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Development and Characterization of an in vitro Four-species Anaerobic Dental Biofilm Model

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DEVELOPMENT AND CHARACTERIZATION OF AN *IN VITRO* FOUR-SPECIES ANEROBIC DENTAL BIOFILM MODEL

by

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Development and Characterization of an *in vitro* Four-species Anaerobic Dental Biofilm Model

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ABSTRACT

Dental caries is the most common chronic disease worldwide. It is characterized by the demineralization of tooth enamel caused by acid produced by cariogenic dental bacteria growing on tooth surfaces, termed bacterial biofilms. Cariogenesis is a complex biological process that is influence by multiple factors and is not attributed to a sole causative agent. Instead, caries is associated with multispecies microbial biofilm communities composed of some bacterial species that directly influence the development of a caries lesion and other species that are seemingly benign but must contribute to the community in an uncharacterized way. Clinical analysis of dental caries and its microbial populations is challenging due to many factors including low sensitivity of clinical measurement tools, variability in saliva chemistry, and variation in the microbiota. Our laboratory has developed an *in vitro* anaerobic biofilm model for dental carries to facilitate both clinical and basic research-based analyses of the multispecies dynamics and individual factors that contribute to cariogenicity. The rational for development of this system was to improve upon the current models that lack key elements. This model places an emphasis on physiological relevance and ease of maintenance and reproducibility. The

uniqueness of the model is based on integrating four critical elements: 1) a biofilm community composed of four distinct and representative species typically associated with dental caries, 2) a semi-defined synthetic growth medium designed to mimic saliva, 3) physiologically relevant biofilm growth substrates, and 4) a novel biofilm reactor device designed to facilitate the maintenance and analysis. Specifically, human tooth sections or hydroxyapatite discs embedded into poly(methyl methacrylate) (PMMA) discs are incubated for an initial 24 hr in a static inverted removable substrate (SIRS) biofilm reactor at 37°C under anaerobic conditions in artificial saliva (CAMM) without sucrose in the presence of 1 X 10^6 cells/ml of each *Actinomyces odontolyticus*, *Fusobacterium nucleatum*, *Streptococcus mutans*, and *Veillonella dispar*. During days 2 and 3 the samples are maintained continually in CAMM with various exposures to 0.2% sucrose; all of the discs are transferred into fresh medium every 24 hr. To validate that this model is an appropriate *in vitro* representation of a caries-associated multispecies biofilm, research aims were designed to test the following overarching hypothesis: *an in vitro anaerobic biofilm composed of four species (S. mutans, V. dispar, A. odontolyticus, and F. nucleatum) will form a stable biofilm with a community profile that changes in response to environmental conditions and exhibits a cariogenic potential.* For these experiments the biofilms as described above were exposed on days 2 and 3 to either CAMM lacking sucrose (no sucrose), CAMM with 0.2% sucrose (constant sucrose), or were transferred twice a day for 1 hr each time into 0.2% sucrose (intermittent sucrose). Four types of analysis were performed: 1) fluorescence microscopy of biofilms stained with Syto 9 and hexidium idodine to

determine the biofilm architecture, 2) quantitative PCR (qPCR) to determine the cell number of each species per cm^2, 3) vertical scanning interferometry (VSI) to determine the cariogenic potential of the biofilms, and 4) tomographic pH imaging using radiometric fluorescence microscopy after exposure to pH sensitive nanoparticles to measure the micro-environmental pH. The qualitative and quantitative results reveal the expected dynamics of the community profile when exposed to different sucrose conditions and the cariogenic potential of this *in vitro* four-species anaerobic biofilm model, thus confirming its usefulness for future analysis of primary and secondary dental caries.

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INTRODUCTION

Dental caries is the most common chronic infectious disease worldwide (1). It is characterized by the demineralization of tooth enamel caused by acid produced by cariogenic oral bacteria growing on tooth surfaces, termed bacterial biofilms and dental plaque (2). Cariogenesis is a complex process that is influenced by multiple factors and is not attributed to a sole causative agent. Instead, caries is associated with multispecies microbial biofilm communities composed of some bacterial species that have a direct influence on the development of a caries lesion (species capable of fermenting a carbon source into acid) and other species that are seemingly benign. Interestingly, the bacterial species associated with dental caries are members of the normal oral microbiota (3-5). A recent study analyzing the cariogenic potential of plaque isolated from individuals, who were either caries free or had a history of caries, determined that *in vitro* microcosms from both sources had cariogenic potentials that were high and statistically significant (4). Furthermore, microbiome studies have determined that caries and periodontitis are linked to microbial communities that are significantly different from those found not linked to disease (6-8). The relationship of a specific disease state and a specific multispecies microbial community is not restricted to oral disease; microbiome studies have linked many human diseases to microbiomes of specific compositions, including colorectal carcinoma, inflammatory bowel disease, childhood-onset asthma, cardiovascular disease, obesity, reflux oesophagitis, and psoriasis (8).

Dental caries is characterized by a decrease in microbial diversity and an enrichment of specific cariogenic microbes (1, 7, 9, 10). This suggests that a

population shift within the normal commensal oral microbial population is directly linked to the development of caries. However, it is not known how and why caries-associated microbial communities reduce their diversity. The roles played by seemingly benign bacterial species associated with the disease-state microbial community is also unclear. This lack of knowledge concerning the direct relationship between pathogenesis and a disease-associated microbiome is shared by many complex human diseases. Decades of caries research has proven successful in identifying some of the bacterial species and processes that directly influence the development of dental caries. In light of the recent microbiome studies and the dearth of available treatment options (2, 11), novel treatment and prevention approaches that consider the multispecies nature of dental caries are needed.

Koch's postulate has served as the intellectual framework (3-5, 9) for most caries research over the past several decades. As a result, the research community was looking for one microbe that could be isolated from a caries lesion and when returned to a similar environment could cause disease. This has focused the field on characterizing the role played by *Streptococcus* species in cariogenesis. The best studied of these species is *S. mutans,* which can have a significant impact on caries development due to its ability to ferment sucrose to lactic acid and synthesize a robust exopolysaccharide (EPS) that promotes biofilm attachment (4, 12, 13). Although the metabolic contributions of the *Streptococcus* species is certainly a major force that drives a healthy microbial population to a disease-associated state, the impact that other bacterial species have on the cariogenic

process and the on the maintenance of a multispecies pathogenic microbial community has been grossly understudied.

Studying multispecies microbial communities is inherently challenging. Designing experiments for dental multispecies microbial communities is especially complicated by the lack of genetic tools, annotated genomic databases, and model systems (6-8, 14). In addition, the requirements of the typical oral microbe for partial or complete anaerobic growth conditions and specific nutrients can be difficult to fulfill. Methods for culturing many oral microbes are not available (8, 15).

It is now clear that to study human health and disease, we must consider the entire microbial genetic potential associated with an individual. Dental caries could be a useful model system to study the microbes associated with disease. It would be useful to focus on dental plaque and to consider it a community of organisms with a genetic potential to cause and maintain a disease state. Understanding how these microbe-microbe interactions contribute to pathogenesis is an important area of investigation.

The work presented here represents our development of a novel *in vitro* anaerobic multispecies biofilm model system to study of the microbial contribution to dental caries. The rational for development of this system was to improve upon the current models that are lacking in certain key elements. This system was developed with an emphasis on incorporating a high degree of physiological relevance to the *in vitro* system, while at the same time making it easy to maintain and reproduce so that it could be used to address clinical and basic-science questions. The uniqueness of our model is based on the incorporation of four

critical elements: 1) the biofilm community composed of four unique representative species typically associated with dental caries, 2) the semi-defined synthetic growth medium designed to mimic saliva, 3) the physiologically relevant biofilm growth substrates, and 4) the novel biofilm reactor device designed to facilitate the maintenance and analysis of the model system.

Four members of a caries-associated microbial community were chosen to ensure that this artificial microbial consortium includes a simplified, yet diverse range of species that are representative of both species composition and spatio-temporal organization. The organisms included are: 1) *Streptococcus mutans*, an early colonizing Gram-positive cocci that represents the major cariogenic element of the model due to its ability to produce lactic acid from sucrose and to synthesize a glycan-rich biofilm matrix that increases the substrate adherence of all community members; 2) *Veillonella dispar*, a Gram-negative cocci that is a seemingly benign in cariogenesis, but is typically found in high density within caries lesions; 3) *Actinomyces odontolyticus*, a Gram-positive rod, that is commonly associated with diseased oral states; and 4) *Fusobacterium nucleatum,* a ubiquitous member of the oral microbiota consider a 'scaffold' organism based on its ability to maintain biofilm structure. These were freshly isolated strains that were generously provided by our collaborator Dr. Gena Tribble, who isolated them in pairs from two patients.

The inclusion in this model of a semi-defined synthetic medium that mimics saliva was an important element that allows the model to simulate the oral environment, so that the microbes will be as physiologically representative as possible. By choosing to grow our consortia in an artificial saliva medium, our

model is markedly different from essentially all biofilm research that uses standard laboratory microbial growth medium at normal or diluted strength. Although the use of standard lab media brings uniformity to research conducted in different labs worldwide, we propose that the development of simple and standardized physiologically relevant growth media may reveal novel insights in bacterial physiology that may not occur when standard lab medium is used. The development and use of physiologically relevant growth medium for the *in vitro* study of multispecies microbial infections that include *Pseudomonas aeruginosa* has proven successful in facilitating studies previously not possible and has revealed novel phenomenon (16).

To validate that the proposed model is a good *in vitro* representation of a caries-associated multispecies biofilm, the following overarching hypothesis was addressed: that an *in vitro* anaerobic biofilm composed of four species (*S. mutans*, *V. dispar*, *A. odontolyticus*, and *F. nucleatum*) will form a biofilm with a stable, but dynamic community profile that changes in response to environmental conditions. A valid *in vitro* model should exhibit behaviors similar to those observed *in vivo* when subjected to environmental *in vivo*-like conditions. Thus, two specific aims were developed to test our hypothesis. Aim one was designed to determine if a stable and responsive four-species biofilm composed of the recent clinical isolates listed above will form under our *in vitro* conditions. Thus, aim one addressed two basic questions: 1) Will our bacterial multispecies consortia attach to a surface and form a biofilm? 2) Will sucrose exposure alter the species composition of the attached biofilm community in a manner that is reflective of *in vivo* communities?

Natural multispecies oral bacterial communities are known to exhibit specific dynamics as a result of changes in the environmental conditions. Furthermore, *in vitro* biofilms derived from clinically isolated oral bacterial samples have been shown to develop a cariogenic state when exposed to nutritional conditions designed to mimic dietary habits that promote dental caries development, such as in Azevedo, et al. (4).

Aim two was designed to determine if sucrose modulations alter the cariogenic potential of the *in vitro* biofilm community. Host dietary habits have a major impact on the development of dental caries. It has been shown by others that the presence of sucrose directly affects the development of a caries (17, 18). Sucrose appears to promote caries lesion development by producing acid that demineralizes tooth enamel (3, 19, 20). Surprisingly, no studies have directly quantitated the amount of acid produced by the bacteria in the local environment and it is clear that the under most situations the saliva bathing the bacteria is neutral or close to neutral (20). Thus, aim two addressed two basic questions: 1) Does our *in vitro* biofilm model have a cariogenic potential that is dependent on the degree of sucrose exposure? 2) Does the degree of sucrose exposure directly influence the production of acid in a local microenvironment within a biofilm? Our results reveal that this *in vitro* model does exhibit behaviors that are representative of the characteristics observed under *in vivo* conditions and that this experimental approach will be useful in future investigations.

LITERATURE REVIEW

Caries epidemiology

Dental caries is the most common bacterial-associated chronic disease worldwide. Surprisingly, despite decades of research the worldwide caries disease incidence is on the rise (1). The rise in disease occurrence is observed mostly in developing nations, however, it is also observed in specific subpopulations in developed countries (1, 21-23). In the U.S. the occurrence has generally declined to an acceptable rate since the adoption in the 1970's of oral health surveillance programs. A population's dental caries status is estimated based on the average number per individual of decayed, missing, or filled teeth (DMFT). The mean DMFT for the U.S. population dropped from a DMFT of 15 as reported in 1999 to 10 according to the latest comprehensive CDC report in 2004 (22). It was also revealed that specific groups among the U.S. population, including children and economically disadvantaged adults, experienced a disproportionate higher occurrence of disease when compared to the general population. Although the incidence of caries continues to decline, it's rate of decline is decreasing (22, 24). This is disappointing news considering the efforts made by U.S. public health agencies to improve the public's oral health status. Many of the oral health improvement programs implemented over the past decades have focused on educating the public about oral hygiene and dietary risk factors, promoting preventative care, and increasing oral fluoride exposure.

The World Health Organization (WHO) has identified the lack of public health programs in countries experiencing a rise in caries disease occurrence as a

contributing factor that exacerbates the problem. It is interesting to note that historically, from the mid 1970s to the early 1990s, when dental caries was considered a significant problem in wealthy developed countries, the occurrence of dental caries in some developing countries was low (25, 26). The implementation of global public health policies is expected to alleviate the rise in dental caries in developing countries.

Reports of a stagnation in public oral health improvements in developed nations from the early 1990s onward are concerning. Although further analysis is required, there seems to be a direct relationship between the increase in childhood obesity and the rise in dental caries. Fortunately, advances in the understanding of caries pathogenesis through basic science research and improvements in clinical practice appear to have improved the prevention and treatment of the disease (1). There is still much to be learned about both microbial and host-associated elements that lead to the development of dental caries. As a result there is a high potential for novel discovery in caries research that addresses the impact that carries-associated multispecies microbial communities have on the development of the disease. It is certain that many treatment and prevention strategies that target larger subsets of the caries-associated oral microorganisms await discovery.

Dental caries etiology

Cariogenesis is a complex process that is caused by specific bacterial species living within multispecies biofilms attached to the tooth surface, also termed plaque. The etiological agents that cause the disease were among the first

microorganisms observed by Antonie van Leeuwenhoek in the late 1600s. The first reported isolation of a specific microorganism from a caries lesion was of *S. mutans* in the mid 1920s. Since then *Streptococcus*-related species have been at the center of much of the work investigating of how these organisms cause disease (9). Most of this work has made great contributions, not only towards developing an understanding of the caries disease process, but it has also produced important insights into the relationship between infectious diseases and the biofilm mode of bacterial growth (27). It is anticipated that novel therapeutic strategies to better control the development of the disease are destined to be discovered (28).

An important area of research that requires further exploration is the multispecies nature of the biofilm communities that cause the disease. Recent advances in sequencing technology have allowed for a more thorough characterization of the microbial communities found on and in the body. Two general but very significant discoveries were made through investigations of the human microbiota: 1) the human microbiota is much more diverse that previously imagined, and 2) the species compositions of an individual's microbiome is associated with health and disease (29). In the case of dental caries, it was discovered that bacteria previously thought to be the causative infectious agents can be a part of a caries-free individual's microbiome (30). This discovery has prompted many to reconsider the use of Koch's postulates as an intellectual framework to study dental caries (31). This classical paradigm in medical microbiology does not account for the cariogenic process, since it is now known that the presence or lack of a suspected pathogenic species does not always lead to a

pathogenic state (7, 9, 32, 33). Instead, a novel paradigm is now being developed that borrows from ideas used in the study of microbial ecology; caries is being seen as a health problem induced by a bacterial community. There are at least two common themes being developed by investigators: 1) development of the disease is associated with distinct microbial communities, and 2) these disease-associated communities are derived from a pre-existing microbial population (8, 29).

Examining the caries problem from this new perspective is prompting investigators to address questions about external and internal forces that drive a non-pathogenic microbial community into a disease state. Previously, the best-characterized 'driving force' was the affect of *S. mutans* on the dental microbial ecology that promotes a pathogenic state (discussed below). No obvious 'internal forces' within a dental biofilm that would promote a disease state are known. However, the effect of dietary sucrose on promoting dental caries is a well-characterized 'external force'.

Another excellent example of how biological systems can be promoted to enter 'disease states' by external factors, specifically the introduction of excess nutrients, is the harmful algal blooms (HAB) that occur in aquatic environments (34). Although algal blooms occur as natural events, HABs have been associated with human actions that lead to high-level deposits of nutrients into the affected aquatic environments. Excess nutrients are thought to promote the proliferation of specific algal species that can devastate a natural ecology by various means. These events cause ecological catastrophes that lead to drastic reductions in aquatic diversity and aquatic death. It seems that similar to environmental biological systems, the

microbial ecology of the human microbiome has important implications on health and disease.

Oral microbial communities associated with health and disease

A well-established component of dental caries development is the enrichment of *Streptococcus* species in multispecies biofilms (9, 32). Interestingly, the specific factors that contribute to cariogenesis mediated by the bacterial species that exist in a cariogenic biofilm are unknown. One of the major attributes of a caries-associated dental microbial community is the great abundance of *Streptococcus* species and a decrease in the abundance of many other species (8, 9, 35, 36). A recent study analyzing the species diversity of dental biofilms in individuals with and without caries found that the microbial community composition is directly dependent of the oral health of the host. Caries-free hosts were found to have oral microbial communities composed of microbial populations were potential cariogenic agents such as *S. mutans*, *S. salivarius*, and *S. sobrinus* were cumulatively represented at 15% of the total population; over 50% of the population was composed of species considered 'normal' members of the oral microbiome, and the remainder were classified as transient or non-significant species. In contrast, hosts with active caries had microbial populations that were dominated by caries-associated species that included with the *Streptococci* listed above at 65% and *V. dispar* was also a dominant member of the caries-associated microbial community (9).

Interestingly, a studying to identify a bacterium that could serve as a predictor for the eventual development of dental caries found that the presence of *V. dispar,* rather than *S. mutans,* served as the strongest indicator for the risk of caries development (9). The role that *V. dispar* plays in cariogenesis is not clear and investigating the inter-species dynamics that exist would be interesting. Clearly, the existence of such inter-species relationships must be identified and their impact on the cariogenic state and the process that mediates this impact must be determined, so that ways of exploiting these interactions for the development of novel treatments can be discovered.

The pathogenic physiology of *S. mutans* is promoted by other bacterial species

The cariogenesis-associated physiology of *S. mutans* is relatively well understood and thus is a good point of reference in understanding the process of caries development. The consumption of dietary sucrose by a host has long been established as a primary external force that promotes a pathogenic state (17, 37). There are three major virulence factors known to contribute to *S. mutans* cariogencity: biofilm formation, acid production, and acid tolerance (38-40). The ability to form biofilms is an important process that most strongly contributes to the maintenance of persistent colonization and thus a chronic infection. The biofilm lifestyle is a sessile mode of bacterial growth that is characterized by four basic steps: 1) the adherence of the bacterium to a surface, 2) the production of a extracellular polymeric substance that forms a matrix that encases the attached

biofilm cells, 3) maturation and persistence of the biofilm, and 4) dispersal of the biofilm cells into the environment when conditions become unfavorable.

The biofilm lifestyle is a powerful mode of bacterial growth, because it confers advantages not present under planktonic conditions, such as an increased resistance to environmental stress (27, 41). The initial steps of attachment to a surface are mediated by adhesion factors that selectively interact with receptors on the substrate surface. One such group of adhesions are serine-rich repeat polypeptides; deficiencies in expression of the gene that codes for this surface exposed glycosylated protein inhibits the adherence of oral *Streptococcus* species to the tooth pellicle, which is a heterogeneous layer of peptides and polysaccharides coating the surface of a tooth (42).

Oral *Streptococci* also produce factors that promote their adherence to other bacterial species (43, 44). The association of *S. mutans* with other specific bacterial species is known to be important during the cariogenic process, since specific associations have been found to either promote or inhibit *S. mutans* virulence. A study analyzing the expression of virulence-related genes by *S. mutans* grown in dual-species cultures found that growth with specific species either promoted or inhibited virulence-related gene expression. Dual-species cultures of *S. mutans* with *Lactobacillus casei* caused a significant decrease in expression of a gene coding for an adhesion that promotes attachment to the tooth pellicle (SpaP) and genes involved in production of and adherence to glucans (38).

The *S. mutans* biofilm matrix is composed of polysaccharides, proteins and extracellular DNA (27, 44). The *S. mutans* EPS is primarily composed of glucans (a

water insoluble complex polysaccharide) and is of particular importance since its synthesis is dependent on the presence of sucrose and is not produced at levels that support the biofilm development in the presence of glucose (45). EPS production is also promoted by the presence of specific bacterial species (44). Expression of genes required for EPS synthesis by *S. mutans* are positively affected by the presence of bacterial species commonly associated with dental caries. In the presence of *A. naeslundii* and *S. oralis* the expression of two glycotransferase genes (*gtfB*, *gtfC*) required for EPS production by *S. mutans* is enhanced (44). These and other studies suggest that inter-species bacterial interactions are critical in mediating the virulence of oral *Streptococcal* species.

One interesting inter-species bacterial relationship is that between *V. dispar* and *S. mutans*. There appears to be natural cross-feeding between *S. mutans* and *V. dispar*, as lactate, which is a primary carbon source for *V. dispar*, is abundant in a mixed microbial community with *Streptococcal* species because they produce lactate by the anaerobic fermentation of sucrose and provide *V. dispar* with an abundance of nutrients for growth (46). It has also been observed that the presence of *V. dispar* promotes the development of *in vitro* two-species and three-species biofilms (46). Interestingly, it was determined in a microbiome study that the presence of *V. dispar*, rather than S. *mutans*, within the oral microbiome of individuals was a strong predictor for the future development of dental caries (9).

MATERIALS AND METHODS

Bacterial strains and growth conditions

The species used in this study were obtained from and isolated by the G.D. Tribble lab. *F. nucleatum* and *S. mutans* and *V. dispar* and *A. odontolyticus* were isolated in pairs from the oral plaque of two individuals. The bacteria were cultured on CDC Anaerobe 5% Sheep Blood agar (BBL, Franklin Lakes, NJ) or anaerobe-tryptic soy broth (A-TSB, BD) enriched with menadione and hemin (medium composition below); the medium was supplemented with 1.5% sodium lactate for culturing *V. dispar*. The cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) in the presence of 85% N_2, 10% CO_2, and 5% H_2. The Coy anaerobe chamber was the generous gift of Dr. Millicent Goldschmidt.

Media

Anaerobe TSB medium

Anaerobe-TSB is a complex medium enriched with factors that promote the growth of anaerobic bacteria. The medium was enriched with the following: hemin (0.005 g/L), menadione (0.0005 g/L), sodium bicarbonate (0.4 g/L), and 5% horse serum. To prepare the medium, stock solutions of TSB supplemented with 0.1% yeast extract and each supplement were prepared. TSB (1 L) was prepared by dissolving 30 g of TSB and 1 g of yeast extract into ddH_2O. A 5 mg/ml stock solution of hemin was used. The 100-ml hemin stock solution was prepared by first dissolving 50 mg of hemin into 1 ml of 1M NaOH and then adjusting the final volume with ddH_2O. $NaHCO_3$ was

prepared as a 10% stock solution in ddH_2O. The TSB, hemin, and $NaHCO_3$ stocks were autoclaved and stored at 4°C. Menadione was prepared as a 5 mg/ml stock in 95% ethanol, filter sterilized, and stored at -20°C.

Artificial saliva: casamino acid mucin medium (CAMM)

Artificial saliva was developed based on the recipe for DMM artificial saliva by Wong et al. (47). All the stock solutions were prepared as described in Wong et al. (47), and the final medium was prepared as follows. To prepare 1 L of medium, a mucin base solution was prepared containing 2.5 g of mucin dissolved into 100 ml of ddH_2O by heating at 80°C for 1 hr while stirring. The mucin solution was incubated at 4°C overnight, followed by two filtrations steps through # 4 and # 1 Whatman filter paper, respectfully. The ion stock solutions (except $CaCl_2$), choline, chloride, sodium citrate, hemin, inositol, and uric acid were added to the mucin base, the final volume was adjusted to 900 ml, and it was autoclaved. Next, the nutrient base was prepared by first dissolving 5 g of vitamin free casamino acids (BBL), which replaces the casein-equivalent amino acids into 50 ml ddH_2O. Then the base salivary amino acid, vitamin, urea, $CaCl_2$ and ascorbic acid stock solutions were added to the nutrient base and stirred at room temperature until the solution became translucent (10 min). The mucin base was brought to room temperature and the nutrient base was filter-sterilized (Corning 0.22 um cellulose acetate membrane filter) directly into the mucin base and stirred with a sterile stir rod. The final artificial saliva, termed casamino acid mucin medium (CAMM), was divided into 50 ml portions and immediately frozen in disposable centrifuge tubes (-20° C) for future use. The pH of CAMM was measure at pH 7.6.

Primer Design

Primer sets for qPCR analysis were designed to amplify unique regions of the 16S rRNA gene that would quantitatively identify the presence of each bacterial species used in the *in vitro* model with similar PCR amplification efficiencies. A primer design software, Allele ID (Premiere Biosoft, Palo Alto, CA) was used. All of the primers were designed to strictly conform to the physical properties (Tm, %CG, BP length) required for this assay. The primers are listed in Table 1.

Bioreactors

Twenty-four well-plate biofilm reactor

A 96-well microtiter plate biofilm reactor is the accepted standard device used for growing and analyzing static biofilms. In the standard system medium inoculated with bacteria is added to the wells of a microtiter plate and allowed to develop into a biofilm on the surfaces of the well (48). In this study a modified version of the standard system was used in which the substrates (described below) are placed at the bottom of a 24 well-plate and the biofilms that developed on the top-facing surface of the substrate were studied. To prepare the biofilm reactor, substrates are placed in each well of a sterile 24-well plate. The substrates are treated and inoculated as described below. Every 24 hr the used medium is replaced with fresh medium by gently removing the medium from each well using a peristaltic pump and replacing it with fresh medium using a pipet.

Table 1. Primer sets used for qPCR analysis

Bacterial Species	Amplicon Size		Sequence
A. odontolyticus	151	Fwd:	5' - GATACTGTGAGGTGGAGCGAATC - 3'
		Rev:	5 '- GTGACGGGCGGTGTGTAC - 3'
S. mutans	135	Fwd:	5' - GCTTACCAAGGCGACGATACAT - 3'
		Rev:	5' - GCGTTGCTCGGTCAGACTT - 3'
F. nucleatum	158	Fwd:	5 '- CCAACAGAAGAAGTGACGGCTAA - 3'
		Rev:	5' - ACAGTTTCCAACGCAATACAGAGT - 3'
V. dispar	142	Fwd:	5' - GCGGATTGGTCAGTCTGTCTT - 3'
		Rev:	5' - CGCCACTGGTGTTCTTCCTAAT - 3'

Static inverted removable substrate (SIRS) biofilm reactor

A novel static inverted removable substrate (SIRS) biofilm reactor was designed and developed to incubate substrates in a static inverted fashion. This system facilitates the introduction of new medium into the system. To transfer the discs into new medium, the lid with the attached discs is removed from one plate and transferred into a new 24-well plate containing wells pre-filled with the appropriate medium.

Biofilm growth conditions

Biofilm substrates

All biofilm substrates were prepared as circular discs 10 mm in diameter and composed primarily or exclusively of poly(methyl methacrylate) (PMMA, Keystone Research and Pharmaceuticals, Cherry Hill, NJ). Three types of substrates were prepared: 1) substrates composed of only PMMA; 2) substrates composed of hydroxyapatite (HAP) encased in PMMA; 3) tooth sections (TS) encased in PMMA. A medical grade PMMA used in clinical dentistry was used. Hydroxyapatite discs measuring 5 mm in diameter (Himed, Old Bethpage, NY) were used. Carries-free human teeth were acquired from the UT Houston Dental School. Tooth sections were prepared by longitudinally sectioning each tooth into quarters to maximize the number of biofilm substrates prepared from a single tooth. The biofilm substrates were attached to the SIRS biofilm reactor and sterilized by ethylene oxide treatment. The substrates were incubated in CAMM at 4˚C overnight in preparation for inoculation.

Biofilm assay

Overnight liquid cultures of each strain were pelleted, washed with phosphate buffered salts (PBS) composed of 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and normalized to 1 X 10^8 cells/ml based on an optical density measurement. The washed cells were used to inoculate the CAMM biofilm medium to a final concentration of 1 X 10^6 cells/ml of each strain. The inoculum was added to the wells of a sterile 24 well plate at a final volume of 500 µl/well and the SIRS substrates were suspended in the medium. The inoculated SIRS plates were placed in the anaerobic chamber and incubated at 37°C for 24 hr before the specific treatments were initiated.

Experimental design: differential sucrose treatments

Differential sucrose treatments were conducted after the initial 24 hr incubation period. Biofilms were subjected to one of three sucrose conditions. Biofilms were either never exposed to sucrose, exposed to CAMM with 0.2% sucrose two times per day for one hr each time, or continuously exposed to CAMM with 0.2% sucrose. Each day two new (sterile) 24-well plates containing wells with either CAMM without sucrose or supplemented with 0.2% sucrose were prepared. All the 24-well plates also contained negative control wells to test for CAMM sterility. All SIRS bioreactors were organized with three replicate substrates for every condition and one substrate as a negative control for bioreactor sterility.

An intermittent sucrose exposure event (performed in the anaerobic chamber) was conducted by moving the SIRS bioreactor lid from the initial incubation plate onto

the treatment-plate. The substrates in the fresh sucrose-containing medium were then incubated at 37°C for 1 hr, and then transferred into the overnight-plate. A 2 hr time period was allowed to elapse before repeating a sucrose exposure event. After the second exposure event the biofilms were incubated in anaerobic conditions at 37°C for 20 hr in CAMM without sucrose. Each day a new sterile treatment plate and overnight plate were prepared. Sucrose events were performed on the 2nd, 3rd, and 4th days.

After the treatments were complete the biofilm substrates were removed from the SIRS bioreactor lid and prepared for analysis. HAP/PMMA and PMMA discs were prepared for either fluorescence imaging analysis of the attached biofilms or community profiling analysis by qPCR. The cariogenesis analysis was conducted on TS substrates exposed to biofilms as described above, except that sucrose exposure events only occurred for 2 days.

Biofilm analysis

Fluorescence imaging of biofilm structure

Biofilms were stained with Syto 9 and hexidium iodide (Life Technologies, Grand Island, NY) in PBS for 15 min and imaged with an Olympus IX81 fluorescence microscope (Olympus America, Center Valley, PA). Fluorescence staining solution (1.5 µl/ml Syto 9; 1.5 ul/ml hexidium iodide) was prepared in PBS from 1000 x fluorophore stock solution as described by the manufacturer. Biofilm substrates were removed from the SIRS reactor lid as needed and inverted (biofilm side facing up) into a well of a 24-well plate filled with 1 ml of PBS to remove un-adhered cells. The substrate was then transferred into a well containing 1 ml fluorescence staining solution and incubated at

room temperature for 15 minutes. The biofilm substrates were then placed (biofilm side down) onto a glass bottom petri dish (Greiner Bio-One, Monroe, NC) and imaged using an Olympus IX81 fluorescence microscope set at the appropriate excitation and emission detection wavelengths. Image acquisition and processing was performed using Slidebook 5 software (Intelligent Imaging Innovations, Denver, CO).

DNA extraction from biofilm cells attached to growth substrate

After completion of the differential sucrose treatment total DNA was extracted from the biofilm cells using a DNeasy Blood and Tissue DNA extraction kit (Qiagen Group). Modifications to the standard method were made to increase the probability of efficient cell lysis and a more quantitative isolation of DNA from all species in the biofilm community. Modifications are as follows: 1) sonication of the substrate samples to assist in the detachment of biofilm cells from the growth substrate, 2) the use of additional lytic enzymes to assist in the lysis of Gram-positive bacteria, and 3) larger reaction volumes to ensure that the substrate remain completely submerged in the lysis solution.

First, the substrates were removed from the SIRS bioreactor and placed into 15 ml conical tubes containing 600 µl of bacterial lysis buffer (20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100). The samples were then sonicated for ten min in a sonicating water bath (Branson, Danbry, CT). A concentrated working stock of enzamatic lysis solution was prepared fresh as follows. First, a stock solution of lysozyme (60 mg/ml) was prepared. Next lysostaphin (100 µg/ml) and lytic enzyme solutions (7.5 µl/ml) (Qiagen) were added to the lysozyme stock. The enzymatic lysis

solution (300 µl) was added to each sample and incubated at 37°C for 1 hr. Next, 100 µl of Proteinase K (20 mg/ ml) followed by 1 ml buffer AL were added to each sample and incubated at 56°C for 1 hr. Molecular grade absolute ethanol (1 ml) was the added to each sample and vortexed until thoroughly mixed. The ethanol/cell lysate solution was then applied to DNA binding columns (provided in the kit) by adding 660 µl of the solution to the column, centrifuging the samples at 6000 x g for one min. The eluted solution was discarded and this step was repeated two more time until all the lysate solution was applied to the column. All the subsequent steps were performed as described in manufacturer's protocol.

Real-time qPCR community analysis

All of the real-time qPCR reactions performed used the iTaq Universal qPCR SYBR Green kit (Bio-Rad, Hercules, CA). The conditions were developed according to the product literature. The real-time qPCR reactions were monitored using an 7500 ABI instrument (Life Technologies). Standard curves were generated using amplicons generated with the primer sets used for qPCR analysis. Reactions were prepared in 20 µl volumes with template DNA (125 pg/µl) and primers (500 nM). For each treatment, two samples of DNA extracted from independent biofilm substrates were examined in duplicate for a total of four values.

qPCR Data analysis

Amplicon copy numbers were derived from the ABI 7500 analysis software. The total number of cells of each organism was extrapolated by dividing the total gene copy number by the predicted number of 16S rRNA genes present in each species'

chromosome. The calculations presented here are based on 16S operon copy number predictions obtained from the Ribosomal RNA Operon Variation Database (49). The following formulas were used: {# cells/qPCR rxn.} = [(16s gene amplicon copy number) / (predicted operon copy #)]; {#cells/biofilm substrate} = ([# cells/qPCR rxn)*(total extracted DNA (ng)/2.5 ng]).

Vertical scanning interferometer

Topographical images using vertical scanning interferometer (VSI) were collected before and after exposure of the substrates to the biofilm using a prototype Zemapper (Zemetrics/Zygo, Tucson, AZ) interferometer with a Mirau objective. Image Metrology's SPIP^{TM} software was used to generate the images and make measurements. Each TS sample prepared for VSI required a reference point that was not subject to degradation. This reference point was generated by applying a roughly 1 mm in diameter drop of silicone-masking polymer (Permatex High Temp RTV silicone gasket maker) to the center of prepared PMMA-embedded tooth surface prior to the biofilm exposure. After the biofilm exposure the samples were sonicated as described above to remove biofilm growth and dried. Prior to viewing the reference mask was removed.

Statistical Analysis

Community profile (qPCR)

The results presented here represent averages of 6 samples (per experimental condition) originating from duplicate (2 biofilm substrates) biofilms from 3 separate experiments. In order to determine if differences between the percentage of each organism within a biofilm community exposed to a specific sucrose treatment

sequence are significantly different, comparisons using ANOVA with tukey post-hoc comparisons were performed. ANOVA analysis was used to determine if differences between the total number of bacterial cells in a given sucrose treatment conditions was statistically different from others.

Cariogenesis analysis

A paired t-test was used to compare the post reaction values to the pre-reaction value for each of the three conditions.

Fluorescence nanoparticle pH analysis

Core-shell silica nano-particle pH sensors (Hybrid Silica Technologies, Cambridge, MA) have been successfully used in a proof of concept report by Hildago, et al. (50) to characterize the spatial distribution of mix-species wastewater biofilms. Measurement of pH is based on the use of two fluorophores (one particle with fluorescence emission that is insensitive to pH and one with fluorescence emission that are directly dependent on pH) encased in single nano-particle sensors. The emission intensity of the pH dependent fluorophore is divided by that of the insensitive fluorophore to generate a ratio, which can be used to determine the pH value based on a standard curve (see below). These core-shell silica sensors are used here for the qualitative characterization of average pH values within individual fluorescence microscopy images from 3D image stacks to investigate differences in pH between areas of the biofilm that are at the biofilm-substrate interface compared to areas that are at the biofilm-medium interface.

The core-shell particles used for this study were composed of a tetramethylrhodamine isothiocyanate (TRITC) core with an emission intensity that is independent of pH and a fluorescein isothiocyanate (FITC) shell with an emission intensity that is directly dependent on pH. The sensors (25 nm in diameter) were supplied by the manufacture in 0.2 µM and 0.4 µM concentrations. Before imaging a standard curve was generated to calibrate sensor responsiveness and to determine the relationship between fluorescence emission ratios and pH. Calibration was performed by determining fluorophore emission ratios of sensors suspended in sodium phosphate saline solution (SPSS) at different pH values. SPSS solutions at different pH values were prepared by mixing appropriate volumes of monobasic SPSS (10 mM NaH_2PO_4, 14 mM NaCl) and dibasic SPSS (0.01 M Na_2HPO_4, 0.14 M NaCl). Individual solutions were prepared at pH values within a linear range between 4.77 and 7.84. The nano-particles sensors were added to 600 µl of the PBS solutions to a final concentration of 22 nM and 175 µl of this solution was added, in triplicate wells of a glass bottom 96 well plate (Greiner Bio-One, Monroe, NC). Fluorescence imaging of the solutions and analysis with a fluorescence plate reader (Biotek, Winooski, VT) confirmed that emission ratios had a linear response to pH. Emission ratios were extrapolated to pH as described in Hildago et al. (50).

For each pH distribution analysis within the *in vitro* biofilms, the biofilms attached to their growth substrate, were prepared as follows: The biofilm substrates were removed from the SIRS bioreactor lid and inverted biofilm side up into a 24-well plate. A 200 µl portion of the nano-particle sensors (0.1 µM) in CAMM with 0.2% sucrose was carefully added to the surface of the biofilm substrate, so that the solution was

maintained on the substrate. The biofilms were incubated for 30 min under aerobic conditions and any sensors that were not absorbed by the biofilm were removed by briefly submerging the substrates into PBS (pH 7.4). The substrates were then inverted onto a glass bottom petri dish (Greiner Bio-One) and 3D imaged using an Olympus IX81 fluorescence microscope set at the appropriate excitation and emission detection wavelengths for the core-shell silica fluorophore particles. Excitation exposure times were determined by setting the imaging capture software, Slidebook 5 (Intelligent Imaging Innovations, Denver, CO) to automatically select the best exposure time for TRITC and setting the same exposure time for FITC. Fluorescence emission values for every pixel of each image from the 3D stacks was exported into excel spreadsheets and the pH value for each pixel was calculated as described by Hildago, et al. (50). All data for pixels with ratios/pH values outside the range of detection were discarded. The percentage of pixels with pH values within the defined value range groups was binned and these values were divided by the total number of pixels within each image.

RESULTS

The current *in vitro* dental biofilm models have proven useful and effective in the characterization of microbial biofilms associated with oral disease. Studies using these models have also contributed significantly towards a better general understanding of microbial-biofilm-related phenomenon in health and disease. Specific *in vitro* model systems are normally designed based on the type of questions being tackled and the analytic techniques required to effectively address these questions. The model presented here was developed to characterize the impact of multispecies microbial communities on the development of dental caries. It was important that this model system be as physiologically relevant as possible, be easily maintained and inexpensive, and that a wide range of analytical techniques be usable for the analysis. After surveying the literature, it was determined that none of the existing models met all of the required criteria. Most of the current models were found to lack species diversity, as a majority are composed of *Streptococcus*-related species. However, a few of the current models were composed of too many species, which would complicate our analysis (20, 51-54). The current models most generally use common standard microbial media for biofilm growth, whereas others use real or artificial saliva (51-53). A wide range of substrates are used including natural substrates such as human teeth (48, 53), but the most common substrate is the wells of a polystyrene microtiter plate.

We have developed a novel model system that is physiologically relevant due to its use of: 1) four representative recently isolated dental species, 2) CAMM, semi-defined artificial saliva, 3) natural and relevant biofilm substrates, and 4) a

novel biofilm reactor and substrate system designed to facilitated the maintenance of biofilms over long growth periods and to allow a variety of analytical techniques for biofilm analysis.

Development of a novel static inverted removable substrate (SIRS) biofilm reactor

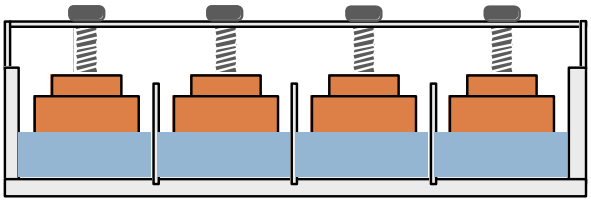
Inverted substrate component of the SIRS biofilm reactor

The static inverted removable substrate (SIRS) biofilm reactor was fabricated using a standard 24-well microtiter plate. The lid of the 24-well plate was modified to accommodate 24 ½ inch 6-32 stainless steel screws. First, using a 7/64 drill bit, 24 holes were drilled into the area of the lid directly centered above each well. A 6-32 tap was used to thread each hole. The stainless steel screws were then threaded into the lid (Fig. 1).

Removable substrate component of the SIRS biofilm reactor

Three types of substrates were fabricated: 1) PMMA only, 2) a tooth section embedded into PMMA, and 3) a HAP disc embedded into PMMA (Fig. 2). All the embedded materials were embedded into the flat biofilm-growth-surface side of a 10 mm dia. PMMA disc that was later exposed to the bacteria and medium (Fig. 2). The opposite side of the substrate contained a narrow diameter internally threaded end. The substrates were cast from PMMA paste and further modified. First, a 5 cc syringe was modified to serve as a mold for casting the substrates. The syringe was cut at the 1.5 cc mark and the tip end was retained and the barrel discarded. A

A



B

C

F**igure 1. The static inverted removable substrate (SIRS) biofilm reactor.** (A) A diagram of a cross-section of the SIRS biofilm reactor. Each disc is suspended from the 24 well-plate lid with a screw that is adjusted so that only 5 mm of the lower part of the disc surface is suspended in the medium. (B) The image shows prepared discs attached to the 24-well plate lid resting on top of a 24-well plate bottom prior to being inverted and placed into the plate for sterilization. (C) This image shows a SIRS biofilm reactor sterilized by exposure to ethylene oxide and ready for use.

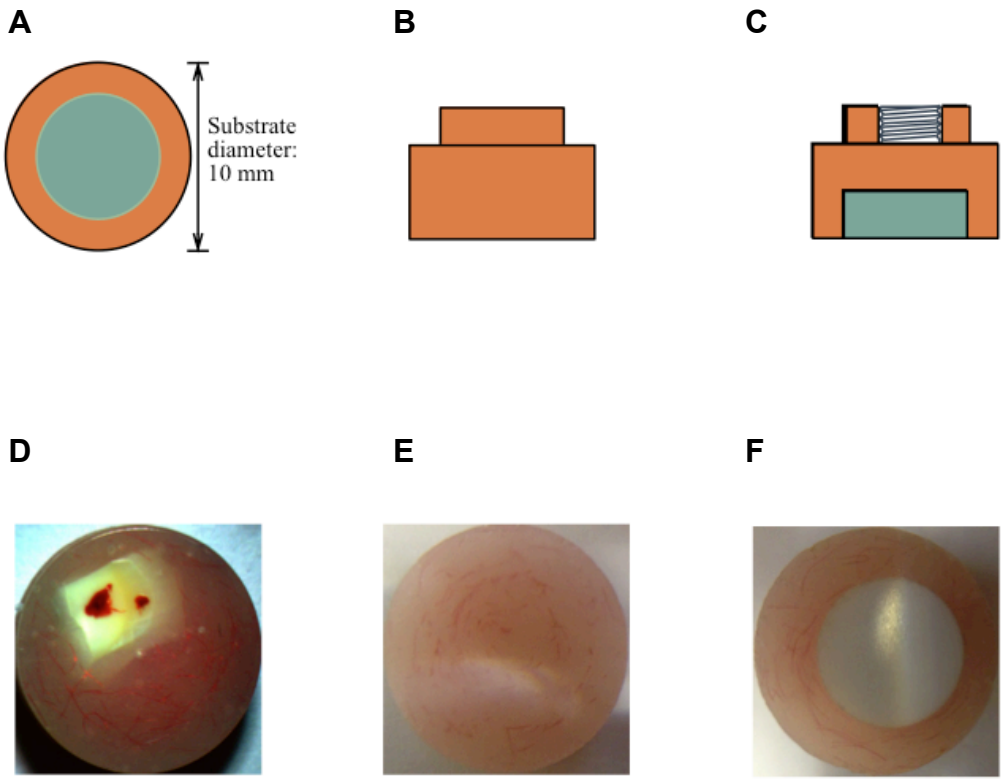


FIGURE 2. Native or artificial tooth sections embedded in poly(methyl methacrylate) (PMMA) are the SIRS biofilm reactor substrates.
(A) Bottom view diagram of the substrate showing the native or artificial tooth section in green and the PMMA in orange. (B) A side-view diagram of the substrate. (C) A cross-sectional view diagram of the substrate. The gray lines represent the internal threads for the screw. (D) An image of the native tooth section embedded in PMMA. The native tooth sections are divided longitudinally into quarters. It is embedded in PMMA to expose the enamel surface by casting in a PMMA (Keystone) paste. After the PMMA hardens, the exposed surfaces are polished. A silicone-masking polymer (Permatex High Temp RTV silicone gasket maker), seen as the red object on the substrate, is applied (1 mm dia.) in the center of the exposed tooth prior to sterilization to serve as the unaffected reference surface. (E) An image of the PMMA substrate with nothing embedded in it. (F) An image of the artificial tooth section embedded in PMMA. The artificial tooth sections are commercially prepared high-density hydroxyapatite (HAP) discs (7 mm dia. X 2.2-2.4 mm thick with a bulk density of >3.0 gm/cm^3 and internal porosity about 5-6%, Himed Corp., Old Bethpage, NY) embedded with the flat disc surface exposed.

6-32 screw was threaded into the tip end of the syringe until it reached the 0.5 cc graduation mark. The modified syringe was then placed (with the open side facing up) in a test tube rack to hold it in place. Next, a two-part dental grade PMMA resin was prepared according the manufacturer's directions. Briefly, the resin was prepared by mixing a liquid acrylate monomer catalyst with a powdered polymer (3 to 1 ratio). This produced a viscous liquid that was poured into the modified syringe mold. The resin was cured for 5 min, so that the PMMA hardened slightly into a gummy soft clay-like consistency that could support the material to be embedded without the material becoming submerged in the liquid polymer. Next, either a HAP disc or a tooth section was placed into the resin-filled mold. The PMMA was allowed to cure overnight. The next day the screw was detached and the PMMA substrate was removed from the plastic syringe mold. Each substrate was then polished with fine grit sand paper to generate a flat surface.

Community profile analysis in response to modulation of the sucrose concentration in the medium.

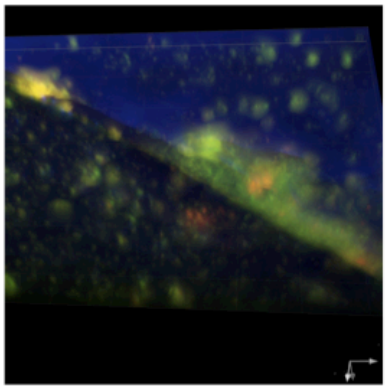
Fluorescence microscopic analysis

To qualitatively examine the growth of the biofilm communities and to evaluate the biofilm architecture, a subset of the treated discs were subjected to analysis with the fluorescence microscope. The fluorescence microscope images of the four-species anaerobic biofilm model grown on an artificial HAP tooth substrate after 72 hr of exposure to different sucrose conditions qualitatively demonstrate the formation of biofilms on the substrate surface and provide an view of the biofilm

architecture (Fig. 3). A fluorescence 'Gram-staining kit' containing Syto 9 and hexidium idodine was used to differentiate between Gram-positive and Gram-negative cells. Both fluorophores label bacteria by binding to DNA and selectively stain the cells based on the unique permeability of each molecule to bacterial cell walls. Syto 9 permeates all bacterial cells and hexidium iodide permeates only Gram-positive cells. Syto 9 dye emits in the green spectrum, so the Gram-negative cells appear green. Hexidium iodide emits in the red spectrum, so the Gram-positive cells appear red.

Figure 3 shows representative images of biofilms grown under different sucrose exposures: without sucrose (Fig. 3A), with intermittent sucrose exposure (0.2% sucrose 2 times per day for 1 hr each time) (Fig. 3B), and continuous exposure (0.2% sucrose) (Fig. 3C). The biofilms grown without sucrose appeared to have the least amount of biofilm formation in comparison to the biofilms grown under the other two conditions; almost all attached cells exhibited only green fluorescence, indicating that the attached cells were mostly Gram-negative cells (Fig 3A). The biofilms exposed to intermittent sucrose conditions appeared to have a greater degree of biofilm attachment, as reflected by a greater amount of fluorescence associate with the substrates. A larger percentage of attached cells exhibited red fluorescence, indicating a larger presence of Gram-positive cells in comparison to the biofilms grown without sucrose (Fig. 3B). The biofilms exposed to continuous sucrose, appeared to have the greatest degree of biofilm formation, as reflected by the most fluorescent associated with the substrate. A larger number

A



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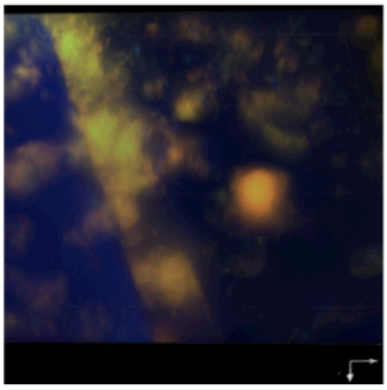

C

Figure 3. Fluorescence microscope images of the four-species anaerobic biofilm model grown on an artificial HAP tooth substrate after 72 hr of exposure to different sucrose conditions.
Each biofilm was grown without sucrose for 24 hr and then grown under different conditions for 72 hr. Before viewing each biofilm the discs were stained with Syto 9 (green emission) and hexidium iodide (red emission) for 15 min. The images were collected with an Olympus XL80i fluorescence microscope fitted with a 60 X objective. (A) Biofilms not exposed to sucrose. (B) Biofilms exposed to 0.2% sucrose 2 times per day for 2 days. (C) Biofilms continuously exposed to sucrose. The scale grid is 50 microns.

of biofilm cells exhibited red fluorescence, indicating a larger presence of Gram-positive cells in comparison to the other conditions (Fig. 3C). A great deal of the fluorescence under the constant sucrose conditions appeared orange. This may be due to over lapping Gram-positive and Gram-negative cells or to fluorescence of the matrix eDNA produced by the large number of *S. mutans* in these biofilms. The fluorescence microscopy analysis qualitatively revealed that the degree of biofilm formation and the presence of Gram-positive bacterial cells was dependent on the degree of sucrose exposure: the more sucrose exposure, the more attached cells and the more Gram-positive cells.

The images also provide a glimpse into the biofilm architecture. It was noted that the cells preferentially grew in the gaps at the substrate interfaces. These gaps were estimated to be 25 μm wide and of unknown depth. In addition, the bacteria were arranged in characteristic patterns in the Z planes in all of the biofilms. *S. mutans* was typically at a lower levels closest to the substrate, whereas *F. nucleatum* was generally found at the top portion of the biofilm; *V. dispar* and *A. odontolyticus* were more dispersed and at higher concentration in the middle (data not shown).

Quantitative PCR analysis

Community profile

The real-time qPCR analysis of the four-species anaerobic biofilm model grown on a natural tooth section substrate after 72 hr of exposure to different sucrose conditions provides the total number of individual bacteria of each of the

four species within the biofilms (Fig. 4). It was determined that the biofilms communities that developed under conditions lacking sucrose (Fig. 4A) were dominated by *V. dispar* at 57% of the population. There was no statistical difference between the levels of *A. odontolyticus* and *F. nucleatum*, which were determined to be present at 28% and 13%, respectively. *S. mutant* was the least represented species at 2% of the total population. The biofilm communities that were exposed to intermittent sucrose (Fig. 4B) were the most diverse, with each species being more equally represented in comparison to the other tested conditions. *S. mutans* was present at 28%, *V. dispar* at 41%, *A. odontolyticus* at 19% and *F. nucleatum* at 12%. Biofilms grown under continuous sucrose conditions (Fig. 4C) exhibited a community profile dominated by *V. dispar* and *S. mutans* at 38% and 43% of the total population, respectively. Although *F. nucleatum* was essentially absent in the population under these conditions, *A. odontolyticus* was present at 18%. Interestingly, *A. odontolyticus* remained at a similar level in the population regardless of the sucrose exposure. The statistical analysis of these data reveal that the percentage of individual species within these biofilm communities is related to the sucrose exposure conditions with the exception of *A. odontolyticus* (see Fig. 4 legend for P-values).

Biofilm bacteria population size

The real-time qPCR analysis of the four-species anaerobic biofilm model grown on HAP/PMMA substrates after 72 hr of exposure to different sucrose conditions provided the average total number of cells of each species per cm^2 within

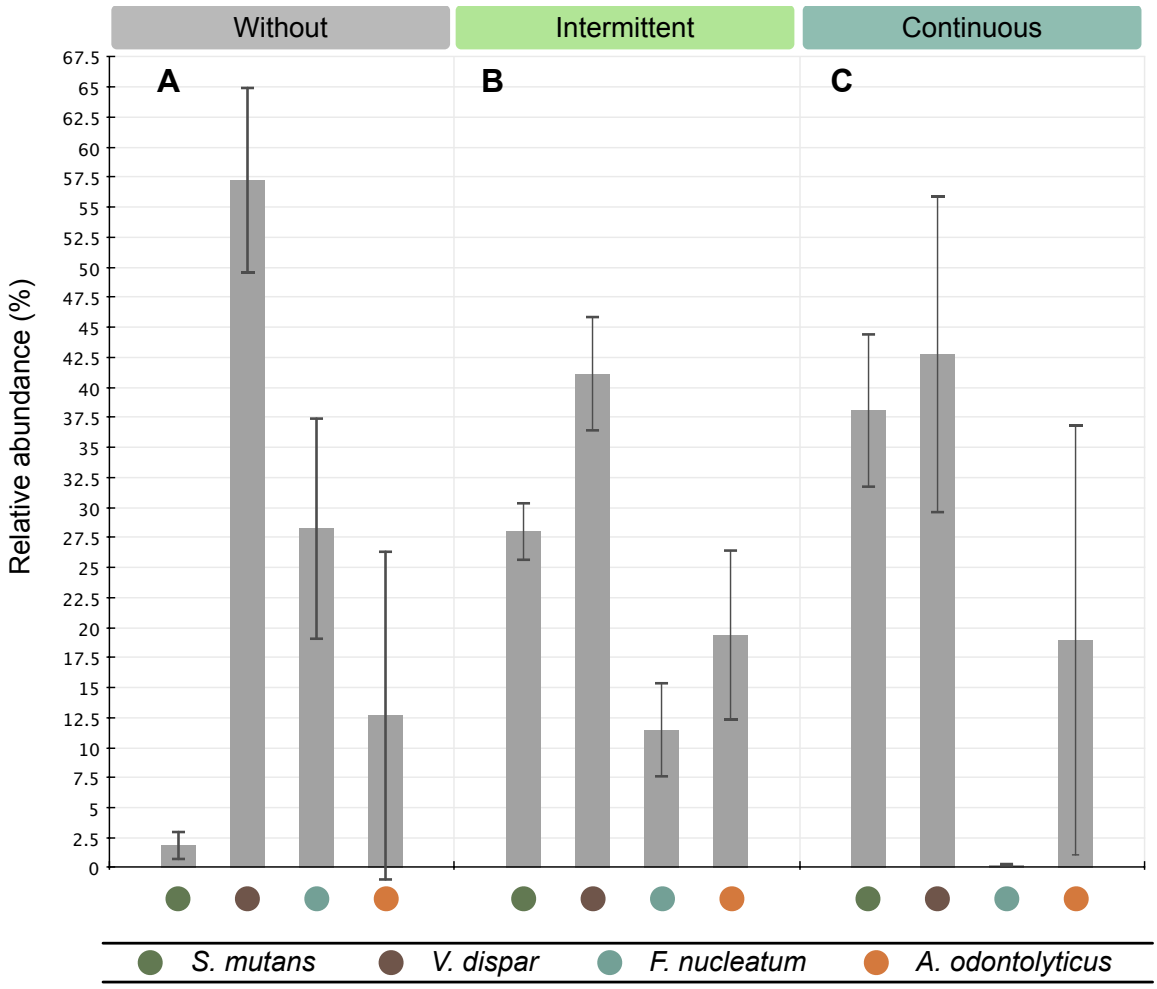

Figure 4. Community profile analysis of the four-species anaerobic biofilm model grown on an artificial HAP tooth substrate and exposure to different sucrose conditions. Each biofilm was grown without sucrose for 24 hr and then grown under different sucrose conditions for 72 hr. Total DNA was quantitatively isolated from each substrate and used to determine the total number of cells by qPCR based on number of 16S rRNA gene copies present. It was determined that the biofilms exposed to different growth conditions contained different amounts of cells. *S. mutans* ($p<0.001$), *V. dispar* ($p=0.015$) and *F. nucleatum* ($p<0.001$) were statistically different, but the differences for *A. odontolyticus* ($p=0.641$) were not significant. Averages represent 6 samples from three replicates (2/replicate) per condition.

each biofilm community (Fig. 5). The biofilms grown without sucrose exposure had the least total number of cells with 1.4 X 10^8 per cm^2 (Fig. 5A). The biofilms grown under intermittent sucrose exposure conditions had about five times the number of cells (7.0 X 10^8 per cm^2) compared to the biofilms that were not exposed to sucrose (Fig. 5B). The biofilms grown under continuous sucrose had the largest cumulative population (1.2 X 10^9 per cm^2), which was about ten times the number of cells in comparison to the biofilms that were not exposed to sucrose (Fig. 5C). Analysis using ANOVA revealed that the biofilms communities that developed under intermittent and continuous sucrose conditions were not statistically different, but that both communities were statistically different from biofilms that developed under conditions lacking sucrose (P=0.001). The qualitative analysis performed by the fluorescence microscopy (Fig. 3) correlates with the qPCR analysis described here (Fig. 4). As a result, the following general conclusions can be made: depending on the sucrose exposure both the total biomass and the proportion of the four species is altered.

Analysis of biofilm cariogenic potential

VSI analysis revealed that the degree of sucrose exposure was directly correlated with the cariogenic potential the four-species anaerobic biofilm model. Figure 6 shows qualitative representations of enamel erosion of the natural tooth section substrates using our *in vitro* model after 72 hr of exposure to different sucrose conditions. Each VSI image is presented as a heat map, with darker colors

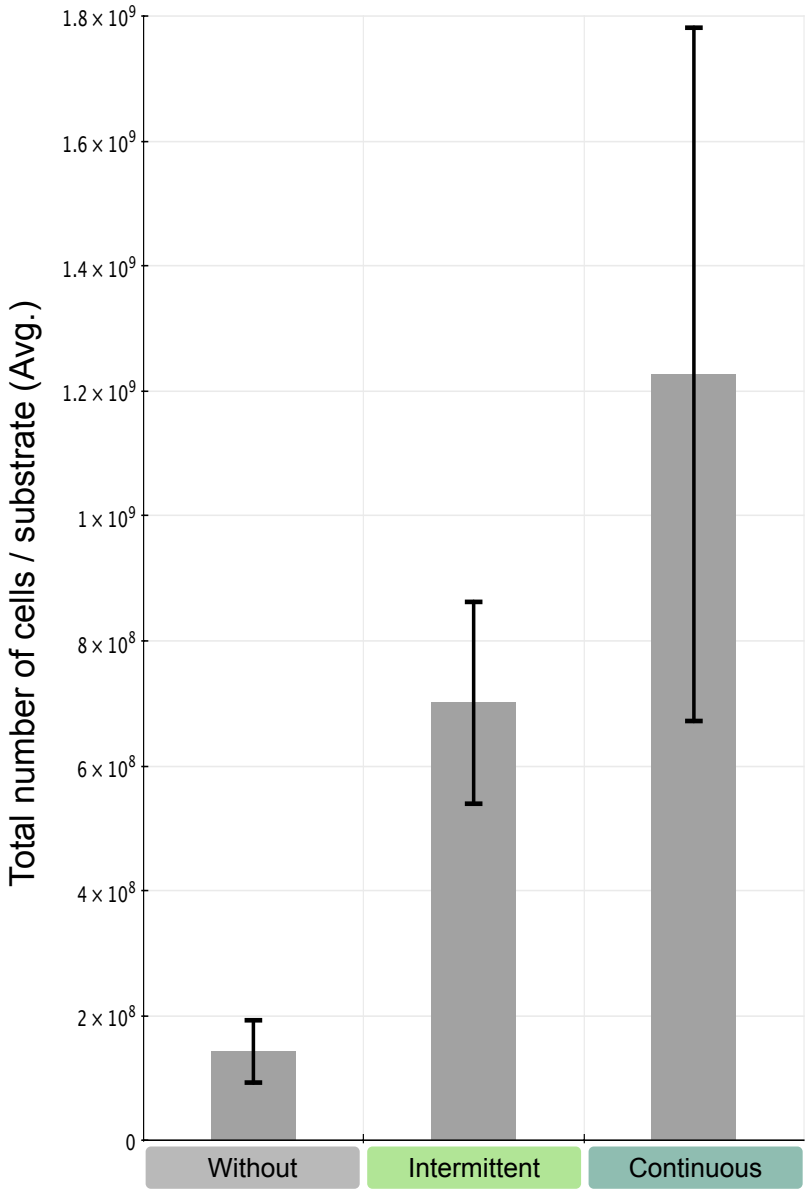

Figure 5. Average total number of bacterial cells detected per biofilm growth substrate of the *in vitro* dental biofilm model grown in different sucrose after 72 hr. of sucrose exposure.

The total number of bacterial cells per substrate was determined by adding the total number of cells of each organism per substrate. An analysis of variance (ANOVA) of the averages (n=6) between groups showed that the total number of cells detected in the intermittent (A) and continuous (B) sucrose conditions were similar but different than the biofilms grown without sucrose (c) (P=001).

representing areas of lower elevation and light colors representing areas of higher elevation. The images of the substrates for which the biofilms were grown in medium without sucrose or with intermittent sucrose exposures were similar (Fig. 6A and 6B). They show a relatively homogenous surface elevation with visible striations resulting from the polishing process. When compared to the masked unreacted surface, the surface exposed to the biofilm and media experienced no detectable enamel erosion or an insignificant change in surface elevation. The images of the tooth sections exposed to continuous sucrose conditions were significantly different in elevation compared to the masked reference area (Fig. 6C). The bottom right hand corner of the image, which was the masked area of the tooth section has a light gold coloration. In contrast, the rest of the image is a dramatically different in elevation. The blue and red arrows on the image highlight that the difference in surface elevation in this cross-sectional area is 10 μm, representing a stepwise dissolution of enamel.

The VSI images were quantitatively analyzed to determine the average amount of enamel dissolution, as measured by an increase in surface roughness, that tooth section substrates experienced under each condition (Fig. 7). No difference in roughness was detected for the tooth section substrates not exposed to sucrose. The tooth sections exposed to intermittent sucrose conditions experienced a slight increase (1.2 times) in surface roughness as compared to the masked unreacted area. The tooth sections exposed to continuous sucrose conditions were the most significantly changed; the surface roughness increased almost ten times. However, a high degree of variability was observed in the data for

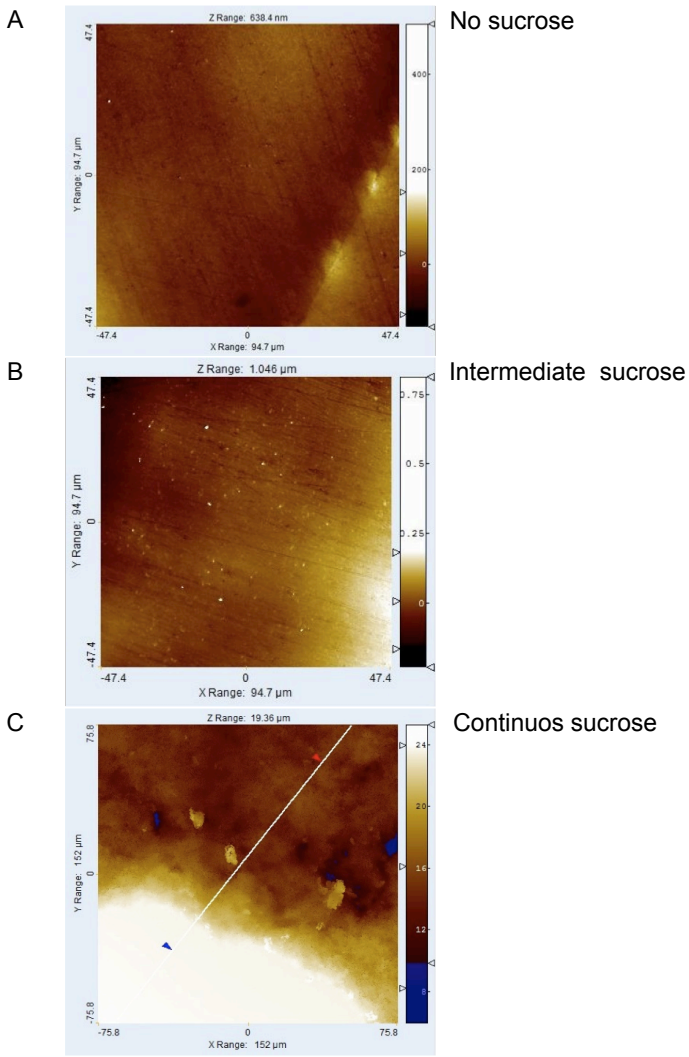

Figure 6. Vertical scanning interferometry (VSI) images of the four-species anaerobic biofilm model grown on native tooth substrate after 72 hr of exposure to different sucrose conditions.

Each biofilm was grown without sucrose for 24 hr and then grown under different conditions for 72 hr. Before viewing the biofilm was removed by sonication of the discs and the silicone mask was also removed. Topographical images were obtained using a prototype Zemapper (Zemetrics/Zygo) interferometer with a Mirau objective. Image Metrology's SPIP^{TM} was used to generate images and make measurements. (A) Biofilms not exposed to sucrose. (B) Biofilms exposed to 0.2% sucrose 2 times per day for 2 days. (C) Biofilms continuously exposed to sucrose. In the heat map white represents the highest coordinate (no erosion) and black the lowest coordinate (most erosion).

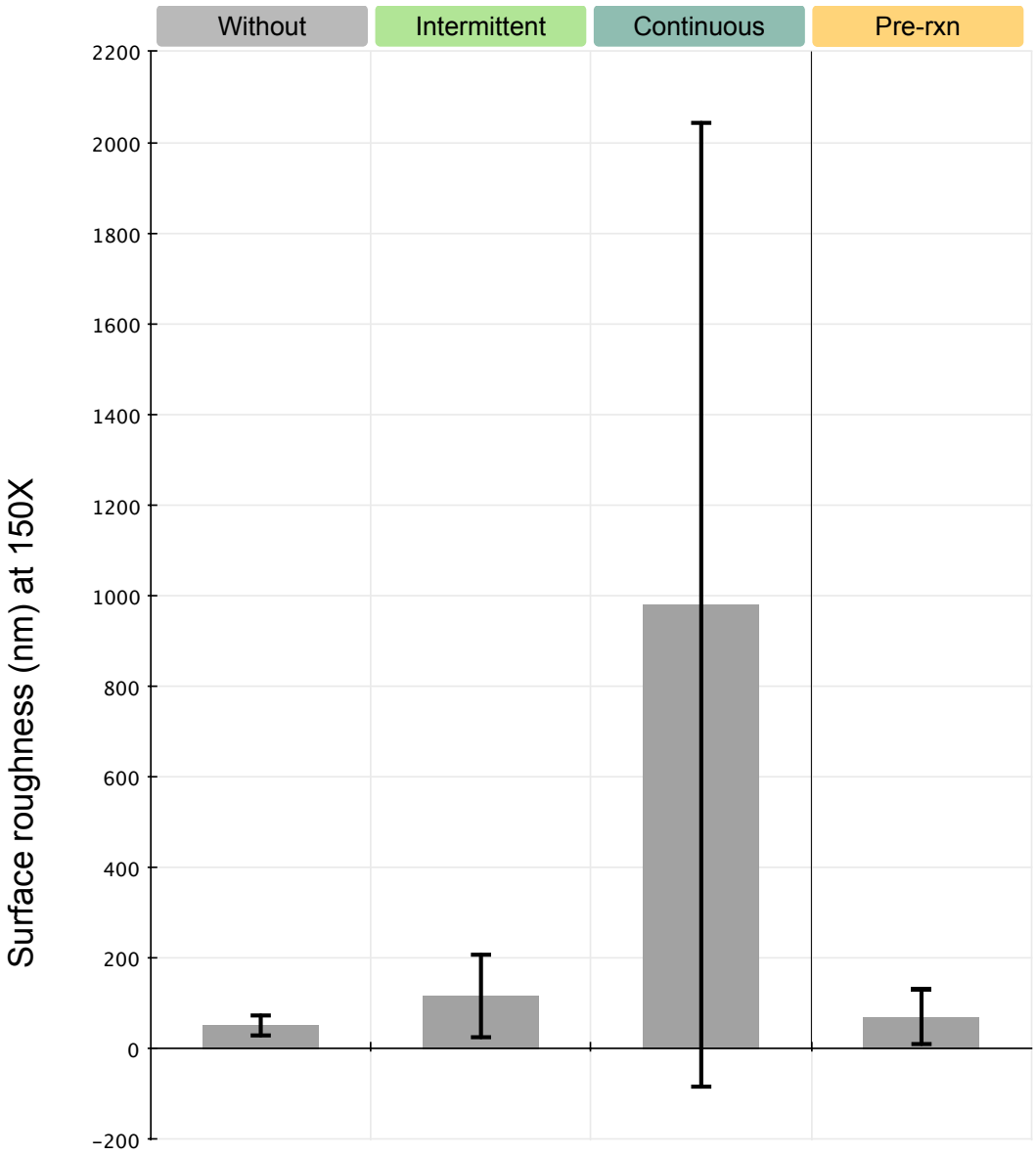

Figure 7. Quantitation of the erosion of tooth sections by the four-species anaerobic biofilm model after 72 hr of exposure to different sucrose conditions.

Average surface roughness of PMMA-embedded tooth samples after 48 hr of differential sucrose exposure. Paired t-test analysis comparing the average pre-reaction surface roughness to post reaction surface roughness measurements showed that TS substrates exposed to biofilms grown under conditions lacking sucrose and intermittent sucrose conditions had insignificant changes in surface roughness [(P=0522);(P=0.212) respectively]. The average increase in surface roughness of the TS substrates exposed to biofilms under continuous sucrose was found to be significantly different from pre-reaction measurements (P=0.002).

the tooth section exposed to continuous sucrose. This is most likely due to the inherent inconsistencies between different teeth and different sections of the same tooth.

Analysis of biofilm micro-environmental pH

The micro-environmental pH of biofilms grown under intermittent and continuous sucrose conditions revealed that the pH profile within the biofilm is dependent on the degree of sucrose exposure. A qualitative analysis of the ratiometric images of the biofilms (Fig. 8) revealed that the biofilms grown under intermittent sucrose conditions were not as densely populated with biofilm compared with the continuous sucrose biofilms. This was evident by the larger areas of black (no biofilm present) in the image of the biofilm exposed to intermittent sucrose conditions (Fig. 8A). In contrast, only a limited area of the image of the biofilm exposed to continuous sucrose condition was black (Fig. 8B), revealing that there was more biofilm material in that sample. In addition, the biofilms exposure to intermittent sucrose had more areas of higher pH compared to the biofilm exposed to continuous sucrose; this was reflected by the greater amount of yellow (pH 6.75 to 7.25) in the image.

A quantitative analysis of the micro-environmental pH of biofilms grown under intermittent and continuous sucrose conditions was performed by examining the pH at every pixel within each Z-plane in the 3D image stacks. The percentage of different pH values in each Z-plane were placed into ranges and represented by different colors (Fig. 9). The pH values of the biofilms exposure to intermittent

sucrose conditions were in four different pH ranges from as low as pH 4.75 to as high as 6.75. Roughly 15% of the biofilm was within the pH range of 4.75 to 5.25, which was the lowest pH range and roughly 5% was at the highest measured pH range of 6.25 to 6.75. Most of the biofilm was either in a pH range of 5.25 to 5.75 (40%) or 5.75 to 6.25 (40%). A slightly higher pH was detected at the biofilm–liquid interface (biofilm top) compared to the biofilm–substrate interface (biofilm bottom).

In contrast, the biofilms grown under the continuous sucrose condition had an overall lower pH that was more uniformly distributed throughout the depth of the biofilm. The pH values of the biofilms exposure to continuous sucrose conditions were in three different pH ranges from as low as pH 4.75 to as high as 6.25. Roughly 22% of the biofilm was within the pH range of 4.75 to 5.25, which was the lowest pH range and roughly 18% was at the highest measured pH range of 5.75 to 6.25. Most of the biofilm (60%) was in a pH range of 5.25 to 5.75. The highest pH values for the continuous sucrose exposed biofilms was a half pH unit lower than the biofilms exposed to intermittent sucrose conditions. No significant levels of neutral pH values (pH 6.75 to 7.25) were detected for either of the biofilms.

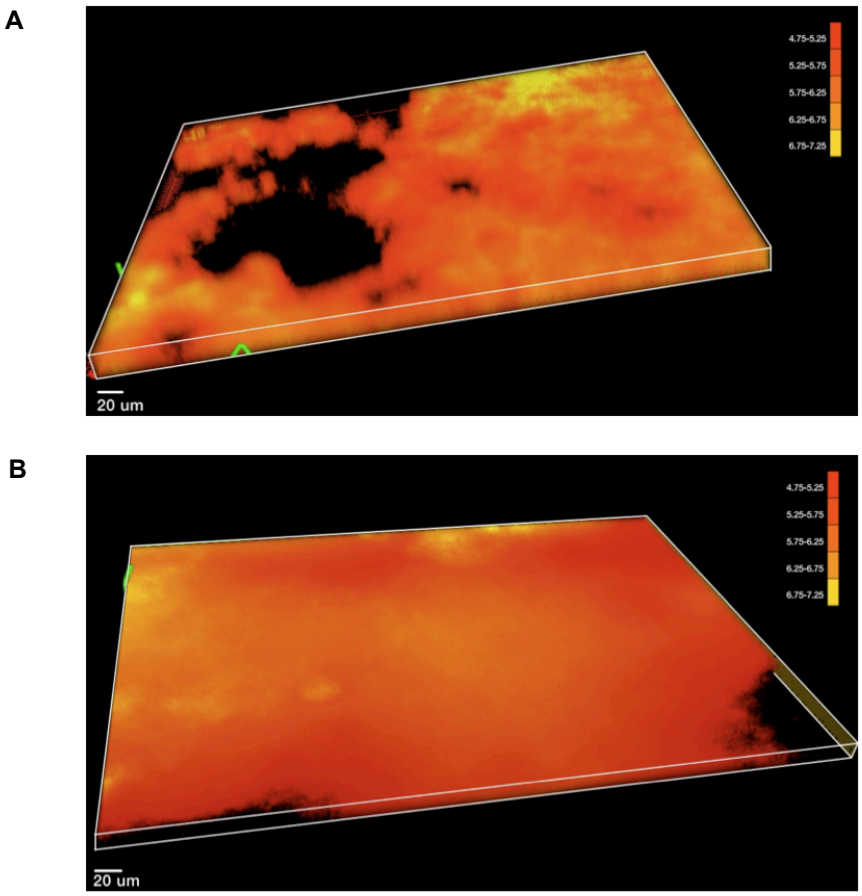

Figure 8. Tomographic pH imaging of the four-species anaerobic biofilm model grown on native tooth substrates after 72 hr of exposure to intermittent and continuous sucrose conditions.

Biofilm were grown without sucrose for 24 hr and then exposed to intermittent and continuous sucrose conditions for 72 hr. Before viewing the biofilms were exposed to the nanospheres and 0.2% sucrose for 30 minutes. Sensor and reference images show the colocalization of the particles in the film. The pH was analyzed ratiometrically based on the sensor and reference images and is expressed as a false spectrum (red-yellow) overlaid on the bright-field image, with the pH scale shown at the right.

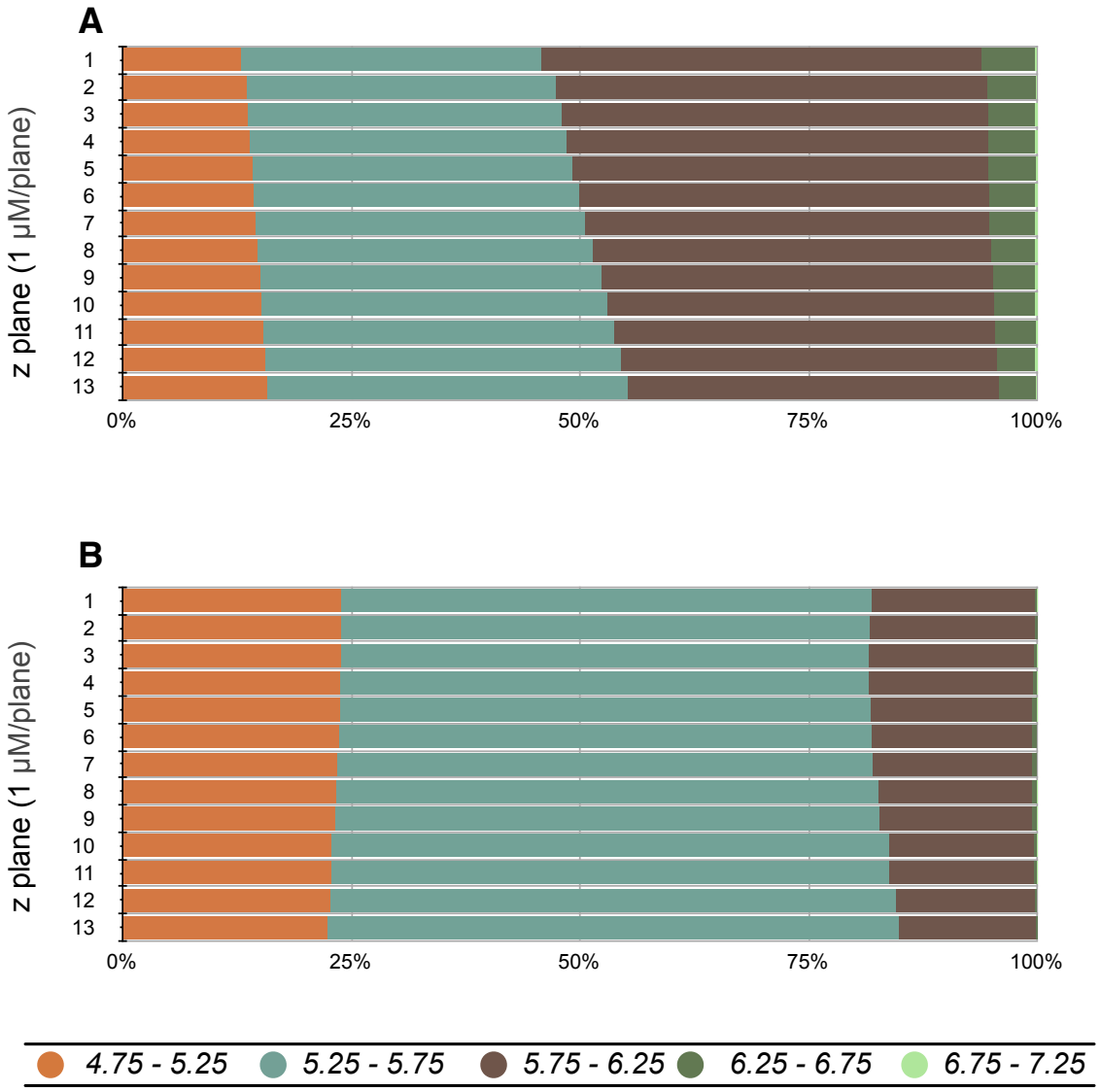

Figure 9. Quantitation of the pH of the four-species anaerobic biofilm model after exposure to 0.2% sucrose for 30 min after growth for 72 hr under intermittent and continuous sucrose conditions. Each biofilm was grown without sucrose for 24 hr and then grown under intermittent and continuous conditions for 72 hr. Before viewing the biofilms were exposed to nanospheres and 0.2% sucrose for 30 minutes. The figure illustrates the percentage of the biofilm at the different pH ranges at each 1 uM slice of the biofilm depth. The biofilm-medium interface is represented by a z value of 1.

DISCUSSION

Dental caries is a worldwide health problem that is growing in significance as global diets include greater levels of refined carbohydrates. A new paradigm is focusing attention on characterizing the impact of multispecies microbial communities on development of dental caries and may lead to the development of novel methods to prevent and treat dental caries. Novel *in vitro* models systems that focus on incorporating physiological relevance and ease of use are required to facilitate these types of studies. We have developed an *in vitro* anaerobic dental multispecies biofilm model with a species composition representative of *in vivo* dental plaque, that uses a physiologically relevant growth medium (CAMM) and substrates of human tooth sections and hydroxyapatite, and a novel biofilm reactor (SIRS) to facilitate growth and analysis. Through an analysis aimed at validating the model in was found that: 1) the multispecies bacterial consortium used in the model forms biofilms on the tested substrates, 2) the biofilm's community profile, population size, cariogenic potential, and spatial distribution of pH is directly dependent on the degree of sucrose exposure, 3) higher degrees of sucrose exposure result in biofilms with larger populations enriched in *S. mutans* and *V. dispar*, 4) continuous sucrose conditions have the highest degree of cariogenic potential, and 5) the spatial distribution of pH throughout the biofilms grown under continuous sucrose conditions was generally lower than the biofilms grown under intermittent sucrose conditions.

Development of a realistic *in vitro* multispecies anaerobic biofilm caries model

The primary goal of this project was to develop a realistic *in vitro* multispecies anaerobic biofilm model to facilitate the study of dental caries that would specifically

incorporate features having a high degree of physiological relevance. We decided, based on our laboratory's previous experience, that including the following five elements would establish this as a unique and exceptionally useful method. These elements are: 1) recent clinical bacterial isolates collected from the related sources, 2) artificial saliva medium, 3) natural and artificial tooth substrates, 4) a static biofilm reactor with daily medium exchange, and 5) qualitative and quantitative analysis.

Our model focuses on using a multispecies microbial community as the causative agents of dental caries, instead of focusing on a single organism. This is especially important in light of recent findings suggesting that the presence of *S. mutans* (the primary etiological focus of decades of dental caries research) in a host's dental microbiota is not directly correlated with caries, nor is it an adequate predictor for the eventual development of the disease (30). Furthermore, other work suggests that the presence of *V. dispar* serves as a better predictor for eventual cariogenesis (9). We decided to include both of these species in our model, particularly due to the studies of the interactions of *V. dispar* and *S. mutans*, which found that a 'cross feeding' occurs, as *V. dispar* uses as a carbon source the lactic acid produced by *S. mutans* from its fermentation of sucrose (46). Furthermore, other studies revealed that *V. dispar* is an important component of *in vitro* caries models; apparently, the lack of this species in *in vitro* biofilm models was found to be inhibitory towards the development of a dental biofilm (46). It will be interesting to use this model to investigate the factors that *V. dispar* and other microbial species contribute to the development of dental biofilms on tooth enamel and to understand the progression that promotes the development of a microbial plaque community with cariogenic potential (46).

Our model includes four diverse and representative members of a typical dental biofilm. It consists of one bacterial species with a known direct influence on the development of caries, *S. mutans*, and three species, *A. odontolyticus*, *F. nucleatum*, and *V. dispar*, also associated with cariogenic dental biofilm. All four species have been identified in 'caries active' and 'caries inactive' dental biofilms. This microbial composition in our model will allow for future studies of how the species profile and interactions in a dental biofilm shifts from a non-cariogenic profile to a cariogenic one in response to environmental conditions.

The synthetic medium designed to mimic saliva was an important feature included to increase the physiological relevance of our *in vitro* model. Using a medium that replicates the *in vivo* conditions to study multispecies communities reduces the risk that novel phenomenon discovered are artifacts of experimental conditions. More importantly, using a relevant and realistic medium can increases the likelihood of discovering the mediators of virulence-associated microbial behavior observed under *in vivo* conditions (16, 55).

Use of a novel toolbox

A problem encountered early in the project was that traditional methods for growing static biofilms was not conductive to growing biofilms in an anaerobic chamber under our conditions, which limited the growth rate, so that the plates require more than 48 hr of incubation. Many methods for growing static biofilms employ the use of a 96-well plate, which serves dual roles as a growth reactor and as a surface substrate (48). For these studies, growth medium inoculated with bacterial cells is incubated in the microtiter plate and the well surfaces of the dish are rinsed and stained with crystal violet

to quantify biofilm cell attachment. These studies are traditionally limited to 24 to 48 hr, because they do not incorporate any exchange of growth medium, which is required for longer incubation times.

As an alternative to the microtiter plate assay, we first grew our biofilms on 1 cm diameter removable discs of PMMA plastic resin or HAP as an artificial proxy for teeth, in each well of a 24-well plate. The substrates were placed at the bottom of each well and were removed from the bioreactor for analysis. As our experimental conditions required incubation for up to 96 hr, the growth medium required changing every 24 hr. This proved to be difficult when manipulating the plates in the anaerobic chamber, since manual dexterity is reduced due to the rubber glove interface. A flow-through system was considered, but ruled out for two reasons: 1) the risk of having a clogged system that would overflow within the chamber, and 2) it would be likely to have a higher flow dynamic than the oral environment.

In response to these issues, we designed a novel static biofilm reactor, designated the static inverted removable substrate (SIRS) biofilm reactor. The inspiration for this reactor was an existing microtiter platform bioFILM PA (Innovotech, Edmonton, AB, Canada), which uses as substrates plastic pegs that are permanently attached to the microtiter plate lid. The SIRS bioreactor developed for this project maintains the principle of an inverted substrate attached to a microtiter plate lid, but differs in that it uses disc-shaped substrates that are detachable. The flat surface of each disc is directly inserted into the microtiter well and partially submerged in the medium. The flat disc-shaped substrates are more versatile than the pegs since the substrates can be analyzed by microscopy or can fit into a 15 ml disposable tube for any

molecular-based analysis. In addition, daily replacement of the growth medium is conducted by replacing the bottom portion of the microtiter plate with a new bottom plate containing fresh medium. The plastic wells of the microtiter plate remain available for traditional assays that analyze biofilms attached to the well surfaces.

The biofilm substrates were designed to incorporate natural surfaces for biofilm development, but in a manner that would maintain a flat surface for analysis and easily attach and detach from the SIRS reactor lid. This goal was accomplished by embedding human tooth sections or hydroxyapatite discs into a puck-shaped PMMA substrate with threaded opposite ends for attachment to the SIRS. This method of developing substrates is versatile, because any type of material can be embedded into PMMA and attached to the bioreactor lid. PMMA and acrylic based polymers are materials commonly used in dentistry and medicine. They are used for fabricating dentures, filling cavities, and as bone cement in orthopedic surgery. The use of PMMA for embedding these natural substrates adds to the incorporation of relevant conditions since it is a material that would normally be encountered by bacterial biofilms in an *in vivo* setting.

The SIRS reactor, was initially tested by growing the biofilms for a total of 96 hr and analyzing the substrates for biofilm attachment. Using fluorescence microscopy it was determined that the biofilms attach well to the inverted substrates. It is noteworthy that the biofilms grown in an inverted manner exhibited a spatial organization more similar to that observed in *in vivo* dental biofilms. In addition, there is little or no carry over of the growth medium; only the biofilm itself is transferred into the new medium.

Analysis of biofilm development and community composition

Fluorecence microscopy using a fluorescence Gram-stain was an ideal choice in initial analysis testing for the development of biofilms on our biofilms substrates, since this method has the ability to verify biofilm development and can also shed light on architectural characteristics. Comparison of imaged biofilms grown under different sucrose conditions revealed significant differences as determined by qualitative assessment. The use of qPCR allowed for the quantitative measurement of the average total number of biofilm cells and the characterization of the different multi-species bacterial communities that developed under different sucrose conditions.

Analysis of biofilm micro-environmental pH

The reduction in the local pH is a critical component of dental caries. We devised an experiment to determine the distribution of the pH within a biofilm. Although the oral environment is generally a neutral pH, the interface between the enamel tissue and a cariogenic biofilm is approximately pH 5. In order to determine the pH distribution within the *in vitro* biofilms, we developed a protocol that used novel core-shell silica pH nano-particles sensors. These sensors function based on the principle of differential excitation/emission potential of fluorophors at different pH values. The particles are composed of two fluorophors, a pH reactive silica shell (FITC - green) and a pH static silicacore (TRITC-red). The silica nano-particles were incubated with the biofilms for 30 min before they were imaged using ratiometric imaging with a fluorescence microscope. It was determined that biofilms exposed to intermittent sucrose conditions had a stratified vertical distribution of pH with about 10% of the horizontal surface area closest to the tooth interface having pH values between 5.0-5.5. Some areas of the biofilm pH

increased to close to neutral at the biofilm/liquid interface. Biofilms exposed to continuous sucrose conditions had a lower pH range. The remaining area was at a pH between 5 and 6.

The community profile's link to carcinogenicity

To verify that the *in vitro* biofilm model is a good representation of dental caries, we designed novel substrates composed of human tooth sections encased in a PMMA disc to be used with the SIRS biofilm reactor and grew the biofilms on them under the conditions described above. The surface was analyzed using vertical scanning interferometry (VSI), which is a microscopy technique that measures changes in the vertical height of a solid reflective surface at nanometer scale resolution. The biofilms grown under continuous sucrose conditions had the greatest and most aggressive cariogenic potential, defined as the ability to erode tooth enamel. Intermittent sucrose exposure conditions and no sucrose exposure produced either an insignificant erosion of enamel or no detectable affect, respectively. In is interesting to note that the biofilm communities exposed to intermittent sucrose conditions developed biofilms with a significant presence of *S. mutans*, but only the biofilms exposed to continuous sucrose conditions were able to directly affect tooth enamel. Intermittent sucrose exposure provides conditions sufficient to promote biofilm formation with a statistically indistinguishable abundance of total bacteria compared to the continuous sucrose exposure, but it is deficient in its cariogenic potential due to carbon source limitations.

Our results are in line with the recent paradigm shifts in caries research that approach the problem from an ecological perspective (3). For the most part epidemiological and basic science research agrees that host dietary factors play a

significant role in the progression of the dental caries. In our model it was only biofilm growth conditions with excess carbon that resulted in the biofilm-mediated erosion of enamel. Perhaps the development of chemotherapies designed to sequester the sucrose should be considered. A multifaceted approach influenced by this new developing ecological/multispecies paradigm for caries is expected to lead to novel discoveries and the development of new treatments for dental caries based on targeting the whole microbial community instead of single etiological agents.

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VITA

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