structures (Azeredo *et al*., 2017). Crystal violet (CV) staining method is the most frequently used method to quantify the biofilms in the MTP-based systems (Azeredo *et al*., 2017). The CV stains both live and dead cells as well as the matrix components, which makes it suitable for quantifying the entire biofilm structure (Pitts *et al*., 2003). The advantages of this method include 1) versatility, as this method is applicable for most bacterial strains, 2) biofilms are stained directly in the well without removal from the substratum, and 3) the assay is quick, so multiple test conditions can be assessed simultaneously. Limitations of the CV staining method includes 1) the assessment of the biofilm biomass is a crude estimate and can be highly subjective, 2) the quantity of live and damaged/dead cells cannot be distinguished, 3) the measured biomass may contain settled planktonic bacteria, and 4) a standardized protocol is still lacking and reproducibility is a major concern (Stepanovic *et al*,. 2007; Peeters *et al*., 2008).

A plate reader can be utilized with CV and other staining methods mentioned above to quantify the biofilms formed over time in a high throughput manner (O'Toole 2011). For this dissertation project, a green fluorescent nucleic acid stain, Syto-9 (Invitrogen, Carlsbad, CA, USA) was used to stain the oral biofilms and quantified using a plate reader (Shin *et al*., 2015; Chapter III).

Confocal Microscopy Method

Microscopy methods in conjunction with computer imaging software such as IMARIS (Bitplane AG), COMSTAT and ImageJ have become popular for biofilm analyses (Heydorn *et al*., 2000; Abramoff *et al*., 2004). Microscopy methods are useful, since qualitative and quantitative types of analysis can be performed. As a base-line technique, light microscopy can be used to detect the presence of a biofilm structure. In addition, when combined with classic staining techniques such as the Gram staining, it can be used to distinguish between the Gram-positive from Gram-negative species. Light microscopy methods are very practical, inexpensive and reliable methods to obtain some general information of the biofilm architecture. However, due to the limited magnification and resolution, detailed quantification analyses are much better with a high resolution microscopes such as the confocal laser scanning microscope (CLSM). For this dissertation project, Leica CLSM was used to quantify the biofilms (SPE, Leica, USA).

The CLSM together with fluorescent staining (ie. syto-9 and propidium iodide) allows the detailed assessment of biofilm properties such as biofilm biovolume, biomass, thickness, roughness and viability (Shin *et al*., 2015). The CLSM serves as a powerful tool for evaluation of the biofilm spatial architecture, distribution and organization of the contained species (ie. with fluorescent in situ hybridization techniques) and the effects of antimicrobials on morphology and cell viability of biofilm-contained cells (Valm *et al*., 2011). Other advantages include high resolution imaging without tampering the biofilm sample, which is a common problem with the ultrafine

electron microscopy methods. Crucially, the biofilm can be visualized as a three-dimensional Z-stack structure for 3D analyses (Schlafer and Meyer, 2016).

The CLSM analysis of biofilms involves several critical factors. Fluorescence staining or hybridization with fluorophores are required. For accurate assessment of the biofilm structure, a well-defined protocol is needed, since inadequate staining can lead to false-positive results due to the background noise and auto-fluorescence. Furthermore, with thicker and dense biofilms, staining protocol must be optimized to allow the stain to penetrate throughout the entire biofilm thickness.

FlowCam® Method – For Coaggregation and Biofilm Dispersion

This sub-section on the novel method to assess bacterial coaggregation and biofilm dispersion is adapted from a published manuscript and a manuscript in preparation (Levin-Sparenberg *et al*., 2016; *Shin et al*., 2017b; Chapter V).

Recently, a new method to evaluate the partner-specific bacterial coaggregation was developed using a real-time particle-analyzing device called the FlowCam®. The FlowCam® device allows the determination of the rates of coaggregation and the size of aggregates formed (Levin-Sparenberg *et al*., 2016). In comparison to the original semi-quantitative visual coaggregation assay method, the FlowCam® approach further validates and quantifies the extent of aggregation and coaggregation interactions (Cisar *et al*., 1979). In the manuscript 'High-throughput quantitative method for assessing coaggregation among oral bacterial species', the authors demonstrated that the

combinatory application of the FlowCam® and confocal microscopy techniques are synergistic in studying coaggregation.

Another novel application of the FlowCam® approach is to evaluate biofilm dispersion. When used alone, the microscopy techniques mentioned above, including CLSM are limited to an end-point assessment of the biofilm. To evaluate the real-time effects of anti-biofilm agents on biofilm dispersion, and to quantify the dispersed biofilm cells, the FlowCam® approach is suitable. For the dissertation work outlined in Chapter V, the Swinnex model was combined with the FlowCam® approach to evaluate the biofilm dispersion effects of L-aginine and different ant-biofilm agents. In Chapter V, a detailed protocol of using the FlowCam® device in combination with the Swinnex biofilm model system is discussed.

Biofilm Community Composition

One of the primary objectives of this dissertation was to develop oral biofilms from our model systems that is compositionally representative (with regard to bacterial composition) of *in vivo* plaque-like communities. Most of prior studies have been using regular bacterial media or artificial media to grow and study oral biofilms (Bjorklund *et al*., 2011; Tian *et al*., 2010; Wong and Sissons, 2001). The community composition of an *in vitro* biofilm can vary based on several factors, which include 1) the inoculum and the growth media, 2) static versus flowing system, 3) duration of biofilm growth period, and 4) environmental conditions. Hence, for the current project, pooled human saliva was used as the biofilm inoculum and the growth supporting media.

Following growth, the biofilms can be harvested for the community composition analysis. For identifying who is present in the multi-species biofilm, several methods are available. The most popular method is using the bacterial 16S rRNA, which is a ribosomal RNA component of the 30S small subunit of the prokaryotic ribosome (Janda and Abbott, 2007). The use of the 16S rRNA genome sequences to study bacterial phylogeny and taxonomy is the current gold standard for identifying bacterial species composition in a multi-species community. The 16S gene is present in all prokaryotes and the function of the 16S rRNA is highly conserved (Janda and Abbott, 2007). Furthermore, the gene is large enough to be analyzed through bioinformatics. For the microbiome sequencing conducted in Chapter V, 16S rRNA next generation sequencing was conducted using the Personal Genome Machine (PGM) Ion-Torrent platform. There are several other sequencing platforms available, however the sequencing platforms such as the PGM Ion-Torrent and Illumina Miseq both provide reasonably high quality resolution (Liu *et al*., 2012). The specific method used for sequencing analyses is further discussed in Chapter V.

Selection of Model system

Ideally, an *in vitro* biofilm model system should mimic the *in vivo* biofilm environment as closely as possible. In addition, for evaluation of therapeutic strategies, a model system should help predict the performance of anti-biofilm agents *in vivo*. However, because of the several limitations for a given model system, no single system is known as the gold standard model for all biofilm studies. For better understanding of different properties of the biofilm, the application of different types of models and the combination of more than one model system can facilitate answering specific research questions. New model systems are constantly emerging with technical and biological improvements. In Chapter II, few of the most well known *in vitro* biofilm model systems were introduced and discussed. Each of the model systems described in this Chapter offers different advantages, but with limitations. Hence, as a biofilm research scientist, the development and application of appropriate *in vitro* model systems are critical for pushing the biofilm research forward.

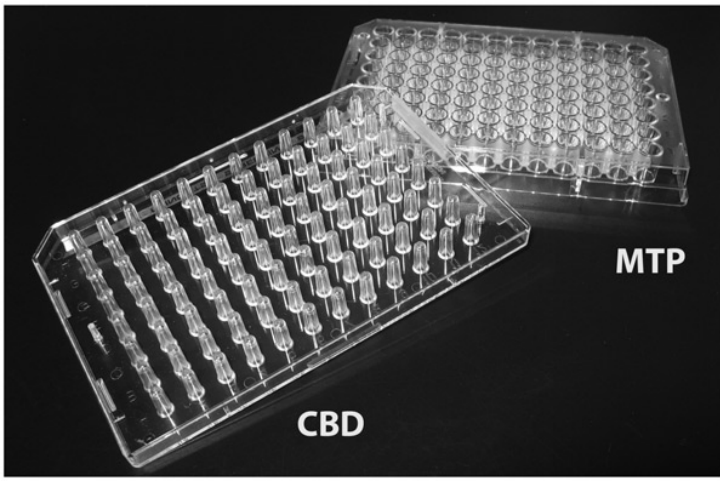
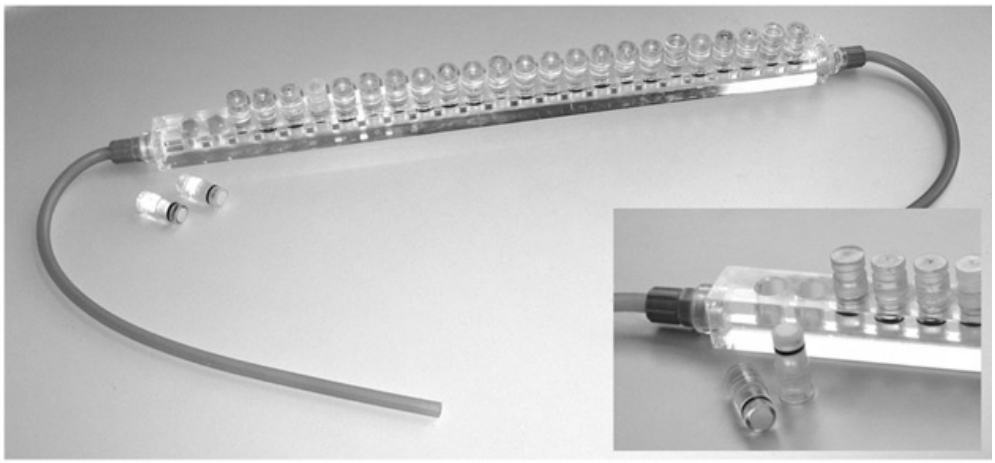


Figure II.1. **Microtiter Plate (MTP) Biofilm Model System**. A classic example of a novel MTP based biofilm model system is the Calgary Biofilm Device (Azeredo *et al*., 2017). Image © Claus Sternberg.

Figure II.2. **Glass Bottomed 24-well Sensoplates**. Image acquired from Greiner Bio-One.

A.



B.

Fig. II.3. Open Flow Biofilm Model Systems. **A**. Modified Robbins Device (Azeredo *et al*., 2017; Image © Claus Sternberg), and **B**. Centers for Disease Biofilm Reactor. Image acquired from Donlan *et al*., 2004.

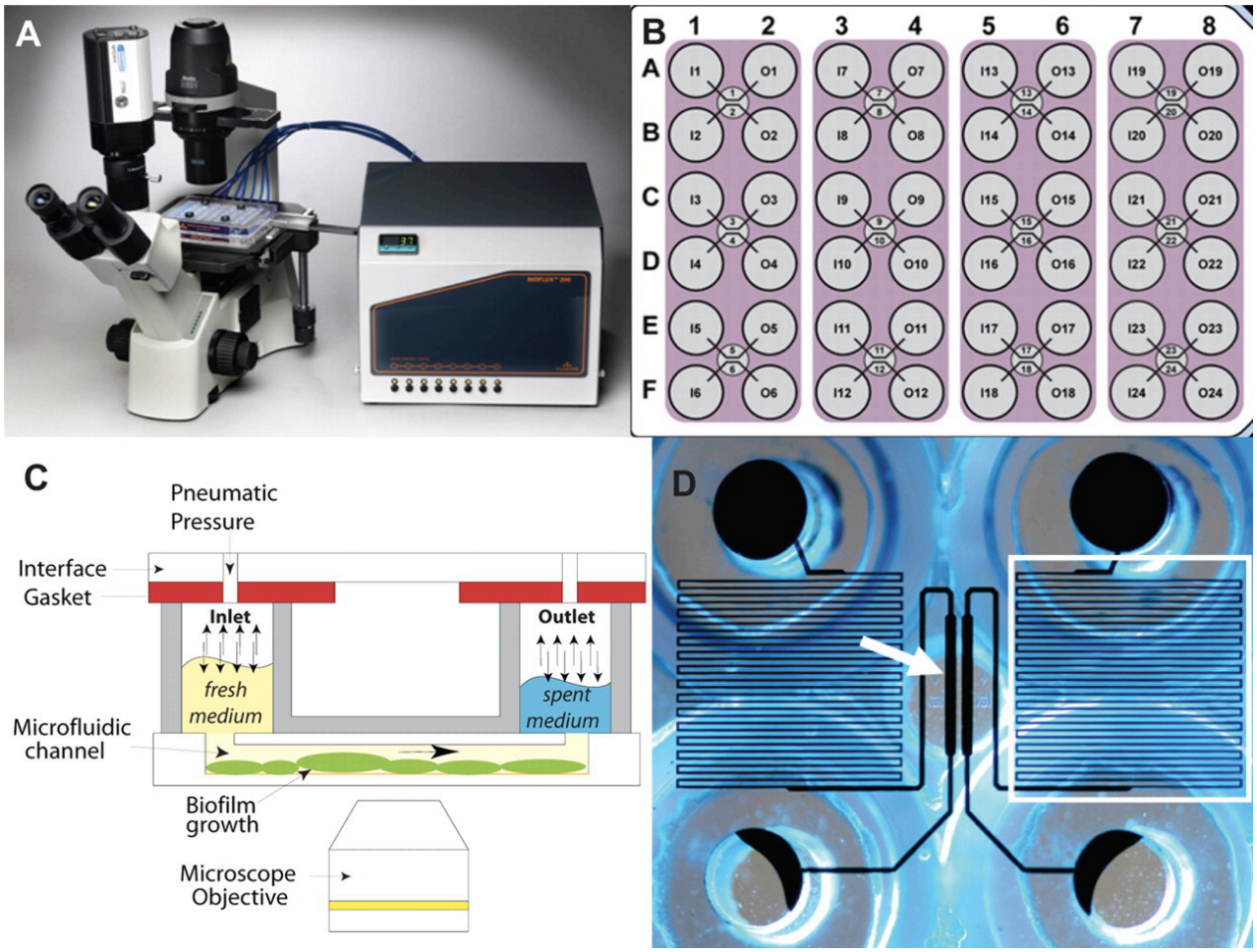


Fig. II.4. **BioFlux Microfluidic Biofilm System**. **A**. A photograph of the BioFlux 200 system along with the pneumatic pump connected directly to the specialized BioFlux plate shown on the microscope stage. **B**. Plate design of a 48-well BioFlux plate is shown. There are 24 inlet and 24 outlet wells, along with 24 individual channels as the biofilm substratum and the viewing port. **C**. The schematic shows the different components of the BioFlux plate design. Each channel requires an inlet and an outlet to provide open flow. **D**. A close up image of two microfluidic channels. Each inlet portion of the channels have a serpentine design to provide sufficient back pressure for more precise control of the shear force. Image acquired from Benoit *et al*., 2010.

Chapter III

Antimicrobial Nisin Acts Against Saliva Derived Multi-Species Biofilms without Cytotoxicity to Human Oral Cells

Chapter III is adapted from a previously published manuscript (Shin *et al*., 2015).

Abstract

Objectives: Nisin is a lantibiotic widely used for the preservation of food and beverages. Recently, investigators have reported that nisin may have clinical applications for treating bacterial infections. The aim of this study was to investigate the effects of ultra pure food grade Nisin ZP (> 95% purity) on taxonomically diverse bacteria common to the human oral cavity and saliva derived multi-species oral biofilms, and to discern the toxicity of nisin against human cells relevant to the oral cavity. **Methods**: The MICs and MBCs of taxonomically distinct oral bacteria were determined using agar and broth dilution methods. To assess the effects of nisin on biofilms, two model systems were utilized: a static and a controlled flow microfluidic system. Biofilms were inoculated with pooled human saliva and fed filter-sterilized saliva for 20-22 h at

37°C. Nisin effects on cellular apoptosis and proliferation were evaluated using acridine orange/ethidium bromide fluorescent nuclear staining and lactate dehydrogenase activity assays.

Results: Nisin inhibited planktonic growth of oral bacteria at low concentrations (2.5 – 50 μg/ml). Nisin also retarded development of multi-species biofilms at concentrations ≥ 1 μg/ml. Specifically, under biofilm model conditions, nisin interfered with biofilm development and reduced biofilm biomass and thickness in a dose-dependent manner. The treatment of pre-formed biofilms with nisin resulted in dose- and time-dependent disruption of the biofilm architecture along with decreased bacterial viability. Human cells relevant to the oral cavity were unaffected by the treatment of nisin at anti-biofilm concentrations and showed no signs of apoptotic changes unless treated with much higher concentrations (> 200 μg/ml).

Conclusions: This work highlights the potential therapeutic value of high purity food grade nisin to inhibit the growth of oral bacteria and the development of biofilms relevant to oral diseases.

Introduction

Dental plaque is an architecturally complex bacterial multi-species biofilm community (Zinge *et al*., 2010; Marsh 2010). Hundreds of species can coexist in these communities and together they can be up to 1000-times more resistant to antimicrobials than their planktonic counterparts (Gilbert *et al*., 2002; Aas *et al*., 2005). Enhanced antimicrobial resistance accounts, in part, for the accumulation of pathogens that are associated with dental caries, periodontal disease, and pulpal infections (Marsh 2003). Strategies to control biofilms and their contained species have met with difficulties, as is evidenced by the public health burden associated with poor oral health. The Centers for Disease Control and Prevention (CDC) estimated that in the United States (US) alone, $108 billion was spent on dental services in 2010 and is therefore constantly on the search for alternative cost-effective preventative strategies (CDC, 2010). Given the public health burden associated with dental plaque, new candidate anti-biofilm technologies are currently being investigated. These include modifications to traditional approaches, such as the development of improved antimicrobial compounds and formulations (zinc, cetylpyridinium chloride, stannous compounds, natural agents) (Allaker *et al*., 2009; Marsh 2010) to more innovative technologies, such as those that display antimicrobial but also anti-biofilm effects (Kaplan 2010). For example, there has been considerable interest in approaches to inhibit cell-cell signaling between bacteria to control oral biofilm formation (Rickard *et al*., 2006; Barrios *et al*., 2006; Raffa *et al*., 2011; Bordi and de Bentzmann 2011; Cuadra-Saenz *et al*., 2012) and to augment cell-cell signaling for enhancing antimicrobial activity (Eckert *et al*., 2006; Ahmed *et al*.,

2009). In addition, there has been substantial interest in approaches to structurally weaken biofilms by targeting bacterially-produced extracellular polymeric substances (EPS) using enzymes such as dispersin B (Izano et al., 2007). One technology that has recently garnered attention is the use of bacteriocins, such as nisin, which is produced by *Lactococcus lactis* (Pepperney *et al*., 2011; Arthur *et al*., 2014).

Lactococcus lactis is a non-pathogenic, Gram-positive, lactic acid bacterium used in food fermentation and found commonly in dairy products (Vuyst *et al*., 1994; Cleveland *et al*., 2001). In addition, *L. lactis* has been proposed as a probiotic agent (Vinderola *et al*., 2003). Studies have implied that lactic acid bacteria can prevent the co-localization of pathogenic bacteria in certain microflora (intestinal, vaginal) by stabilizing the complex biofilm community (Todorov *et al*., 2007; Kindrachuck *et al*., 2013). These processes may be mediated by bacteriocins like nisin. Nisin is a bacterially secreted polypeptide composed of 34-amino acids that exists as two natural variant forms; nisin A and Z. The two variants differ only by a single amino acid at position 27; histidine in nisin A and asparagine in nisin Z (Mulders *et al*., 1991). Nisin has amphipathic and cationic properties, and is classified as a Type A (I) lantibiotic (Gross and Morell 1971; Asaduzzaman and Sonomoto 2009). Lantibiotics like nisin are known for their broad-spectrum Gram-positive antimicrobial activities, high potency, low association with cytotoxicity and lack of stable and transmissible antimicrobial resistance (Willey *et al*., 2007; Smith and Hillman 2008). As a food preservative, nisin has been granted GRAS (Generally Regarded as Safe) status by the US Food and Drug Administration (FDA) for use in pasteurized, processed cheese spreads and is currently licensed in 48 countries (Cotter *et al*., 2005). Nisin acts by altering the

structure of the cellular membrane by forming short-lived pores and binding to the bacterial cell-wall precursor lipid II with high affinity (Breukink *et al*., 1999; Wiedemann *et al*., 2001). This initial interaction facilitates further modes of action to inhibit the cell wall biosynthesis, spore outgrowth and activation of autolytic enzymes (Peschel and Sahl 2006). Thus, the lack of bacterial resistance towards nisin likely stems from its interaction with a number of distinct targets.

Investigators from multiple fields have shown promising results for the use of nisin to treat bacterial infections, such as mastitis in humans and cows (Cao *et al*., 2007; Wu *et al*., 2007; Fernández *et al*., 2008), *Staphylococcus aureus* infections in atopic dermatitis (Valentia et al., 1996), respiratory tract infections (Bush and Macielag 2000; De Kwaadsteniet *et al*., 2009) and experimental gingivitis in dogs (Howell *et al*., 1993). Recent in vitro and in vivo evidence has even indicated a role for nisin as an anticancer agent (Joo *et al*., 2012). Given the high therapeutic potential of nisin, we hypothesize that nisin can be utilized as an antimicrobial and anti-biofilm agent to prevent or treat oral biofilm associated diseases. Thus, the aim of this work was to determine the effectiveness of nisin in inhibiting the formation and maintenance of saliva derived multi-species oral biofilms. In addition, we examined the ability of nisin to inhibit the growth of common oral bacteria found in the human oral cavity, including both Gram-positive and Gram-negative, and aerobic and anaerobic bacteria. Lastly, we examined the potential cytotoxicity of nisin on human cells relevant to the oral cavity.

Materials and Methods

Nisin Preparation

An ultra pure food grade (> 95%) form of the nisin Z, referred to here as nisin ZP, was purchased from Handary (S.A., Brussels, Belgium), a primary manufacturer of nisin in the food industry. From here forward, nisin ZP will be referred to as nisin. Nisin powder was stored at 4°C in a vacuum desiccator (Thermo Scientific, Waltham, MA, USA). The stock solution was prepared at a concentration of 5 mg/ml in water, filter sterilized, and stored at 4°C for a maximum of 5 days for use in experiments.

Bacterial Strains and Growth Conditions

Streptococcus oralis 34 (kindly donated by P.E. Kolenbrander, National Institutes of Health, Bethesda, MA, USA), *Streptococcus gordonii* DL1 (kindly donated by P.E. Kolenbrander), *Streptococcus mutans* UA159 (kindly donated by M.C. Peters, University of Michigan School of Dentistry, Ann Arbor, MI), *Streptococcus mutans* ATCC 25175, and *Aggregatibacter actinomycetemcomitans* Y4 were grown on Brain Heart Infusion agar (BHI, Difco, Sparks, MD, USA) and cultured in BHI broth media. *Actinomyces odontolyticus* ATCC 17982, *Prevotella intermedia* (clinical isolate, kindly donated by W. Loesche, University of Michigan School of Dentistry, Ann Arbor, MI), *Fusobacterium nucleatum* ATCC 25586, were grown on Fastiduous Anaerobe Agar (Acumedia, Lansing MI, USA) containing 5% sheep blood and cultured in BHI broth media supplemented with hemin (5 µg/ml) and Vitamin K (1 µg/ml). *Treponema denticola* ATCC 35405 was grown in TYGVS broth (Ohta *et al*., 1986; Fenno 2005). All

bacterial species mentioned above were incubated at 37˚C under appropriate atmospheric conditions in either an anaerobic chamber (Coy, Grass Lake, MI, USA) or a 5% CO2 incubator. For each strain, a single colony was inoculated into 5 ml of culture medium (as indicated above) and incubated to exponential growth phase. For use in experiments, the optical density at 600 nm (OD) of each culture was adjusted approximately to 0.15 to correspond to a bacterial concentration of 10^9 CFU/ml in culture medium.

MIC and MBC of Planktonic Oral Bacteria

The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of nisin against oral Gram-positive and Gram-negative bacterial strains were determined using the Clinical and Laboratory Standards Institute (CLSI) standards with slight modifications as described below (Wikler 2006; Wiegand *et al*., 2008). A total volume of 200 µl with different concentrations of nisin and the bacterial culture suspended in BHI or TYGVS broth medium was added to a 96-well microplate (Costar, Corning Inc., NY, USA). Of the total volume, each well contained 150 µl of bacterial culture and 50 µl of nisin. As mentioned above, the initial optical density of the bacterial cultures were calibrated approximately to 0.15 (OD_{600}) to achieve 109 CFU/ml. The final working concentrations of nisin were 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15, 25, 50, 100, 200 µg/ml. The microplates were incubated at 37°C for 24 h under aerobic or anaerobic conditions according to the bacterial growth requirements. *T. denticola* was cultured under anaerobic conditions in TYVGS media up to six days to determine the MIC. The determined MIC was the lowest concentration of nisin that inhibited the visible

growth of bacteria compared to the zero time point, indicated by an increase (≤ 0.05) in optical density (OD_{600}). For determination of MBC, 10 µl of these bacterial samples were removed from wells that had bacterial concentrations equivalent to and higher than the MIC, and inoculated on appropriate agar plates or in TYGVS broth medium. The MBC was defined as the lowest concentration of nisin that killed at least 99.9% of the bacteria in a given time.

Human Saliva Collection and Preparation: Multi-species inoculum and nutrient source

A saliva collection protocol was reviewed and given “not regulated status” by the University of Michigan Institutional Review Board (IRB) for Human Subject Research (HUM00095026). This protocol has been used previously (Nance *et al*., 2013; Samarian *et al*., 2014). Briefly, for the collection of pooled human whole saliva, the protocol required that at least 6 healthy individuals donate saliva. These individuals had not consumed any food or beverages besides water during the two hours prior to donation. All donors were non-smokers and had not been prescribed antibiotics for the preceding three months. Collected saliva was prepared for one of two purposes: to be used as a cell-containing saliva (CCS) inoculum or to be used as a cell-free saliva (CFS) nutrient source for biofilm growth. CCS was prepared by mixing native, pooled saliva with glycerol in a 75%/25% ratio, respectively, and then separated into 3 ml aliquots for storage at - 80˚C. CFS was prepared by adding 2.5 mM dithiothreitol (DTT) to the saliva then allowing it to stand for 10 min on ice followed by centrifugation at 17000 x g for 30 min. The resulting supernatant was mixed with distilled water to a final concentration of

25% and filter sterilized through 0.2-µm syringe filter with a cellulose polyethersulfone membrane (VWR, Radnor, PA, USA). Aliquots of 30 ml were stored at - 80˚C.

Effect of Nisin on Biofilm Development in a Static Model System

Twenty-four well glass bottom Sensoplates (Greiner Bio-One, Monroe, NC, USA) were used for the static model system (Kolderman *et al*., 2015). 15 µl of CCS was inoculated into each well with 1.5 ml of CFS with or without nisin (0.5 – 50 µg/ml). Upon inoculation, the biofilms were incubated for 20-22 h at 37°C for overnight growth. Following overnight incubation, the CFS that remained in each well was aspirated. All wells were then washed with 500 µl of phosphate buffered saline solution (PBS) three times. Following the wash, PBS in each well was aspirated, and biofilms were stained using formulated BacLight LIVE/DEAD bacterial viability staining solution (Invitrogen, Carlsbad, CA, USA) or Syto-9 nucleic acid stain (Invitrogen, Carlsbad, CA, USA) prepared according to the manufacturer's instructions. Syto-9 stain was used to quantify the total DNA content of the biofilm. 250 µl of LIVE/DEAD or 250 µl of Syto-9 solutions were added to each well and incubated for 45 min at room temperature and washed with 500 µl of PBS three times. For capturing 3D images of biofilms, confocal laser scanning microscopy was used as described below. The total DNA content of the biofilm was measured using a Victor X3 2030 Multi-label reader (PerkinElmer, Waltham, MA, USA) by detecting fluorescence intensity at 530 nm.

Effect of Nisin on Biofilm Development in a Controlled flow Microfluidic System

Forty-eight-well BioFlux microfluidic plates (Fluxion, San Francisco, CA, USA) in conjunction with the BioFlux 200 system (Fluxion, San Francisco, CA, USA) were used for the microfluidic model system (Nance *et al*., 2013; Samarian *et al*., 2014). Prior to adding the CCS, the microfluidic channels were first pre-treated with CFS for cell attachment, salivary pellicle formation and biofilm development. 100 µl of CFS was added to each outlet well, then flowed towards the inlet well at 1.0 dyn/cm^2 (equivalent to the flow rate of 93 µl/h, corresponding to a shear of 100 s-1 through the channel) for 2 min at room temperature. Flow was then stopped and the plate was incubated at room temperature for 20 min. Once pre-treatment incubation was completed, the CFS remaining in each outlet well was transferred to the corresponding inlet well, then 100 µl of CCS was added to each outlet well. To introduce bacterial cells into the plate channel for viewing biofilm growth, the CCS was flowed from outlet to inlet wells at 1.0 dyn/cm^2 for 6 s at 37°C. Upon inoculation of bacterial cells into the growth/viewing channels, cell seeding was confirmed visually with a Nikon Eclipse TCS-100 inverted light microscope equipped with a 20x0.40 NA PH1 infinity-corrected objective. The plate was then set to incubate at 37°C for 40 min to allow cell adherence and initial growth of the biofilm prior to the nutrient flow. Following incubation, CCS was aspirated from each outlet well and 800 µl of CFS with or without nisin (0.5 – 50 µg/ml) was added to each inlet well. Plates were then incubated at 37°C for 20-22 h with controlled flow of 0.2 dyn/cm^2 (flow rate of 19 µl/h, corresponding to a shear of 20 s-1) for overnight biofilm growth. Following overnight incubation, CFS in each inlet and outlet well was aspirated. One hundred microliter of PBS was added to each inlet well and flowed at room temperature for 20

min at 0.2 dyn/cm^2 to remove remaining treatment solution. Following the wash, PBS in the inlet wells was aspirated, and biofilms were stained using formulated BacLight LIVE/DEAD bacterial viability staining solution (Invitrogen, Carlsbad, CA, USA). One hundred microliter of formulated BacLight solution was added to each inlet wells and flowed at 0.2 dyn/cm^2 for 45 min at room temperature to allow staining of the biofilms. Once the staining was complete, the remaining solutions in the inlet wells were aspirated and 100 µl of PBS was added to each inlet well and flowed at 0.2 dyn/cm^2 for 20 min at room temperature to remove any excess stain in the BioFlux channels. For capturing 3D images of biofilms, confocal laser scanning microscope was used as described below.

Disruption of Pre-formed Biofilms by Nisin

To study the effect of nisin on pre-formed biofilms and to precisely control the exposure times, biofilms were inoculated and grown in twenty-four well Sensoplates (Greiner Bio-One, Monroe, NC, USA) as described above using CCS and CFS for 20-22 hrs at 37°C. Following overnight growth, the biofilms were treated with nisin (10, 50 µg/ml) with short exposure times (1, 5, 10 minutes). Following the treatment of pre-formed biofilms with or without nisin in CFS, all wells were washed with PBS three times. The same biofilm staining protocol was followed as described above.

Confocal Laser Scanning Microscopy and Quantitative Analysis of Biofilms

After nisin treatment, biofilms were imaged using Leica confocal laser scanning microscopy (CLSM, SPE, Leica, IL, USA) with a 40X1.25 NA HCX PL APO infinity-

corrected oil or a HCX PL APO 40X/0.85 CORR CS objective. All biofilms were stained with the Baclight Live/Dead Bacterial Viability kit (Invitrogen, Carlsbad, MA, USA), which contains the nucleic acid stains Syto-9 (green signal) and propidium iodide (red signal) as described above. Once the microscopy images were taken, biofilms were rendered as three-dimensional (3D) structures with Imaris (Bitplane, Zurich, Switzerland) computer imaging software. Image stacks were treated equally and the signal intensity of rendered 3D biofilm structures were confirmed using histograms generated in Imaris. Imaris allowed for the visualization of biofilm architecture in three dimensions, penetration of nisin into biofilms (inferred by the extent and degree of the red signal) and the preparation of 3D files for the quantification of biofilm structure using the computer software program Comstat2. For detailed biofilm analysis, Comstat2 was used to determine the biofilm biovolume (total amount of space/biomass occupied by a biofilm), average thickness (thickness of each biofilm extending from the bottom to the top of the growth/viewing channel surface), and roughness (a measure of heterogeneity in biofilm architecture). The degree of killing, based on green (Syto-9; live) and red (PI; dead/damaged) pixel intensity for every pixel in all three-dimensional planes were evaluated using ImageJ (National Institutes of Health). The percentages of live to dead/damaged cells was determined by first multiplying the total number of pixels by the level of intensity (0 – 255) and then summing the total value for both the LIVE and DEAD signals from each image stack recorded. All renderings and quantification analyses were performed on a dedicated laptop computer equipped with an Intel Core i5 CPU with 8 GB RAM, 64-bit operating system (MSI Computer Corp., CA, USA).

Cell Culture

A direct cell outgrowth technique was used to obtain primary periodontal ligament (PDL) cells and gingival fibroblast (GF) cells as previously described (Scanlon *et al*., 2011). These cells were maintained in minimum essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% fungizone (Gibco, Life Technologies, Grand Island, NY, USA). Passage 3 or 4 PDL and GF cells were used for experiments. Primary human oral keratinocytes (OK) were purchased from Science Cell Research Laboratories (Carlsbad, CA, USA) and maintained in keratinocyte growth medium (KGM) supplemented with OK growth supplements (OKGS) and 1% penicillin/streptomycin. Passage 1 or 2 OK cells were used for experiments. Osteoblast-like cells were purchased from American Type Culture Collection (MG63; Manassas, VA, USA) and maintained in α-MEM supplemented with 10% FBS, 1% penicillin/streptomycin and 1% fungizone. Passage 2 to 4 MG63 cells were used for experiments.

Apoptosis Staining and Microscopy

To assess the effects of nisin on cell viability and nuclear morphology, an acridine orange/ethidium bromide (AO/EB) staining assay was used as described previously (Ribble *et al*., 2005; Kasibhatla et al., 2006). Acridine orange (AO, Acros Organics, Geel, Belgium) and ethidium bromide (EB, Bio-rad laboratories, Berkeley, CA, USA) are fluorescent DNA binding dyes that can be combined to assess apoptotic cellular changes and cell membrane integrity. Using a 96-well microplate (Thermo Scientific, Waltham, MA, USA), cells (GF, PDL, OK, and MG63 cells) were plated at 2 x

10^4 cells/cm^2 and allowed to adhere and spread overnight. After 24 h, cell cultures were exposed to nisin (final concentrations: 1, 10, 100, 200, 400, 800 µg/ml) or left untreated and incubated for a following 24 to 48 h at 37°C. Following each treatment period, the cells were stained with the AO/EB dye solution for one minute under gentle agitation. The AO/EB dye reagent was comprised of 100 µg/ml of ethidium bromide and 100 µg/ml of acridine orange in PBS. The dye solution was removed and the cells were viewed and imaged using an epifluorescence microscope (Eclipse 50i, Nikon, Melville, NY, USA). For cell counting, each well was divided into two halves where a minimum of 150 cells was counted per half at 4X magnification. Cell viability, apoptosis, and necrosis were assessed as described previously (Ribble *et al*., 2005; Kasibhatla et al., 2006). Experiments were performed in triplicate.

Cell Proliferation Assay

The effect of nisin on cell proliferation was assessed by measuring the level of intracellular lactate dehydrogenase (LDH) activity using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) per manufacturer's recommendations. Using a 96-well microplate (Thermo Scientific, Waltham, MA, USA), the cells (GF, PDL, OK, and MG63 cells) were plated at 2 x 10^4 cells/cm^2 and allowed to adhere and spread overnight. The subsequent day, the cells were treated with different concentrations of nisin (10 – 800 µg/ml) for 24 to 48 h. After 24 or 48 h, 10 µl of the CCK-8 solution was added to each well and incubated at 37°C for 3 hours, then absorbance was measured at 450 nm using a microplate reader (Spectra Max M2, Molecular Devices, CA, USA) to determine

the LDH activity. Cell proliferation was evaluated based on the LDH activity measured as absorbance values at 450 nm.

Statistical Analysis

Values are expressed as mean values ± standard deviation. Independent t-tests were used to compare the control (nisin-free) with nisin-treated samples. The difference between the viability, apoptosis and necrosis was analyzed by ANOVA, using Dunnett's method. Individual P values for each data set are indicated either individually or as a group in each figure. All experiments were repeated at least three times. Values of $P < 0.05$ were considered significant, and $P < 0.01$ were considered highly significant.

Results

Antimicrobial Activity of Nisin on Oral Biofilm Colonizers

The MICs and MBCs of nisin on oral bacterial species are listed in Table 1. The MIC and MBC of nisin ranged from 2.5 to 50 μg/ml and 15 to 200 μg/ml respectively for early, middle, and later colonizers of dental plaque (Table 1). *S. mutans*, a cariogenic Gram-positive bacteria, showed a 1.5 to 3 fold higher sensitivity to nisin when compared to the two commensal organisms *S. gordonii* and *S. oralis* (Fig. 1, Table 1). *F. nucleatum*, a Gram-negative bacteria known for its prominent role in coaggregation with both early and later colonizers of dental plaque showed the least susceptibility amongst the tested species with a MIC of 50 μg/ml (Fig. 1, Table 1). In addition, nisin exerted antimicrobial activities against the known Gram-negative periodontal pathogens, including *P. gingivalis*, *P. intermedia*, *A. actinomycetemcomitans* and *T. denticola* (Fig. 1, Table 1) at nisin concentrations between 2.5 to 20 μg/ml. Amongst the later colonizers of oral biofilms, *T. denticola* exhibited the highest sensitivity to nisin with a MIC of 2.5 μg/ml and MBC of 15 μg/ml.

The Anti-biofilm Effects of Nisin on Formation of Multi-Species Biofilms

Saliva derived multi-species biofilms were grown with or without nisin using static and microfluidic model systems. Under the static growth condition, early signs of biofilm membrane damage was observed at nisin concentrations ≥ 0.5 μg/ml (Fig. 2A). From confocal microscopy imaging and quantitative analysis, the biofilm growth was significantly reduced at nisin concentrations ≥ 4 μg/ml (Fig. 2C). In the static system, the

average biofilm biomass and thickness of the control biofilms were 27.25 $\mu m^3/\mu m^2$ and 29.33 μm (Fig. 2B). The average biofilm biomass and thickness of the nisin treated biofilms at 4 μg/ml were 3.26 $\mu m^3/\mu m^2$ and 3.05 μm (Fig. 2B). At 8 μg/ml nisin, the biofilm growth and architecture was abrogated when compared to the control conditions (Fig. 2A).

Under controlled flow microfluidic growth conditions, the anti-biofilm effects of nisin were exerted at concentrations ≥ 0.5 μg/ml (Fig. 3A). The average biofilm biomass and thickness of the control biofilms were 30.88 $\mu m^3/\mu m^2$ and 31.02 μm (Fig. 3B). The average biofilm biomass and thickness of the nisin treated biofilms at 1 μg/ml were 5.06 $\mu m^3/\mu m^2$ and 7.17 μm (Fig. 3B). The formation of biofilms was absent at 4 μg/ml (Fig. 3A,B).

When evaluated under both biofilm model systems, biofilm biomass and thickness were significantly reduced when the biofilms were grown in presence of nisin at concentrations ≥ 1 μg/ml (Fig. 2B, 3B). The roughness coefficient values of the biofilms increased in a dose-dependent manner, suggesting an increase in heterogeneity within the biofilm architecture due to membrane associated structural damages. Collectively, our data suggest that nisin concentrations > 8 μg/ml can inhibit the formation of saliva derived multi-species biofilms.

The Anti-biofilm Effects of Nisin on Preformed Multi-Species Biofilms

Saliva derived multi-species biofilms formed overnight (20 – 22 h) under static conditions, then treated with two different concentrations of nisin (10, 50 μg/ml). The biofilms were treated with nisin for short exposure times (1, 5, and 10 min). At both

concentrations and at all three exposure times, the 3D rendered confocal microscopy images of biofilms demonstrated structural damage and dissociation within the biofilm architecture (Fig. 4A). When compared to the control biofilms treated with PBS, nisin treated biofilms exhibited reduced biofilm biomass and thickness (Fig. 4B). This reduction in the biomass and thickness of the biofilms occurred in a dose- and time-dependent manner. At a nisin concentration of 10 μg/ml, the viability of the biofilms was only slightly reduced for all exposure times (although statistically significant). However at 50 μg/ml, much higher biofilm killing was observed at 5 and 10 min exposure times, as indicated by the confocal microscopy imaging and Live/Dead signal quantification of the biofilms (Fig. 4A and C). In addition, the roughness coefficient values of the nisin treated biofilms were significantly increased compared to the control biofilms treated with PBS. The increase in the roughness coefficient values occurred in a time-dependent manner at 50 μg/ml, but it did not follow a specific time-dependent trend with 10 μg/ml.

The Effects of Nisin on Viability of Human Cells Relevant to the Oral Cavity

The effect of nisin on cell viability was assessed in human cells that are relevant to the oral cavity. After a 24 h treatment, GF, PDL, OK and MG63 cells were highly tolerant to nisin treatments (Fig. 5B). With nisin treatments of up to 400 μg/ml, GF, PDL, OK and MG63 cells exhibited normal cell viability levels (> 95%) and cell phenotypes with minimal apoptotic characteristics. After a 48 h treatment, GF, PDL and OK cells exhibited normal cell viability with nisin up to 200 μg/ml. Osteoblast-like MG63 cells exhibited the highest tolerance against nisin (Fig. 5B). At antimicrobial and anti-biofilm

concentrations (< 100 μg/ml), nisin treated cells maintained a normal cell shape and nuclear phenotype compared to the untreated control cells. Only at concentrations ≥ 200 μg/ml and after 48 h, cells started showing low levels of apoptosis with chromatin condensation and nuclear fragmentation (Fig. 5A).

The Effects of Nisin on Cell Proliferation

The effect of nisin on cell proliferation was assessed by measuring the innate lactate dehydrogenase activity of the cells (GF, PDL, OK, MG63). After 24 h with treatment of up to 800 μg/ml of nisin, GF, PDL and MG63 cells exhibited normal cell proliferation compared to the control (Fig 6). In control experiments, nisin (10 - 800 μg/ml) incubated in media alone exhibited negligible levels of lactate dehydrogenase activity (data no shown). Only OK cells exhibited reduced cell proliferation after a 24 h nisin treatment at concentrations > 400 μg/ml. In addition, GF and PDL exhibited normal cell proliferation with nisin concentrations up to 800 μg/ml, and OK and MG63 exhibited normal cell proliferation with nisin concentrations up to 400 μg/ml (Fig. 6). At 800 μg/ml, OK cells were unable to attach to the cell surface and proliferate. At antimicrobial and anti-biofilm concentrations (< 100 μg/ml), all cells exhibited normal cell attachment and proliferation.

Discussion

The data presented here demonstrate the potential for nisin as an antimicrobial and anti-biofilm agent against oral pathogens. The peptide structure of nisin is characterized by the presence of five intra-molecular rings formed by the thioether amino acids lanthionine and 3-methyllanthionine (Wiedemann *et al*., 2001). Due to its unique chemical features, it has been hypothesized that nisin has various modes of antimicrobial action (Peschel and Sahl 2006) and exerts multiple antimicrobial activities based on the interaction with multiple cellular targets (Bierbaum and Sahl, 1985; Chan *et al*., 1996; Pag and Sahl 2002). However, when used in a clinical setting, there is potential risk of developing nisin resistance. Although there have been few examples of naturally occurring lantibiotic resistance, certain bacteria have been noted to possess innate anti-lantibiotic mechanisms. For example, nisinase is a dehydropeptide reductase that can inactivate nisin (de Freire Bastos *et al*., 2014; Draper *et al.*, 2015). Nisinase activity has been associated with *Lactobacillus platarum* (Kooy 1952), *Streptococcus thermophiles* (Alifax and Chevalier, 1962), *Clostridium botulinum* (Rayman *et al*., 1983), *Lactococcus lactis* sub-species cremoris, Enterococcus faecalis and Staphylococcus aureus (Carlson and Bauer, 1957). Thus, future characterization of specific genetic or protein components that may contribute to nisin resistance is needed to understand any potential resistance issues in a clinical setting.

Previously, studies have implicated that nisin has broad-spectrum activity against Gram-positive bacteria with limited potency on Gram-negative bacteria (Delves-Broughton *et al*., 1996; Severina *et al.*, 1998; Cleveland *et al*., 2001). Our findings

demonstrate that the high purity form of nisin Z (nisin ZP, > 95% purity) exhibits antimicrobial activity against both Gram-positive and Gram-negative oral bacteria. In comparison to recent studies that used a low content nisin (Tong *et al*., 2010; Corbin *et al.*, 2011), our data suggests that nisin ZP (> 95% purity) is much more potent than low content nisin A (2.5% purity) in inhibiting cariogenic oral bacteria and oral biofilms. These earlier studies utilized low purity nisin, which is generally less soluble than the high purity form. In addition, when considering the preparation of nisin solutions, nisin A (2.5% purity) requires approximately pH 2 whereas nisin ZP can be prepared at more neutral pH conditions (Pag and Sahl 2002). Nisin ZP can be easily prepared in water at pH 7 as a colorless, odorless and tasteless solution. Hence, the utilization of high purity nisin ZP as a potential oral anti-microbial rinse has promising features.

Commensal oral bacteria, such as *S. gordonii* and *S. oralis*, exhibited lower sensitivity to nisin than the pathogenic species *S. mutans* (Table 1). From the single species experiments, the most interesting data resulted from the Gram-negative anaerobes. Periodontal disease is strongly associated with pathogenic bacteria such as *P. gingivalis*, *P. intermedia*, A. *actinomycetemcomitans* and *T. denticola*. These anaerobic pathogens are believed to gain access to the periodontal tissues and thereby mediate tissue damage by a complex array of host-pathogen interactions, including modulation of inflammatory host response mechanisms (Haffajee and Socransky 1994; Pihlstrom *et al*., 2005; Darveau 2009). In our study, nisin at low concentrations < 20 µg/ml inhibited the growth of these periodontal pathogens (Table 1). Amongst these pathogens, *T. denticola* was highly susceptible to nisin treatment, requiring only 2.5 µg/ml to inhibit its growth up to six days (Fig. 1). *T. denticola* comprises up to 30% of the

microbiota in diseased gingival pockets and is associated with tissue and bone destructive mechanisms in periodontitis (Riviere *et al*., 1992; Baehni *et al*., 1992; Gopalsami *et al*., 1993, Choi *et al*., 2003). Thus, our findings suggest that nisin can inhibit the growth of both Gram-positive and Gram-negative disease-associated oral bacteria.

Dental plaque is a complex of multi-species biofilm community (Marsh 2010). Collectively, the contained species can withstand the constantly changing conditions of the oral cavity and can be highly resistant to the treatment of antimicrobial agents (Jakubovics and Kolenbrander 2010). Since these biofilms can rapidly form and mature to develop additional pathogenic traits, anti-biofilm agents that inhibit the formation of and disperse established biofilms would benefit the prevention and treatment of oral diseases (Marsh 2010). Using biologically relevant human saliva as the multi-species biofilm inoculum and growth media, our results demonstrated that nisin exerted anti-biofilm properties. Overnight incubation of nisin within saliva caused substantial inhibition of biofilm formation. Specifically, nisin concentrations ≥ 0.5 and 1 μg/ml caused a significant reduction in biofilm biomass and thickness of biofilms developed in static and microfluidic systems respectively (Figs. 3B and 2B). The resulting biofilms were highly disintegrated and lacked coaggregative behavior expressed in the control untreated biofilms (Fig. 2A, 3A).

Similar to preventing biofilm development, the anti-biofilm properties of nisin against pre-formed biofilms occurred in a dose- and time-dependent manner. As inferred by observed changes in biofilm architecture, biofilms seemingly dispersed and sloughed into smaller aggregates after a 1 min exposure to 10 and 50 μg/ml of nisin.

(Fig. 4A). At 50 µg/ml and after a 5 min exposure, biofilms exhibited cell death and membrane damage indicated by the Live/Dead signal quantification (Fig. 4C). However, at 10 µg/ml, significant bacterial killing was not observed regardless of the treatment time. The antimicrobial action of nisin may be either bacteriostatic or bactericidal depending on multiple factors, such as nisin concentration, bacterial concentration, physiological state of the bacteria, and the prevailing conditions (Delves-Broughton *et al*., 1996). Thus, our data support the premise that nisin acts as a fast-acting anti-biofilm agent with both biofilm-static and biofilm-killing properties.

Coaggregation of different bacterial species is considered critical to maintaining the stability of the architecture and species composition of dental plaque (Hojo *et al.*, 2009; Katharios-Lanwermeyer *et al*., 2014). Coaggregation interactions promote the development of multi-species biofilms by enabling bacterial communication and colonization of initial, middle and later colonizers (Kolenbrander *et al*., 2010). Previously, Smith *et al*. demonstrated that cationic antimicrobials, such as chlorhexidine digluconate and cetylpyridinium chloride, can selectively inhibit coaggregation interactions of later colonizers of dental plaque (Smith *et al*., 1991). As a cationic and a membrane acting bacteriocin, the modes of action of nisin may be to disrupt the coaggregation process needed to form a stable biofilm. Present data are suggestive that the likely role of nisin in inhibiting coaggregation may be at least in part responsible for its anti-biofilm effects. Assuming that the coaggregation process is critical for developing and maintaining the biofilm complex, studies are in progress to determine the ability of nisin to inhibit specific coaggregation interactions between oral biofilm colonizers.

After more than thirty years of use as an antiplaque agent in the dental profession, chlorhexidine is still considered the gold standard (Jones 1997; Baehni and Takeuchi 2003). However, frequent or long-term use of chlorhexidine, although still employed clinically, is associated with negative effects (Flötra *et al*., 1971; Gagari, E. and Kabani 1995). Previously it was reported that direct exposure to as little as 0.0025 to 0.01% of chlorhexidine can significantly affect cell morphology and cell attachment of cultured gingival fibroblasts (Cline and Layman 1992). In addition, chlorhexidine is highly cytotoxic to neutrophils, human epithelial cells, PDL cells, fibroblasts and HeLa cells (Goldschmidt *et al*., 1977; Chang *et al.*, 2001). Our study demonstrates that orally relevant human cells are highly tolerant to direct contact by nisin. Our data indicate that nisin at antimicrobial and anti-biofilm concentrations (< 100 μg/ml) is not cytotoxic to these cells (oral keratinocytes, gingival fibroblasts, periodontal ligament cells, and osteoblast-like cells) that play an integral role in the maintenance of healthy gingival tissues. It is essential to preserve the cellular characteristics of primary oral keratinocytes, since these cells act as the first line of defense against the oral pathogens (Weinberg *et al*., 1998; Hans and Hans 2014). When cells were incubated with nisin for 24 to 48 h, cell apoptosis was nearly absent unless treated with nisin at > 200 μg/ml, which is more than 10-fold greater than the minimum concentration displaying anti-biofilm effects. In addition, all cell types exhibited normal cell attachment and proliferation when treated with up to 400 μg/ml of nisin after a 24 h incubation. Cationic antimicrobial peptides are unique in that they are hypothesized to play an important role in the immune system or to exert different effects on eukaryotic cells (Hancock and Diamond 2000). Although bacterially secreted, nisin is known to trigger

immune responses in host eukaryotic cells (Hancock and Diamond 2000; Bedge *et al*., 2011). As an immunogenic agent, nisin was reported to elevate the T-cell population (CD4 and CD8) while reducing the B-cell population (Pablo *et al*., 1999). In addition, nisin has been shown to modulate the innate immune response through the induction of chemokine synthesis and suppression of lipopolysaccharide induced pro-inflammatory cytokines, both in vitro and in vivo (Kindrachuck *et al*., 2013). Thus, at certain non-toxic concentrations to the oral cells, nisin could potentially aid in the induction of innate defense mechanisms to help clear the oral pathogens.

The work presented here demonstrates that nisin is a promising candidate for development as an oral therapeutic anti-biofilm agent. High purity food grade nisin (> 95%) exerted anti-biofilm effects against saliva derived multi-species biofilms without causing cytotoxic effects to the human oral cells. In addition to its long history and utilization as a food preservative, nisin possesses great potential for other applications involving treating clinical bacterial infections and inhibiting biofilm growth. Further investigation of the clinical role of nisin in modulating the microbiome of the biofilm community and its immunomodulatory role in human oral cells are necessary to determine its potential as a therapeutic or prophylactic agent against oral diseases.

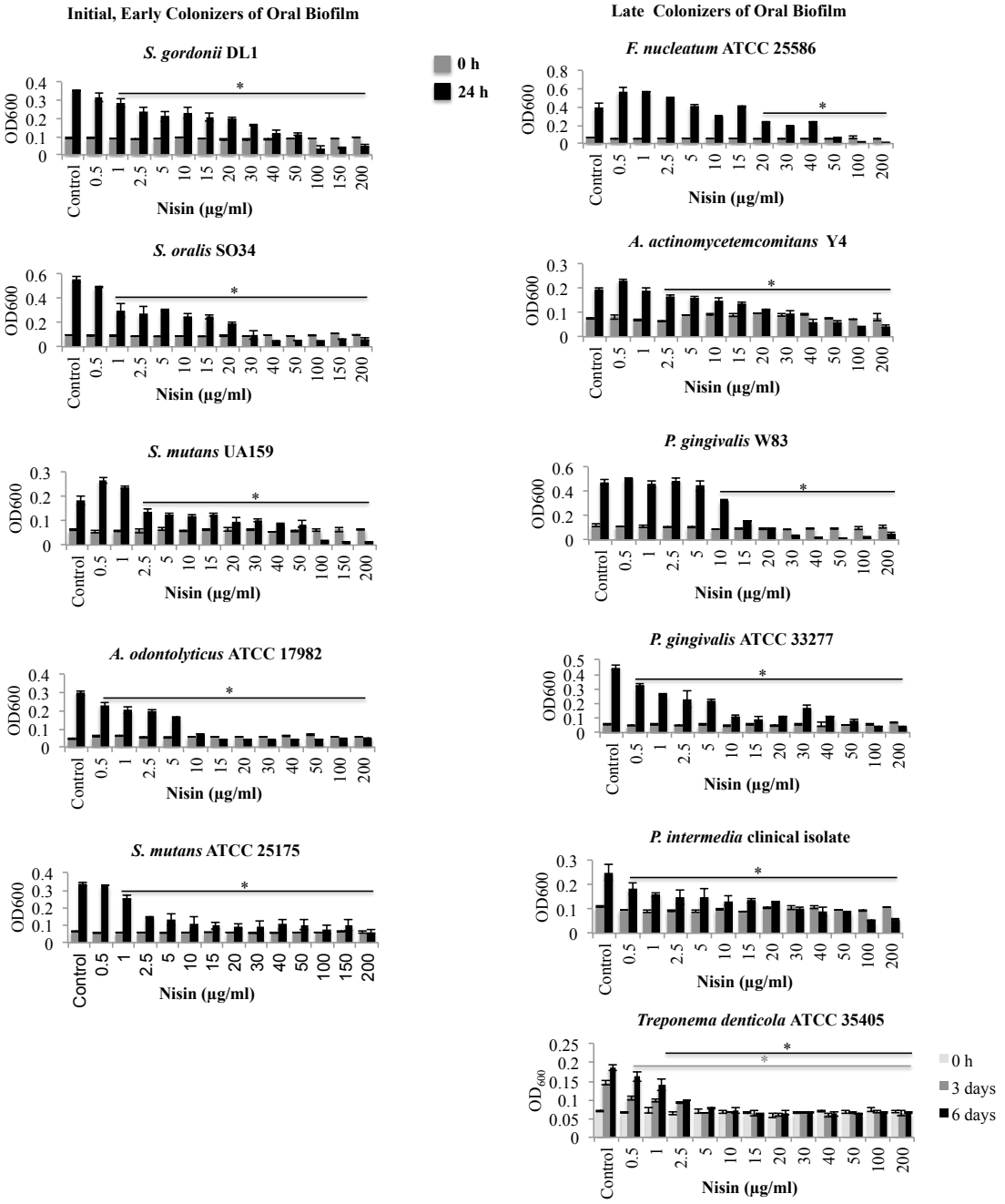
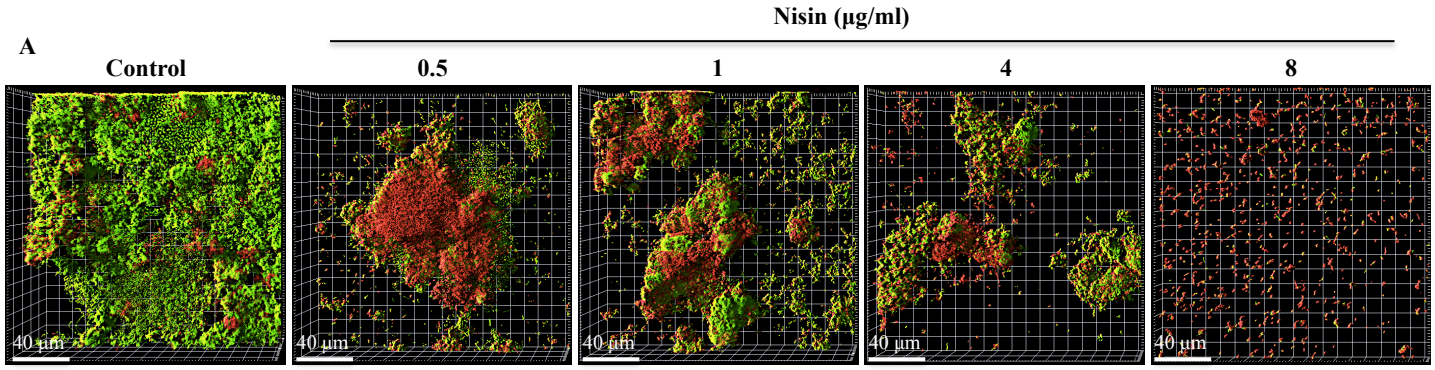


Figure III.1. **Nisin Inhibits the Growth of Cariogenic and Periodontal Pathogens**. Using a broth dilution method, S. gordonii DL1, S. oralis SO34, S. mutans UA159, A. odontolyticus ATCC 17982, S. mutans ATCC 25175, F. nucleatum ATCC 25586, A. actinomycetemcomitans Y4, P. gingivalis W83, P. gingivalis ATCC 33277, P. intermedia clinical isolate and T. denticola ATCC 35405 was cultured with or without nisin (0.1 – 200 µg/ml) on a microplate for 24 h at 37°C, under aerobic or anaerobic conditions. The determined MIC was the lowest concentration of nisin that inhibited the visible growth (≤ 0.05 increase in OD600 after 24 h growth) of the inoculated bacteria. *P < 0.05: significant differences from the control (nisin-free).



B

Biofilm parameter	Control	Nisin (µg/ml) 0.5	1	4	8
Biomass ($\mu m^3/\mu m^2$)	**27.25** (5.37)	**11.2** (1.95)**	**10.16** (3.22)**	**3.26** (1.63)**	**1.23** (0.83)**
Thickness (µm)	**29.33** (7.41)	**10.62** (1.98)**	**9.9** (2.93)**	**3.05** (1.41)**	**1.18** (0.95)**
Roughness	**0.41** (0.16)	**1.03** (0.11) **	**1.09** (0.19) **	**1.56** (0.15) **	**1.53** (0.22) **

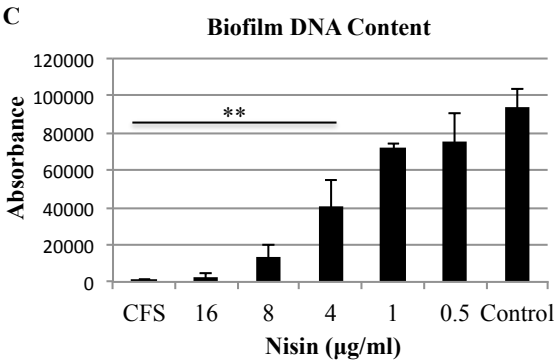
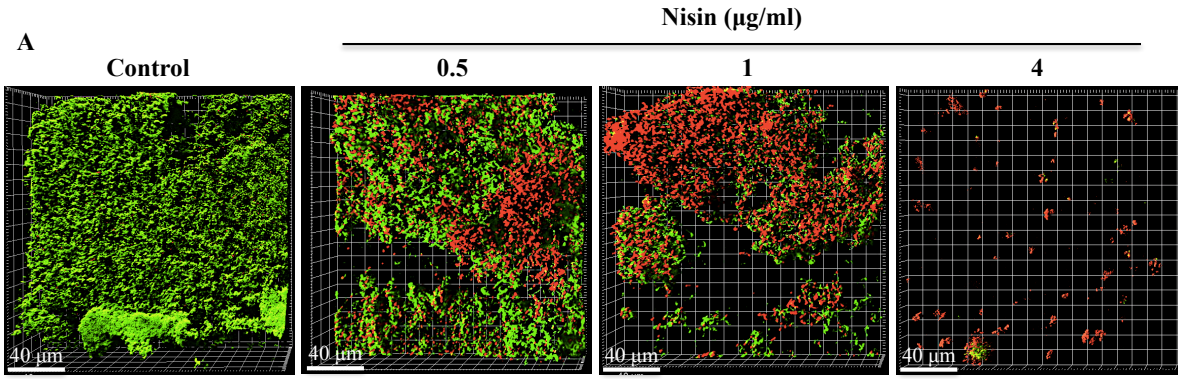


Figure III.2. **Nisin Inhibits the Formation of Multi-Species Biofilms in a Static Model System**. Cell-containing saliva (CCS) was inoculated in filter sterilized cell-free saliva (CFS) for 20-22h at 37°C with or without nisin. (A) Confocal microscopy images are represented in the x-y plane. A green signal indicates viable live cells (Syto 9), a red signal indicates damaged/ dead cells (propidium iodide) (B) Biofilm biomass, thickness, and roughness [mean (standard deviation)] were derived from imaging of at least three separate wells (experiments) (C) DNA content of the biofilms was quantified by absorption spectroscopy at fluorescence intensity of 530 nm. *P < 0.05 and **P < 0.01: significant differences from the control (nisin-free).

A

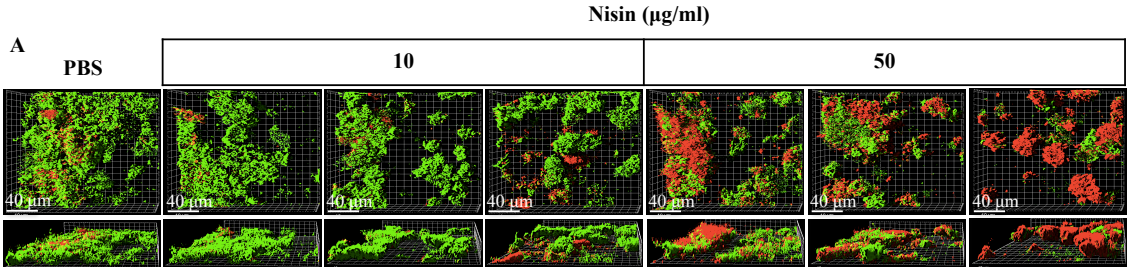


B

Biofilm parameter	Control	Nisin (µg/ml) 0.5	1	4
Biomass ($\mu m^3/\mu m^2$)	**30.88** (9.45)	**22.74** (10.13)*	**5.06** (2.85)**	**0.4** (0.13)**
Thickness (µm)	**31.02** (6.39)	**27.44** (8.6)	**7.17** (4.08)**	**0.24** (0.12)**
Roughness	**0.39** (0.27)	**0.38** (0.21)	**1.16** (0.37) **	**1.82** (0.09) **

Figure III.3. **Nisin Inhibits the Formation of Multi-Species Biofilms in a BioFlux Controlled Flow Microfluidic Model System**. Cell-containing saliva (CCS) was added, then fed filter sterilized cell-free saliva (CFS) for 20-22h at 37°C with or without nisin (A) Confocal microscopy images are represented in the x-y plane. A green signal indicates viable live cells (Syto 9) and a red signal indicates damaged/dead cells (propidium iodide). (B) Biofilm biomass, thickness, and roughness [mean (standard deviation)] were derived from imaging of at least three separate channels (experiments). *P < 0.05 and **P < 0.01: significant differences from the control (nisin-free).

A



B

Biofilm parameter	PBS	1 min	5 min	10 min	1 min	5 min	10 min
Biomass (µm³/ µm²)	**30.72** (6.16)	**18.59** (5.2) **	**12.54** (2.73) **	**13.08** (3.17) **	**11.52** (3.34) **	**9.67** (3.46) **	**5.37** (1.93) **
Thickness (µm)	**30.14** (5.43)	**21.78** (4.29)**	**14.58** (2.54)**	**14.31** (2.97)**	**14.84** (2.67)**	**13.71** (3.38)**	**7.36** (1.92)**
Roughness	**0.38** (0.11)	**0.62** (0.17)**	**0.98** (0.14)**	**0.84** (0.28)**	**0.97** (0.17)**	**0.99** (0.15)**	**1.27** (0.13)**

C

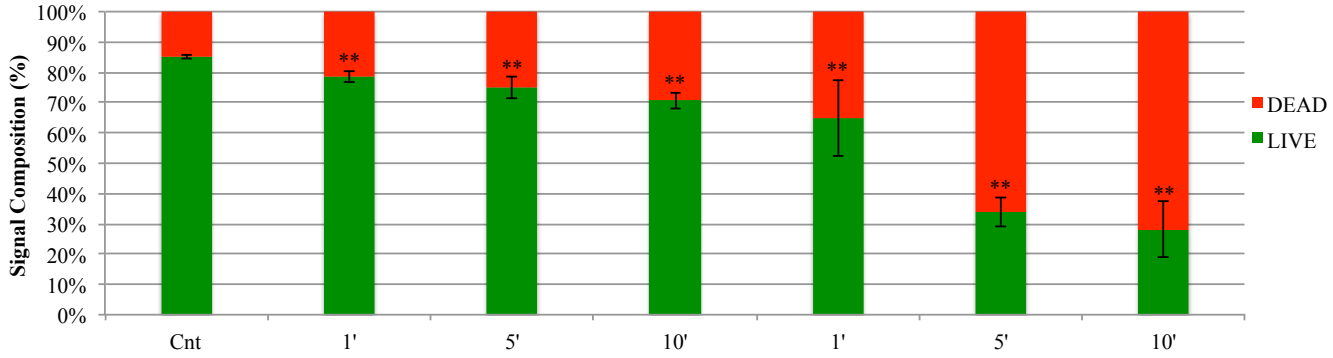


Figure III.4. **Nisin Disrupts the Maintenance of Three-Dimensional Architecture of Pre-Formed Biofilms**. Cell-containing saliva (CCS) was inoculated in filter sterilized cell-free saliva (CFS) for 20-22h at 37°C and treated with PBS solution (control) or nisin at different concentrations and incubation times (A) Confocal microscopy images are represented in the x-y and x-y-z plane. A green signal indicates viable live cells (Syto 9) and a red signal indicates damaged/dead cells (propidium iodide) (B) Biofilm biomass, thickness, and roughness [mean (standard deviation)] were derived from imaging of at least three separate wells (experiments) (C) An average percentage signal from the biofilms was determined by the Live/viable signal (green) and the Dead/damaged signal (red) in relation to the total signal captured for both. *P < 0.05 and **P < 0.01: significant differences from the control (nisin-free).

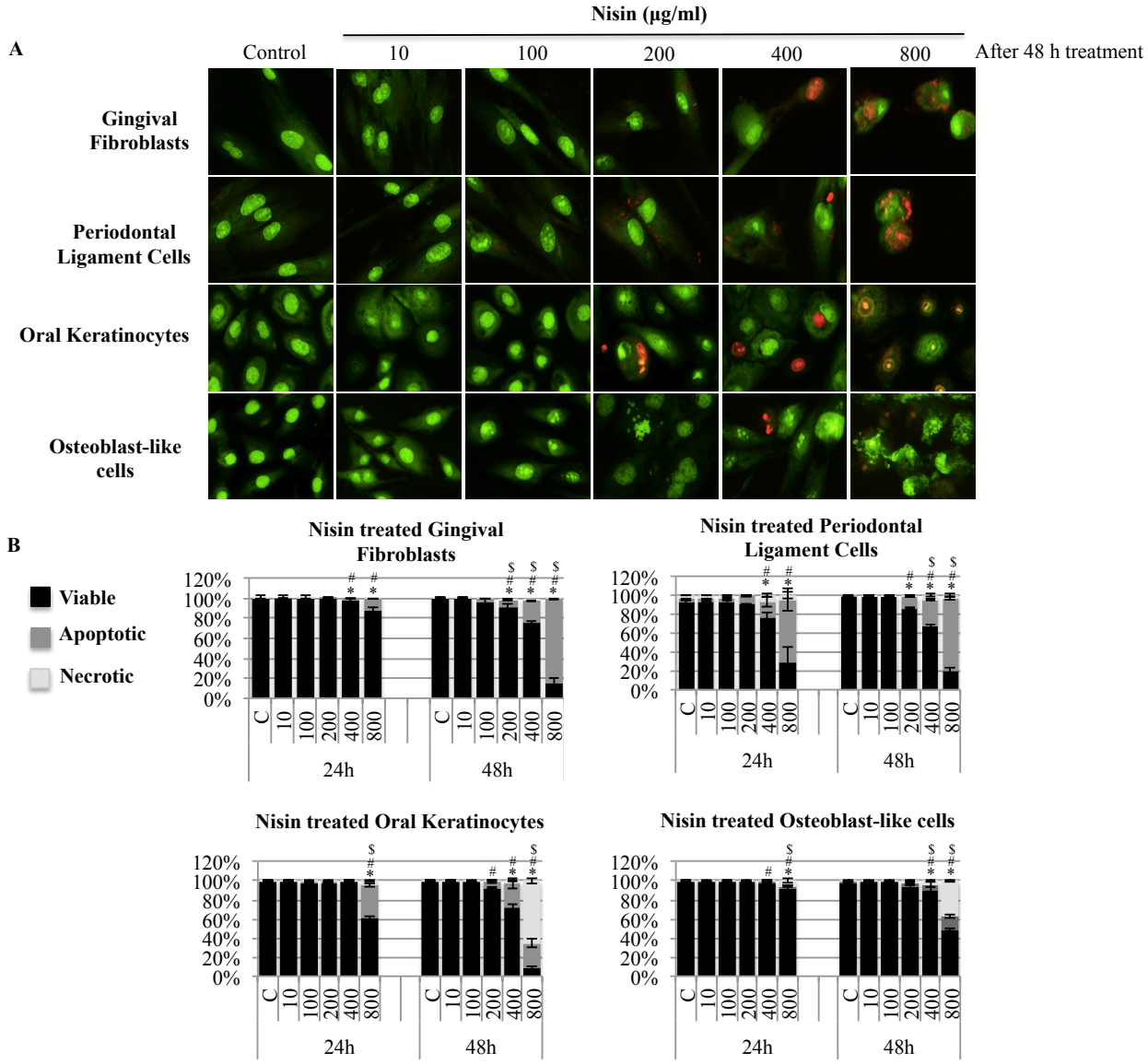


Figure III.5. **Nisin has Minimal Cytotoxicity to Human Cells Relevant to the Oral Cavity**. Primary human gingival fibroblast cells, periodontal ligament cells, oral keratinocyte cells and osteoblast-like cells were incubated with nisin (1 – 800 µg/ml) for 24-48 h on a 96-well microplate at 37°C. Cells were then stained with acridine-orange/ethidium-bromide (AO/EB) to evaluate cell viability, apoptosis and necrosis using epifluorescence microscopy. (A) Images of the cells after 48 h incubation period with nisin at different concentrations (B) The cytotoxicity of nisin was quantified by counting viable, apoptotic and necrotic cells and expressed as bar graphs with heights representing mean % and error bars representing standard deviation. AO (green) stained cells with intact membrane integrity. Early apoptotic cells stained green but contained bright green dots in the nuclei due to chromatin condensation and nuclear fragmentation. Late apoptotic cells stained with EB (orange) with apoptotic phenotypes. Necrotic cells stained orange but the nuclear morphology resembled the viable cells with absence of chromatin condensations. Mean values were calculated with standard deviations. *,#,$ represent $P < 0.05$: significant differences from the control (nisin free) for viability, apoptosis and necrosis respectively.

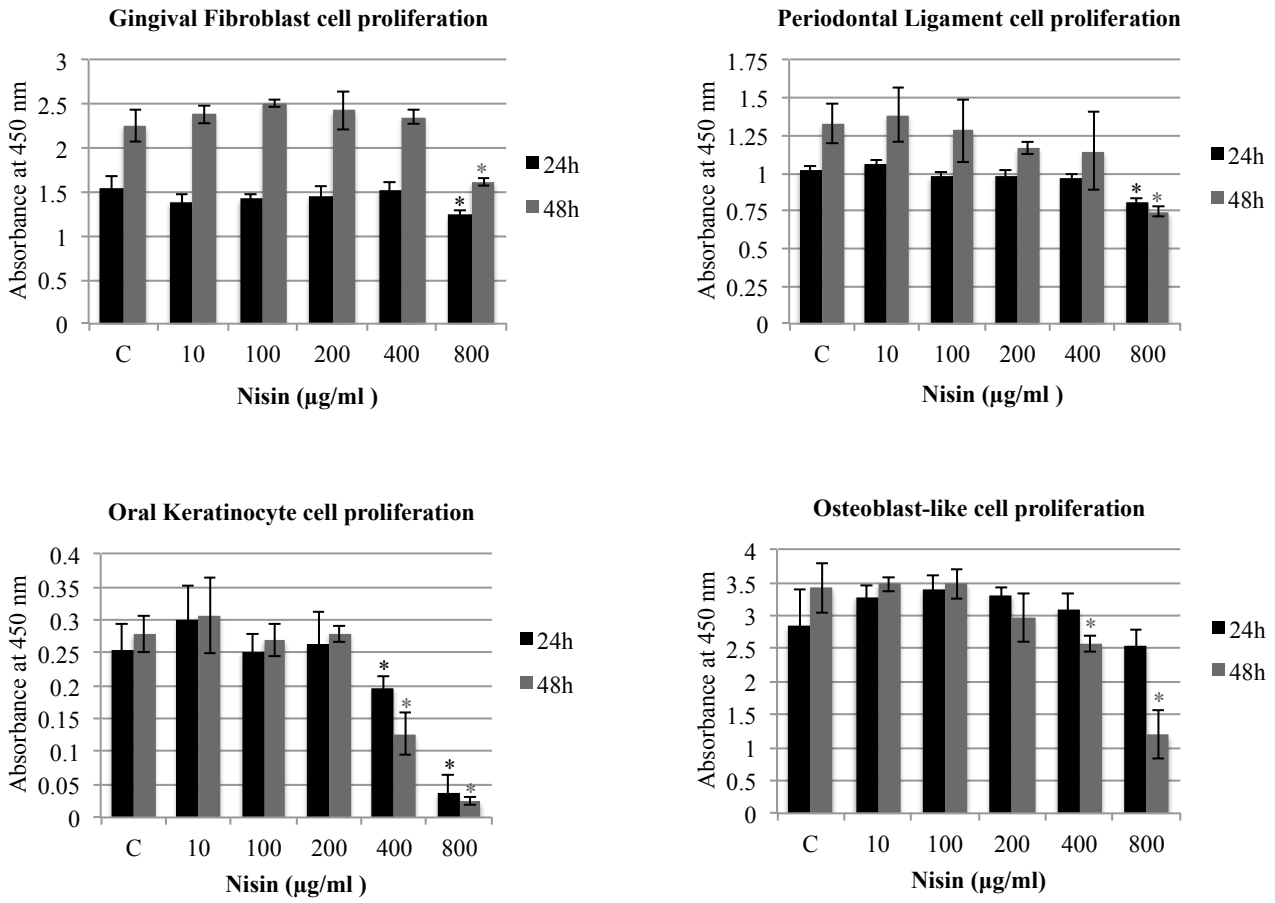


Figure III.6. **Nisin Does Not Effect Cell Proliferation of Human Cells**. The effect of nisin on cell proliferation was assessed using a Cell Counting Kit-8 measuring the lactate dehydrogenase (LDH) activities in cells. Using a 96-well microplate, gingival fibroblasts, periodontal ligament cells, oral keratinocytes and osteoblast-like cells were plated at 2 x 104 cells/cm2 and incubated for 24 to 48 h in the presence or absence of nisin (1 – 800 µg/ml). At 24 and 48 h time points, LDH levels were measured using absorption spectroscopy at 450 nm. Mean values were calculated with standard deviations. *P < 0.05: significant differences from the control (nisin-free).

Oral Microorganisms	MIC	MBC	MBC/MIC
Gram positive – Early, Middle Colonizers of Oral Biofilm			
Streptococcus gordonii DL1	40 μg/ml	150 μg/ml	3.75
Streptococcus oralis SO34	30 μg/ml	150 μg/ml	5
Streptococcus mutans UA159	20 μg/ml	100 μg/ml	5
Actinomyces odontolyticus ATCC 17982	10 μg/ml	30 μg/ml	3
Streptococcus mutans ATCC 25175	10 μg/ml	200 μg/ml	20
Gram negative – Late Colonizers of Oral Biofilm			
Fusobacterium nucleatum ATCC 25586	50 μg/ml	150 μg/ml	3
Aggregatibacter actinomycetemcomitans Y4	15 μg/ml	100 μg/ml	6.67
Porphyromonas gingivalis W83	20 μg/ml	100 μg/ml	5
Porphyromonas gingivalis ATCC 33277	15 μg/ml	100 μg/ml	6.67
Prevotella intermedia clinical isolate	10 μg/ml	150 μg/ml	15
Treponema denticola ATCC 35405	2.5 μg/ml	15 μg/ml	6

Table III.1. **MICs and MBCs of Planktonic Oral Pathogens**. MBC/MIC ratio of > 4 indicate that nisin has a bacteriostatic effect. MBC/MIC ratio of < 4 indicate that nisin has a bactericidal effect (Pankey and Sabath 2004).

Chapter IV

Biomedical Applications of Nisin

Chapter IV is adapted from a previously published manuscript (Shin *et al*., 2016a).

Abstract

Nisin is a bacteriocin produced by a group of Gram-positive bacteria that belongs to *Lactococcus* and *Streptococcus* species. Nisin is classified as a Type A (I) lantibiotic that is synthesized from mRNA and the translated peptide contains several unusual amino acids due to post-translational modifications. Over the past few decades, nisin has been used widely as a food biopreservative. Since then, many natural and genetically modified variants of nisin have been identified and studied for their unique antimicrobial properties. Nisin is an FDA approved and GRAS (generally regarded as safe) peptide with recognized potential for clinical use. Over the past two decades the application of nisin has been extended to biomedical fields. Studies have reported that nisin can prevent the growth of drug-resistant bacterial strains, such as methicillin

resistant *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococci* and *Clostridium difficile*. Nisin has now been shown to have antimicrobial activity against both Gram-positive and Gram-negative disease-associated pathogens. Nisin has been reported to have anti-biofilm properties and can work synergistically in combination with conventional therapeutic drugs. In addition, like host defense peptides, nisin may activate the adaptive immune response and have an immunomodulatory role. Increasing evidence indicates that nisin can influence the growth of tumors and exhibit selective cytotoxicity towards cancer cells. Collectively, the application of nisin has advanced beyond its role as a food biopreservative. Thus, this review will describe and compare studies on nisin and provide insight into its future biomedical applications.

Nisin: A Bacterially-Derived Antimicrobial

Nisin is an antimicrobial peptide produced by certain Gram-positive bacteria that include *Lactococcus* and *Streptococcus* species (Lubelski *et al*., 2008; de Arauz *et al*., 2009). Nisin was first identified in 1928 in fermented milk cultures and commercially marketed in England in 1953 as an antimicrobial agent (Rogers and Whittier, 1928; Delves-Broughton *et al*., 1996). In 1969, nisin was approved by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) as a safe food additive. Currently, nisin is licensed in over 50 countries, and it has made a significant impact in the food industry as a natural biopreservative for different types of foods (de Arauz *et al*., 2009). In the United States (US), nisin was approved by the Food and Drug Administration in 1988 and was given a generally regarded as safe (GRAS) designation for use in processed cheeses (Cotter *et al*., 2005).

The originally described variant of nisin, known as nisin A, is composed of 34 amino acids and is produced by *Lactococcus lactis* (Gross and Morell, 1971). Nisin belongs to a group of cationic peptide antimicrobials collectively called Type A (I) lantibiotics (Smith and Hillman, 2008). Nisin and other lantibiotics have gained considerable attention due to their potent and broad spectrum activity, low likelihood of promoting the development of bacterial resistance, and low cellular cytotoxicity at antimicrobial concentrations (Asaduzzaman and Sonomoto, 2009; Van Heel *et al*., 2011; Cotter *et al*., 2013; Shin *et al*., 2015). Similar to other lantibiotics, nisin contains several unusual amino acids as a result of enzymatic post-translational modifications (Sahl *et al*., 1995). Nisin contains dehydrated amino acid residues (serine and threonine) and thioether amino acids that form five lanthionine rings, which are characteristic of nisin and lantibiotics (Karakas *et al*., 1999; Wiedemann *et al*., 2001). As a food biopreservative, nisin serves as a broad-spectrum bacteriocin against mostly Gram-positive foodborne bacteria (Delves-Broughton *et al*., 1996; Severina *et al*., 1998; Cleveland *et al*., 2001). However, research has now shown that the antimicrobial action of nisin can extend to a range of non-food related bacteria (Blay *et al*., 2007; Shin *et al*., 2015). Studies have demonstrated that purified nisin and nisin in combination with other antibiotics can be effective against Gram-negative pathogens and that certain bioengineered nisin variants can enhance the activity against both Gram-positive and Gram-negative pathogens (Kuwano *et al*., 2005; Naghmouchi *et al*., 2010; Field *et al*., 2012). In addition, with recent improvements in biotechnology, researchers from interdisciplinary fields have bioengineered newer forms of nisin variants that have

therapeutic potential for human diseases (Piper *et al*., 2011; Field *et al*., 2012; Rouse *et al*., 2012; Balciunas *et al*., 2013; Field *et al*., 2015).

Since its discovery, nisin has garnered significant influence in the food industry as an alternative biopreservative. However, with demonstrated safety over the past 40 years, the use of nisin has begun to expand to include a diverse array of unrelated applications, such as those related to the biomedical industry (Fig. 1). Many lantibiotics (and more broadly, other bacteriocins) have been reported to possess additional biological activities beyond their antimicrobial activities (Asaduzzaman and Sonomoto, 2009; Benmechernene *et al*., 2013; Kamarajan *et al*., 2015). For example, nisin has beneficial properties in the context of biomedical applications, including bacterial infections, cancer, oral diseases and more. This review will provide a comprehensive overview of the latest findings by focusing on the advances in nisin bioengineering and the new discoveries in biomedical applications of nisin.

Natural and Bioengineered Variants of Nisin

Several other naturally-occurring variants of nisin have been reported. These variants have been identified from a range of taxonomically distinct organisms isolated from a broad range of environments. Nisin A was first discovered in *L. lactis*, an organism that is commonly found in dairy products and is the most widely studied nisin variant (Fig. 2) (Gross and Morell, 1971). Nisin Z, the closest variant of nisin A, was isolated from L. lactis NIZO22186 (Mulders et al., 1991). Nisin Z differs from nisin A by a single amino acid residue at position 27, asparagine instead of histidine (Fig. 2; Table 1) (Mulders *et al*., 1991). Nisin A and Z share similar properties as antimicrobials, but nisin

Z has a superior rate of diffusion and solubility under neutral pH conditions (De Vos *et al*., 1993). Nisin F was isolated from *L. lactis* F10 in the feces of a freshwater catfish in South Africa and differs from nisin A by two amino acid residues (De Kwaadsteniet *et al*., 2008). Nisin F has two amino acid substitutions at position 27 and 30 (Fig. 2; Table 1). Nisin Q was isolated from *L. lactis* 61-14 that was cultured from a river in Japan (Zendo *et al*., 2003). Nisin Q contains four substitutions at position 15, 21, 27 and 30 (Fig. 2; Table 1). Nisin A, Z, F and Q have antimicrobial activity against a range of *Staphylococcus aureus* targets (Piper et al., 2011). Nisin U and U2 are more distantly related variants that were isolated from *Streptococcus uberis*, an organism that commonly inhabits the lips, skin, and udder tissues of cows and is found in raw milk (Wirawan *et al*., 2006). Nisin U and U2 contain nine and ten amino acid substitutions respectively, compared to nisin A (Table 1). Recently, nisin H was isolated from a *Streptococcus hyointestinalis* strain derived from porcine intestine (O'Connor *et al*., 2015). The amino acid sequence of nisin H has similarities to nisin peptides produced by both *lactococcal* and *streptococcal* strains (Table 1). Nisin H maintains the terminal amino acids found in nisin A, Z, F, and Q, while harnessing features of nisin U and U2, including a dehydroaminobutyric acid substitution at position 18 (Table 1). Furthermore, nisin P was identified by genome mining techniques in *Streptococcus gallolyticus subsp. Pasteurianus*, an organism found in the alimentary tract of ruminants (Zhang *et al*., 2012). The protein sequence of nisin P closely resembles that of nisin U2 but differs from it by two substitutions at position 20 and 21 (Fig. 2; Table 1). Thus far, based on published reports, there are at least eight nisin variants that have been isolated, identified and sequenced for cross-analysis.

The potential for utilizing genetic tools to modify the activity of bacteriocins has been recognized for several decades (Gillor *et al*., 2005). In addition to the naturally occurring nisin variants, there are bioengineered forms of nisin that have been developed in attempts to enhance the efficacy and stability of nisin under different physiologic conditions, and to enhance its pharmacokinetic properties for a variety of biological applications (Field *et al*., 2015). Here, we describe several bioengineered nisin variants that have been recently identified. Nisin Z N20K and M21K were derived from the genetic modification of *L. lactis* NZ9800 and first reported by Yuan and colleagues. These genetically modified nisin variants exhibited enhanced activity against pathogenic Gram-negative bacteria, such as *Shigella*, *Pseudomonas* and *Salmonella* species (Yuan *et al*., 2004). Nisin Z N20K and M21K contain substitutions in the flexible hinge-region of the peptide backbone structure of nisin Z (Table 1). Furthermore, these variants displayed greater thermal stability at higher temperatures and solubility at neutral or alkaline pH (Yuan *et al*., 2004). The hinge region of nisin, which consists of three amino acids, asparagine-methionine-lysine, is located between the first three and the last two lanthionine-constricted rings of nisin. Modifications in the hinge region have been studied extensively because this region is important for the insertion of nisin into the bacterial membrane (Hasper *et al*., 2004; Lubelski *et al*., 2009; Ross and Vederas, 2011). Healy and coworkers demonstrated that mutants of the hinge region exhibited enhanced activity against specific indicator strains such as *L. lactis* HP, *Streptococcus agalactiae* ATCC 13813, *Mycobacterium smegmatis* MC2155 and *S. aureus* RF122 (Healy *et al*., 2013). In addition, Zhou and colleagues demonstrated that by altering the length of the hinge region of nisin, the efficacy of nisin against a panel of

test microorganisms can be altered in a temperature and matrix dependent manner (Zhou *et al*., 2015). Recently, a wide range of bioengineered nisin peptides with greater activity and enhanced therapeutic properties against foodborne and clinical pathogens began surfacing in the literature. The newly bioengineered variants include nisin A K22T, A N20P, A M21V, A K22S, S29A, S29D, S29E and S29G (Table 1) (Field *et al.,* 2008; Field *et al*., 2012). Field and colleagues applied site-directed and site-saturation mutagenesis to the hinge region residues of nisin A to successfully identify variants that displayed enhanced bioactivity and specificity against a range of Gram-positive drug-resistant, clinical veterinary and food-borne pathogens (Field *et al*., 2012). Thus, based on emerging reports, bioengineered variants of nisin appear to be promising candidates for future applications in health care.

Nisin and Treatment of Infectious Diseases

Certain human infectious diseases, such as antibiotic-resistant skin and soft tissue infections and especially biofilm-associated infections can be difficult to prevent and/or treat (Mah and O'Toole, 2001; Gilbert *et al*., 2002; Fauci and Morens, 2012). While conventional medical treatments that are based on antibiotics and antivirals have been used for bacterial and viral infections, the emergence of drug resistance has led to the search for alternative or adjunctive methods to treat these drug resistant diseases (Zetola *et al*., 2005). With decades of safe usage in the food industry, investigators have started exploring nisin as a potential alternative agent for infectious diseases, including drug-resistant infections, thereby also decreasing the use of antibiotics (Table 2) (Balciunas *et al*., 2013).

Methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin resistant enterococci (VRE) has become a major medical problem in hospitals around the world. The difficulty in treating these infections has been extensively documented (Huycke *et al*., 1998; Chambers, 2001; Köck *et al*., 2010; Ahire and Dicks, 2015). MRSA and VRE are leading causes of bacterial nosocomial infections, urinary tract infections, and are known to be resistant to many standard therapies. For example, MRSA infections account for up to 70% of the *S. aureus* infections in intensive care units (Sahm et al., 1999; Diekema *et al*., 2001). Both MRSA and VRE infections can manifest as skin infections and in medical settings as bacteremias, pneumonia, and post-surgical infections (Huycke *et al*., 1998; Center for Disease Control and Prevention, MRSA Infections, 2015). Numerous studies have been published regarding the efficacy of nisin as an antimicrobial therapeutic (Piper *et al*., 2009; Dosler and Gerceker, 2011; Okuda *et al*., 2013; Singh *et al*., 2013; Ahire and Dicks, 2015). Piper and coworkers reported that nisin was especially effective against antibiotic resistant *staphylococci*, and that further research into nisin and other lantibiotic compounds could result in promising antimicrobial alternatives (Piper *et al*., 2009). Dosler and colleagues investigated the *in vitro* effects of nisin against MRSA strains, and concluded that nisin was a good candidate for further research by itself or in combination with conventional antibiotics, such as vancomycin or ciprofloxacin (Dosler and Gerceker, 2011). Other studies have shown that nisin in combination with conventional antibiotics can promote synergistic effects (Brumfitt *et al*., 2002; Singh *et al*., 2013). An earlier study by Severina and colleagues demonstrated that nisin exhibited bactericidal effects against a large panel of Gram-positive bacteria including *MRSA*, *VRE* and *S. pneumoniae* (Severina *et al*.,

1998). In addition, nisin producing *L. lactis* strain was shown to reduce the intestinal colonization of *VRE* in a mouse infection model (Millette *et al*., 2008).

Bacteria that adhere to implanted medical devices or damaged tissue can form a biofilm and cause chronic infection (Stewart and Costerton, 2001). Biofilms are surface associated communities of microorganism that can be up to 1,000-times more resistant to antimicrobials. Treatment of these biofilms accounts for over a billion dollars in healthcare costs each year in the US (Mah and O'Toole, 2001; Gilbert *et al*., 2002). Okuda and colleagues investigated the antibiofilm effects of nisin against MRSA biofilms on medical devices and reported that nisin A compared to two other bacteriocins (lacticin Q and nukacin ISK-1) was most effective in the prevention of biofilm formation (Okuda *et al*., 2013). Recently, Ahire and Dicks demonstrated that a combination therapy of 2,3-dihydroxybenzoic acid, an antibiotic extracted from Flacourtia inermis fruit, and nisin resulted in an increase in iron concentrations that reduced biofilm formation of the *MRSA* Xen 31 strain (Ahire and Dicks, 2015).

The potential for using nisin to treat local site-specific infections has also been explored. For example, the antimicrobial effects of nisin against mastitis, respiratory, gastrointestinal, and skin infections has been reported (Table 2). In respiratory tract infections, although viral etiologies are common, these can progress to bacterial infections that further compromise the health status (Hament *et al*., 1999). The upper and lower respiratory tract is primarily infected by *S. aureus* (Micek *et al*., 2007; Weber *et al*., 2007; Bosch *et al*., 2013). De Kwaadsteniet and colleagues reported that nisin F safely inhibited the growth of *S. aureus* in the respiratory tract of immunocompromised rats (De Kwaadsteniet *et al*., 2009). Furthermore, studies have shown that nisin can

exert synergistic effects when combined with lysozyme and lactoferrin, which are both antimicrobial proteins and normally secreted in the human respiratory tract (Nattress *et al*., 2001; Murdock *et al*., 2007; De Kwaadsteniet *et al*., 2009). It was proposed that while nisin deters cell growth by binding to the lipid II precursor of the cell wall, lysozyme and lactoferrin can further damage the glycosidic bonds in the peptidoglycan wall, and sequester iron necessary for cellular respiration, respectively (Arnold and Cole, 1977; Ganz, 2004; De Kwaadsteniet *et al*., 2009).

Superficial and invasive skin and soft tissue infections are commonly caused by *S. aureus* (Fridkin *et al*., 2005; Daum, 2007). MRSA skin infections are relatively uneventful but failure to treat effectively can result in death (Dakota, 1999). Heunis and coworkers investigated the efficacy of nisin using an electrospun nanofiber wound dressing containing nisin, which diffused active nisin onto skin wounds (Heunis *et al*., 2013). In a murine excisional skin infection model, the nisin-containing wound dressing significantly reduced the *S. aureus* colonization as analyzed by bioluminescence. In addition, the wound showed signs of accelerated healing (Heunis *et al*., 2013). Mastitis is a common inflammatory disease in lactating women that causes breastfeeding cessation (Foxman *et al*., 2002). *S. aureus* and *S. epidermidis* are two common etiologic agents that cause mastitis-associated infections (Foxman *et al*., 2002). Considering the potent antimicrobial properties of nisin against *staphylococcal* strains, investigators have explored using nisin as a clinical therapeutic for mastitis. Cao and colleagues reported that a nisin-based formulation was effective in the treatment of clinical mastitis in lactating dairy cows caused by several different mastitis pathogens (Cao *et al*., 2007). In addition, Wu and coworkers demonstrated that nisin Z was

effective in treatment of subclinical mastitis caused by multiple mastitis pathogens in lactating dairy cows (Wu *et al*., 2007). Recently, Fernandez and others reported that topical nisin treatment alleviated clinical signs of mastitis and significantly reduced the *staphylococcal* count in breast milk of nisin treated women (Fernandez *et al*., 2008). Overall, as an alternative to conventional antibiotics, the latest research suggests that nisin has potential as a therapeutic against certain infectious pathogens and disease conditions.

Nisin and Oral Health

The pervasiveness of oral diseases, such as caries and periodontal diseases, remains high in developed and developing countries (Marcenes *et al*., 2013). Oral diseases are considered a major public health burden due to their high prevalence and incidence (Petersen, 2003). Therefore, research on new strategies to prevent and treat oral diseases are a focus of industry and many academic, and government institutions (Centers for Disease Control and Prevention, Chronic Disease Prevention and Health Promotion, 2015). Oral biofilms, including dental plaque, play a key role in the etiology and the progression of biofilm-associated oral diseases (Marsh, 2010; Zijnge *et al*., 2010). Enhanced antimicrobial resistance is associated with the accumulation of pathogens that cause dental caries and periodontal disease (Marsh, 2003; Aas *et al*., 2005). Nisin's potential as an oral antimicrobial was first described by Johnson and colleagues, who demonstrated that there were fewer numbers of *streptococci* in the dental plaque of monkeys that received nisin in their foods (Johnson *et al*., 1978). Later, Howell and coworkers demonstrated that a nisin-based antimicrobial mouthrinse

exhibited promising clinical results in prevention of plaque build-up and gingival inflammation in beagle dogs (Howell *et al*., 1993). Thus, the idea of using nisin to improve oral health has been around for some time.

Emerging evidence continues to support the antimicrobial properties of nisin against oral pathogenic bacteria relevant to caries and periodontal diseases. Tong and colleagues demonstrated that nisin A can inhibit the growth of cariogenic bacteria, including *Streptococcus mutans* (Tong *et al*., 2010). Scanning electron microscopy confirmed that nisin exerted bactericidal activity by forming small pores on the surface of cells (Tong *et al*., 2010). Furthermore, investigators have reported that nisin in combination with poly-lysine and sodium fluoride displayed synergistic properties in inhibiting planktonic and biofilm forms of *S. mutans* (Najjar *et al*., 2009; Tong *et al*., 2011). Nisin A has been shown to inhibit the growth of Gram-positive oral bacteria such as *Streptococcus sanguinis*, *Streptococcus sobrinus* and *Streptococcus gordonii* (Tong *et al*., 2010). In addition, Shin and coworkers demonstrated that high purity nisin Z can inhibit the growth of Gram-negative oral colonizing pathogens, including *Porphyromonas gingivalis*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* (Shin *et al*., 2015). Shin and colleagues also reported that nisin exerted anti-biofilm effects on saliva derived multi-species biofilms without causing cytotoxicity to human oral cells (Fig. 3) (Shin *et al*., 2015). As a cationic bacteriocin, nisin's mode of action may include inhibition of coaggregation of oral colonizers. Indeed, cationic antimicrobials can selectively inhibit coaggregation interactions of oral biofilm species (Smith *et al*., 1991).

In addition to dental caries and periodontal disease, nisin's potential application to other oral diseases has been explored. Investigators have reported that nisin can inhibit *Enterococcus faecalis*, which is an opportunistic Gram-positive pathogen frequently recovered from infected root canals of teeth (Stuart *et al*., 2006). In an ex vivo root canal system, nisin successfully eradicated the colonization of *E. faecalis* (Turner *et al*., 2004). Nisin, when paired with MTAD (a common intracanal irrigant, consisting of 3% doxycycline, 4.5% citric acid, and 0.5% polysorbate 80 detergent), improved the post-antibiotic sub-MIC effects of MTAD against *E. faecalis*, and made it less resistant to alkaline environments (Tong *et al*., 2014). Another potential oral application of nisin was demonstrated in the treatment of oral candidiasis. *Candida albicans* is one of the most prevalent pathogens that causes mucosal fungal infections (Pfaller *et al*., 2002; Trick *et al*., 2002). The invasion of candida species into oral epithelial cells is a signature of oropharyngeal candidiasis (Eversole *et al*., 1997; Drago *et al*., 2000; Farah *et al*., 2000). Le Lay and colleagues reported that nisin Z can significantly reduce the growth and transition of *C. albicans* (Le Lay *et al*., 2008). In addition, nisin Z has the potential to work synergistically with oral gingival cells to provide greater resistance against *C. albicans* infections (Akerey *et al*., 2009). Thus, with recent reports highlighting the therapeutic potential of nisin in oral diseases, future studies will be essential to further evaluate the potential clinical role of nisin.

Bacteriocins and Cancer: Nisin as a Cancer Therapeutic

The potential for using bacterially-derived compounds to control infectious disease also extends to controlling cancers (Frankel *et al*., 2002; Lundin and Checkoway, 2009; Nobili *et al*., 2009). For example, antimicrobial peptides have been indicated to exhibit cytotoxic effects on cancer cells and thus may have therapeutic potential (Meyer and Harder, 2007; Boohaker *et al*., 2012). Specifically, purified bacteriocins, including pyocin, colicin, pediocin, microcin, and nisin have shown inhibitory properties against neoplastic cell lines and in xenograft mouse models (Cornut *et al*., 2008; Lagos *et al*., 2009; Yates *et al*., 2012; Shaikh *et al*., 2012; Yang *et al*., 2014). This is relevant because current treatment strategies have yet to reduce cancer-related deaths below a half million per year in the USA alone (Centers for Disease Control and Prevention, Leading Causes of Death, 2015). Cancer is a complex disease characterized by the dysregulated growth of abnormal cells. Significant progress has been made in the treatment of cancers, however the majority of treatments involve surgery and chemo- and radiation therapy, which are detrimental to normal cells and tissues and cause further morbidity (Patel *et a*l., 2014; DeSantis *et al*., 2014).

Recently, Joo and colleagues explored the cytotoxic and antitumor properties of nisin A and discovered that it blocks head and neck squamous cell carcinoma (HNSCC) tumorigenesis (Joo *et al*., 2012). Nisin mediated these effects by inducing preferential apoptosis, cell cycle arrest, and reducing cell proliferation in HNSCC cells compared to primary oral keratinocytes. Nisin also reduced HNSCC tumorigenesis *in vivo* in a mouse model (Joo *et al*., 2012). Mechanistically, nisin exerted these effects on HNSCC, in part, through cation transport regulator homolog 1 (CHAC1), a proapoptotic cation transport

regulator and through a concomitant CHAC1-independent influx of extracellular calcium (Mungrue *et al*., 2009; Joo *et al*., 2012). Nisin can interact with the negatively charged phospholipid heads of the cell membrane, thereby mediating its reorganization and forming pores that allow an influx of ions (Giffard *et al*., 1996; Moll *et al*., 1997). Since HNSCC cells and primary oral keratinocytes differ in their lipid membrane composition and function, and response toward calcium fluxes, the ability of nisin to differentially alter the transmembrane potential and membrane composition of HNSCC cells may explain its predominant effects on these cells (Ponec *et al*., 1984; Ponec *et al*., 1987; Tertoolen *et al*., 1988; Eckert, 1989; Gasparoni *et al*., 2004; Tripathi *et al*., 2012). Indeed, recent reports support this premise as the basis for the nisin-mediated differential apoptotic cell death and reduced proliferation of HNSCC cells compared to primary keratinocytes (Schweizer, 2009).

Recently, Kamarajan and coworkers focused on investigating the translational potential of a high purity form of nisin Z for the treatment of HNSCC (Kamarajan *et al*., 2015). The data support the role of nisin as an alternative therapeutic for HNSCC, since nisin promoted HNSCC cell apoptosis, suppression of HNSCC cell proliferation, inhibition of angiogenesis, inhibition of HNSCC orasphere formation, inhibition of tumorigenesis *in vivo*, and it prolonged survival *in vivo* (Fig. 4) (Kamarajan *et al*., 2015). Considering that the FDA has approved 83.25 mg/kg in humans as the no-observed-effect-level (NOEL) for nisin (66.7 mg/kg was used in mice as a cancer therapeutic dose), this study demonstrated the promising potential for nisin as an anti-cancer agent. In addition, Preet and colleagues demonstrated that combining doxorubicin, a conventional cancer drug, with nisin can potentiate the effectiveness of the treatment in

terms of decreasing tumor severity in skin carcinogenesis (Preet *et al*., 2015). Therapeutic strategies for utilizing nisin alone or in combination with other conventional drugs to treat cancer are still at an early stage. However, the few studies that have been reported demonstrate the significant anti-cancer potential of using nisin as a promising alternative or adjunctive therapeutic. Furthermore, increasing evidence suggests an etiologic linkage between the microbiome and cancers (Wroblewski *et al*., 2010; Bultman, 2014). Recent studies indicate that certain bacteria (i.e. oral bacteria) may promote carcinogenesis in humans (Ahn *et al*., 2012; Michaud and Izard, 2014). In these scenarios, it is possible that nisin may have dual benefits by altering or disrupting the microbiome and inhibiting the growth of cancer cells. Thus, nisin may be a useful therapeutic since it exerts both antimicrobial/biofilm and anti-cancer properties.

Immunomodulatory Role of Nisin

Host-defense peptides (HDPs) are ubiquitous in nature. HDPs are small amphiphilic cationic peptides, which play an essential role in the innate immune response (Sahl and Bierbaum, 2008). Almost all living organisms use antimicrobial peptides or HDPs as an innate defense mechanism. Interestingly, despite differences in size and native structure, HDPs and bacterially secreted bacteriocins share similar physicochemical properties (Hancock and Sahl, 2006). Nisin is both a cationic and amphiphilic peptide, and thereby mediates diverse effects on membrane processes similar to HDPs (Cotter *et al*., 2005). Pablo and colleagues demonstrated that short-term dietary administration of nisin (as Nisaplin, containing 2.5% nisin A, 77.5% NaCl and non-fat dried milk) resulted in an increase in CD4 and CD8 T-lymphocytes, while

decreasing the B-lymphocyte levels (Pablo *et al*., 1999). In addition, prolonged administration of nisin resulted in a return to normal levels of both B- and T-lymphocytes (Pablo *et al*., 1999). This study provided the first evidence of nisin's influence on the immune system of mice. Recently, Begde and coworkers reported that nisin was able to activate neutrophils, and suggested that nisin may be influencing multiple subsets of host immune cells (Begde *et al*., 2011). Considering that nisin appears to behave similar to HDPs, it is possible that the immunomodulatory properties associated with HDPs may also apply to nisin (Kindrachuck *et al*., 2013). Bacteriocins were once thought to have a very limited role in disrupting bacterial membranes and exerting bactericidal activity. However, Kindrachuck and colleagues demonstrated that purified nisin Z was capable of modulating the innate immune response by inducing chemokine synthesis and suppressing LPS-induced pro-inflammatory cytokines in human peripheral blood mononuclear cells (Kindrachuck *et al*., 2013). Furthermore, nisin Z promoted immunomodulatory responses within both *ex vivo* and *in vivo* model systems (Kindrachuck *et al*., 2013). These reports underscore nisin's significant potential for use in a variety of human diseases that are mediated by the host immune response and pathogenic biofilms, like periodontal disease. Given that the periodontal lesion is characterized by an initial burst of neutrophils that is followed by a B- and T-cell mediated immune response in its later stages, nisin could play a significant therapeutic role in modifying both the immune and biofilm signature of this lesion (Page and Schroeder, 1976). The ability of nisin to alter the host immune response provides yet another opportunity for its potential use within health care settings. Since information

regarding the role of nisin in modulating the host immune response is limited, this area merits further examination.

Resistance to Nisin

Bacteriocins have different modes of action when compared to antibiotics (Cleveland *et al*., 2001; Cotter *et al*., 2005). Specifically, lantibiotic bacteriocins, such as nisin, require a docking molecule (lipid II), through which they target cells by forming pores in the membrane. This depletes the transmembrane potential and/or the pH gradient and results in the leakage of cellular materials (Peschel and Sahl, 2006). Although binding of lipid II is similar to other antibiotics such as vancomycin, nisin is unique in that it can span the entire membrane by using the pyrophosphate cage as the anchoring point (Hsu *et al*., 2004). Some evidence suggests that resistance against nisin can arise from mutations that induce changes in the membrane and cell wall composition (thickening of the cell wall to prevent the nisin binding to lipid II), reducing the acidity of the extracellular medium to stimulate the binding of nisin to the cell wall and induce degradation, prevent the insertion of nisin into the membrane, and transport or extrude nisin out across the membrane (Mantovani and Russell, 2001; Kramer *et al*., 2006; Kramer *et al*., 2008). These changes may occur independently or together and have been described as physiological adaptations (Sun *et al*., 2009). The cellular mechanisms of resistance to nisin are, however, still not well understood. One key reason for this stems from the fact that only a few examples of nisin resistance have emerged and only under laboratory conditions.

Lipid II plays an essential role in bacterial cell wall biosynthesis and growth, and nisin initiates its mode of action by binding to lipid II with high affinity (Breukink *et al*., 1999; Wiedemann *et al*., 2001). Kramer and colleagues tested whether nisin resistance could result from differences in the lipid II levels of Gram-positive bacteria (Kramer *et al*., 2004). Those studies suggested that there was no direct role for lipid II in nisin resistance as there was no correlation with the amount of lipid II present and increase in resistance (Kramer *et al*., 2004). It was recently reported that lack of antibiotic resistance to a newly described antibiotic was due to its targeting the highly conserved lipid II component of bacteria; nisin may be working in the same way (Ling *et al*., 2015).

Nisinase is a dehydropeptide reductase that can inactivate nisin through an enzymatic reaction (de Freire Bastos *et al*., 2014; Draper *et al*., 2015). Nisinase activity has *been* detected in *Lactobacillus plantarum*, *Streptococcus thermophiles*, *Clostridium botulinum*, *L. lactis* sub-species cremoris, *E. faecalis* and *S. aureus* (Kooy, 1952; Carlson and Bauer, 1957; Alifax and Chevalier, 1962; Rayman et al., 1983). However, despite all of the reports suggesting the presence of nisinase in several different species, there has not been a conclusive study indicating the presence of nisinase in *L. lactis* (Pongtharangku and Demirci, 2007). In addition, Sun and coworkers reported that nisin resistance protein (NSR) is a nisin degrading protease that non-nisin producing bacteria can produce as a novel mechanism for nisin resistance (Sun *et al*., 2009). NSR was capable of proteolytically cleaving the C-terminal tail of nisin, thereby inactivating and reducing nisin's antimicrobial activity by a 100-fold (Sun *et al*., 2009).

Currently, the majority of studies on the mechanisms of nisin resistance have been focused on single foodborne pathogens, such as *Listeria monocytogenes*

(Crandall and Montville, 1998). A number of mechanisms are now known to contribute to and affect nisin resistance, including environmental stress and specific genetic components (Mantovani and Russell, 2001; Gravesen *et al*., 2001; Thedieck *et al*., 2006; Begley *et al*., 2010). As the applications of nisin expand even further into the biomedical field, it will be critical to study and monitor the development of nisin resistance in pathogenic organisms and cells relevant to disease processes. Antibiotic resistance is not an uncommon phenomenon, however, bacteriocins such as nisin are distinctly different from conventional antibiotics in both their synthesis and mode of action (Cleveland *et al*., 2001). Thus, characterization of specific genetic or protein components that may contribute to nisin resistance will be important to better understand any potential resistance issues that may arise in clinical settings.

Concluding Remarks: Outlook

In recent years, nisin research has shown its potential use in a broad range of fields, including food biopreservation and biomedical applications. Among different classes of lantibiotics, nisin is the most well-known and best-studied lantibiotic (Benmechernene *et al*., 2013). Considering that variants of nisin are now available in high purity forms from numerous commercial vendors, it is projected that more studies on different applications of nisin will be published. In addition, the mode of action of nisin in the context of human systems and disease will be better understood for newer biomedical applications. Currently, antibiotic resistance is a major concern in the food and biomedical industries. Until now, nisin has shown promising laboratory and clinical results as a useful therapeutic agent. Furthermore, different variants and forms of nisin

may be combined with conventional drug(s) to promote synergistic outcomes. Further validation of nisin's usefulness in biomedical fields will require *in vivo* studies to evaluate its efficacy. Although nisin has been associated with the development of minimal resistance, it will be critical to continue surveying for potential novel mechanisms of nisin resistance *in vitro* and *in vivo*. There is still much knowledge to be gained, however current findings support the incorporation of nisin and/or other bacteriocins into a variety of disease therapies.

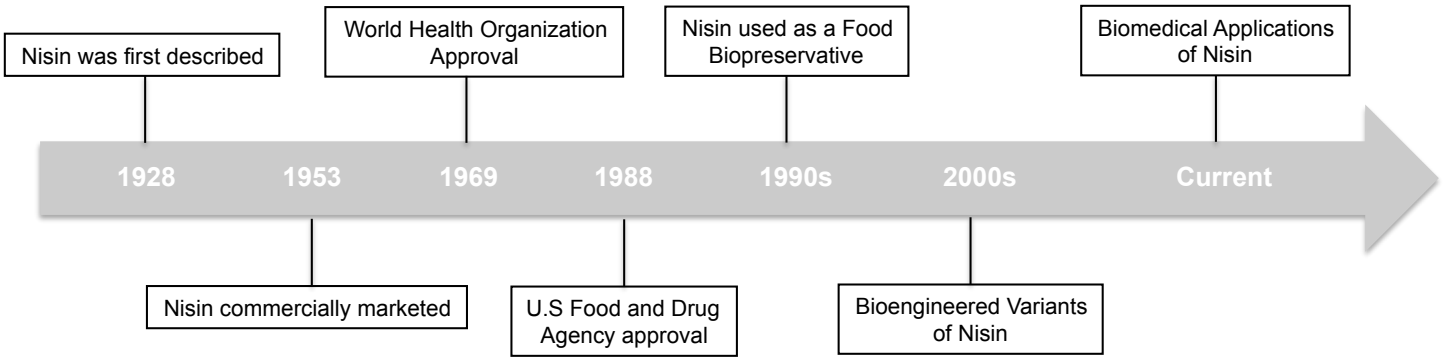


Figure IV.1. Timeline of Nisin Development

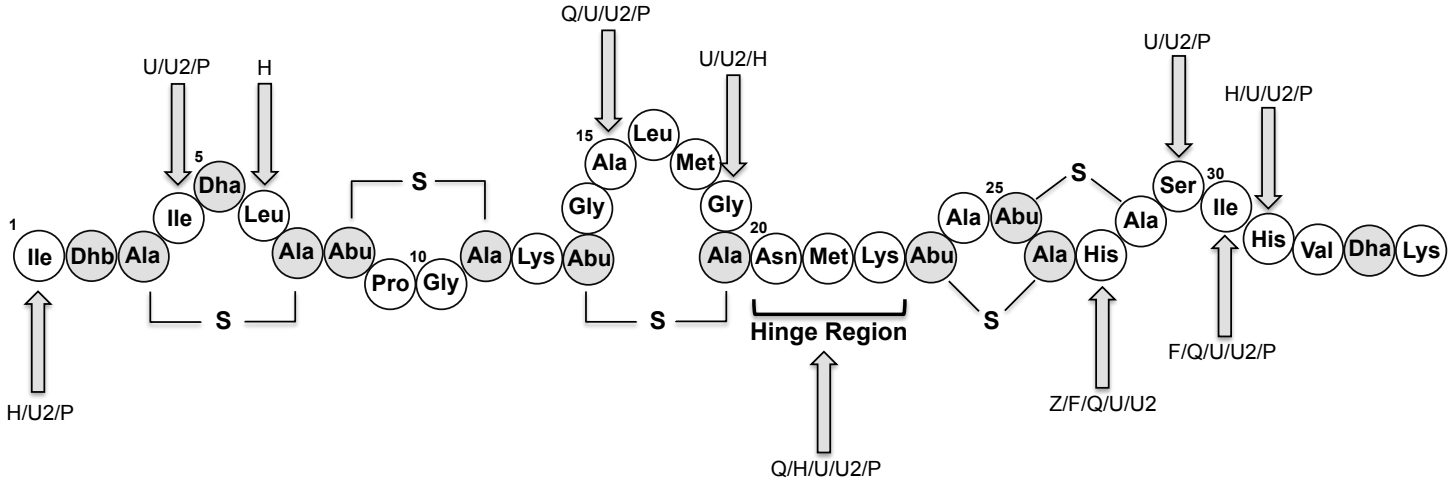


Figure IV.2. Peptide Structure of Nisin. Modified amino acids are colored gray with black letters. Dha, dehydroalanine (from Alanine); Dhb, dehydrobutyrine (from Threonine); Ala-S-Ala, lanthionine; Abu-S-Ala; β-methyllanthionine. The hinge region is composed of Asparagine-Methionine-Lysine. Arrows indicate the sites of amino acid substitutions for natural variants.

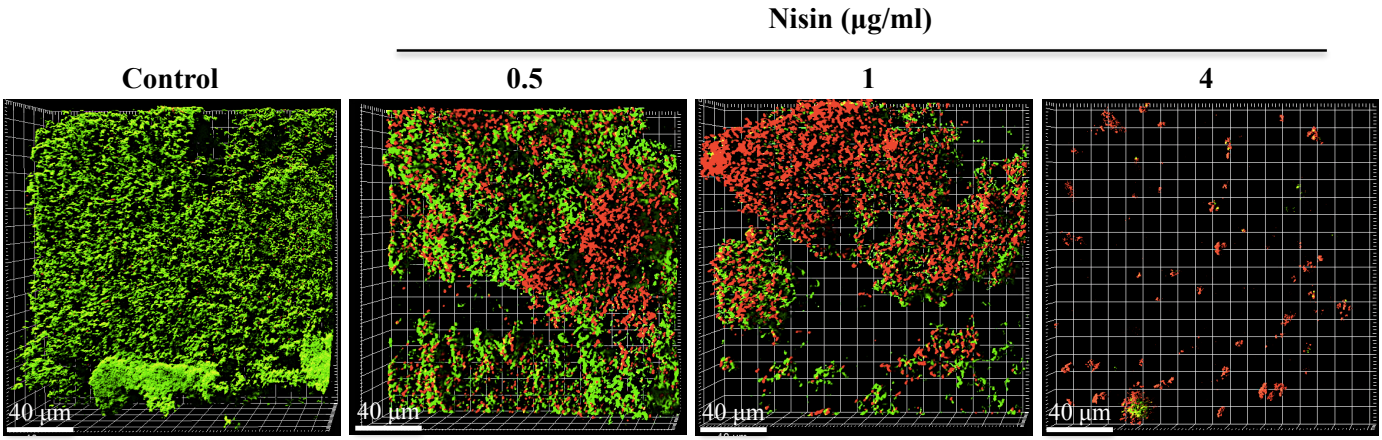


Figure IV.3. Nisin Inhibits the Formation of Multi-Species Biofilms. Cell containing saliva was added, then fed filter sterilized cell free saliva for 20–22 h at 37°C with or without nisin. Confocal microscopy images are represented in the x–y plane. A green signal indicates viable live cells (Syto 9) and a red signal indicates damaged/dead cells (propidium iodide). These images were previously published (Shin *et al*., 2015).

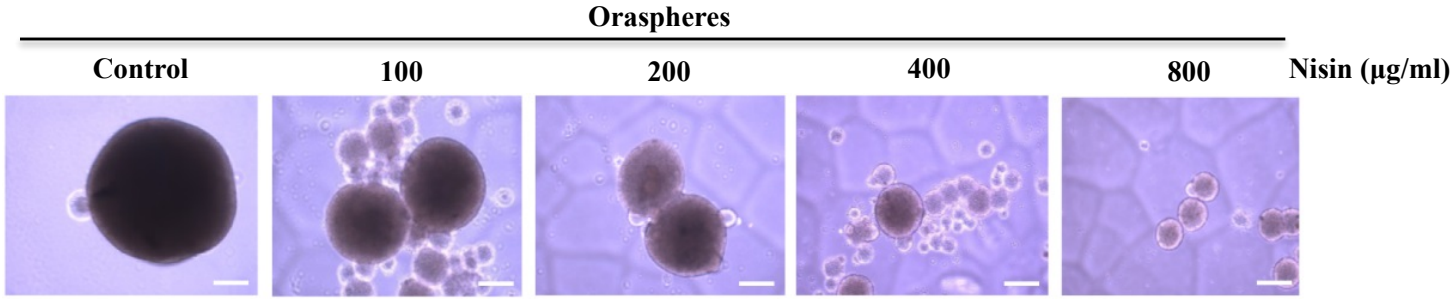


Figure IV.4. Nisin Z Inhibits Orasphere Formation in HNSCC Cells. Phase contrast images of oraspheres in HNSCC cells (UM-SCC-17B) cultured under suspension conditions and treated with control media or media containing nisin Z (100 to 800 µg/ml) for 36 h. These images were previously published (Kamarajan *et al*., 2015)

Natural Variants	Unmodified Amino Acid Sequences	Origin
Nisin A	ITSISLCTPGCKTGALMGCNMKTATCHCSIHVSK	*Lactococcus lactis* strains (Gross *et al*., 1971)
Nisin Z	ITSISLCTPGCKTGALMGCNMKTATCNCSIHVSK	*Lactococcus lactis* NIZO 22186 (Mulders *et al*., 1991)
Nisin F	ITSISLCTPGCKTGALMGCNMKTATCNCSVHVSK	*Lactococcus lactis subsp. lactis* F10 (de Kwaadsteniet *et al*., 2008)
Nisin Q	ITSISLCTPGCKTGVLMGCNLKTATCNCSVHVSK	*Lactococcus lactis* 61-14 (Zendo *et al*., 2003)
Nisin H	FTSISMCTPGCKTGALMTCNYKTATCHCSIKVSK	*Streptococcus hyointestinalis* (O'Connor *et al*., 2015)
Nisin U	ITSKSLCTPGCKTGILMT CPLKTATCGCHFG	*Streptococcus uberis* (Wirawan *et al*., 2006)
Nisin U2	VTSKSLCTPGCKTGILMT CPLKTATCGCHFG	*Streptococcus uberis* (Wirawan *et al*., 2006)
Nisin P	VTSKSLCTPGCKTGILMT CAIKTATCGCHFG	*Streptococcus galloyticus subsp. Pasteurianus* (Zhang *et al*., 2012)
Bioengineered Variants		
Nisin A S29A	ITSISLCTPGCKTGALMGCNMKTATCHCAIHVSK	*L. lactis* NZ9800 (Field *et al*., 2012)
Nisin A S29D	ITSISLCTPGCKTGALMGCNMKTATCHCDIHVSK	*L. lactis* NZ9800 (Field *et al*., 2012)
Nisin A S29E	ITSISLCTPGCKTGALMGCNMKTATCHCEIHVSK	*L. lactis* NZ9800 (Field *et al*., 2012)
Nisin A S29G	ITSISLCTPGCKTGALMGCNMKTATCHCGIHVSK	*L. lactis* NZ9800 (Field *et al*., 2008)
Nisin A K22T	ITSISLCTPGCKTGALMGCNMTTATCHCSIHVSK	*L. lactis* NZ9800 (Field *et al*., 2008)
Nisin A N20P	ITSISLCTPGCKTGALMGCPMKTATCHCSIHVSK	*L. lactis* NZ9800 (Field *et al*., 2008)
Nisin A M21V	ITSISLCTPGCKTGALMGCNVKTATCHCSIHVSK	*L. lactis* NZ9800 (Field *et al*., 2008)
Nisin A K22S	ITSISLCTPGCKTGALMGCNMSTATCHCSIHVSK	*L. lactis* NZ9800 (Field *et al*., 2008)
Nisin Z N20K	ITSISLCTPGCKTGALMGCKMKTATCNCSIHVSK	*L. lactis* NZ9800 (Yuan *et al*., 2004)
Nisin Z M21K	ITSISLCTPGCKTGALMGCNKKTATCNCSIHVSK	*L. lactis* NZ9800 (Yuan *et al*., 2004)

Table IV.1. Natural and Bioengineered Variants of Nisin. Amino acids in white letters indicate the flexible hinge region. Yellow highlights indicate amino acid substitutions compared to nisin A. Please note that this table does not contain all variants that have been reported to date.

Disease	Nisin	Model	Results	References
Infections associated with drug resistant pathogens				
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin exhibited bactericidal effect against a large panel of Gram-positive bacteria including MRSA, *S. pneumoniae* and *enterococci*	Severina *et al*., 1998
	Nisaplin (2.5% w/w purity)	*In vitro*	Nisin exhibited bactericidal effects against clinical isolates of *S. pneumoniae*, including penicillin- and other drug-resistant strains	Goldstein *et al*., 1998
	Nisin A (> 95% purity)	*In vitro*	Nisin was active and highly bactericidal against *C. difficile*. Nisin was not absorbed by the gastrointestinal tract and did not have indiscriminate activity against all bowel flora or all anaerobes	Bartoloni *et al*., 2004
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin was active against drug resistant *S. aureus*	Piper *et al*., 2009
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin exhibited bactericidal effect against both MSSA and MRSA strains. In addition, it enhanced the activity of ciprofloxacin and vancomycin when used in combination	Dosler *et al*., 2011
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin exhibited bactericidal activity against both MRSA and other staphylococcal biofilms grown on medical devices	Okuda *et al*., 2013
	Nisaplin (2.5% w/w purity)	*In vitro*	Nisin incorporated with 2,3-dihydroxybenzoic acid in nanofibers inhibited formation of MRSA biofilms	Ahire and Dicks, 2015
Gastrointestinal Infections				
	Nisin A (> 95% purity)	*In vitro*	Nisin did not disrupt the intestinal epithelial integrity, suggesting that it may be suitable for the treatment of gastrointestinal tract infections	Maher and McClean, 2006
	Nisin A and Z (> 95% purity)	*In vitro*	Nisin A and Z exhibited similar inhibition effect against a broad range of intestinal Gram-positive bacteria	Blay *et al*., 2007
	Nisaplin	*In vitro*	Nisin was tableted with a	Ugurlu *et al*.,

	(2.5% w/w purity)		pectin/HPMC mixture to form an enzymatically controlled delivery system for potential colonic drug delivery	2007
	Nisin Z	*In vitro* and Mice	Nisin producing strain *L. lactis* modulated the intestinal microbiota and reduced the intestinal colonization of vancomycin-resistant *enterococci* in infected mice.	Millette *et al*., 2008
	Nisin A and Z (unknown purity)	*Ex vivo* using jejunal chyme from fistulated dogs	Nisin was insensitive to degradation by the components of the jejunal chyme	Reunanen and Saris, 2009
	Nisin F (Purity in arbitrary units)	*In vitro* and Mice	Nisin may have a stabilizing effect on the bacterial population of the gastro intestinal tract	van Staden *et al*., 2011
Respiratory Infections				
	Nisin F (Purity in arbitrary units)	*In vitro* and Rats	Nisin was used to control intranasal *S. aureus* infection	De Kwaadsteniet *et al*., 2009
	Nisaplin (2.5% w/w purity)	*In vitro* and Mice	Low blood and tissue levels of nisin were sufficient to prevent the death of mice infected with *S. pneumoniae*	Goldstein *et al*., 1998
Skin and Soft Tissue Infections				
	Nisaplin (2.5% w/w purity)	*In vitro* and Mice	Nisin-containing nanofiber wound dressings significantly reduced *S. aureus* induced skin infections	Heunis *et al*., 2013
Mastitis				
	Nisin Z (18000 IU/mg)	Cows	Intramammary administration of nisin was effective in the treatment of mastitis in lactating dairy cows	Cao *et al*., 2007 Wu *et al*., 2007
	Nisin A (Approximately 6 µg/ml)	*In vitro* and Human	Topical treatment of nisin was effective in the treatment of staphylococcal mastitis	Fermandez *et al*., 2008
Cancer				
	Nisin A (2.5% w/w purity)	*In vitro* and Mice	Nisin reduced HNSCC tumorigenesis by inducing preferential apoptosis, cell cycle arrest, and reducing cell proliferation in HNSCC cells	Joo *et al*., 2012
	Nisin A (2.5% w/w purity)	*In vitro* and Mice	Combination of nisin and doxorubicin decreased tumor severity in skin carcinogenesis	Preet *et al*., 2015
	Nisin AP and ZP (* P stands for pure;	*In vitro* and Mice	Nisin promoted HNSCC cell apoptosis, suppression of HNSCC	Kamarajan *et al*., 2015

	95% purity)		cell proliferation, inhibition of angiogenesis and cancer orasphere formation. Nisin inhibited tumorigenesis and prolonged survival of mice	
Oral Health				
	Nisaplin (2.5% w/w purity)	Monkeys	Nisin reduced the numbers of *streptococci* in the dental plaque of monkeys that received nisin in their foods	Johnson *et al*., 1978
	Nisin (Ambicin N) (unknown purity)	Dogs	Nisin based mouthrinse prevented plaque build-up and gingival inflammation in beagle dogs	Howell *et al*., 1993
	Nisin A (2.5% w/w purity)	*Ex vivo* using the root canals of human teeth	Nisin eradicated the colonization of *E. faecalis*	Turner *et al*., 2004
	Nisin Z (unknown purity)	*In vitro*	Nisin significantly reduced the growth and transition of C. albicans	Le Lay *et al*., 2008
	Nisin Z (unknown purity)	*In vitro*	Nisin may work synergistically with oral gingival cells to provide greater resistance against C. albicans infections	Akerey *et al*., 2009
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin inhibited the growth of cariogenic bacteria, including *S. mutans*	Tong *et al*., 2010
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin in combination with poly-lysine and sodium fluoride displayed synergistic effects in inhibiting planktonic and biofilm forms of *S. mutans*	Najjar *et al*., 2009; Tong *et al*., 2011
	Nisin A (2.5% w/w purity)	*In vitro*	Nisin paired with MTAD improved post-antibiotic sub-MIC effects of MTAD against *E. faecalis*	Tong *et al*., 2014
	Nisin ZP (* P stands for pure; 95% purity)	*In vitro*	Nisin inhibited growth of Gram-positive and Gram-negative oral pathogens and saliva derived multi-species biofilms without cytotoxicity to human oral cells	Shin *et al*., 2015

Table IV.2. Overview of Biomedical Applications of Nisin.

Chapter V

The Effect of L-Arginine on Destabilization of Oral Biofilms Developed in a Swinnex Model System

Chapter V is adapted from a manuscript in preparation (Shin *et al*., 2017b).

Abstract

Objectives: *In vitro* model systems are essential for studying biofilms and anti-biofilm strategies. For this chapter, we developed a novel microfluidic biofilm model, the modified Swinnex biofilm system, to evaluate the effects of L-arginine and other anti-biofilm agents on multi-species oral biofilms.

Methods: Biofilms were cultured for 40 h in Swinnex using pooled human saliva and were treated with water, L-arginine (Arg; 50 – 400 mM), L-lysine (Lys; 400 mM), or cetylpyridinium chloride (CPC; 0.075%). Biofilm parameters such as biovolume, thickness, roughness, viability and biomass of the sloughed aggregates (B_{ABD}) were captured using confocal microscopy and FlowCam®. In addition, water and Arg-treated biofilms and dispersed bacterial cells were characterized using 16S rRNA sequencing.

Results: After the short exposure with Arg, a dose-dependent reduction in biofilm biovolume and thickness was observed. Significant biomass reduction was observed for biofilms treated with 400 mM Arg compared against water and Lys-treated samples. Increasing the Arg concentrations led to a greater dispersal of the biofilm cells relative to their biomass and the number of aggregates dispersed. No antimicrobial effects were observed in Arg-treated biofilms. Short-term exposure of Arg did not significantly alter the biofilm community composition relative to control. This suggests that the observed physicochemical effect of Arg on biofilms is non-specific, however, Arg can significantly impact the biofilm architecture to promote biofilm destabilization.

Conclusions: The Swinnex model allows the study of various aspects of biofilm biology and is particularly useful in studying biofilm dispersion in conjunction with confocal microscopy and FlowCam®. High concentrations of Arg promote biofilm destabilization and de-adhesion through non-specific biofilm dispersion mechanism.

Introduction

Oral biofilms can constitute small microcolonies of cells or be as visually conspicuous dental plaque. The biofilm architecture provides a microenvironment for cell-to-cell interactions (ie. autoaggregation and coaggregation), nutrients and signal sharing (ie. quorum sensing), and protection against different environmental stresses (ie. shear force, physical and mechanical and chemical) (Stoodley *et al*., 2004). In comparison to the planktonic species, biofilm-associated bacteria are highly resistant to antimicrobial challenges (Mah and O'Toole, 2001). Based on several factors such as location, species composition and biomass, the accumulation of cells and the associated development of biofilms can lead to dental caries, periodontal disease and systemic infections (Marsh 2003; Li *et al*., 2010). Thus, to reduce the health burden levied by oral biofilms, the use of novel anti-biofilm agents to hinder development would offer a valuable alternative or adjunct to antimicrobial treatments.

One approach to prevent biofilm development is to promote destabilization. To promote biofilm destabilization, several strategies can be implemented. For example, dispersin B is a matrix-degrading enzyme that causes the biofilm structure to break apart (Kaplan 2010). Oral biofilm inactivating agents such as chlorhexidine gluconate (CHX) and cetylpyridinium chloride (CPC) interact with the membrane to not only have antimicrobial effects but to also promote biofilm destabilization (Hope and Wilson, 2004; Rao *et al*., 2005). Recently, evidence has shown that L-arginine (Arg) can alter the architecture of the oral biofilms by interfering with interbacterial coaggregation and shift the biofilm composition (Kolderman *et al*., 2015). In addition to the destabilizing effects,

Arg has also been shown to enhance the ecological benefit of fluoride by enriching the growth of alkali-generating bacteria in the mixed-species biofilm community (Zheng *et al*., 2015).

Previously, studies have mostly focused on the long-term effects of Arg on biofilm ecology (Nascimento *et al,*. 2014). The extended exposure of high concentrations of Arg have been shown to prolong the destabilization effects and prevent growth of specific pathogens (Kolderman *et al*., 2015; He *et al*., 2016). In this study, we explored the short-term effects of Arg on the multi-species oral biofilm. The *in vitro* complex dental plaque-like biofilm communities were developed using the modified Swinnex biofilm model system (Fig. 1). Furthermore, as an innovative approach, the biofilm dispersion was directly measured through a real-time particle analyzer (FlowCam®; Levin-Sparenberg *et al*., 2015). Understanding the anti-biofilm mechanisms of Arg can lead to new drug development and formulations for improving oral health. The aims of this study was to i) develop and validate a novel *in vitro* microfluidic model system to study the multi-species biofilm physiology, ii) study the effects of Arg and other anti-biofilm agents on oral biofilms, iii) evaluate and quantify the biofilm dispersion effects of Arg in real-time, and lastly iv) characterize the biofilm communities and the bacterial cells that were removed by Arg treatment through 16S rRNA next generation sequencing.

Materials and Methods

L-Arginine, L-Lysine and CPC Preparation

L-arginine monohydrochloride (Arg; Sigma Alrich, USA), L-lysine monohydrochloride (Lys; Sigma Alrich, USA) stock solutions were prepared in sterile distilled water or molecular grade pure water (used for sequencing). Arg concentrations were adjusted to 50, 100 and 400 mM. Water was used as a negative control. L-lysine (400 mM) and cetylpyridinium chloride monohydrate (CPC; 0.075%; EMD Millipore, USA) was prepared in sterile distilled water as positive controls.

Biofilm inoculum and nutrient collection and preparation

Using a protocol reviewed by the University of Michigan Health Sciences and Behavior Sciences Institutional Review Board (HUM00042954), human saliva was pooled from 5 individuals and prepared as the biofilm inoculum and growth medium. The saliva collection and preparation protocol was adapted from a previous study (Shin *et al*., 2015). The exclusion criteria for the saliva donors were non-smokers who have not taken antibiotics in the past three months, had not consumed any food or beverages besides water at least 2 h prior to collection. The pooled saliva was prepared for one of two purposes: cell-containing saliva (CCS) to grow the biofilms and cell-free saliva (CFS) as the nutrient growth media.

Swinnex Biofilm Model System

A 13 mm Swinnex filter holder (EMD Millipore, USA) was used as the biofilm reactor (Fig. 1A). Brown, hydrophilic polycarbonate (PC) membrane with 0.22 µm pores (EMD Millipore, USA) was used as the biofilm substratum (Fig. 1A). The Swinnex filter holder was connected to a 5 or 10 ml syringe (Becton Dickinson, USA) operated by an automated syringe pump (Chemyx Inc., USA). Up to 10 syringes connected to the Swinnex filter holder were used per run. White nylon male and female luer style thread connectors (Cole Parmer, USA) were used to connect the Swinnex filter holder to the syringe via clear peroxide-cured silicon precision pump tubing (Masterflex, USA). Biofilms were grown at a constant 37 degree Celsius in an incubator (Model 132000, Boekel Scientific, USA).

Following the assembly of O-ring, PC membrane, and the Swinnex filter holder, the Swinnex was autoclaved prior to each use (Fig. 1B). Two hundred microliter of CFS was used to pre-treat the substratum surface for 20 minutes to allow salivary pellicle formation, and to promote cell attachment and biofilm development (Fig. 1C). Two hundred microliter of CCS was then added, and connected to the syringe pump with CFS-containing syringes (Fig. 1D). At a flow rate of 5 µl/min, biofilms were grown for 20 h in an upright position (Fig. 1D). Following the initial growth, the Swinnex was re-oriented for second growth period for another 20 h, in an upside down position to remove unattached planktonic bacteria and cellular debris (Fig. 1E). The biofilm growth protocol was adapted after optimization of growth parameters needed for generating reproducible biofilms using human saliva in the Swinnex biofilm model system.

Following the 40 h growth period, biofilms were treated with water, L-lysine (400 mM), L-arginine (50, 100, 400 mM), or CPC (0.075%) solution for up to 1 or 10 minutes at a flow rate of 20 µl/min. Biofilms were either stained with Live/Dead Bacterial Viability kit (Syto-9 and Propidium iodide, Invitrogen, USA) for confocal microscopy or connected to the FlowCam® for real-time analysis of spent biofilm aggregates and cells (Fig. 1G, H).

Confocal Laser Scanning Microscopy and Quantitative Analysis of Biofilms

The effects of Arg on biofilm architecture were quantified using confocal microscopy and image analysis. The biofilm was carefully mounted on a 24 x 60 mm micro cover glass (VWR, USA) and washed to remove planktonic bacteria and debris. Following different treatments, biofilms were stained with Syto-9 (green signal) and propidium iodide (red signal) to visualize the biofilm under a confocal laser scanning microscope (CLSM) with a HCX PL APO 40X/0.85 CORR CS objective (SPE, Leica, USA). Experiments were conducted in duplicates and three representative images were captured for each biofilm (n=12). Independent experiments were repeated at least twice. Microscopy images were rendered with Imaris (Bitplane, Switzerland) and the image stacks were quantified using Comsat2 (Heydorn *et al*., 2000) to determine the biofilm biovolume (total amount of space/biomass occupied by a biofilm), average thickness (total thickness of a biofilm from the substratum to the glass surface) and average roughness (heterogeneity in biofilm thickness). The degree of cell damage/death was evaluated using ImageJ (National Institute of Health) based on the ratio of pixel intensity between Syto-9 and PI as previously reported (Nance *et al*., 2013; Shin *et al*., 2015).

FlowCam® Imaging System

The FlowCam® VS series model was used for this study (Fluid Imaging, USA). For each experiment, a single Swinnex filter holder was connected to a syringe on the syringe pump and a flow cell unit of the FlowCam®. Olympus UPlanFL N10X/0.30 objective (Olympus, USA) was used for imaging cells. Images were acquired at a rate of 10 frames per second with a flash duration of 8 µSec. The particle size was measured as area based diameter (ABD), which is the diameter of the sphere that has the same surface area as a given particle, referred here as the biomass of the bacterial aggregates (B_{ABD}). The filter gating was set at 10 to 5000 µm. Biofilms were treated with water, L-lysine (400 mM), L-arginine (50, 100, 400 mM), or CPC (0.075%) solution for 1 min at a flow rate of 20 µl/min. Four independent biofilm samples were tested for each treatment condition. Only the cells that were properly focused were considered for data analysis.

Microbiome Analysis

The samples that were collected for the 16S rRNA sequencing included 1) Control, water-treated biofilms, 2) Arg-treated biofilms, 3) dispersed bacterial cells from the control biofilms, and 4) dispersed bacterial cells from the Arg-treated biofilms. DNA was extracted from the samples and was normalized to 5 ng/µl per sample prior to running polymerase chain reactions (PCR). The sequencing was performed based on the V4 hypervariable region of the 16S rRNA gene (Caporaso *et al*., 2011). For the genome sequencing, Ion Torrent Personal Genome Machine (PGM) was utilized as previously reported (www.mrdnalab.com, USA; Fernandez *et al*., 2017). The sequenced

raw 16S data were processed with QIIME 1.9.0. Samples with read counts less than 20000 reads preprocessing were excluded for the microbiome analysis. Standard operating procedures were followed as previously reported for the sequence processing (Caporaso *et al*., 2010; McMurdie and Holmes, 2013; Fernandez *et al*., 2017).

Downstream analytics included Shannon alpha diversity, community relative abundance, and weighted UniFrac beta diversity. Outcomes were measured within the Phyloseq package and graphical output generated with R's ggplot package. Additionally, beta diversity was visualized in 2-dimensional space with principal component analysis (PCA) using R's built-in prcomp function. Log-transformed read counts of the operational taxonomic unit (OTU) table were used as input for PCA.

Statistical Analysis

The data values for the biofilm biovolume and thickness are expressed as mean values ± standard deviation. The B_{ABD} data values are expressed as mean ± standard error. Independent one-way t-tests were used to compare the controls (water, L-lys, CPC) with Arg-treated samples. Data values were considered significant if the P value was < 0.05 and highly significant if < 0.01.

Differences in the Shannon diversity index and the Euclidean distance for between groups were tested using a non-parametric Kruskal-Wallis test. Differences in the phyla and genus abundances were evaluated using a non-parametric Mann-Whitney U test. An alpha significance threshold of 0.05 was used for the Kruskal-Wallis test and an alpha significance threshold of 0.01 was used for the Wald negative

binomial test. For the PCA, an Adonis test that fits linear models to weighted UniFrac distance matrices was performed with R's vegan package.

Results

Validation of the Swinnex Biofilm Model System

Saliva derived oral biofilms were developed successfully using the Swinnex model system (Fig. 1). After 40 h growth period, abundant biofilm biomass was obtained on the surface of the PC membrane (Fig. 2A). To validate the model system for evaluating the effects of anti-biofilm agents, the biofilms were treated with water (control) or CPC (0.075%) for 10 min. The control biofilms exhibited significantly greater biofilm biovolume and thickness compared to the CPC-treated biofilms (Fig. 2A and B). The roughness was significantly increased in CPC-treated biofilms (Fig. 2B). In addition, there was a significant increase in the antimicrobial activity in the CPC-treated biofilms as indicated by the amount of cell damage occurred (red signal) in the biofilms (Fig. 2A and B). The average viability of biofilms were 94.29% to 21.33% for the control and CPC-treated biofilms, respectively. (Fig. 2B)..

The Effects of L-Arginine on Oral Biofilms

After a 10 min exposure, the biofilms treated with Arg (50, 100 and 400 mM) showed a decrease in biofilm biovolume and thickness in a dose-dependent manner. The mean biovolume and thickness of the control biofilms were 605.58 μm^2, 19.01 $\mu m^3/\mu m^2$ and 31.54 μm (Fig 3A and B). The average biovolume was significantly deceased for biofilms treated with Arg (50, 100, 400 mM) and Lys (400 mM). Arg and Lys treated biofilms exhibited a significant decrease in biofilm thickness (Fig. 3B). Only the 400 mM Arg-treated biofilms exhibited a significant increase in roughness (Fig. 3B).

When comparing the Arg and Lys at 400 mM for their anti-biofilm effects, Arg was superior in reducing the biofilm biovolume and thickness (Fig. 3B). Although the viability was statistically lower in both the Arg and Lys-treated biofilms compared to the control biofilms, the biofilms exhibited minimal membrane damage as indicated by the qualitative and quantitative biofilm analysis (Fig. 3B).

The Dispersion Effect of L-Arginine on Oral Biofilms

The Swinnex filter holder was directly connected to the FlowCam® device to capture the biofilm aggregates and cells dispersed upon different treatment conditions (water, Arg, Lys and CPC). The rate of biofilm dispersion was measured for 1 min. The average B_{ABD} of biofilm aggregates of water-treated biofilms were 133.02 µm (Fig. 4A and B). The average B_{ABD} of biofilm aggregates of Arg-treated biofilms were 153.50, 163.46, and 814.44 µm for Arg concentrations of 50, 100 and 400 mM, respectively (Fig. 4A and B). The average B_{ABD} of biofilm aggregates for Lys- and CPC-treated biofilms were 425.68 and 246.05 µm, respectively (Fig. 4A and B). Four hundred millimolar Arg-treated biofilms exhibited significantly greater B_{ABD} than the water- and CPC-treated biofilms (Fig. 4A and B). For the average B_{ABD}, although not statistically significant (for Lys versus Arg at 400 mM; p-value = 0.055), Arg-treated biofilms dispersed much larger aggregates compared to the Lys-treated biofilms (Fig. 4A and B). Furthermore, the total biofilm biomass dispersed for 400 mM Arg-treated biofilms were significantly greater than the control-, Lys- and CPC-treated biofilms (Fig. 4B). Four hundred millimolar of Lys also exhibited significant dispersion effect on biofilms compared to the water-treated biofilms (Fig. 4A and B). Both the 400 mM Arg and CPC

resulted significant increase in the total number of biofilm aggregates that were detached from the biofilm substratum (Fig. 4B).

Characterization of the Microbial Community of the Remaining Biofilm and the Dispersed Cells

The bacterial cells that were dispersed from the biofilms following treatment with water or with Arg (400 mM) were compared for differences in the microbial community composition (Fig. 5 and 6). Based on the community analysis at the phyla and genus levels, there were notable differences between the control and Arg-treated samples but were not statistically significant (Fig. 5A and B). Both types of samples exhibited a diverse microbial community structure (Fig. 5 and B). The major phyla groups in both the biofilms and dispersed cells were *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Fusobacteria* and *Proteobacteria* (Fig. 6A). For both conditions (control and Arg), and the biofilm and biofilm cells, the most abundant genus groups included *Aggregatibacter*, *Fusobacterium*, *Haemophilus*, *Neisseria*, *Parvimonas*, *Porphyromonas*, *Rothia*, *Streptococcus* and unknown genus groups (Fig. 6B). Notably, the relative abundance of *Prevotella* and *Aggregatibacter* increased in the biofilm cells that were dispersed from the biofilms treated with Arg (Fig. 6B). While *Neisseria* and *Porphyromonas* populations were less abundant in the biofilm cells dispersed from the Arg-treated biofilms (Fig. 6B).

There were no significant differences in the alpha diversity of all sample types based on the pair-wise comparisons ($p = 0.102$) (Fig. 6A). Based on the Shannon diversity index, the Arg-treated biofilm cells exhibited the lowest alpha diversity (Fig. 6A). For the beta diversity comparisons, a statistical difference was found between the

control cells and the arginine cells ($p < 0.01$) (Fig. 6B). Based on the PCA, it was more evident that the biofilm cells that were dispersed upon Arg treatment resulted in an increase in beta diversity (Fig. 6C). The control water-treated biofilm communities exhibited the greatest clustering, whereas the Arg-treated biofilm cells exhibited the least clustering (Fig. 6C).

Discussions

The prolonged accumulation of oral biofilms on teeth and gingival surfaces can lead to biofilm-associated oral and systemic diseases (Beikler and Flemming, 2011). Thus, the development of novel biofilm model systems and new strategies to modulate or eradicate biofilms are essential to prevent or treat biofilm-associated diseases. The evolution of *in vitro* biofilm systems has accelerated over the last decade (Azeredo *et al*., 2017). In this study, we constructed and modified a Swinnex biofilm model system, which uses a filtering apparatus as the biofilm reactor (Fig. 1). Generally, the Swinnex filter holder is used for vacuum filtration of liquids for microbial and particle analysis. Here, we developed saliva derived multi-species biofilms using the Swinnex model system (Fig. 1).

In the past, the Swinnex biofilm model was used to study the growth rate and the pharmacokinetic response to antibiotics on single-species biofilm (Gander and Gilbert, 1997). Additionally, the Swinnex has been used to study the growth and release patterns of biofilm cells of different organisms including oral bacteria and as a filter mating apparatus for bacterial conjugation (Kaplan and Fine, 2002; Hmelo *et al*., 2015). In this study, the biofilm dispersion was studied in real-time and at an end point using the Swinnex together with the FlowCam® and confocal microscopy. Given that our main interest was to explore the destabilization/dispersion properties of Arg on oral biofilms, the modified Swinnex model was ideal to address our proposed research questions (McBain 2009).

In the Swinnex model, the 40 h growth period was adequate to develop a mature biofilm with sufficient biovolume and thickness to be tested with different anti-biofilm

agents. Before we explored the anti-biofilm properties of Arg, we compared the anti-biofilm effects of water and CPC (a well known anti-biofilm agent) to validate the model system (Fig. 2). In comparison to the water-treated, the CPC-treated biofilms clearly exhibited a greater loss of biofilm biomass through enhanced detachment of biofilm structures from the substratum (Fig. 2 and 4). Furthermore, the antimicrobial effects of CPC was evident from the significant membrane damage resulted following the short-term exposure (Fig. 2).

Recently, Arg was shown to destabilize the architecture of oral biofilms (Kolderman *et al*., 2015). In addition, along with Arg, Lys has been indicated to inhibit autoaggregation and coaggregation of oral bacteria (Takemoto *et al*., 1995; Merritt *et al*., 2009). Arg and Lys are both amino acids that have basic side chains, however Arg has a guanidium group instead of an amine group as a functional side chain. Guanidine based compounds have been shown to prevent biofilm formation and promote disassembly of existing biofilm structure (Bottcher *et al*., 2013; Rabin *et al*., 2015). In addition, Arg/guanidium composition can increase the potency of antimicrobial peptides (Cutrona *et al*., 2015). Unlike Lys, Arg can self-dimerize and form Arg-Arg crystal structures in solution (Prell *et al*., 2009). Furthermore, Arg-Arg clustering interaction has been reported to play a key role in inhibiting protein-protein interactions and protein aggregation (Das *et al*. 2007; Lange *et al*., 2009; Shukla and Trout, 2010; Gao *et al*., 2013). Here, we observed that shorter-term exposure of Arg at > 50 mM significantly destabilized the biofilm structure and enhanced biofilm de-adhesion (Fig. 3 and 4).

For biofilm dispersion, there are at least few known mechanisms. Active biofilm dispersion is initiated by the bacterial species within the biofilm, whereas the passive dispersion is initiated by the external factors such as shear force and anti-biofilm agents (Kaplan 2010). Biofilm 'sloughing' is a dispersion mechanism where large clusters of aggregates of biofilm cells are dispersed from the substratum (Kaplan 2010). Our results demonstrated that Arg was causing enhanced biofilm de-adhesion through passive sloughing mechanism. Arg molecules may be small enough to be incorporated into the biofilm architecture to induce a direct effect on disrupting the bacterial coaggregation interactions and cause biofilm destabilization (Fig. 7).

He and collaegues demonstrated that the long-term exposure of 1.5% Arg (wt/vol) modified the exopolysaccharide matrix (EPS) of mixed-species oral biofilms and selectively inhibited the outgrowth of *Streptococcus mutans* (He *et al*., 2016). Furthermore, continuous supplementation of Arg at > 5 mM dramatically reduces the growth of *Streptococcus gordonii* biofilms (Jakubovics *et al*., 2015). However, to our knowledge, the short-term effects of Arg on the community composition of the multi-species oral biofilms have not been investigated. Here, we assessed both the biofilm and the bacterial cells that detached from the substratum through 16S rRNA sequencing (Fig. 5). The microbial community that was developed in the Swinnex model system resembled a subgingival biofilm community with a high abundance of anaerobic genus groups such as *Fusobacterium*, *Porphyromonas* and *Aggregatibacter* (Fig. 5B). The phyla composition was limited to 5 major groups, most likely due to the extended 40 h growth period in the Swinnex (Fig. 5B). Compared to water, Arg did not induce a major shift in the biofilm composition (Fig. 5B). However, the measured beta diversity

was greatest in the bacterial cells detached following Arg treatment, which we suspect is due to the enhanced non-specific biofilm de-adhesion (Fig. 6B). The short-term exposure of Arg may have caused the biofilm structures to rapidly dissociate and be released from the attached substratum due to the Arg cluster mechanism (Fig. 7).

In this study, we report that a novel *in vitro* biofilm model system, the Swinnex, can be used to develop a complex multi-species oral biofilms. Furthermore, the Swinnex model can be combined with the confocal microscopy and the FlowCam® for end-point and real-time biofilm quantification analyses. In addition to the mechanical control of dental plaque, chemically induced dispersion of oral biofilms can slow down and prevent the re-growth of recalcitrant biofilms. In our model system, Arg significantly enhanced biofilm de-adhesion to promote sloughing of the biofilm structures. In conclusion, Arg is a unique amino acid that can destabilize architecture of the multi-species oral biofilms.

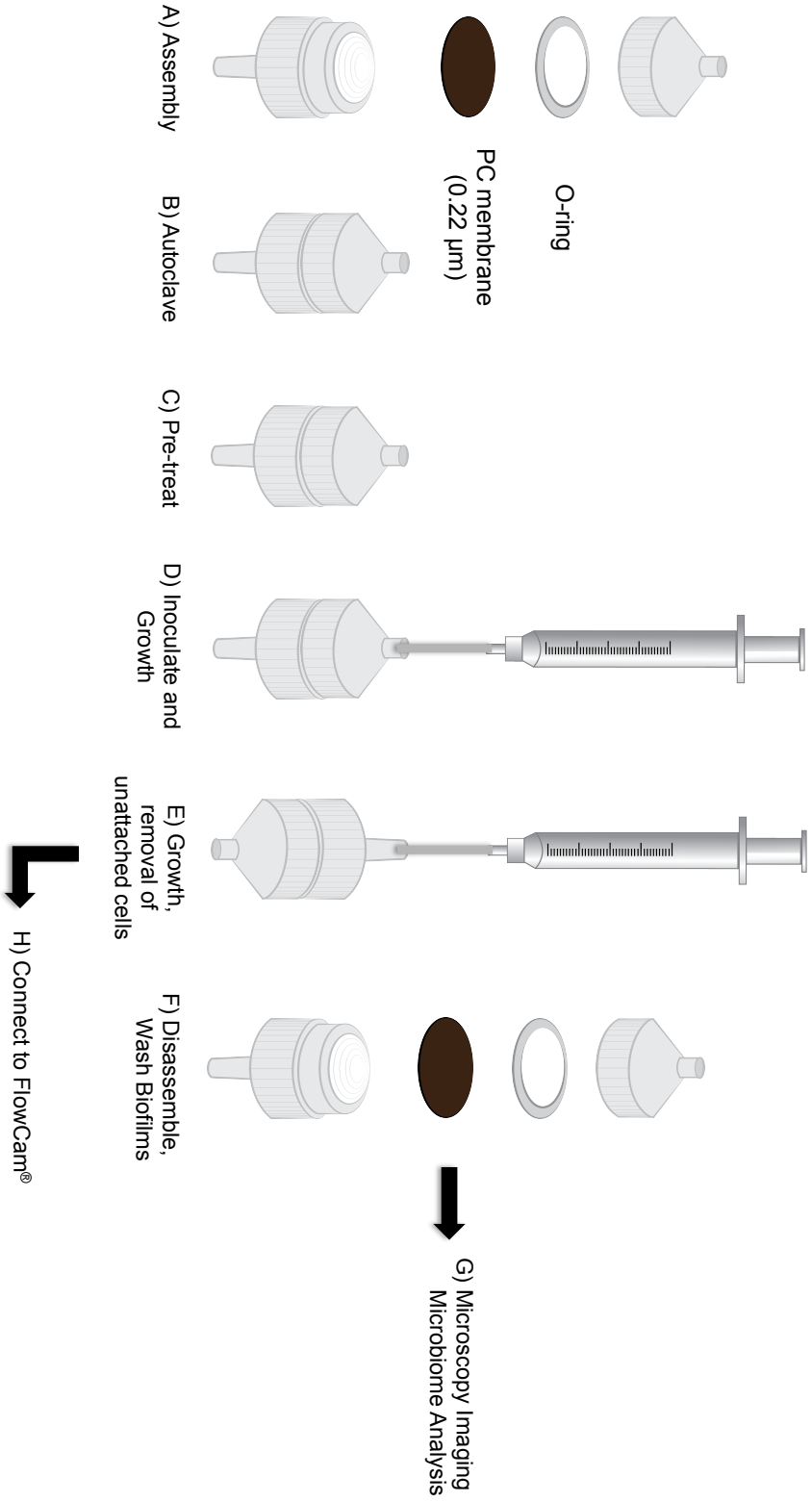
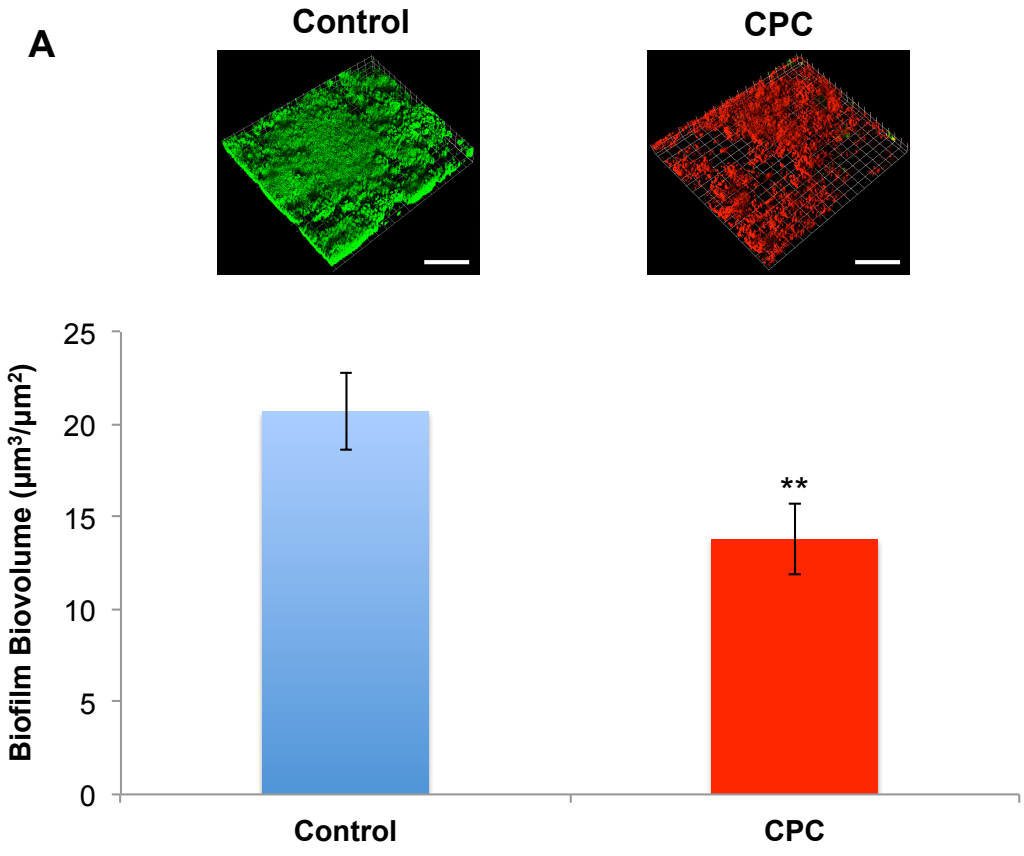


Figure V.1. A Schematic Diagram of the Swinnex Biofilm Model System. (A – H) describes the protocol for the development of multi-species oral biofilms and biofilm analyses.

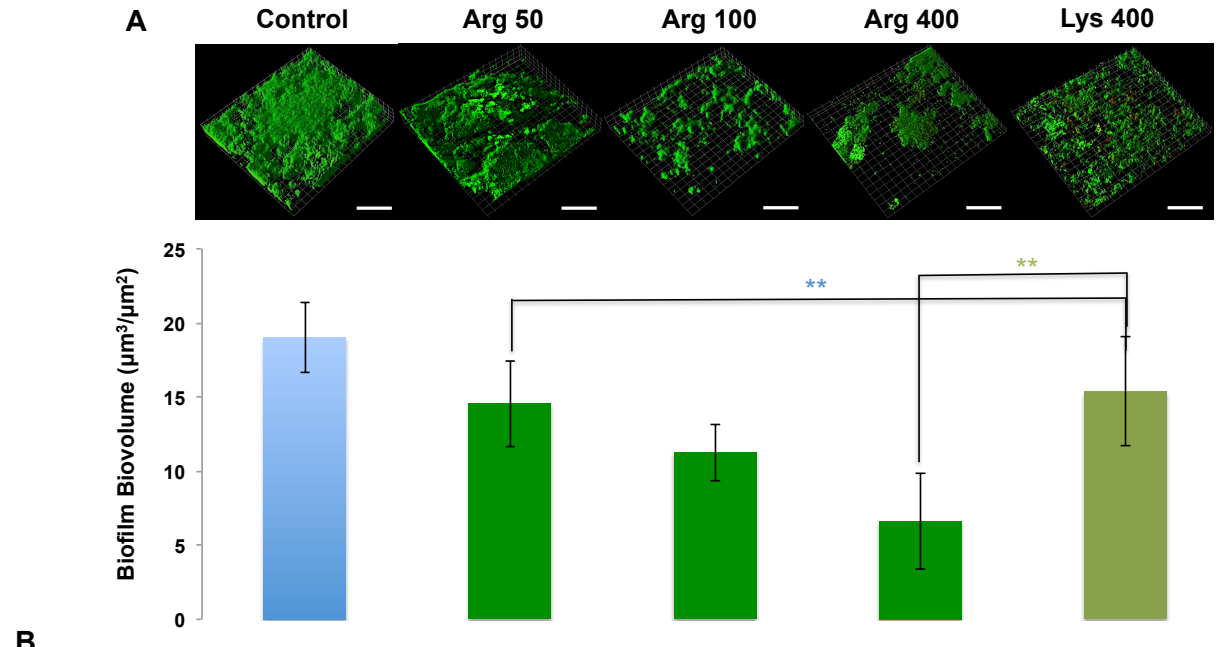
A



B

Biofilm treatment	Control (H_2O)	CPC
Biovolume ($\mu m^3/\mu m^2$)	20.69 (2.08)	13.80 (1.91)**
Thickness (μm)	34.75 (3.91)	17.17 (1.85)**
Roughness	0.01 (0.005)	0.09 (0.24)**
Viability (%)	94.29 (3.92)	21.33 (12.89)**

Figure V.2. Validation of the Swinnex Model System. Multi-species oral biofilms were grown for 40 h in the Swinnex model system. The biofilms were treated with water (Control) or CPC (0.075%) for 10 min at a shear rate of 20 μl/min. **(A)** Representative confocal microscopy images of biofilms treated with water (Control) or CPC. Biofilm biomass is shown in μm^2. **(B)** Biofilm parameters including biomass (μm^2), biovolume ($\mu m^3/\mu m^2$), thickness (μm), roughness and viability (%) for control and CPC-treated biofilms are shown. n=12 per condition. ** p-value < 0.01.

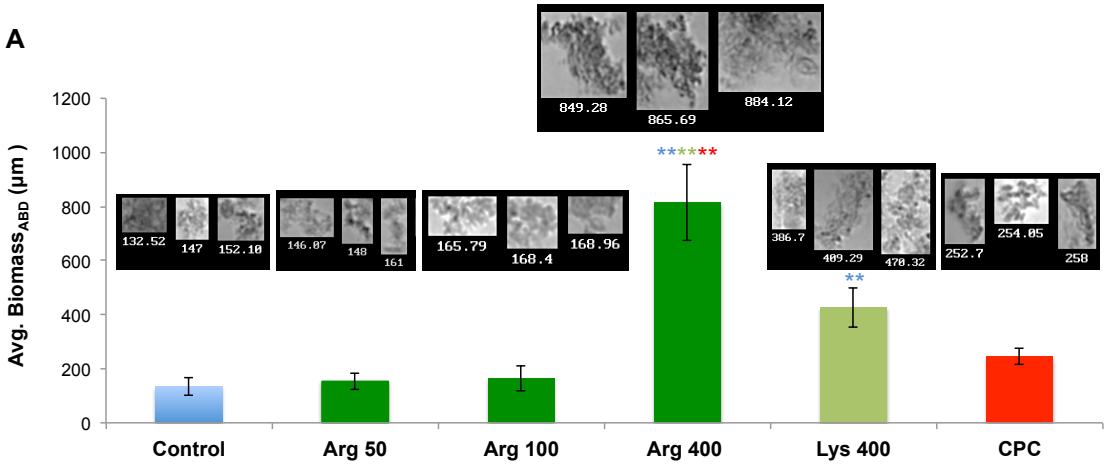


B

Biofilm treatment	Control (H_2O)	Arg 50 mM	Arg 100 mM	Arg 400 mM	Lys 400 mM
Biovolume ($\mu m^3/\mu m^2$)	19.01 (2.37)	14.77 (2.90)**	11.26 (1.90)**	6.62 (3.23)****	15.36 (3.69)**
Thickness (μm)	31.54 (4.59)	27.82 (3.96)*	21.71 (2.81)**	14.15 (5.79)****	20.00 (3.09)**
Roughness	0.03 (0.03)	0.02 (0.17)	0.07 (0.01)	0.54 (0.48)**	0.04 (0.03)
Viability (%)	97.97 (1.25)	98.94 (0.20)*	99.01 (0.22)**	97.70 (1.15)**	86.00 (4.33)**

Figure V.3. The Effects of L-Arginine on Oral Biofilms. Multi-species oral biofilms were grown for 40 h in the Swinnex model system. The biofilms were treated with water (Control), Arg (50 – 400 mM) or Lys (400 mM) for 10 min at a shear rate of 20 μl/min. **(A)** Representative confocal microscopy images of biofilms treated with water (Control), Arg (50 – 400 mM) or Lys (400 mM). Biofilm biomass is shown in μm^2. (B) Biofilm parameters including biomass (μm^2), biovolume ($\mu m^3/\mu m^2$), thickness (μm), roughness and viability (%) for control, Arg (50 – 400 mM) and Lys (400 mM) - treated biofilms are shown. n=12 per condition. * p-value < 0.01 and ** ** p-value < 0.05.

A



B

Biofilm treatment	Control (H_2O)	Arg 50 mM	Arg 100 mM	Arg 400 mM	Lys 400 mM	CPC
Biomass$_{ABD}$ (µm)	133.02 (33.35)	153.50 (32.30)	163.46 (45.01)	814.44 (139.72)****	425.68 (71.54)**	246.05 (29.94)
Total Biofilm Biomass Dispersed	9639 (6483)	20704 (24475)	22496 (10527)*	141013 (51957)****	58730 (33480)*	43244 (28360)*
Number of Biofilm Aggregates Captured	70 (30)	116 (34)*	174 (89)*	193 (31)**	132 (58)	168 (46)**

Figure V.4. The Dispersion Effects of L-Arginine on Oral Biofilms. Multi-species oral biofilms were grown for 40 h in the Swinnex model system. Following growth, the Swinnex filter holder was directly connected to the FlowCam® device. The biofilms were treated with water, Arg (50 – 400 mM), Lys (400 mM), or CPC (0.075%) to monitor the dispersion effect for 1 min. **(A)** Representative FlowCam® images are shown of biofilm treated with water, Arg (50 – 400 mM), Lys (400 mM), or CPC (0.075%) are shown along with the average Biomass$_{ABD}$ in µm. **(B)** Biofilm parameters including Biomass$_{ABD}$, total biofilm biomass dispersed and number of biofilm aggregates captured are shown. n=4 per condition. ** ** ** p-value < 0.05.

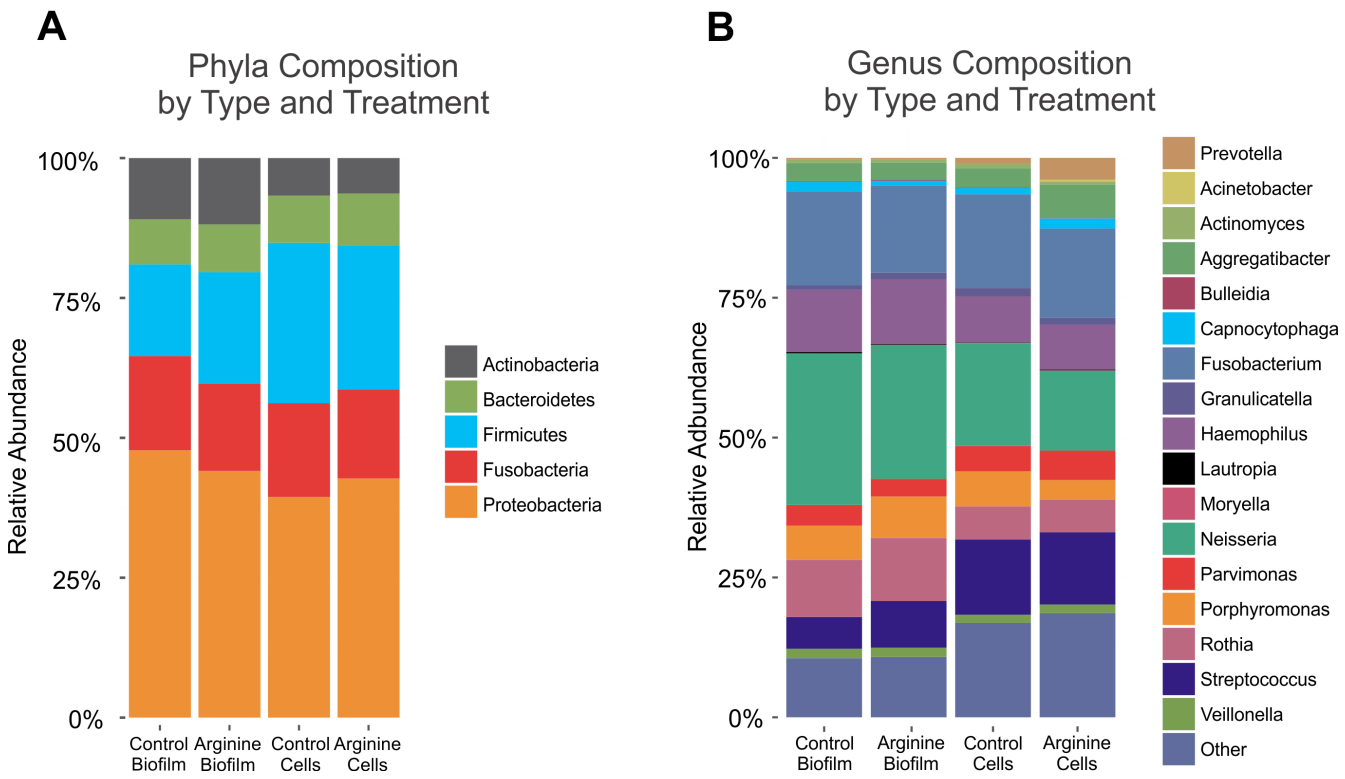


Figure V.5. Community Composition of the Biofilms and the Dispersed Biofilm Cells. Multi-species oral biofilms were grown for 40 h in the Swinnex model system. Following growth, the biofilms were treated with water (Control) or Arg (400 mM) for 10 min at a shear rate of 20 µl/min. The bacterial cells from the intact biofilms and the dispersed biofilm cells were harvested for 16S rRNA sequencing. (A) The phyla composition by Type and Treatment, and (B) The genus composition by Type and Treatment of the Control biofilm, Arginine biofilm, Control cells and Arginine cells.

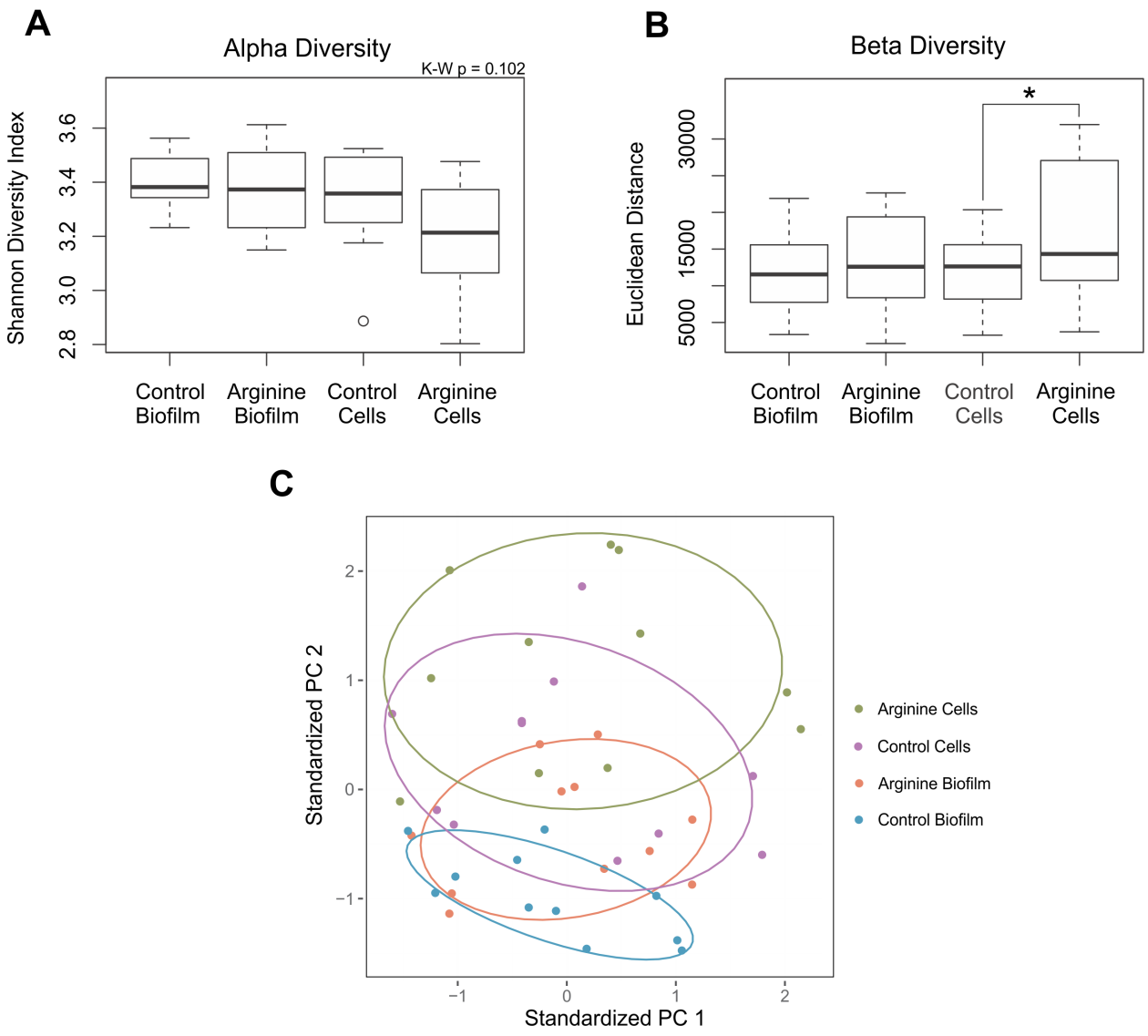


Figure V.6. The Diversity of the Communities Found in the Biofilms and the Dispersed Biofilm Cells. Microbial community analyses of the Control biofilm, Arginine biofilm, Control cells and Arginine cells were conducted using the Shannon diversity index (for Alpha diversity) and the Euclidean distance (for Beta diversity). Principal component analysis (PCA) was conducted using the log-transformed read counts of the operational taxonomic units (OTUs) **(A)** Alpha diversity showed no significant differences between all groups, (B) Beta diversity was the greatest for the Arginine cells and a significant difference was detected compared to the Control cells. (C) Based on the PCA, there were no significant differences between the Control and Arginine biofilms (p=0.57) and the Control and Arginine cells (p=0.70). PCA.

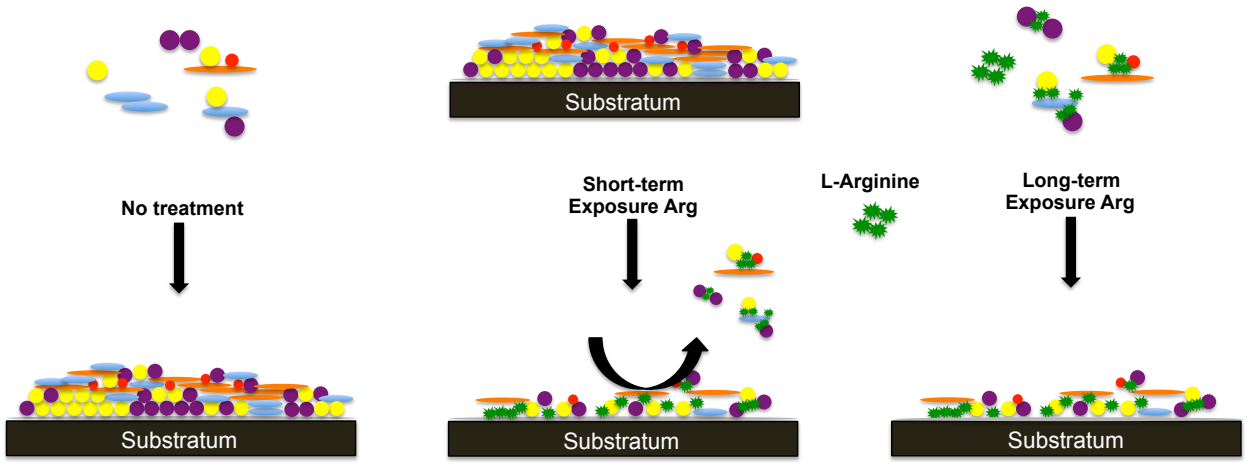


Figure V.7. A Proposed Model for the Short and Long-term Effects of High Concentrations L-Arginine on Biofilm Destabilization. The model shows the potential interaction of Arg with the planktonic bacteria and the developed multi-species biofilm community. We hypothesize that the clustering effect of Arg can rapidly destabilize the oral biofilm structure to enhance dispersion/de-adhesion of biofilms. In addition, long-term exposure of Arg can inhibit biofilm formation. The proposed model is based on the previous work published in our lab (Kolderman *et al*., 2015) and the data presented in this dissertation project (Chapter V).

Chapter VI

Conclusions and Future Directions

Conclusions

Biofilm–associated oral diseases are a global health concern (Chronic Disease Prevention and Health Promotion, 2017). Hence, the development of novel therapeutic anti-biofilm agents and strategies to prevent and treat biofilm-associated diseases are highly relevant for improving human oral health.

In this dissertation project, nisin and L-arginine were studied for their therapeutic potential as novel anti-biofilm agents. For multi-species biofilm development, three model systems were utilized, 1) a static MTP based system, 2) high throughput microfluidic BioFlux system, and 3) the Swinnex biofilm model system. For biofilm quantification methods, confocal microscopy and a newly developed FlowCam® approach was utilized. Furthermore, the development of the *in vivo*-plaque like biofilm communities in the Swinnex biofilm model was validated through the usage of next generation sequencing.

High purity nisin, nisin ZP exhibited strong antimicrobial and anti-biofilm properties against oral bacterial species and saliva derived oral biofilms. Nisin at very low concentrations was effective in inhibiting biofilm development and disrupting the

pre-formed mature biofilm structures. Nisin at the active anti-biofilm concentrations did not exhibit cytotoxic effects against human oral cells. Additionally, our recent study have demonstrated that nisin ZP alone or in combination with sodium hypochlorite can effectively inhibit the growth of planktonic and biofilm populations of an endodontic pathogen, *Enterococcus faecalis* (Kajwadkar and Shin *et al*., 2017). In summary, nisin is a safe broad-spectrum bacteriocin that has been used for decades and has great therapeutic potential for numerous biomedical applications. Through the work highlighted in Chapter III and IV, we propose that nisin is a promising candidate as a novel oral anti-biofilm agent.

In Chapter V, we demonstrated that the high concentrations of L-arginine promoted the destabilization of the multi-species oral biofilms. Short-term treatment of the mature biofilms with L-arginine significantly reduced the biofilm biomass and thickness through rapid dispersion, sloughing of the biofilm structure. Compared to L-lysine and cetylpyridinium chloride, L-aginine exhibited significantly greater biofilm dispersion effect. The microbial community compositions of the treated biofilms did not change following the short-term exposure to L-arginine. However, the beta diversity of the sloughed aggregates and cells were significantly increased in the L-arginine treated biofilms compared to the water treated control cells. We propose that this increase is due to the enhanced non-specific dispersion effect caused by L-arginine on the biofilm. In summary, the work presented in Chapter V indicates that L-arginine has high potential to be incorporated in mouthrinses and dentifrices to improve the effectiveness of the current anti-biofilm formulations.

Future Directions

As indicated by our data, nisin and L-arginine have different modes of action on biofilms. As a cationic peptide, nisin interferes with the negatively charged membranes to prevent biofilm development and maintenance, whereas, the high concentrations of L-arginine cause non-specific and rapid dispersion of the biofilm structure. Thus, as two potent anti-biofilm agents with distinct mechanisms, the combination of two agents may provide synergistic activities against oral biofilms. For example, using the Swinnex biofilm system, whether the nisin + L-arginine exerts greater anti-biofilm effects than nisin or L-arginine alone can be evaluated. In addition, the application of these two agents in combination with other anti-biofilm agents should be considered for the development of new mouthrinse and dentifrice formulations.

Bacterial coaggregation has been validated as a critical process in the development of oral biofilms (Rickard *et al*., 2003). Direct inhibition of coaggregation can prevent the initial adhesion of bacteria to one another and to the substratum surface. Nisin alone was demonstrated to inhibit the growth of a variety of oral commensal and pathogenic bacteria (Shin *et al*., 2015). Coaggregation studies are planned to further evaluate the role of nisin in inhibiting coaggregation between a diverse-spectrum of oral bacteria. Previously, L-arginine and L-lsine were shown to inhibit autoaggregation of selective strains of *Fusobacterium nucleatum* (Merritt *et al*., 2009). However, L-arginine and L-lysine were only tested at a single concentration (50 mM) and their effect on intergeneric coaggregation was not assessed. Whether L-arginine inhibits a specific coaggregation interaction or is non-specific, future studies are needed. For examples, isomers of L-arginine and coaggregation defective mutants can

be used to determine the specificity of L-arginine's anti-coaggregation mechanisms. In addition, for future studies assessing coaggregation interactions, it will be beneficial to combine the traditional visual coaggregation method along with the confocal microscopy and the FlowCam® method (Levin-Sparenberg *et al*., 2016).

In this dissertation project, the Swinnex biofilm model was revived and modified to develop and study oral biofilms. Instead of using pooled saliva, plaque samples can be used as an alternative biofilm inoculum. Recently, our lab has demonstrated that based on the type of the multi-species inoculum and shear force, the communities developed in a microfluidic device can be artificially shaped within a model system (Fernandez *et al*., 2016). Using the Swinnex model, it would be interesting to change the inoculum and alter the environmental parameters to develop different types of *in vivo*-like biofilms (ie. supragingival, subgingival, tongue). Furthermore, in addition to the microbiome sequencing, global transcriptomic analyses will allow us to understand other possible mechanisms of L-arginine on destabilization of oral biofilms. As of now, it is still unclear whether the high concentration of L-arginine is strictly causing a physicochemical effect (ie. clustering effect) or also has an effect at the molecular level to promote biofilm destabilization. By utilizing the omics approach, it should allow for more specific research questions to be answered.

Lastly, as emphasized in this dissertation thesis, the development of novel model systems are critical for biofilm research. Currently, the gold standard model for studying host-biofilm interactions is lacking. To better understand the biological effect of a biofilm in human host and the response to biofilm-modulating agents *in vivo*, models that mimic the host-biofilm interactions are much needed. The BioFlux system and the Swinnex

model system described in this thesis can be used to study the host-biofilm interactions. In a recent study, monolayers of mammalian cells have been co-cultured with biofilms in the microfluidic channels of the BioFlux plates (Trembley *et al*., 2015). By utilizing my knowledge in mammalian cell biology and microbiology, I am hoping that I can develop a model system for better studying the host-biofilm interactions. If a model system can re-create an environment for the host cells (ie. gingival epithelial cells, periodontal ligament cells) and the biofilms (ie. multi-species oral biofilm) to co-exist as in reality, such model system will be extremely valuable and highly relevant for biofilm-related research (Parsek and Singh, 2003; Lebeaux *et al*., 2013).

APPENDICES

Appendix A

High Purity Nisin Alone or in Combination with NaOCl is Effective Against Planktonic and Biofilm Populations of *Enterococcus faecalis*

Appendix A is adapted from a manuscript that has been published (Kajwadkar and Shin *et al*., 2017). Co-1st authors.

Abstract

Introduction: Nisin, a broad-spectrum bacteriocin, has recently been highlighted for its biomedical applications. To date, no studies have examined the antimicrobial and antibiofilm properties of high-purity (>95%) nisin (nisin ZP) on *Enterococcus faecalis* and biofilms formed by this species. We hypothesize that nisin can inhibit *E. faecalis* and reduce biofilm biomass, and combinations of nisin and sodium hypochlorite (NaOCl) will enhance the antibiofilm properties against *E. faecalis* biofilms.

Methods: Using broth cultures, disc diffusion assays, and biofilm assays, we examined the effects of nisin on various *E. faecalis* growth parameters and biofilm properties (biovolume, thickness, and roughness). Confocal microscopy was used in conjunction

with Imaris and Comstat2 software (Kongens Lyngby, Copenhagen, Denmark) to measure and analyze the biofilm properties.

Results: Nisin significantly decreased the growth of planktonic *E. faecalis* dose dependently. The minimum inhibitory concentrations against *E. faecalis* strains OG-1 and ATCC 29212 were 15 and 50 µg/mL, and the minimum bactericidal concentrations were 150 and 200 µg/mL, respectively. A reduction in biofilm biovolume and thickness was observed for biofilms treated with nisin at 10 µg/mL for 10 minutes. In addition, the combination of nisin with low doses of NaOCl enhanced the antibiofilm properties of both antimicrobial agents.

Conclusions: Nisin alone or in combination with low concentrations of NaOCl reduces the planktonic growth of *E. faecalis* and disrupts *E. faecalis* biofilm structure. Our results suggest that nisin has potential as an adjunctive endodontic therapeutic agent and as an alternative to conventional NaOCl irrigation.

Introduction

One of the most common and persistent bacteria associated with failed endodontic cases is *Enterococcus faecalis* (Molander *et al*., 1998; Sundqvist *et al*., 2014). *E. faecalis*, a gram-positive cocci, has an inherent antimicrobial resistance and ability to form biofilms that adapt to harsh environmental conditions, which contribute to its role in endodontic failures (Lin *et al*., 1992; Stuart *et al*., 2006). Biofilms are highly organized structures of bacterial cells enclosed within an exopolymeric matrix attached to living and nonliving surfaces (Costerton 1999). For removal of pathogenic bacteria and their biofilms, several irrigating solutions and techniques are used during an endodontic procedure. Sodium hypochlorite (NaOCl) is a conventional endodontic irrigant that is widely used and has strong antimicrobial activity (Bystrom and Sundqvist, 1983). However, inadvertent exposure to NaOCl can cause extensive soft tissue and nerve damage and even airway compromise (Zhu *et al*., 2013). From a clinical perspective, an endodontic irrigant that can effectively remove persistent bacteria with minimal host cytotoxicity would be a valuable addition to the endodontic armamentarium (Turner *et al*., 2004).

Bacteriocins are bacterially secreted antimicrobial peptides with diverse applications, including uses in food preservation, treatment of pathogen-associated diseases, cancer therapy, and maintenance of human health (Shin *et al*., 2016; Yang *et al*., 2014). Nisin is a cationic bacteriocin that can interact with the negatively charged cell membranes and form pores to promote cell death (Giffard *et al*., 1996; Lubelski *et al*., 2008). In addition, bacteriocins like nisin exhibit various modes of action to target a

wide range of bacterial species (Peschel and Sahl, 2006; Shin *et al*., 2015). Nisin has been granted a generally regarded as safe status by the Food and Drug Administration, and it exhibits minimal cytotoxicity on human oral cells (Cotter *et al*., 2005; Shin *et al*., 2015). Thus, the clinical applications of nisin for endodontic infections could be promising as an adjunct or alternative antimicrobial and antibiofilm agent.

To date, there have been no studies on the antimicrobial properties of high-purity nisin on planktonic and biofilm populations of *E. faecalis*. Furthermore, the effects of nisin and NaOCl combinations on these populations have not been explored. The purpose of our study was to test the antimicrobial and antibiofilm properties of high-purity, food-grade nisin Z, herein referred to as nisin ZP, alone and in combination with low concentrations of NaOCl against *E. faecalis* and its biofilms.

Materials and Methods

Nisin ZP Preparation

High-purity (>95%), food-grade nisin ZP was purchased from Handary SA (Brussels, Belgium) and used for this study. For experimental use, a stock solution was prepared in a sterile tube by dissolving 5 mg nisin ZP in 1 mL filtered distilled water and re-filtered using a sterile 0.22-μm filter. A fresh stock solution was prepared for each experiment.

Bacterial Culture Conditions

E. faecalis strains OG-1 (gift from Dr Don Clewell, Professor Emeritus, University of Michigan, Ann Arbor, MI) and ATCC 29212 (American Type Culture Collection, Manassas, VA) were used for this study. *E. faecalis* strains were grown at 37°C in brain-heart infusion broth (BHI; Becton, Dickinson and Company, Franklin Lakes, NJ) or trypticase soy agar supplemented with 5% sheep blood (Remel, Lenexa, KS). For use in experiments, a single isolated colony from a trypticase soy agar supplemented with 5% sheep blood plate grown from frozen stock cultures was inoculated in BHI medium and incubated overnight.

Broth Dilution/Turbidity Assay

Freshly prepared overnight cultures of *E. faecalis* were adjusted to an optical density at 600 nm (OD_{600}) of 0.8 and then diluted 1:10 in BHI for use in experiments. In each well of a 24-well cell culture plate (Sigma-Aldrich, St Louis, MO), the adjusted

bacterial suspension (0.9 mL) and nisin stock solution (0.1 mL, diluted in distilled water to appropriate concentrations) were added so that the total volume in each well was 1 mL, and the nisin concentrations obtained were 0.5, 1.0, 2.5, 5.0, 10, 15, 20, 30, 40, 50, 100, 150, and 200 µg/mL, respectively. The bacterial culture plus phosphate-buffered saline (PBS) was used as a negative control (NC), and bacterial culture plus 4.125% NaOCl was used as a positive control (PC). For minimum inhibitory concentration (MIC) determination, 0.1 mL of the test samples was transferred into 96-well plates and incubated for 24 hours at 37 C. The optical density at 600 nm was recorded and obtained before and after 24 hours of incubation at 37°C. For minimum bactericidal concentration (MBC) determination, 0.1-mL aliquots of the test samples were spread on trypticase soy agar plates incubated for 24 hours at 37°C.

Disc (Agar) Diffusion Assay

The disc diffusion assay, also known as the Kirby-Bauer method, was used as an additional measure of the inhibitor effect of nisin on *E. faecalis* (Boney *et al*., 2008). Freshly prepared *E. faecalis* OG-1 and ATCC 29212 (0.1 mL of an overnight culture adjusted to OD_{600} of 0.8 and then diluted 1:10) were spread on trypticase soy agar plates. Six-millimeter Whatman filter paper discs (Sigma-Aldrich) were then placed on the agar plates, and 20 m L of the nisin test solutions of various concentrations (10, 50, 75, 100, 150, and 200 µg/mL) was added onto each of the discs. Twenty microliters of PBS and 4.125% NaOCl (20 µL each) were used as the NC and PC, respectively. After incubation (24 hours at 37°C), the diameter of the zone of growth inhibition was measured using a vernier caliper, and images of all discs were captured using a digital

camera (D5200 24.1-Megapixel DX-format with CMOS image sensor; Nikon, Tokyo, Japan).

Biofilm Development and Treatment with Nisin Alone or Nisin-NaOCl Combinations

E. faecalis biofilms were grown in 24-well glass bottom sensoplates (Greiner Bio-One, Monroe, NC). Fifteen microliters of fresh E. faecalis cultures (standardized and diluted as described previously) was inoculated into each well with 1.5 mL BHI and incubated for 48 hours at 37°C. This procedure was performed separately for *E. faecalis* OG-1 and ATCC 29212 strains. Biofilm growth was visually confirmed by the presence of an intact biofilm on the glass wells. All wells were then washed 3 times with 0.5 mL PBS. The biofilms were treated with 0.5 mL PBS or various concentrations of the nisin solution (10, 50, 100, and 200 µg/mL) for 10 minutes. After treatment with nisin, all wells were washed 3 times with 0.5 mL PBS in order to prepare the biofilms for staining as described later. To evaluate the effect of nisin-NaOCl combinations, biofilms were treated for 10 minutes with 0.5 mL of combinations containing 50 µg/mL nisin with 0.05%, 0.5%, and 1% NaOCl, PBS (NC), or 4.125% NaOCl (PC). The same protocol described earlier was used for the washing steps.

Biofilm Staining and Quantitative Analysis

Biofilm staining and quantitative analysis of biofilm biovolume (biomass volume divided by the area of substratum, µm3 /µm2), thickness (µm), roughness (measures the variability within the biofilm architecture and thickness), and viability (using the

BacLight Live/Dead Viability Kit; Invitrogen, Carlsbad, CA) were performed using confocal microscopy and Imaris (Bitplane, Zurich, Switzerland) and Comstat2 (Kongens Lyngby, Copenhagen, Denmark) software as previously described (15) .

Statistical Analyses

Data values are expressed as mean values ± standard deviations. The statistical significance of the results for MIC and MBC values was determined using independent t-tests and 1-tailed determination of P-values. The significance between different concentrations of nisin alone and nisin-NaOCl concentrations for the biovolume, thickness, roughness, and viability was analyzed by analysis of variance using the Tukey method. All experiments were repeated at least 3 times in triplicate. Differences were expressed as not significant, significant ($P < .05$), or very significant ($P < .01$).

Results

Effects of Nisin on the Growth of *E. faecalis*

Broth dilution assays revealed that nisin inhibited the planktonic growth of OG-1 and ATCC 29212 strains (Fig. 1A and B). The MIC and MBC of OG-1 were 15 and 150 µg/mL (Fig. 1A). A bimodal distribution in the MIC was observed with ATCC 29212 at 2.5 and 50 µg/mL with an MBC of 200 µg/mL (Fig. 1B). Both strains of *E. faecalis* responded in a dose-dependent manner in the disc diffusion assays (Fig. 1C and D). A significant zone of inhibition was observed starting at 50 and 100 µg/mL for ATCC 29212 and OG-1, respectively.

Effects of Nisin on *E. faecalis* Biofilms

E. faecalis biofilms were sensitive to 10-minute treatments with nisin (Fig. 2). In the absence of nisin, OG-1 and ATCC 29212 formed robust biofilms with an average biofilm biovolume and thickness of 5.12 µm3 /µm2 and 14.93 µm and 8.34 µm3 /µm2 and 15.92 µm, respectively (Fig. 2A and B). Compared with the control biofilms, biofilms treated with nisin for 10 minutes exhibited significant decreases in biovolume and thickness at all concentrations tested (10–200 µg/mL, Fig. 2A and B). At lower concentrations, in the range of 10 and 50 µg/mL, biofilm viability started showing decreases for both OG-1 and ATCC 29212. However, at concentrations >100 µg/mL, biofilm viability was significantly decreased as shown by the viability quantification data and the confocal microscopic images (Figs. 2 and 3A and B). The roughness of the biofilms was increased in all nisin-treated groups, suggesting a disturbed architecture in

treated biofilms (Fig. 2A and B).

Combination of Nisin and NaOCl on *E. faecalis* Biofilms

E. faecalis biofilms were treated for 10 minutes with 50 µg/mL nisin in combination with 0.05%, 0.5%, or 1%NaOCl, respectively. Nisin in combination with low doses of NaOCl exhibited significant decreases/alterations in biofilm biovolume, thickness, and roughness compared with PBS treatment for OG-1 and ATCC 29212 biofilms (Fig. 4A and B). Biofilms that were treated with 4.125% NaOCl exhibited negligible biofilm biovolume for both strain types (Fig. 4A and B). Furthermore, 0.5% and 1% nisin-NaOCl combinations were as effective in removing biofilms from the adherent substratum compared with those treated with 4.125% NaOCl. At the lowest nisin-NaOCl combination (0.05% NaOCl), biofilm cells were still intact but exhibited a significant reduction in biofilm biovolume, thickness, and viability (Figs. 3C and D and 4). Thus, the combination of low concentrations of NaOCl with nisin was more effective than the nisin-alone treatments and just as effective as the 4.125% NaOCl-alone treatment in eradicating *E. faecalis* biofilms.

Discussions

The most common reason for the failure of endodontic treatments and retreatments is the persistence of pathogenic bacteria within the root canal systems (Lin *et al*., 1992). Neither irrigation nor current intracanal medicaments can render the root canals completely free of bacteria (Haapasalo *et al*., 2005). Thus, the discovery of new root canal irrigating agents with improved antimicrobial properties could benefit both the patient and the clinician. *E. faecalis* can persist within root canals by forming antimicrobial resistant biofilms and surviving harsh environmental conditions (Dahlen *et al.*, 2000; , Portenier *et al*., 2005). Furthermore, certain species of enterococci possess bimodal or trimodal distribution of MICs against antimicrobials (Bywater *et al*., 2005). In endodontics, NaOCl is the most commonly used irrigant, and other agents alone or in combination, including calcium hydroxide, EDTA, and chlorhexidine, have shown evidence of improved activity and function (Dunavant *et al*., 2006; Haapasalo *et al.*, 2005). However, combining different chemical reagents can also produce toxic by-products. For example, when NaOCl is combined with chlorhexidine, this can produce a carcinogenic by-product, which can occlude the dentinal tubules and affect the seal within the root canal (Bui *et al*., 2008). In our study, we showed that combinations of nisin with low concentrations of NaOCl exerted improved antibiofilm effects compared with treatment with nisin alone (Figs. 2 and 4). Future studies can be performed to assess the cytotoxicity of this combination on human oral cells compared with the standard 4.125% NaOCl solution. Recently, there has been a growing interest in using nisin for biomedical applications and for targeting oral biofilms and resistant

microorganisms (Shin *et al*., 2016). The two most well-known natural variants of nisin are nisin A and Z, which differ by a single amino acid substitution at position 27 (Vuyst and Vandamme, 1994). Previously, Tong et al (Tong *et al*., 2014a) reported that the MIC and MBC against *E. faecalis* was 1 and 2 mg/mL using nisin A (2.5%, Sigma-Aldrich), which is at least 10-fold higher than that reported for nisin ZP in this study (Fig. 1A and B). In addition, Shin et al (Shin *et al*., 2015) showed that low concentrations of nisin ZP exhibited strong antibiofilm properties against saliva-derived multi-species biofilms. Thus, our data support that nisin ZP has superior antimicrobial efficacy compared with low-purity nisin A. Furthermore, studies have shown that the antimicrobial efficacy of common antibiotics and intracanal medicaments are substantially enhanced when combined with nisin (Tong *et al*., 2014a; Tong *et al*., 2014b). Our results are in agreement with these findings because combinations of nisin with low doses of NaOCl enhanced the antibiofilm properties of these antimicrobial agents (Fig. 4). As an irrigating agent, NaOCl is stable at a pH of 11, whereas nisin A is only soluble at an acidic pH, thereby limiting the compatibility of these agents at their optimal pH (Rollema *et al*., 1995; Zehnder 2006). However, because nisin ZP is stable at a neutral and basic pH, this allows preparations of nisin-NaOCl combinations for potential use as irrigating solutions (Rollema *et al*., 1995; Shin *et al*., 2015). As a unique bacteriocin, nisin has minimal cytotoxicity on mammalian cells and an absence of stable and transmissible resistance properties (Sahl and Bierbaum, 1998; Smith and Hillman, 2008; Willey and Van der Donk, 2007). Here, we showed that nisin ZP exerts antimicrobial and antibiofilm properties against *E. faecalis*. In addition, when combined with low concentrations of NaOCl, nisin's antibiofilm effects were enhanced in removing

E. faecalis biofilms from their substratum. In conclusion, because novel bacteriocins, such as nisin ZP, can potentially improve the prognosis of endodontic therapy, their exploration is important for future clinical advancement.

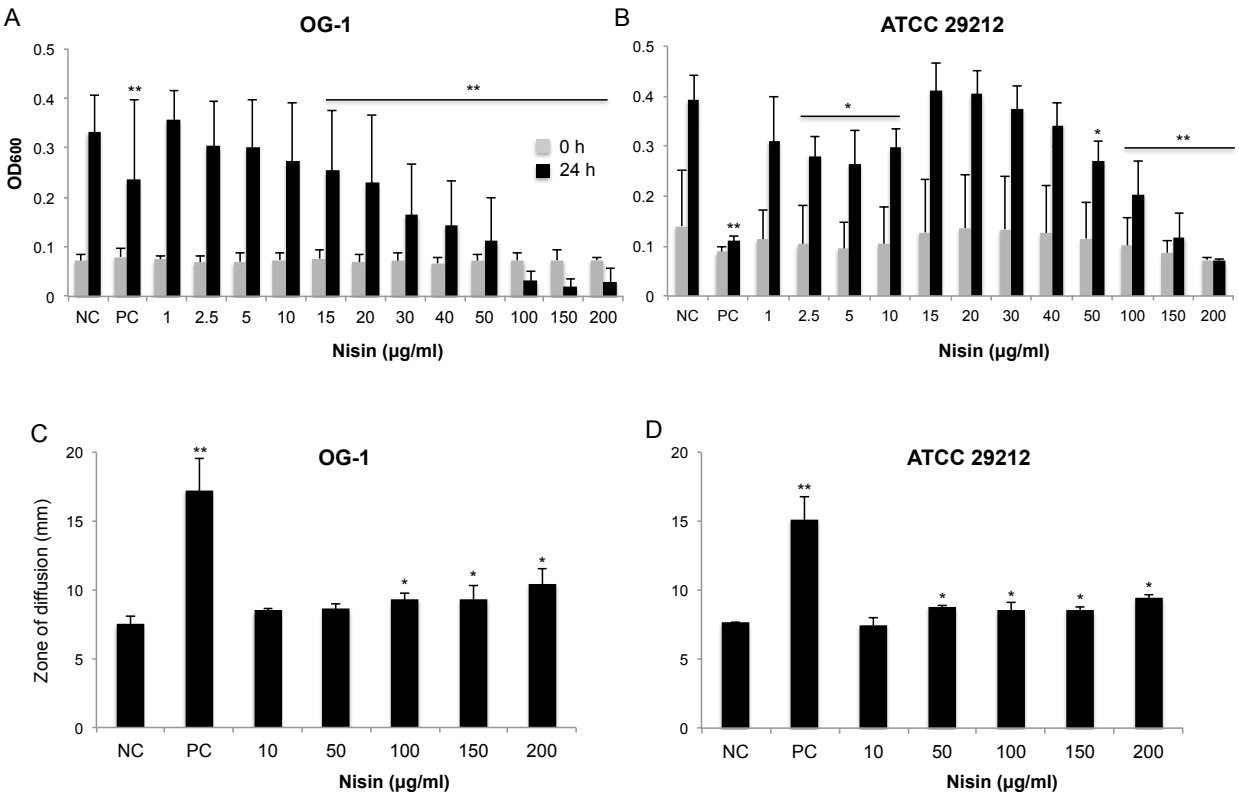
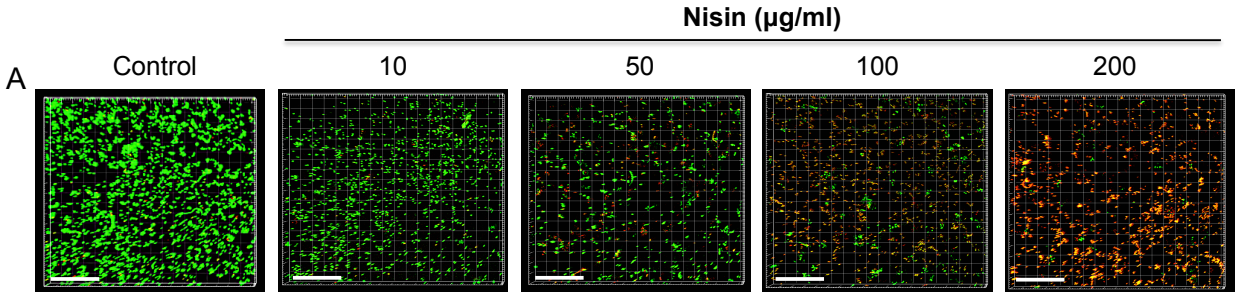
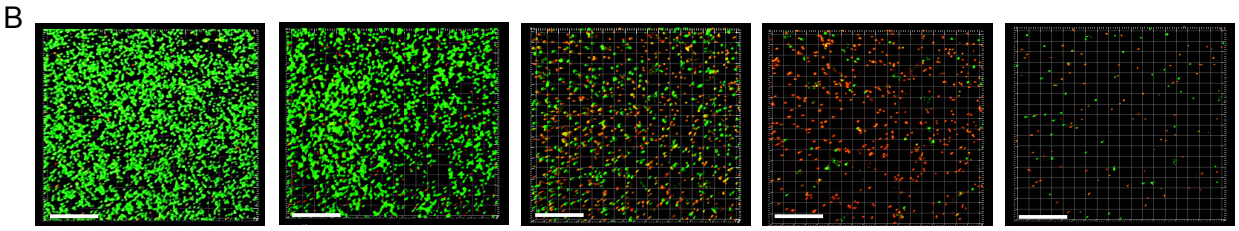


Figure A.1. Antimicrobial effects of nisin on *E. faecalis*. *E. faecalis* strains **(A)** OG-1 and **(B)** ATCC 29212 were grown under planktonic conditions with or without nisin (0.5 – 200 µg/ml). In addition, (C) OG-1 and (D) ATCC 29212 were grown on Whatman filter paper discs on agar with or without nisin (10 – 200 µg/ml). * $P < 0.05$ and ** $P < 0.01$ compared to the PBS treated negative control (NC). 4.125% NaOCl was used as a positive control (PC).

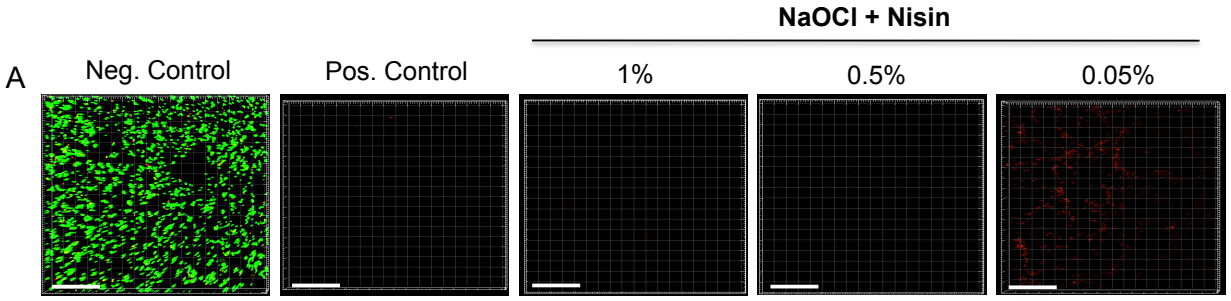


Biofilm Parameters	Control	10	50	100	200
Biovolume ($\mu m^3/\mu m^2$)	5.12 (2.34)	2.65 (0.93)**	2.49 (0.75)**	1.52 (0.82)**	1.48 (0.96)**
Thickness (µm)	14.93 (4.79)	7.89 (2.70)**	6.14 (1.63)**	3.38 (2.32)**	3.54 (2.93)**
Roughness	0.89 (0.36)	1.77 (0.67)	2.05 (0.55)**	2.68 (0.67)**	2.50 (1.10)**

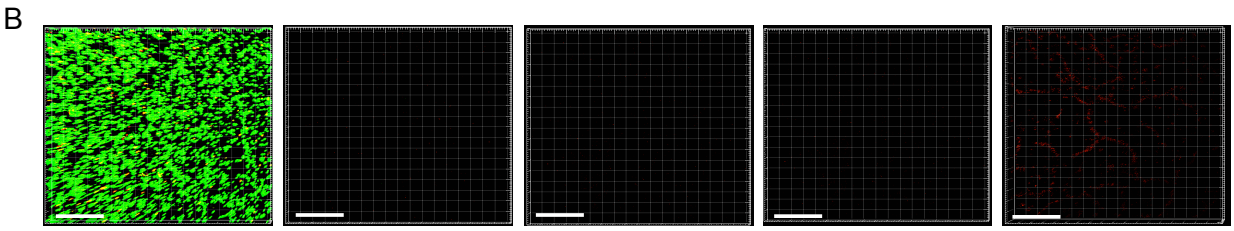


Biofilm Parameters	Control	10	50	100	200
Biovolume ($\mu m^3/\mu m^2$)	8.34 (3.51)	2.81 (1.13)**	1.95 (1.30)**	1.71 (1.67)**	1.76 (1.29)**
Thickness (µm)	15.92 (7.83)	6.60 (2.03)**	4.17 (2.34)**	3.82 (3.72)**	3.83 (2.96)**
Roughness	0.26 (0.19)	1.70 (0.78)**	2.04 (1.01)**	2.27 (0.97)**	2.02 (1.28)**

Figure A.2. Anti-biofilm effects of nisin on *E. faecalis* biofilms. Biofilms formed from **(A)** OG-1 and **(B)** ATCC 29212 was treated with PBS (Control), or different concentrations of nisin (10 – 200 µg/ml). Confocal microscopy images are shown in the x-y plane. Green indicates viable cells stained with Syto-9 and red indicates damaged/dead cells stained with propidium iodide. Biofilm parameters were averaged from triplicates of three separate experiments. Scale bar represents 40 µm. * $P < 0.05$ and ** $P < 0.01$ compared to the control.



Biofilm Parameters	NC	PC	NaOCl conc. combined with 50 µg/ml Nisin		
			1%	0.5%	0.05%
Biovolume ($\mu m^3/\mu m^2$)	11.07 (1.85)	0.16 (0.14)**	0.05 (0.03)**	0.22 (0.13)**	0.68 (0.24)**++
Thickness (µm)	17.33 (2.97)	1.04 (0.56)**	0.04 (0.03)**++	0.39 (0.25)**	1.67 (1.10)**++
Roughness	0.17 (0.11)	3.71 (0.18)**	3.91 (0.10)**+	3.77 (0.25)**	3.11 (0.25)**++



Biofilm Parameters	NC	PC	NaOCl conc. combined with 50 µg/ml Nisin		
			1%	0.5%	0.05%
Biovolume ($\mu m^3/\mu m^2$)	12.50 (2.42)	0.05 (0.02)**	0.05 (0.03)**	0.23 (0.09)**	1.25 (0.19)**++
Thickness (µm)	17.87 (1.26)	0.04 (0.02)**	0.05 (0.04)**	0.46 (0.20)**+	1.89 (0.46)**++
Roughness	0.13 (0.09)	2.62 (1.13)**	3.94 (0.04)**++	3.74 (0.24)**++	2.58 (0.18)**

Figure A.3. Anti-biofilm effects of low concentrations of NaOCl with nisin on *E. faecalis* biofilms. Biofilms formed from **(A)** OG-1 and **(B)** ATCC 29212 was treated with PBS (NC), 4.125% NaOCl (PC), or low concentrations of NaOCl (0.05 – 1%) in combination with 50 µg/ml of nisin. Confocal microscopy images are shown in the x-y plane. Green indicates viable cells stained with Syto-9 and red indicates damaged/dead cells stained with propidium iodide. Biofilm parameters were averaged from triplicates of three separate experiments. Scale bar represents 40 µm. * $P < 0.05$ and ** $P < 0.01$ compared to the NC; + $P < 0.05$ and ++ $P < 0.01$ compared to the PC.

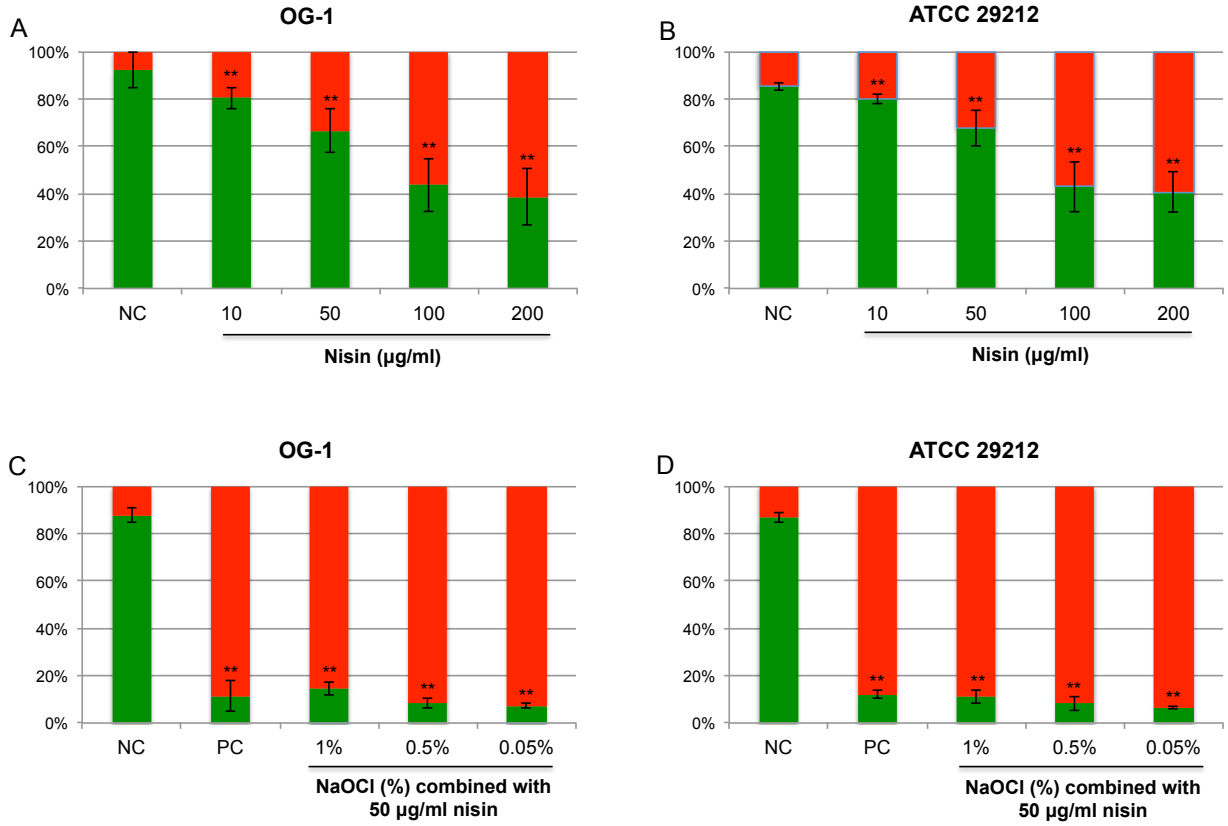


Figure A.4. Viability of *E. faecalis* biofilms after being treated with nisin-alone or in combination with low concentrations of NaOCl. Quantification of viability was determined by the average percent signal between green (Syto-9) and red (propidium iodide) signal in relation to the total signal captured. **(A, B)** OG-1 and ATCC 29212 treated with nisin. **(C, D)** OG-1 and ATCC 29212 treated with NaOCl and nisin.

Appendix B

Metabolomics of Head and Neck Cancer: A Mini-Review

Appendix B is adapted from a manuscript that has been published (Shin, *et al*., 2016b).

Abstract

Metabolomics is used in systems biology to enhance the understanding of complex disease processes, such as cancer. Head and neck cancer (HNC) is an epithelial malignancy that arises in the upper aerodigestive tract and affects more than half a million people worldwide each year. Recently, significant effort has focused on integrating multiple 'omics' technologies for oncological research. In particular, research has been focused on identifying tumor-specific metabolite profiles using different sample types (biological fluids, cells and tissues) and a variety of metabolomic platforms and technologies. With our current understanding of molecular abnormalities of HNC, the addition of metabolomic studies will enhance our knowledge of the pathogenesis of this disease and potentially aid in the development of novel strategies to prevent and treat HNC. In this review, we summarize the proposed hypotheses and conclusions from publications that reported findings on the metabolomics of HNC. In addition, we

address the potential influence of host-microbe metabolomics in cancer. From a systems biology perspective, the integrative use of genomics, transcriptomics and proteomics will be extremely important for future translational metabolomic-based research discoveries.

Introduction

The incidence of head and neck cancer (HNC) exceeds half a million cases annually worldwide and accounts for approximately three percent of adult malignancies (Johnson *et al*., 2011; National Cancer Institute, 2013). HNC is defined as epithelial malignancies that arise in the aerodigestive tract (paranasal sinuses, nasal and oral cavity, pharynx and larynx) and can metastasize to different locations (Rezende *et al*., 2010). About seventy-five percent of HNCs are oral cancers and ninety percent of oral cancers are diagnosed as oral squamous cell carcinomas (OSCC) (National Cancer Institute, 2013; Rezende *et al*., 2010). Despite therapeutic and technological advances, the prognosis for HNC has not improved in decades due to its malignant and recurrent properties (Forastiere *et al*., 2001; Mao *et al*., 2004). The most widely accepted risk factors for HNC include tobacco (smoked or chewed), alcohol use, and human papillomavirus (HPV) infection (Gillison, 2004; Schmidt *et al*., 2004). However, these risk factors alone cannot explain the observed incidence and pathogenesis of HNC, since some patients are not in these risk categories. Thus, it is likely that other unknown factors play important roles in tumorigenesis, tumor progression and metastasis of HNC.

There has been an increasing trend to incorporate ‘omics’ technology, including metabolomics, into oncological research (Cho, 2013; Vucic *et al*., 2012; Armitage and Barbas, 2014; Yu and Snyder, 2016). Investigators have explored different technologies and analytical methods to better understand the metabolomic properties of cancers, including HNC (Bathen *et al*., 2010; Blekherman *et al*., 2011; Beger, 2013; Liesenfeld *et*

al., 2013; Olivares *et al*., 2015). As more independent reports on metabolomics of HNC are being published, a comprehensive meta-analysis of these large 'omics' data sets will be of potential value in the near future to enhance translational studies. Specifically, metabolomic studies can help to potentially identify clinically relevant biomarkers that may be useful in early detection of cancer, to enhance the accuracy of diagnosis and prognosis, and to aid in the development of new drug targets to help improve therapeutic outcomes (Olivares *et al*., 2015; Yu and Snyder, 2016).

The objective of this mini-review is to summarize and discuss the published studies on HNC metabolomics. We will discuss the different technological tools utilized in metabolomics, and focus on the findings from studies that used different types of patient samples (i.e. saliva, serum, blood, urine, tissues). In addition to the host-metabolomic profiles, we discuss the potential relationship and influence of the microbial metabolome in cancers. By coupling metabolomics data with other omics data, we can achieve a greater understanding of complex cancer processes and derive new information that may help to better target aggressive and malignant cancer types, such as HNC.

Biological samples used for head and neck cancer metabolomics

A broad array of biological fluids, such as saliva, blood and urine have been used in metabolomic-based studies (Gowda *et al*., 2008; Psychogios *et al*., 2011; Bouatra *et al*., 2013; Dame *et al*., 2015). These biofluids contain hundreds to thousands of detectable metabolites that can be obtained non- or minimally invasively (Beger, 2013). In addition, cell and tissue extracts can be a source of samples for metabolomic-based

studies (Beger, 2013). With current diagnostic procedures requiring a tissue biopsy, a portion of the tissue samples can be harvested for further metabolomic analyses. The following discussion will focus on the findings, postulated hypotheses, and conclusions from the published metabolomic studies that used different biofluids and cell/tissue extracts to study HNC metabolomics.

Saliva metabolomics

Saliva is an important biological fluid required for multiple functions, including speech, taste, digestion of foods, antiviral and antibacterial protection, to maintain adequate oral health (Loo *et al*., 2010; Spielmann and Wong, 2011). Saliva is readily available, and the collection process is simple and non-invasive. Thus, saliva has been a popular medium for "omics' based research studies (Zhang *et al*., 2012; Cuevas-Cordoba and Santiago-Garcia, 2014). Two types of saliva that can be used for metabolomics studies are stimulated and unstimulated whole saliva. These two saliva types vary in their chemical composition, so it is important to identify the specific type of saliva that was used for the study (Humphrey and Williamson, 2001; Carpenter, 2013; Cuevas-Cordoba and Santiago-Garcia, 2014).

Amongst different HNC types, OSCC is associated with a high morbidity rate and a poor 5-year survival rate of less than 50% (Epstein *et al*., 2002; Mao *et al*., 2004). To improve the prognosis for HNC, investigators have proposed using saliva metabolites to differentiate between precancerous and malignant lesions. Using hierarchical principal component analysis (PCA) and discriminate analysis algorithms, Yan and colleagues were able to distinguish between OSCC and its precancerous lesions oral lichen planus

(OLP) and oral leukoplakia (OLK) (Yan *et al*., 2008; Table 1). Although the OLP and OLK groups were not as well separated in the PCA plot, the OSCC group showed a clear separation from the healthy and precancerous groups (Yan *et al*., 2008). In addition, Wei and others used ultra-performance liquid chromatography coupled with quadrupole/time-of-flight spectrometry (UPLC-QTOFMS) analysis to identify a signature panel of salivary metabolites that could distinguish OSCC from healthy controls (Wei *et al*., 2011; Table 1). Wei selected a panel of five salivary metabolites, which included γ-aminobutyric acid, phenylalanine, valine, n-eicosanoic acid and lactic acid. This combination of metabolites accurately predicted and distinguished OSCC from the control samples, suggesting that metabolomic approaches could complement the clinical detection of OSCC for improved diagnosis and prognosis (Wei *et al*., 2011).

Work presented by Almadori and colleagues discovered that salivary glutathione (antioxidant), but not uric acid (antioxidant), was significantly increased in patients with oral and pharyngeal SCC compared to healthy controls (Almadori *et al*., 2007; Table 1). However, although there were significant alterations in the glutathione levels potentially due to metabolism of malignant cells, the concentrations were too inconsistent to suggest glutathione as a definitive SCC diagnostic marker (Almodori *et al*., 2007). Furthermore, Sugimoto and colleagues identified 28 metabolites that correctly differentiated oral cancers from control samples in their study (Sugimoto *et al*., 2010). Among these differentially expressed metabolites, salivary polyamine levels were markedly higher in oral cancer samples compared to other cancer samples (breast and pancreatic) and controls (Sugimoto *et al*., 2010). Polyamines are small molecules derived from amino acids that are essential for many biological functions (Dimery *et al*.,

1987; Pegg 2009). Increased polyamine levels have been associated with increased cell proliferation, decreased apoptosis and elevated expression of genes affecting tumor invasion and metastasis (Gerner and Meyskens, 2004). Thus, it is hypothesized that polyamine homeostasis is important for regulation of cancer related functions, such as cell proliferation and apoptosis.

Based on published studies that analyzed the salivary metabolome of HNC, there is a general consensus that unique metabolites specific to HNC exist. However, due to differences in detection and analytical methods, the current data still lacks coherency, and a common HNC metabolomic signature has yet to be identified.

Blood and urine metabolomics

In addition to saliva, blood and urine are commonly used for metabolomic-based studies (Psychogios *et al*., 2011; Bouatra *et al*., 2013). Blood is divided into plasma - a cellular portion containing red and white blood cells and platelets, and serum - a non-cellular protein-rich liquid separately obtained following blood coagulation. Both plasma and serum contain a wide variety of metabolites, and current studies suggest that plasma and serum are similar in terms of metabolite content within the aqueous phase (Psychogios *et al*., 2011). Importantly, numerous studies have demonstrated that an altered chemical and protein metabolic composition can now be detected in blood samples obtained from subjects with pathology or diseases, such as cancer (Psychogios *et al*., 2011; DeBerardinis and Thompson 2012). Tiziani and colleagues reported that OSCC patients exhibited abnormal metabolic activity in blood serum, wherein altered activity related to lipolysis, the TCA cycle and amino acid catabolism

was detected (Tiziani *et al*., 2009; Table 1). For example, there was an increased level of ketone bodies present in OSCC samples, suggesting that increased lipolysis was a backup mechanism for energy production (Tiziani *et al*., 2009). Furthermore, a common signature for many cancers includes a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by a comparatively low rate of glycolysis followed by oxidation of pyruvate in the mitochondria, known as the "Warburg effect". Similarly in HNC, Tiziani demonstrated that OSCC tumors relied heavily on glycolysis as a main energy source (Warburg, 1956; Tiziani *et al*., 2009).

Yonezawa and others identified several metabolites that were altered in serum and tissue samples of HNSCC patients who experienced relapse (Yonezawa *et al*., 2013). The four metabolites that were significantly altered were glucose, methionine, ribulose and ketoisoleucine (Yonezawa *et al*., 2013). Interestingly, when the authors compared the metabolomic profiles of the OSCC serum and tissue samples, an inverse relationship was observed in the differentially expressed metabolites (Yonezawa *et al*., 2013; Table 1). Metabolites associated with glycolytic pathways (i.e. glucose) were lower in the tissues, whereas amino acids (i.e. valine, tyrosine, serine, methionine) were expressed in higher levels in the tissues than the serum (Yonezawa *et al*., 2013). In addition, the serum metabolomic profiles differed between patients with or without HNSCC relapse (Yonezawa *et al*., 2013). Several other studies further support that serum and plasma samples from HNC subjects possess distinct metabolomic profiles. For example, elevated levels of choline-containing compounds were detected in OSCC samples in numerous studies (Maheshwari *et al*., 2000; El-Sayed *et al*., 2002; Bezabeh *et al*., 2005; Zhou *et al*., 2009; Tiziani *et al*., 2009). Choline is an important constituent

of phospholipid metabolism in cellular membranes and is considered a biomarker for cancer cell proliferation, survival and malignancy (Ackerstaff *et al*., 2003; Glunde *et al*., 2006; Glunde *et al*., 2011). Through our comprehensive analysis, choline was identified as one of the metabolites that was consistently over expressed in HNC samples regardless of sample types (Fig. 1B). Studies have suggested a link between cancer feedback cell signaling and choline metabolism (Aboagye and Bhujwalla, 1999; Ackerstaff *et al*., 2003; Janardhan *et al*., 2006; Glunde *et al*., 2011; Ridgway, 2013). Thus, an abnormal choline metabolism in cancer has gained much attention and is regarded as a metabolic hallmark for tumor development and progression (Glunde *et al*., 2011).

The use of urine samples in HNC metabolomic studies is not as common compared to the other types of biofluids mentioned above. However, urine is widely used by metabolomic researchers for other conditions or diseases due to its ease of collection and the wide coverage of metabolites that is possible with urine samples (Bouatra *et al*., 2013). Thus far, there has only been a single study reported on HNC metabolomics using urine. From patient urine samples, Xie and colleagues identified a panel of differentially expressed metabolites and demonstrated their utility by logistic regression (LR) modeling (Xie *et al*., 2012; Table 1). When two metabolites, valine and 6-hydroxynicotic acid, were inputted together in the LR prediction model, the authors were able to identify OSCC with a 98.9% accuracy, and a greater than 90% sensitivity, specificity and positive predictive value (Xie *et al*., 2012). However, similar to saliva and blood metabolomics, the use of urine samples for HNC metabolomics will require further validation through more independent studies.

Cell and tissue metabolomics

The current gold standard for diagnosis of HNC is a scalpel-obtained biopsy and subsequent histopathological interpretation. However, the current procedure is subjective and does not capture the full heterogeneic properties of neoplastic processes, as it is difficult to distinguish between precancerous from cancerous and malignant lesions (Rezende *et al*., 2010; Yu and Snyder, 2016). Early studies with magnetic resonance spectroscopy (MRS) using patient tissue samples demonstrated that a higher choline to creatine ratio was observed in HNC samples compared to healthy controls (Mukherji *et al*., 1997; El-Sayed *et al*., 2002; Table 1). In addition, Mukherji and colleagues reported that elevated levels of amino acids, such as alanine, glutathione, histidine, isoleucine, valine, lysine and polyamines were more likely found in tumors compared to controls, and similar metabolites, such as glutathione and polyamines were also elevated in saliva associated with HNC (Mukherji *et al*., 1997; Almadori *et al*., 2007; Sugimoto *et al*., 2010). Srivastava and others used proton high-resolution magic angle spinning magnetic resonance (HR-MAS MR) spectroscopy to identify the metabolic perturbations of OSCC tumors compared to healthy controls. The data revealed higher levels of lactate, phosphocholine, choline and amino acids, and decreased levels of PUFA and creatine in OSCC samples compared to non-malignant samples (Srivastava *et al*., 2011). As previously mentioned, higher levels of detected choline in HNC tissues may indicate increased cancer cell proliferation and membrane biosynthesis, as a result of reciprocal interactions between oncogenic signaling and choline metabolism (Glunde *et al*. 2011). The reduced level of creatine could also be an

indication of increased energy metabolism in tumors (Mukherji *et al*., 1997; El-Sayed *et al*., 2002).

Somashekar and colleagues reported that tumorous tissues biopsied from different anatomical locations (tongue, lip, oral cavity and larynx) displayed similar metabolomic profiles between one another, suggesting that HNSCC tissues share similar metabolic activity during malignant transformation (Somashekar *et al*., 2011; Table 1). Primary and metastatic HNSCC tissues both showed increased/altered levels of branched chain amino acids, lactate, alanine, glutamine, glutamate, glutathione, aspartate, creatine, taurine, phenylalanine, tyrosine and choline compounds, with decreased levels of triglycerides (Somashekar *et al*., 2011; Table 1). In addition, Tripathi and others demonstrated that the cell extracts of HNSCC displayed comparable metabolic phenotypes as observed in the HNSCC tissues (Tripathi *et al*., 2012; Table 1). Thus, based on published reports, the metabolites associated with malignant transformation of HNC are associated with multiple dysregulated metabolic pathways, including glycolysis, glutaminolysis, oxidative phosphorylation, energy metabolism, TCA cycle, osmo-regulatory and anti-oxidant mechanisms (Fig 1.; Somashekar *et al*., 2011; Tripathi *et al*., 2012; Wang *et al*., 2014).

Influence of microbial metabolomics

The human body is a host to taxonomically diverse multi-species microbial communities. In particular, the oral cavity and the gut are home to hundreds of transient and resident microbial species (Eckburg *et al*., 2005; Dewhirst *et al*., 2010). Several publications suggest that the microbiota that colonize the human body (particularly the

oral cavity and gut) contribute to the etiology of different types of cancers because of their ability to alter the community composition and induce inflammatory reactions, DNA damage and apoptosis, and an altered metabolism (Meurman, 2010; Chen *et al*., 2012; Farrell *et al*., 2012; Louis *et al*., 2014). Thus, when considering cancer-associated metabolomics, the influence of the microbiota and its repertoire of metabolites should also be considered, since the microbiota are profoundly abundant in the human body and cancerous tissues.

Colorectal cancer (CRC), like HNC, is associated with risk factors that include diet and lifestyle (Gingras and Béliveau, 2011). Specific bacterial genera, like *Fusobacterium,* are found in greater abundance in patients diagnosed with CRC, colorectal adenomas, pancreatic cancer and HNC (Castellarin *et al*., 2012; Kostic *et al*., 2012; Farrel *et al*., 2012; McCoy *et al*., 2013). Accumulated data suggest that diverse polymicrobial communities can produce a wide range of metabolites by metabolic fermentation (Tang 2011). For instance, gut microorganisms can secrete a variety of metabolites that may play a role in the etiology and prevention of complex diseases (Heinken and Thiele, 2015). These microbial metabolites can directly regulate and modulate the host-tumor cell metabolism (Fig. 1A); bacteria isolated from the gut can produce metabolites that are protective or detrimental to the host tissues and cells. For example, short-chain fatty acids (SCFAs) like butyrate, acetate, and propionate function in the suppression of inflammation and cancer, whereas other metabolites, such as polyamines, are toxic and cancer-promoting at high levels (Louis *et al*., 2014). Alterations in microbial diversity and function due to known risk factors for HNC (alcohol

and tobacco use) and unknown factors could actively contribute to HNC tumorigenesis (Schwabe and Jobin, 2013; Fig. 1A).

Concluding Remarks

The complement of 'omics' based approaches could significantly enhance our understanding of the complex processes of HNC tumorigenesis. Although it is extremely complex, progress has been made in integrating two or more omics data sets to study cancer (Cho, 2013). For example, studies have examined the molecular differences between HPV+ and HPV- HNCs by comparing the differences in their genomic, transcriptomic and proteomic profiles (Sepiashvili *et al*., 2015). Since Otto Warburg's first hypothesis of the altered metabolism of cancer cells, the field of cancer metabolomics has rapidly expanded and revealed intriguing new data regarding metabolic pathways associated with cancers (Warburg 1956). With fast-moving advancements in technology and bioinformatics, the quality of data output and the ability to detect small molecular metabolites has significantly improved. Thus, investigators will likely soon be able to transition from untargeted global metabolomic approaches to more focused targeted and mechanistic-based metabolomic studies. In addition, with the availability of growing public databanks, investigators can now search for specific omics variations that characterize different types of cancers and phenotypes of a cancer (Cho, 2013).

From the clinical perspective, understanding the metabolic pathways associated with life threatening conditions, such as cancer, could be extremely valuable in decreasing the burden of disease. With saliva-based DNA screening tests already

available for chair-side use in dentistry for HNC, we can envision a saliva-based screening or diagnostic test that incorporates omics that replaces the surgical biopsy and provides a more individualized and robust patient health, disease, or risk profile. Here, we discussed the metabolomics of both the host (normal and cancerous conditions) and co-existing microbiota (Fig. 1A). In addition, we organized the differentially expressed metabolites from previous publications by sample types (saliva, blood and urine, cells and tissues) and detection methods (Fig. 1B, C). The full integration and routine inclusion of metabolomics in the clinic has yet to be implemented, however, continued research and translational efforts will reinforce the promise of this evolving technology and science. Studies to date have been conducted with relatively small patient sample sizes, with different sample types and detection methods. In the future, it will be critical to follow up with larger, more comprehensive population studies to confirm the validity of the current findings. In addition, sharing detailed sample collection and analytical methods between investigators will be essential to conduct sound HNC metabolomics research. From the systems biology perspective, the integration of other omics data with metabolomics data will be required for a greater understanding of cancer biology.

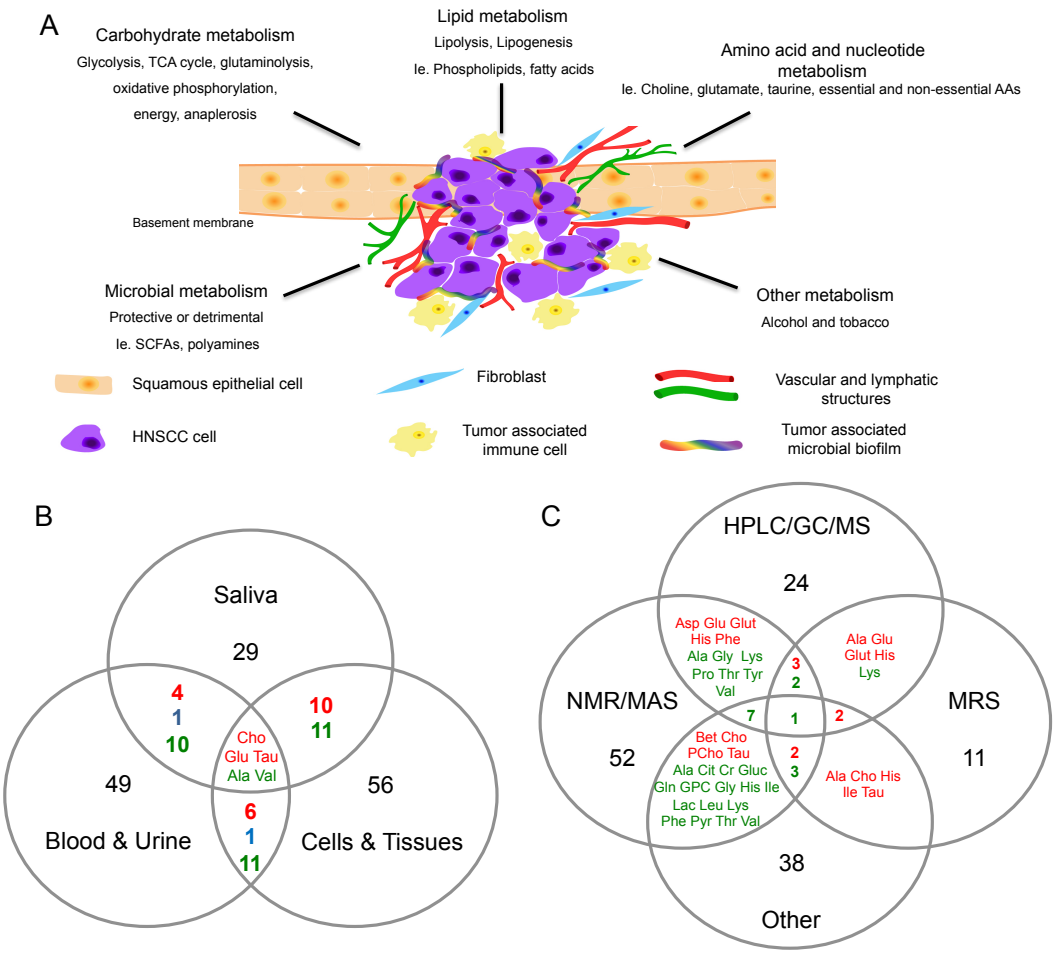


Figure B.1. **Head and neck cancer metabolism**. A) Proposed schematic representation of HNC tumor microenvironment. Altered metabolism in HNC can result in differential expression of metabolites associated with carbohydrates, lipids, amino acids and nucleotide metabolism. The co-inhabiting microbiota of the TME can further result in altered metabolic activity. In addition to the genomic transformation of cancer cells, diet and lifestyle (alcohol, tobacco) are risk factors contributing to the altered cancer metabolism. B-C. Venn diagrams showing, B) overlap of differentially expressed metabolites identified in HNC in saliva, blood and urine, and cells and tissues. C) overlap of differentially expressed metabolites in HNC identified by different detection methods such as HPLC/GC/MS, NMR/MAS, MRS and other. Metabolites were selected and compiled from studies in Table 1.
Red – detected in increased levels; Blue – detected in decreased levels; Green – detected in increased and decreased levels

Abbreviations: Ala(alanine); Asp (aspartate); Bet (betaine); Cit (citrate); Cr (creatinine); Cho (choline); Glu (glutamate); Gluc (glucose); Gln (glutamine); Glut (glutathione); Gly (glycine); GPC (glycerophosphocholine); His (histidine); Ile (isoleucine); Lac (lactate); Leu (leucine); Lys (lysine); PCho (phosphocholine); Phe (phenylalanine); Pro (proline); Pyr (pyruvate); Tau (taurine); Thr (threonine); Tyr (tyrosine); Val (valine).

Subjects	Cancer	Sample	Detection Method	Metabolomic findings	Reference
50 HNSCC 77 healthy	HNSCC	Saliva	HPLC	Increased: Glutathione	Almadori *et al*., 2007
20 OSCC, 20 OLP 7 OLK 11 healthy	OSCC OLP OLK	Saliva	HPLC/MS	Metabolic profiling data distinguished between OSCC, OLP and OLK	Yan *et al*., 2008
69 oral cancer patients 87 healthy	Oral cancer	Saliva	CE-TOF-MS	28 differentially expressed metabolites were detected and was used to predict oral cancer outcome	Sugimoto *et al*, 2010
37 OSCC 32 oral leukoplakia 34 healthy	OSCC Oral leukoplakia	Saliva	UPLC-QTOFMS	41 metabolites distinguished OSCC from control, 61 distinguished OSCC from OLK, and 27 distinguished OLK from control	Wei *et al*., 2011
33 OSCC 5 OLK 28 healthy	OSCC OLK Healthy	Blood (plasma)	^{1}H NMR	At least 17 metabolites were differentially expressed and differentiated OSCC from healthy	Zhou *et al*., 2009
15 OSCC 10 healthy	OSCC	Blood (serum)	1D ^{1}H and 2D ^{1}H J-resolved NMR	Altered energy metabolism: Lipolysis (increased levels of ketone bodies) TCA cycle (i.e. ↓citrate, succinate, formate) Amino acid catabolism (i.e. ↑ 2-hydroxbutyrate, ornithine, asparagine	Tiziani *et al*., 2009
25 HNSCC (Of these patients, 17 used for serum and 19 used for tissue analysis)	HNSCC	Blood (serum) Tissues	GC/MS	Serum: ↑ Glycolysis, ↓ Amino acids Tissues ↑ Amino acids, ↓ Glycolysis	Yonezawa *et al*., 2013
37 OSCC 32 OLK 34 healthy	OSCC OLK	Urine	GC-MS	Increased: Alanine, tyrosine, valine, serine, and cysteine Decreased: Hippurate and 6-hydroxynicotic acid Regression model based	Xie *et al*., 2012

				on valine and 6-hydroxynicotic acid yielded an accuracy of 98.9%, sensitivity of 94.4%, specificity of 91.4%, and positive predictive value of 91.9% in distinguishing OSCC from the controls.	
In vitro: 19 HNSCC 13 healthy 3 metastatic cervical lymph node SCC cell line *In vivo*: 7 HNSCC 7 healthy	HNSCC	Tissues	^{1}H MRS	Mean choline/creatine ratio was higher in HNSCC samples. Several amino acids including alanine, isoleucine, glutathione, histidine, valine, lysine and polyamine were differentially found in HNSCC samples.	Mukherji *et al*., 1997
85 HNSCC 50 healthy	HNSCC	Tissues	^{1}H MRS	Increased: Taurine, choline, glutamic acid, lactic acid, lipid	El-Sayed *et al*., 2002
159 OSCC (Tumor and neighboring margins and bed tissues)	OSCC	Tissues	HR-MAS NMR	Increased: Acetate, glutamate, lactate, choline, phosphocholine, glycine, taurine, leucine, isoleucine, valine, lysine, and alanine Decreased: Creatine, polyunsaturated fatty acids	Srivastava *et al*., 2011
22 HNSCC (matched samples divided into 18NAT, 18 tumor and 7 LN-Met)	HNSCC	Tissues	HR-MAS ^{1}H NMR	HNSCC and LN-Met tissues showed elevated levels of lactate, amino acids and decreased levels of triglycerides.	Somashekar *et al*., 2011
5 HNSCC cell lines 3 primary normal human oral keratinocytes	HNSCC	Cells	^{1}H NMR	21 differentially expressed metabolites: Increased: Lactate, isoleucine, valine, alanine, glutamine, glutamate, aspartate,	Tripathi *et al*., 2012

from patients				glycine, phenylalanine, tyrosine, choline-containing compounds, creatine, taurine, glutathione Decreased: Triglycerides	
2 cell lines (HNSCC cells and stem-like cancer cells)	HNSCC	Cells	Cap IC-MS	Changes in energy metabolism pathways: Glycolysis and TCA cycle	Wang *et al*., 2014

Table B.1. Summary of metabolomic-based studies on head and neck cancers

Abbreviations:

Cap IC-MS: Capillary anion exchange ion chromatography-mass spectrometry
CE-TOF/MS: Capillary electrophoresis-time-of-flight mass spectrometry
GC/MS: Gas chromatography/mass spectrometry
^{1}H-NMR: Proton nuclear magnetic resonance
HR-MAS: High resolution magic angle spinning
^{1}H-MRS: Proton magnetic resonance spectroscopy
HPLC: High performance liquid chromatography
LC/GC: Liquid chromatography/gas chromatography
NMR: Nuclear magnetic resonance
UPLC-QTOFMS: Ultra-performance liquid chromatography coupled with quadrupole/time-of-flight spectrometry

LN-Met: lymph node metastasis

Appendix C

Microbial Communities Associated with Primary and Metastatic Head and Neck Squamous Cell Carcinoma – A High *Fusobacterial* and Low *Streptococcal* Signature

Appendix C is adapted from a manuscript that is under revision.

Shin, J. M., Luo, T., Kamarajan, P., Fenno, J. C., Rickard, A. H. and Kapila, Y. L. (2017) Microbial Communities Associated with Primary and Metastatic Head and Neck Squamous cell Carcinoma – A High *Fusobacterial* and Low *Streptococcal* Signature. *Nat Sci Rep* 'under revision'

Abstract

Given the potential relationship between head and neck squamous cell carcinoma (HNSCC) and microbial dysbiosis, we profiled the microbiome within healthy normal and tumorous (primary and metastatic) human tissues from oral cavity, larynx-pharynx, lymph nodes using 16S rRNA sequencing. Alpha and beta diversity analyses revealed that normal tissues had the greatest richness in community diversity, while the metastatic populations were most closely related to one another. Compared to the normal, the microbiota associated with tumors supported altered abundances in the phyla *Fusobacteria*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*. Most notably, the

relative abundance of *Fusobacterium* increased whereas *Streptococcus* decreased in both primary and metastatic samples. Principal coordinate analysis indicated a separation and clustering of samples by tissue status. However, random forest analysis revealed that the microbial profiles alone were a poor predictor for primary and metastatic HNSCC samples. Here, we report that the microbial communities residing in the tumorous tissues are compositionally distinct compared to the normal adjacent tissues. However, likely due to the smaller sample size and sample-to-sample heterogeneity, our prediction models were not able to distinguish by sample types. This work work provides a foundation for future studies aimed at understanding the role of the dysbiotic tissue microbiome in HNSCC.

Introduction

With greater than 48,000 new cases each year in the United States and >500,000 cases diagnosed annually worldwide, head and neck squamous cell carcinoma (HNSCC) levies a major public health burden (Rezende *et al*., 2010; Siegel *et al*., 2016). Furthermore, the prognosis and the five-year survival rate of HNSCC have been constant for decades (Argiris *et al*., 2008). The known primary risk factors for HNSCC include tobacco and alcohol use, and infection by certain human papillomavirus (HPV) genotypes (Gillison, 2004; Ragin and Taioli, 2007; Schmidt *et al*., 2004). However, these risk factors alone have not been sufficient to explain the incidence and the mechanisms of tumorigenesis, and it is likely that other undescribed factors are playing important roles in HNSCC tumor development, progression and metastasis.

The human microbiome maintains a dynamic relationship with the human host (Cho and Blaser, 2012). For example, if the microbiome experiences an ecological imbalance, also known as dysbiosis, disease processes can emerge (Carding *et al*., 2015; Blumberg and Powrie, 2012). Alternatively, changes in the human host, such as changes in the host adaptive immunity, can alter the associated microbiome (Zhang *et al*., 2015). Numerous studies have now reported that microbial dysbiosis is linked to cancer (Michaud and Izard, 2014; Schwabe and Jobin, 2013; Sobhani *et al*., 2011; Xuan *et al*., 2014). For example, imbalances in the gut microbiota promote altered host-microbial interactions that mediate colorectal cancer (CRC) tumorigenesis (Kostic *et al*., 2013; McCoy *et al*., 2013; Nugent *et al*., 2014). Genomic analysis of the microbiome of CRC patients have revealed a significant enrichment in *Fusobacterium* species with

depletion in species from the phyla *Bacteroidetes* and *Firmicutes* relative to the normal healthy controls (Kostic *et al*., 2013; Tahara *et al*., 2014). Furthermore, Schmidt and colleagues reported that alterations in the oral microbiota were strongly associated with oral cancer (Schmidt *et al*., 2014).

Close interactions between host cells and the microbiota will cause a variety of physiological responses in both the host and the host's microbial inhabitants, including changes in individual microbes or in the collective microbial community. These interactions can be beneficial, neutral, or detrimental to the host. For example, bacterial communities within the gut maintain a mutually beneficial relationship with the human intestinal cells but in CRC, the increased abundance of certain bacteria (ie. *Fusobacterium nucleatum*) and their metabolic byproducts can potentiate and promote tumor growth by eliciting tumor promoting immune and host cell responses (Jobin, 2012; Nugent *et al*., 2014; Shreiner *et al*., 2015). Accordingly, studies have suggested that local or distant cancer-associated microbiota can influence the cancer cells to exhibit cancer-specific inflammatory, immune and metabolic responses, or vice versa (Arthur *et al*., 2012; Burns *et al*., 2015). In this study, we hypothesized that the local microbiota of HNSCC tissues have a distinct bacterial community profile compared to the healthy normal tissues. To understand whether bacterial organisms contribute to HNSCC development and progression or whether the abundance of bacterial organisms is altered in response to HNSCC development and progression, it is important to identify and analyze the associated microbial communities. If the tumor environment favors a specific microbial population or vice versa, further research is warranted to better understand these interactions in the development and progression of HNSCC.

Materials and Methods

Study Design and Human Subject Information

Normal and HNSCC human tissue specimens were obtained from ProteoGenex (ProteoGenex, USA). All clinical specimens were obtained following standard protocols and with appropriate Institutional Review Board/Independent Ethics Committee (IRB/IEC) approval by University of Michigan and ProteoGenex. Tissue samples were acquired based on availability. Tissues were snap-frozen in liquid nitrogen immediately following surgical removal and preserved at -80°C until needed. In total, 72 tissue samples (normal, primary, metastatic) originating from the oral cavity, larynx, pharynx and lymph nodes of 34 HNSCC subjects (32 males and 2 females with an age range of 48-83 years and mean age of 59 ± 5.6 years) were used for this study. Among the collected tissue samples, i) matched normal adjacent, primary and metastatic HNSCC tissues were obtained from 14 subjects, ii) matched normal adjacent and primary HNSCC tissues were obtained from 10 subjects, and metastatic-only tissues were obtained from 10 subjects. We used each human subject as his/her own control (except the 10 metastatic non-matched samples). The subject specific information, including gender, age, tumor anatomic location, clinical diagnosis, TNM staging (extent of the tumor (T), extent of spread to the lymph nodes (N), the presence of distant metastasis (M) and tumor grade as established by histopathological evaluation), are included in Table 1.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from the tissue samples using the RNeasy mini, RNA isolation kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA was then synthesized using the high-capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems, USA).

Microbiome Sequencing and Analysis

cDNA was normalized to 5 ng/µl per sample prior to running polymerase chain reactions (PCR). Targeted amplification and sequencing of the V4 variable region of the 16S rRNA gene was conducted in a single-step 30 cycle PCR using PCR primers 51/806 (Caporaso *et al*., 2011). The HotStarTaq Plus Master Mix Kit (Qiagen, Valentia, CA, USA) was used at the following conditions: 94°C for 3 minutes, followed by 28 cycles (5 cycle used on PCR products) of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. Genome sequencing was performed at MR DNA (www.mrdnalab.com, USA) on an Ion Torrent Personal Genome Machine (PGM) following the manufacturer's guidelines.

Raw 16S data sequences were processed with QIIME 1.9.0. Samples with read counts less than 3000 preprocessing were excluded for microbiome analysis. Of 72 total samples collected, 71 samples had read counts over 3000. Sample M32 was excluded. Sequences with any ambiguous base calls, average Phred quality score below 25, max homopolymer length of > 6, primer mismatch exceeding 0, or sequence length below 200bp were discarded. All sequences that remained after filtering had primers,

adaptors, and linker sequences truncated. Operational taxonomic units (OTUs) were clustered by 97% identity using the Uclust method. An open-reference OTU picking strategy was used where sequences that do not cluster against a reference database of sequences are clustered *de novo*. GreenGenes 13.8 was used as the 16S reference database. Sequences were aligned with GreenGenes-aligned sequences as template using PyNast. Taxonomy was assigned using the RDP Classifier in QIIME (Wang *et al*., 2007). Singleton OTUs were filtered out as part of the default QIIME parameters. Additionally, OTUs constituting less than 0.05% of total reads were filtered out. The final OTU table was analyzed with QIIME and the Phyloseq package in R (Caporaso *et al*., 2010; McMurdie and Holmes, 2013).

Downstream analytics included Shannon alpha diversity, community relative abundance, weighted UniFrac beta diversity, differential OTUs between HNSCC and healthy tissue samples. Outcomes were measured within the Phyloseq package and graphical output generated with R's ggplot package. Additionally, beta diversity was visualized in 2-dimensional space with principal coordinate analysis using R's built-in prcomp function. Log-transformed read counts of the OTU table was used as input for principal coordinate analysis.

Random Forest Analysis

Random forest analysis (RFA) was used to predict normal or HNSCC status. The random forest regression modeling is a nonparametric approach, which accounts for the nonlinearities and interactions within the dataset to identify a subset of OTUs that are predictive of HNSCC. Another advantage of this approach is that cross-validation is built

into the model generation procedure to limit the risks of over-fitting the model to the data (Breiman, 2001). Both primary and metastatic HNSCC tissues were considered different sample types that can be predicted by RFA. Ten RFA iterations were performed with random seeds 1-10 for each dataset where each iteration selected a random subset of 2/3 of the 41 samples to designate as the training dataset. The remaining 1/3 of the samples were designated as the testing dataset where random forest makes its best predictions on the sample types based on the training dataset.

Statistical Analysis

Differences in Shannon alpha diversity as well as Euclidean distance for beta diversity between groups were tested using a non-parametric Kruskal-Wallis test. Differences in phyla abundances were evaluated using a non-parametric Mann-Whitney U test. Differential OTUs were detected using a Wald negative binomial test with the DEseq2 package in R. An α significance threshold of .05 used for the Kruskal-Wallis test and an α significance threshold of .01 was used for the Wald negative binomial test. A more conservative α threshold was selected for differential OTU tests to reduce the number of false positives that would be expected testing a large quantity of OTUs. To test whether microbial communities differ by HNSCC tissue type, an Adonis test that fits linear models to weighted UniFrac distance matrices was performed with R's vegan package. Significance threshold indicating dissimilar communities was set at an α level of .05.

Results

Alpha and Beta diversity of Normal, Primary and Metastatic Tissue Samples

To compare the diversity captured from our samples, we conducted alpha and beta diversity analyses. Alpha diversity was calculated based on the Shannon diversity index, which measures the ecosystem biodiversity. The Shannon alpha diversity algorithm accounts for species richness and species evenness. Normal adjacent tissues had the greatest richness in community diversity compared to the primary and metastatic HNSCC tissue samples (Fig. 1A). The Kruskal-Wallis rank sum p-value was .005, indicating at least one pairwise comparison for Shannon alpha diversity was significant. The pairs driving significance were normal versus primary and normal versus metastatic (Fig. 1A).

For beta diversity, samples were clustered by each category level based on sample groups (normal, primary, metastatic) and each pairwise sample-to-sample dissimilarity was measured using Euclidean distance. Comparing across three sample types, the metastatic microbial taxa populations were more closely related to each other than to those in both the normal versus normal, and the primary versus primary HNSCC tissue samples (Fig. 1B). The p-values comparing within normal versus within primary, within normal versus within metastatic, and within primary versus within metastatic were all < .001 (Fig. 1B).

Phylum Distribution of the Normal, Primary and Metastatic HNSCC Tissue Samples

Tissues were harvested from the oral cavity (lip and tongue), larynx and pharynx, and the mandibular lymph node (Table 1). To account for the differences in the microbiome profiles based on anatomic locations, community analyses were conducted by sample type and location (Fig. 2). Compared to the normal tissues from the oral cavity, primary HNSCC tissues showed increased abundance in *Bacteroidetes, Proteobacteria, Spirochaetes and Fusobacteria* (Fig. 2A). In addition, *Firmicutes* and *Actinobacteria* showed a marked decrease in abundance in the tumor tissues compared to the normal controls (Fig. 2A). Larynx and pharynx also exhibited prominent differences between the normal and the tumorous tissues, where *Fusobacteria* increased and *Firmicutes* decreased in relative abundance (Fig. 2A). As for the metastatic lymph node samples, the increased abundance in *Fusobacterium* species belonging to the phyla *Fusobacteria* and decreased abundance in *Streptococcus* species belonging to the phyla *Firmicutes* was consistent with that in tumor tissues from other locations. However, metastatic tissues selectively exhibited a higher prevalence of *Proteobacteria* (Fig. 2A). The community composition of the oral cavity (n=8) and the larynx-pharynx (n=40) region differed significantly, since *Firmicutes* and *Actinobacteria* flourished much more in the oral cavity (Fig. 2A). However, the power to detect statistical significance in the normal versus primary tumor group was hampered by the smaller sample size for each group. Overall, the microbiota of the tissues collected from the oral cavity exhibited greatest OTU richness (data not shown).

Based on the analysis conducted by tissue status, microbial species from *Firmicutes* and *Actinobacteria* were less abundant in both primary and metastatic HNSCC tissues compared to normal adjacent tissues (Fig. 2B). The relative abundance of *Firmicutes* was significantly lower in both primary and metastatic samples compared to the normal tissue samples (Table 2). The relative abundance of *Actinobacteria* was only significantly lower in primary HNSCC samples when compared to the normal samples (Table 2). The abundance of Fusobacterial populations was increased in both primary and metastatic tumor tissues compared to normal tissues (Fig. 2B); however, only the primary versus normal comparison was statistically significant (Table 2). Statistically significant differences in the abundance of Proteobacterial populations was present when comparing primary and metastatic tumor samples but not when comparing normal and primary tissue samples (Fig. 2B; Table 2). There was no significant difference in *Spirochaetes* abundance in each pairwise comparison by tissue type. The phyla *Tenericutes*, *Synergistetes*, *SR1* and *Thermi* represented less than 1% of the overall composition and their relative abundances were most disparate when comparing metastatic versus normal tissue samples (Table 2).

Genus Distribution of the Normal, Primary and Metastatic HNSCC Tissue Samples

Notable genus groups that increased in abundance in the oral cavity HNSCC tumor samples compared to the normal samples included *Fusobacterium* and *Treponema* (Fig. 2C). A marked decrease in *Streptococcus* and *Actinomyces* were observed in the HNSCC tissues (Fig. 2C). For larynx and pharynx samples, an increase

in *Fusobacterium*, *Prevotella*, *Neisseria* and *Capnocytophaga* was observed, while a decrease in *Streptococcus* was observed (Fig. 2C). Furthermore, the genus *Lactobacillus*, *Parvimonas*, *Peptoniphilus*, *Rothia* and *Veillonella* were differentially abundant in the primary HNSCC samples collected from the larynx and pharynx compared to the normal samples (Table 2).

When the samples were pooled by status, compared to the normal to the primary HNSCC samples, the abundance of *Fusobacterium*, Prevotella, and *Capnocytophaga* increased whereas, *Streptococcus*, *Veillonella*, *Parvimonas*, *Lactobacillus* and *Rothia* significantly decreased (Fig. 2D, Table 2). In comparison to the normal to the metastatic samples, the abundance of *Fusobacterium*, *Neisseria* and other unknown genus groups increased, and *Streptococcus*, *Veillonella*, *Parvimonas* and *Lactobacillus* decreased (Fig. 2D; Table 2). In addition, other significantly altered genus included *Bacteroides*, *Campylobacter*, *Capnocytophaga* and *Rothia* (Table 2).

Relative Abundance of *Fusobacterial* and *Streptococcal* Populations by Sample Types

The relative abundance of *Fusobacterium* and *Streptococcus* was compared by sample types. Matched samples were divided into two groups, 20 (N, P) samples and 41 (N, P, M) samples (Fig. 3). Ten non-matched samples were metastatic tissue samples (Fig. 3). In both matched groups, normal samples expressed greater abundance of *Streptococcus* species than primary HNSCC samples (Fig. 3). In both matched groups, the relative abundance of *Fusobacterium* species in primary HNSCC samples was much greater than the normal samples (Fig. 3). Matched metastatic

samples exhibited more *Streptococcus* species than the primary samples, but were much less than the normal samples (Fig. 3). Both matched and non-matched metastatic samples exhibited lower abundance for both genera compared to the normal and primary samples (Fig. 3).

Differential OTUs Detected between Normal, Primary and Metastatic HNSCC Tissue Samples

Wald negative binomial testing was performed to detect differentially abundant OTUs in 71/72 samples that contained more than 3,000 reads. In the primary versus normal tissue samples, there were 37 differentially abundant OTUs detected, with most belonging to the genera *Streptococcus* (24)*, Fusobacterium* (4) and *Neisseria* (3)*.* The direction of change was consistent across these 3 genera: Normal tissue samples had more *Streptococcus*, less *Fusobacterium*, and less *Neisseria* (see Supplementary Table 1).

In the primary versus metastatic tissue samples, there were 60 differentially abundant OTUs. *Streptococcus* (26), *Actinomyces* (7), and *Fusobacterium* (5) genera constituted a significant portion of the differential OTUs. All of the *Streptococcus* and all of the *Actinomyces* OTUs were differentially abundant in the same direction: all were more abundant in the metastatic tissue samples. *Fusobacterium* OTUs (4/5) were less abundant in the metastatic samples. There was 1 *Fusobacterium* OTU that was more abundant in the metastatic tissue samples compared to primary tissue samples.

The comparison between normal versus metastatic tissue samples was the most disparate in terms of quantity of differential OTUs, which resulted in 104 OTUs. Some

genera of note were *Streptococcus* (23 OTUs, and all of them were more abundant in normal samples), *Fusobacterium* (10 OTUs, with 8 that were more abundant in metastatic samples), and *Actinomyces* (6 OTUs, with all 6 more abundant in normal samples).

Principal Coordinate Analysis (PCoA) Based on OTUs

Forty-one matched samples (normal, primary, metastatic) were used for the PCoA. A clustering pattern exhibited a left to right transition for a normal to primary then to a metastatic tissue status (Fig. 4). Across the sample population, the greatest clustering of communities was observed in the metastatic group, where the distances between samples were the smallest (Fig. 3). The ellipsoid boundaries of all 3 types of samples overlapped with one another. However, there was more substantial overlap in the microbial communities with most of the metastatic samples, such that the metastatic samples' ellipsoid co-localized within the primary samples' ellipsoid (Fig. 4). The separation between the normal versus primary ($p=0.11$), normal versus metastatic ($p=0.194$), and primary versus metastatic samples ($p=0.966$) was not statistically significant as assessed by the Adonis test.

Random Forest Regression Model to Predict HNSCC Using Microbial OTUs

The predictive accuracy of the random forest analysis was 39% (54/140). The majority of misclassifications were metastatic samples misclassified as primary tumor samples and primary tumor samples misclassified as metastatic samples (Table 3). When we categorized primary-metastatic samples as unhealthy, random forest analysis

was able to better differentiate between the two groups. The predictive accuracy of correctly identifying an unhealthy (primary and metastatic) and normal sample increased to 76% and 59%, respectively. In aggregate, the predictive accuracy increased to 98/140 (70%) when the primary and metastatic tumors were combined under one umbrella as “tumor” tissue samples. Based on the RFA results, the outcomes coincided with the intersecting normal, primary and metastatic samples’ ellipsoids shown in the PCoA plot (Fig. 4).

Discussions

Bacteria in the human host often exist as compositionally diverse biofilm communities (Human Microbiome Project Consortium, 2012). The environment created by the host influences the composition of the bacterial community, which is further shaped by other parameters, including temperature, oxygen tension, pH, substratum properties, nutrient availability, and exposure to cell and immune signaling (Cho and Blaser, 2012; Costello *et al*., 2012). In this study, we evaluated the microbial communities of HNSCC tissues and their normal tissue counterparts. Analysis of alpha diversity revealed that normal tissues are significantly more diverse compared to the tumorous (primary and metastatic) tissues (Fig. 1A). Recently, Guerrero-preston and others reported that the saliva of HNSCC patients had significantly lower bacterial richness and diversity (Guerrero-Preston *et al*., 2016). In this study, the beta diversity was greater in the primary HNSCC tissue samples and lower in the metastatic tissue samples (Fig. 1B). However, since the primary tumor tissues were harvested from different anatomic locations compared to the metastatic samples (lymph node), the greater beta diversity may partly reflect the differences in the microbiome profiles based on different biofilm habitats.

Based on our community analyses, the two major differences that were detected in these tissues were related to the abundance of members of the phyla *Fusobacteria* and *Firmicutes* (Fig. 2A and B). Compared to the normal tissue samples, an alteration of these two phyla was clearly observed within primary and metastatic tissue samples regardless of tissue status and location (Fig. 2A and B; Table 2). In both primary and

metastatic HNSCC samples, *Fusobacteria* levels were increased, whereas *Firmicutes* and *Actinobacteria* were decreased compared to the normal samples (Fig. 2B; Table 2). In addition, a significant increase in *Proteobacteria* was observed in the metastatic samples (Fig. 2B). These data demonstrate that the host tumor microenvironment (TME) supports or is influenced by an altered microbial community.

Members of the genera *Fusobacterium* and *Streptococcus* are both highly abundant in the oral cavity (Dewhirst *et al*., 2010). In addition, *Fusobacterium* species are highly associated with periodontal disease (Signat *et al*., 2011). *Fusobacterium nucleatum*, a Gram-negative anaerobe, is well known to coaggregate with both aerobic and obligate anaerobic bacterial species (Bradshaw *et al*., 1998). This strong coaggregative behavior elicited by *Fusobacterium* species is likely to provide additional benefits to the interacting species, beyond assisting with adherence and facilitating multi-species biofilm formation (Kolenbrander *et al*., 2010). For example, *F. nucleatum* has the ability to adhere to and invade human gingival epithelial cells and help other bacteria to enter host cells by altering endothelial integrity (Fardini *et al*., 2011; Han *et al.*, 2000). In solid tumors such as HNSCC, *Fusobacterium* species may play a role in providing protection for the tumor cells from the circulating immune cells. Gur and colleagues demonstrated that the presence of *F. nucleatum* inhibited tumor cell killing through inhibitory protein-receptor interactions with the immune cells (Gur *et al*., 2015). The bacteria and tumor relationship is multifactorial and studies are starting to reveal clues about the specific role of bacteria in cancer. With the significant enrichment of a *Fusobacterial* population in primary HNSCC, these bacteria may be i) providing tumor cell immunity, ii) shaping the microbial community structure, and iii) providing benefits to

the tumor cells by residing in the TME through bacteria-tumor cell interactions. Currently, studies are ongoing in our lab to further investigate the cellular mechanism of *Fusobacterium* species such as *F. nucleatum* in promoting HNSCC tumorigenesis.

Microbial community structure in a habitat is determined by the available nutrients, environmental conditions and the available colonizing species (Kolenbrander *et al*., 2010). A hypoxic and pro-inflammatory TME may promote increases in abundance of certain bacterial populations, such as *Fusobacteria* and *Bacteroidetes*, while limiting the abundance of others like *Firmicutes* and *Actinobacteria* (Fig. 1A). In this study, an inverse relationship in the abundance of *Fusobacterium* and *Streptococcus* species was observed in the tumor tissues versus normal controls (Fig. 3). Schmidt and colleagues also demonstrated that this relationship was present in oral swab samples collected from oral cancer patients (Schmidt *et al*., 2014). A significant reduction in the abundance of *Streptococcus* species and an increase in the abundance of *Fusobacterium* species was observed in oral cancer samples relative to the anatomically matched clinical normal samples (Schmidt *et al*., 2014). In contrast, Gong and colleagues reported that *Streptococcus* dominated over *Fusobacterium* in the mucosa samples of laryngeal SCC patients (Gong *et al*., 2013). Although these studies used different sample types (oral swabs, mucosal tissues, complex tissues) and different sequencing platforms (MiSeq, pyrosequencing, PGM), the inverse relationship relative to abundance between *Fusobacterium* and *Streptococcus* appears to remain robust.

In this study, we hypothesized that HNSCC tissues have distinct microbial communities compared to their normal healthy counterparts. If this community change

occurs as the tissue transition from pre-malignant to malignant, these distinct microbial phenotypes might serve as risk indicators or predictors of disease status. For example, *Treponema denticola* is an oral *Spirochaete* that is normally found in low abundance in the oral cavity. In this study, the *Treponema* species were selectively increased in the primary tumor samples of the oral cavity (Fig. 2C). Frequent and preferential abundance of *T. denticola* has been associated with periodontal disease and esophageal tumor tissues (Holt *et al*., 2005; Narikiyo *et al*., 2004). The oral treponemes are known to be resistant to host antimicrobial peptides (ie. human β-defensins), which can enhance the initial adhesion of other bacterial species to form the multi-species biofilm structures (Sela, 2001). In addition, *Treponema* species are capable of inducing destruction of the host basement membrane structures through their innate proteases, which can further contribute to the tumor development and progression (Grenier *et al*., 1990).

Over the years, identification of strong risk factors, such as tobacco and alcohol use, and HPV infection, have proven to be useful indicators for HNSCC. Smoking can alter the bacterial acquisition and colonization of oral biofilms, and alter the composition of bacterial communities in saliva and biofilms in the subgingival pockets (Bizzarro *et al*., 2013; Kumar *et al*., 2011). In addition, Thomas and colleagues reported that bacterial richness was significantly reduced as a consequence of tobacco or alcohol use.[47] Recently, strong evidence has pointed to microbial dysbiosis as a causative or contributing factor to different types of cancer (Schwabe *et al*., 2013). Alternatively, this dysbiosis may be the result of tumor development and progression. According to our PCoA plot, although not statistically significant, a microbial transition from a healthy to 'HNSCC' status can be seen with ellipsoids moving from a left to right direction along

the PCoA 1 axis (Fig. 4). The primary and metastatic HNSCC samples pooled together with a tighter clustering pattern compared to the normal samples, and the greatest clustering was noted within the metastatic samples (Fig. 4). Further research is warranted to more closely examine the potential relationship between microbial shifts and HNSCC, and the specific role of microbial dysbiosis in HNSCC.

Recently, in a colon cancer mouse model system, RFA was successfully used to predict the behaviors of colon tumors. Specifically, Zackular and others predicted the final number of tumors based on the changes that occurred in the composition of the gut microbiota (Zackular *et al*., 2016). In this study, we utilized RFA to determine if we can predict the HNSCC (primary or metastatic) outcome by using the tissue microbiome data. Our results demonstrated that the accuracy was low in predicting primary and metastatic samples (19%, 37%), but greatly improved when we grouped the primary and metastatic samples into a single group as 'unhealthy' (76%; Table 2). The use of differential OTUs to predict the HNSCC outcome might not be sufficient, since the majority of the OTUs were shared between the normal and the HNSCC tissue types. In addition, unlike the Zackular study that generated predictions from a controlled animal model scenario, the current analysis was applied to human samples, which exhibit a greater level of heterogeneity, and therefore likely explain the lower predictive accuracy. However, it has been demonstrated that differential OTUs can successfully discriminate HNSCC tumor from control samples (Guerrero-Preston *et al*., 2016).

The ideal clinical approach to improve the poor prognosis of HNSCC is through prevention and early detection and treatment. HNSCC is a complex multifactorial disease that is often only detected at advanced stages (Argiris *et al*., 2008). Hence,

understanding the changes that occur in the microbiome associated with HNSCC tumors may provide a foundation for discovering new risk factors for early detection and diagnosis. In addition, a variety of omics biomarkers may be useful as early diagnostic tools for HNSCC (Shin *et al*., 2016). In this study, we report on the alterations in microbial communities observed in primary and metastatic HNSCC tissues. Limitations of this study were i) relatively small sample size, ii) limited subject diversity and patient health history (specifically for tobacco usage, alcohol consumption and HPV status), and iii) heterogenic nature of microbial community based on tissue geography. In this study, we present findings that can serve as a key baseline data for future validation studies. If the altered microbiome is an important risk factor for HNSCC, it will be critical to understand its contributions along with those of other known risk factors. Although it is unclear whether the changes in the microbial composition cause or promote HNSCC or are the result of changes in the cellular activity of cancer cells, more comprehensive analyses involving tissue transcriptomics, proteomics, metabolomics and the microbiome will help better understand the role of host-microbial interactions in cancer.

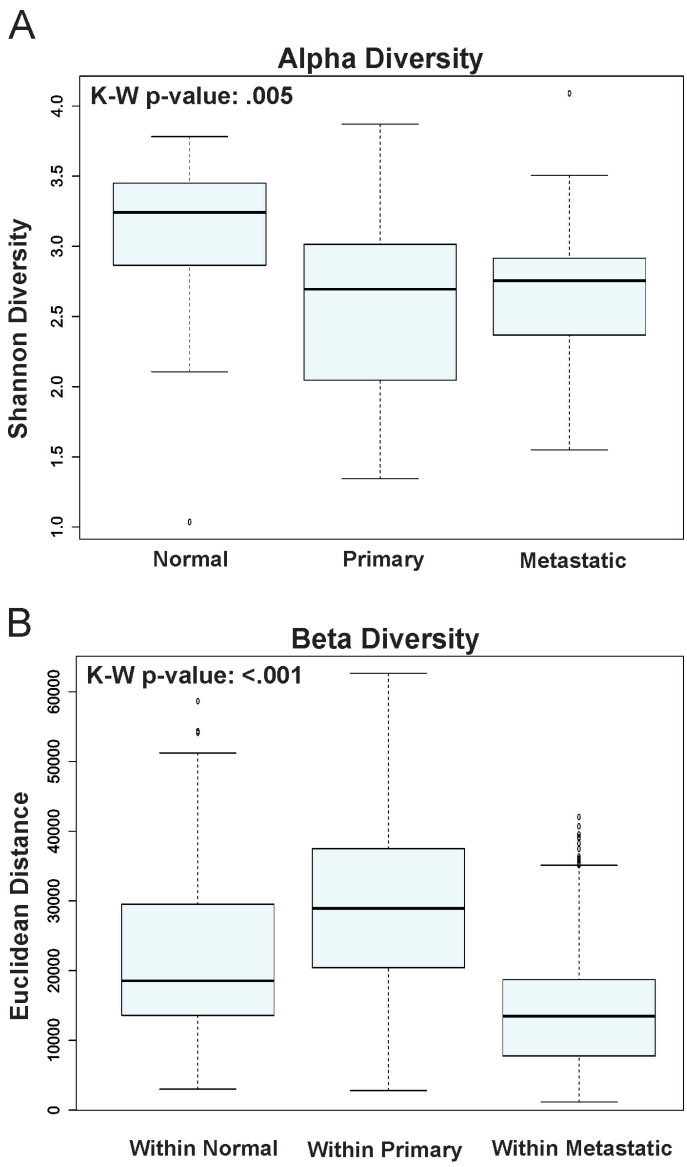


Figure C.1. Alpha and Beta diversity of Normal, Primary and Metastatic Tissue Samples. (A) Alpha diversity based on the Shannon diversity index is shown for normal, primary and metastatic HNSCC tissue samples. (B) Beta diversity was measured by Euclidean distance for normal, primary and metastatic HNSCC tissue samples.

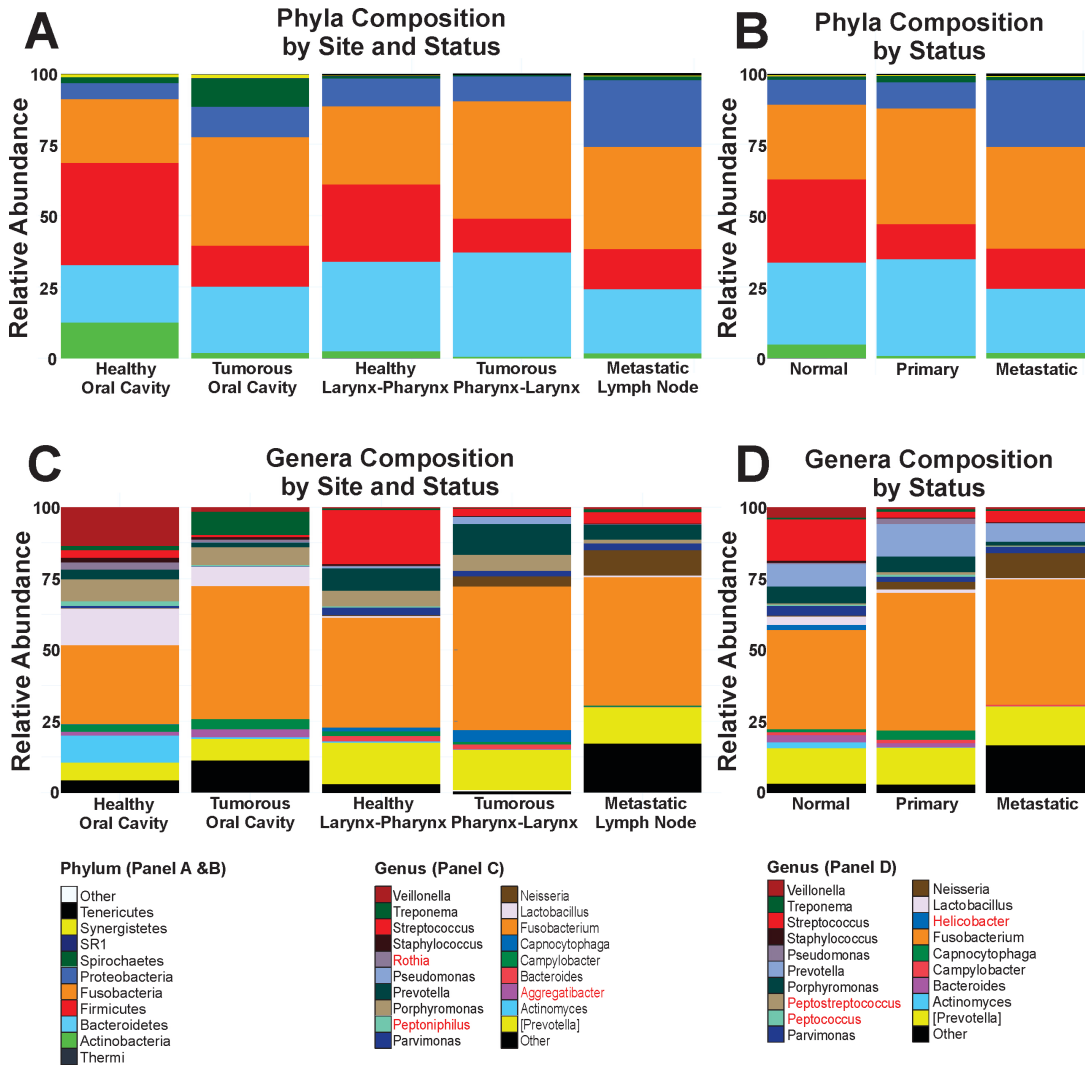


Figure C.2. Phylum and Genus Distribution of the Normal, Primary and Metastatic HNSCC Tissue Samples. (A) The relative distribution of phyla based on anatomical locations is shown for normal, primary and metastatic HNSCC tissue samples. Matched samples were used for analysis. (B) The relative distribution of phyla based on tissue status. Matched and non-matched samples were pooled for analysis. (C) The relative distribution of genus based on anatomical locations is shown for normal, primary and metastatic HNSCC tissue samples. Matched samples were used for analysis. (D) The relative distribution of genus based on tissue status. Matched and non-matched samples were pooled for analysis. *Rothia*, *Peptoniphilus*, *Aggregatibacter* were detected in the top 20 genera by site and status not by status alone (Panel C), whereas, *Peptostreptococcus*, *Peptococcus* and *Helicobacter* were in the top 20 genera by status alone but not by site and status (Panel D).

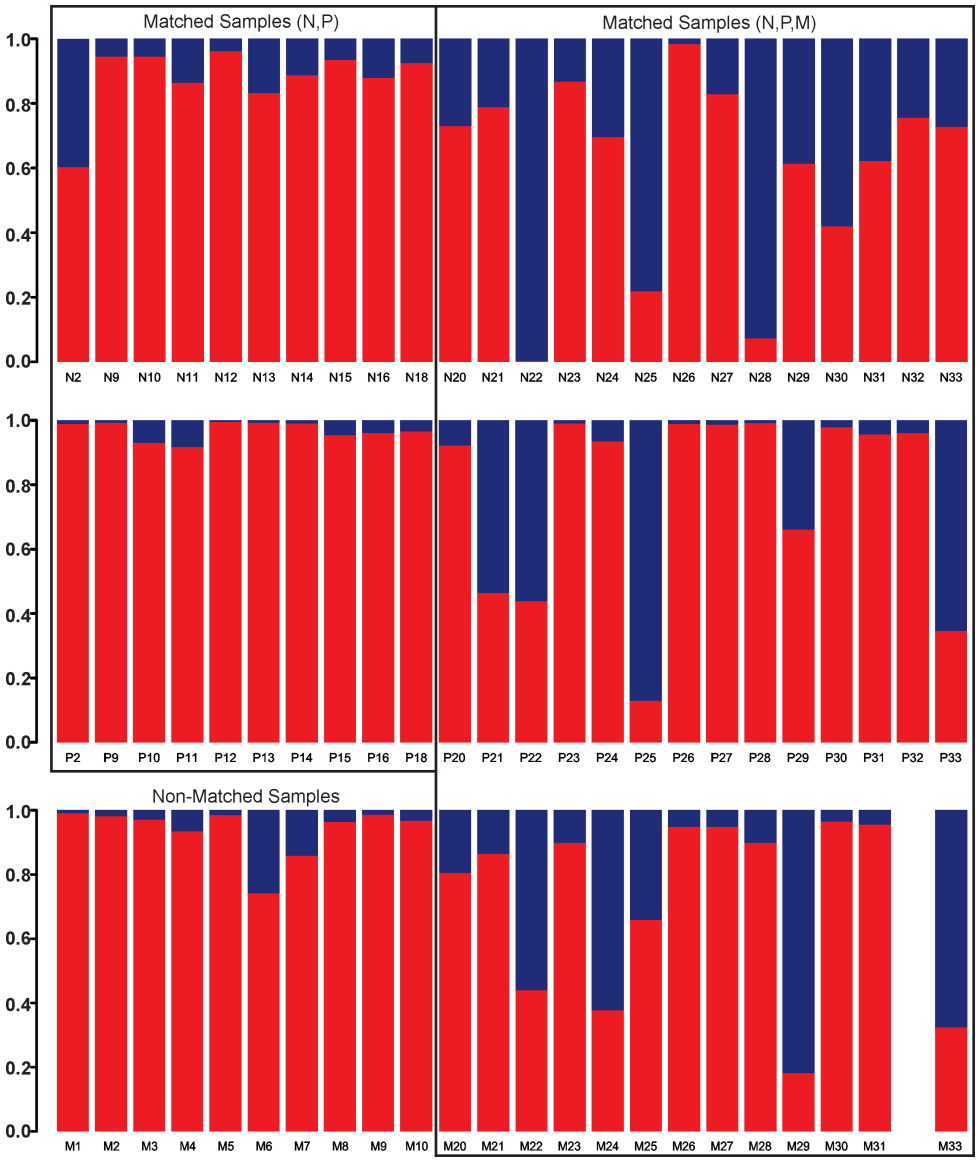


Figure C.3. Relative Abundance of *Fusobacterium* and *Streptococcus* Population by Sample Types. The relative abundance of *Fusobacterium* and *Streptococcus* is shown for matched and non-matched samples. Matched groups are divided into two groups, one containing (N, P) and other (N, P, M) samples. The non-matched group contains only the metastatic samples. Red bars represent the relative abundance of *Fusobacterium* and the blue bars represent the relative abundance of *Streptococcus*.

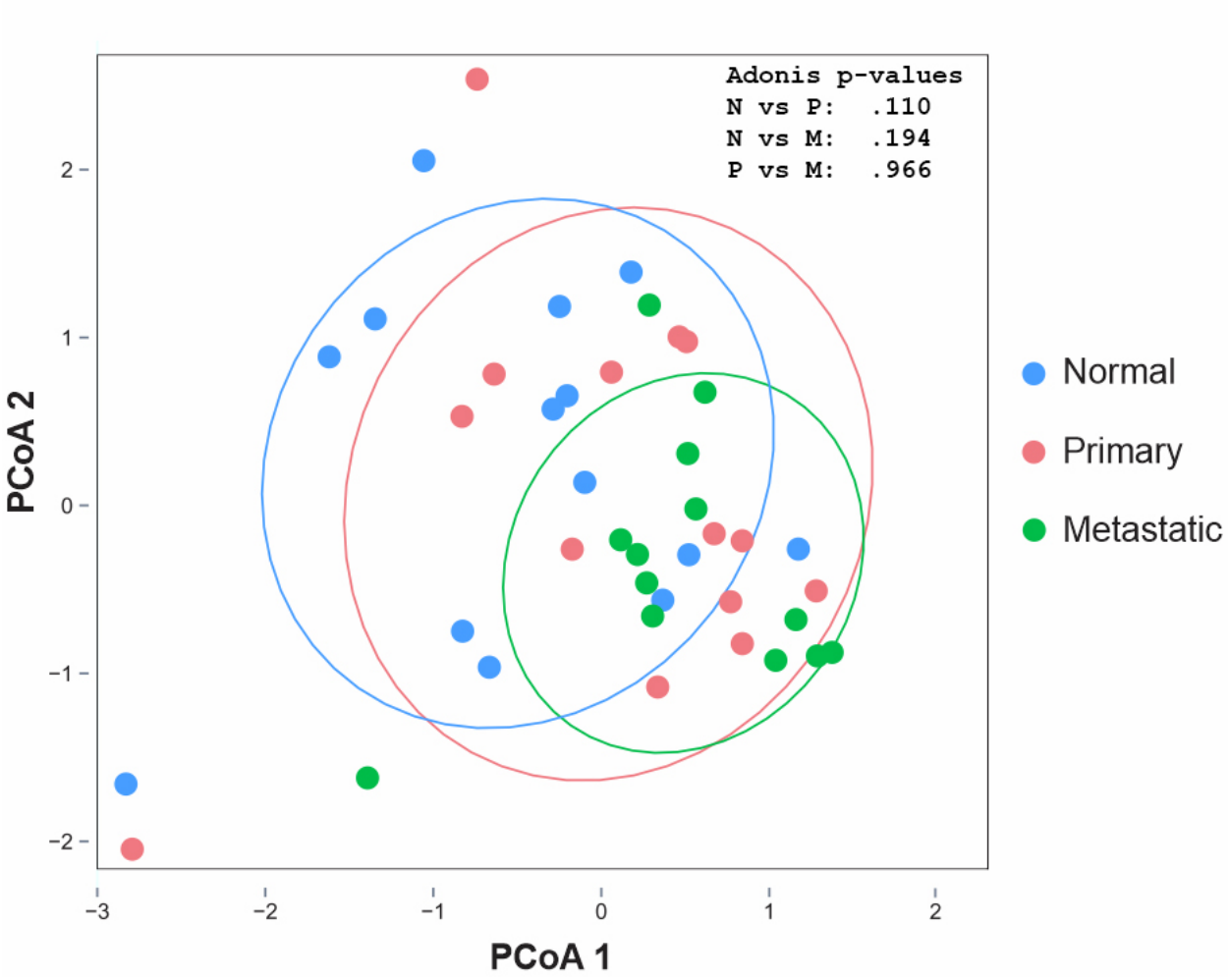


Figure C.4. Distinguishing Normal and HNSCC Samples. Principal Coordinate Analysis (PCoA) was conducted based on the log-transformed read counts of the OTUs. Matched samples (Normal, Primary, Metastatic) were used for the PCoA.

Subject	Gender	Age	Location	TNM*	Grade	Sample type**
20	M	64	Larynx, Lymph node	T2N2bM0	G3	N, P, M
21	M	53	Larynx, Lymph node	T2N2bM0	G2	N, P, M
22	M	56	Larynx, Lymph node	T2N2M0	G3	N, P, M
23	M	54	Larynx, Lymph node	T3N2M0	G1	N, P, M
24	M	59	Oral cavity, Lymph node	T4aN1M0	G3	N, P, M
25	M	63	Laryngopharynx, Lymph node	T3N2M0	G3	N, P, M
26	M	71	Larynx, Lymph node	T4aN2bM0	G2	N, P, M
27	M	60	Pharynx, Lymph node	T3N2bM0	G3	N, P, M
28	M	53	Larynx, Lymph node	T4aN2cM0	G3	N, P, M
29	M	61	Larynx, Lymph node	T3N2cM0	G2	N, P, M
30	M	60	Larynx, Lymph node	T4aN2bM0	G3	N, P, M
31	M	57	Larynx, Lymph node	T3N2aM0	G2	N, P, M
32	M	54	Larynx, Lymph node	T4aN2cM0	G1	N, P, M
33	M	56	Larynx, Lymph node	T4aN2bM0	G3	N, P, M
2	M	62	Oral cavity	T3N0M0	G2	N, P, M
9	M	57	Oral cavity	T4N0M0	G1	N, P
10	F	83	Oral cavity	T3N0M0	G3	N, P
11	M	59	Larynx	T3N0M0	G2	N, P
12	M	50	Larynx	T3N0M0	G3	N, P
13	M	70	Larynx	T3N0M0	G2	N, P
14	M	59	Larynx	T3N0M0	G1	N, P
15	M	67	Larynx	T1N2bM0	G2	N, P
16	M	67	Larynx	T3N1M0	G2	N, P
18	M	60	Larynx	T3N0M0	G3	N, P
M1	M	51	Lymph node	T3N1M0	G2	M
M2	M	68	Lymph node	Recurrent	G2	M
M3	M	59	Lymph node	T3N1M0	G2	M
M4	M	48	Lymph node	T2N1M0	G1	M
M5	M	58	Lymph node	Recurrent	N/A	M
M6	M	58	Lymph node	Recurrent	N/A	M
M7	M	64	Lymph node	Recurrent	N/A	M
M8	M	62	Lymph node	Recurrent	N/A	M
M9	M	61	Lymph node	T3N2cM0	G2	M
M10	F	61	Lymph node	T2N2bM0	G1	M

Table C.1. Human Subject Information. All head and neck tumor samples examined in the study were clinically diagnosed and confirmed as squamous cell carcinoma.
The numbers in the 'Subject' column are for sample identification without any special meaning.
*TNM – TNM classification of malignant tumors is a cancer staging notation system; T describes the size of the original tumor and whether it has invaded nearby tissue; N describes the extent of lymph node involvement; M describes the presence of distant metastasis.[24]
**N – normal; P – primary tumor; M – metastatic tumor

Phylum	Normal Vs. Primary	Normal Vs. Metastatic	Primary Vs. Metastatic	Normal Vs. Primary (Oral Cavity)	Normal Vs. Primary (Larynx-Pharynx)
[Thermi]	.0672	**.0338***	.7808	N/A[a]	.0640
Actinobacteria	**.0045***	.1290	.1134	.2000	**.0094***
Bacteroidetes	.9593	**.0459***	**.0298***	.6857	.8831
Firmicutes	**.0021***	**.0138***	.2666	.1143	**.0042***
Fusobacteria	**.0337***	.1086	.6351	.4857	.0634
Proteobacteria	.3514	.1345	**.0372***	.3429	.1738
Spirochaetes	.4552	.1334	.3221	.4857	.2423
SR1	.0655	**.0044***	.4072	1.000	**.0385***
Synergistetes	.1662	**.0260***	.2746	.8857	.0923
Tenericutes	.4258	**.0225***	.1443	.8857	.4886
Genus					
[Prevotella]	.6903	.9580	.7121	.8857	.5648
Actinomyces	.0271	.8398	.0527	.2000	**.0375**
Aggregatibacter[b]	.8930	.6153	.4999	.3094	.9675
Bacteroides	.1904	**.0017**	**.0345**	.8857	.1478
Campylobacter	.4803	**.0077**	.3922	.0571	.2012
Capnocytophaga	.1472	**.0102**	.1474	.8857	.1022
Fusobacterium	.0533	**.0022**	.2951	.4857	.0965
Helicobacter[c]	.6609	.8574	.5445	.4533	.4989
Lactobacillus	**.0401**	.7738	.1032	.4857	**.0498**
Neisseria	.0926	.0683	.8726	.3429	.2034
Parvimonas	**.0302**	.4795	.0535	.6857	**.0283**
Peptococcus[c]	.8524	.3385	.2519	.4857	.9567
Peptoniphilus[b]	.0832	.8555	.0700	.8845	**.0285**
Peptostreptococcus[c]	.2199	.5198	.0866	.4857	.2977
Porphyromonas	.1253	.0508	.6053	1.000	.0634
Prevotella	.9756	.7438	.8744	.3429	.8410
Pseudomonas	.9753	.4628	.4125	.6857	.8181
Rothia[b]	**.0003**	**.0186**	.3312	.4857	**.0002**
Staphylococcus	.3514	.1039	**.0094**	.4857	.1572
Streptococcus	**.0002**	.1521	**.0088**	.2000	**.0014**
Treponema	.4188	.2130	.5160	.4857	.2211
Veillonella	**.0182**	.0719	1.000	.4857	**.0210**

Table C.2. The p-value matrix indicates the differential abundance of each phylum between tissue samples by status. The average phylum abundance for each tissue sample by status compared to average phylum abundance for each status (normal, primary and metastatic) is shown. Differences in abundance were examined using the Mann-Whitney U-test in R. Boldface* indicates a p-value < .05.

[a] Indicates no reads belonging to the phylum [Thermi] in both comparison groups.

[b] Top 20 genera when stratified by site and status, but not by status alone.

[c] Top 20 genera when stratified by status alone, but not by site and status.

48 Primary Samples	**43 Metastatic Samples**	**49 Normal Samples**	**140 Total Samples**
9(19%) predicted correctly	16(37%) predicted correctly	29(59%) predicted correctly	54 (39%) predicted correctly
21(44%) misclassified as metastatic	23(53%) misclassified as primary	14(29%) misclassified as primary	86 (61%) misclassified
18(37%) misclassified as normal	4(10%) misclassified as normal	6(12%) misclassified as metastatic	
91 Unhealthy Samples		**49 Normal Samples**	**140 Total Samples**
69 (76%) predicted correctly		29 (59%) predicted correctly	98 (70%) predicted correctly
22 (24%) misclassified as Normal		20 (41%) misclassified as unhealthy	42 (30%) misclassified

Table C.3. **Random forest analysis (RFA) was conducted to predict the tissue status by OTUs.** Matched samples (Normal, Primary, Metastatic) were examined to assess the accuracy of using microbial diversity to predict normal and HNSCC tissue conditions.

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