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An Evaluation of Novel Approaches to Antimicrobial Control Of Oral Biofilms

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An Evaluation of Novel Approaches to Antimicrobial Control of Oral Biofilms

Introduction: *Streptococcus mutans* (SM) and *Haemophilus actinomycetemcomitans* (HA) are important pathogens in the development of caries and periodontitis. This thesis investigated several novel strategies aimed at preventing their growth and biofilm forming capacity. **Methods:** SM and HA biofilms were challenged with numerous antimicrobial solutions and assessed for biofilm retention and bacterial survival. Antibiotic susceptibility profiling was performed and the ability of antimicrobial-impregnated silicone disks and *Streptococcus salivarius* probiotic strains K12 and M18 to inhibit the pathogens was investigated. **Results:** Silicone oil effectively disrupted HA biofilms but did not affect either pathogen's viability. Neither HA nor SM showed any relevant antibiotic resistance and polyvinyl siloxane impregnated with triclosan and chlorhexidine demonstrated antibacterial activity against both strains. Finally, both probiotics inhibited the growth of HA but not SM via differential antagonism.

Conclusions: This work supports the widening application of silicone-based products and probiotics in maintaining oral health and fighting disease.

Keywords: *Actinomycetemcomitans, Streptococcus mutans, Streptococcus salivarius*, K12, M18 , Biofilm, Oral, Antimicrobial

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List of Abbreviations

SM: *Streptococcus mutans*

HA*: Haemophilus actinomycetemcomitans*

BMM: Basal Mucin Media

CHL: chlorhexidine

TCN: triclosan in methanol

SCS: spent culture supernatant

PMN: polymorphonuclear leukocytes

LPS: lipopolysacharides

VFs: virulence factors

SO: polydimethylsiloxane- silicone oil

BHYE: Brain Heart Infusion broth and agar supplemented with 0.5% yeast extract

TS+ : buffered media (Tryptic Soy media + 10% yeast extract + 2.5% calcium carbonate).

Mix: [0.12% CHL, 20% ethanol, 25% SO]

THY: thymol

RT: room temperature

HT: preheated to 50°C

PBS: phosphate buffered saline

VWR: antimicrobial susceptibility test discs

ZOI: zone if inhibition

O/N: overnight

LYZ: lysozyme

MeOH: methanol

DDA: disk diffusion assays

DA: differential antagonism

WD: well diffusion

LAP: localized aggressive periodontitis

Introduction

Caries and periodontal disease are two of the most prevalent infectious diseases of mankind (1) and decades ago, a causal relationship between plaque and these diseases was established (2,3). While the oral microflora generally consists of a great diversity of microorganisms including viruses, bacteria and fungi, bacteria are the predominant component and may include 300-350 different species (4). Bacteria in the oral cavity can exist in both planktonic (free floating) and sessile (biofilm) states, although the majority of organisms reside in the latter as dental plaque. Bacteria readily form biofilms both in nature and in clinical settings as it permits them to remain in favourable environmental conditions while protecting them from any detrimental factors present such as antimicrobials and host immunity (5). In the mouth, biofilms provide bacteria protection from the shear stress of saliva, consumed liquids and rinses, as well as the antimicrobial effects of mouthwashes, toothpastes and host immune factors (1,3). Indeed, oral biofilms are so successful with respect to growth and persistence that without continuous debridement both caries and periodontitis would flourish (1).

Tooth biofilm development is a complex process involving physical interactions between the bacteria themselves, tooth surfaces, host saliva components (e.g. proline-rich proteins) and food (6). Since bacteria harbour numerous appendages (e.g. pili) and surface proteins that

adhere to multiple substrates, films can develop under a plethora of different oral environments. In addition, biofilms are highly dynamic, modifying their nutrient environments, growth rates and composition over time (7). The initial stage involves the attachment of active cells to a solid surface and their immobilization on that surface. Following this irreversible attachment, cells begin replicating and secreting exopolysaccharides and other factors that result in the development of an adherent microcolony, which then expands over time to form a multi-layered, mature biofilm. The final size, architecture and composition of a mature oral biofilm will depend upon multiple factors including the organism(s) involved, the local nutrient and gaseous environment, host immunity and level of host oral hygiene. Thus, both the host and microorganism(s) play vital roles in the development of biofilms and their potential clinical impact.

Although many different bacterial species can survive and grow within the oral cavity, certain organisms are recognized for their increased presence and pathogenicity. For example, initial plaque is largely dominated by *Streptococcus* species such as *Streptococcus mutans* (SM). Immediately following debridement by brushing or professional dental prophylaxis, the tooth surface will be recoated by salivary pellicle and these "early" bacterial colonizers (8). SM is a Gram-positive, facultatively anaerobic bacterium strongly implicated as one of the major

causative organisms of dental caries. Caries pathophysiology suggests that SM strongly adheres to the teeth within biofilms and releases acids by fermenting plaque carbohydrates, leading to tooth demineralization and cavitation (caries) (2). There are three SM virulence traits considered critical to the initiation and progression of caries. First is their metabolic breakdown of dietary carbohydrates to produce lactic acid (acidogenicity). Second is their ability to grow and survive in this low pH environment (aciduricity) and finally, their ability to utilize dietary sugars to produce glucan polymers and form plaque (9). This final attribute can be considered the most critical to SM's success in the oral cavity as it provides protection from transient changes in the oral environment including host attack, and as well as mechanical and antimicrobial forces applied during brushing and rinsing. In fact, SM is so attuned to the biofilm lifestyle that it is not found in the mouths of people without teeth or dentures for it to adhere to (9).

Following SM establishment, an environment develops promoting secondary or more "later" colonizers such as *Haemophilus actinomycetemcomitans* (HA - previously known as *Actinobacillus actinomycetemcomitans*) to grow and persist (6). Indeed, a study examining the microbial composition of supra and subgingival plaque in subjects with periodontitis revealed that actinomyces species were the most prevalent taxa in both habitats (10). HA is a Gram-negative,

facultatively anaerobic coccobacillus that grows well in 5% CO_2 in air or under strict anaerobic conditions, but poorly in air (11,12). Importantly, this organism has been implicated in the etiology of juvenile periodontitis (localized aggressive periodontitis) (11). HA has been shown to produce multiple virulence factors (VFs) capable of influencing host attachment and defence. Cell surface carbohydrates interact both specifically and non-specifically in host cell adherence. Leukotoxins lyse human polymorphonuclear leukocytes (PMNs) and monocytes and PMN chemotaxis-inhibiting factor decreases their migration to the active site(s) of infection, reducing bacterial killing (12). Lipopolysacharides on the bacterial surface promote macrophage activation, platelet aggregation, and bone resorption (12), and along with epitheliotoxin result in local tissue damage, allowing HA to penetrate the sulcular epithelium and gain access to the underlying connective tissue (12). Finally, two additional VFs have been implicated in the extensive loss of collagen from the gingival connective tissue, a key attribute of localized aggressive periodontitis (12). The first, collagenase, can account for the specific collagen breakdown that occurs while the second, a fibroblast-inhibiting factor, inhibits the growth and proliferation of human fibroblasts, thereby preventing tissue rebuilding and new collagen formation (13,14). Based upon their ability to not only survive but thrive within the oral cavity and cause disease, both SM and HA can be considered critical pathogens

within this clinical setting and should be a major focus into the prevention and treatment of these conditions.

Control of dental plaque (biofilms) is the primary target of prevention for dental caries and periodontal disease (15). Studies reveal that gingivitis can be prevented by meticulous oral hygiene, including toothbrushing with combined interdental cleaning (15). Chemical agents including antibiotics are a valuable complement to this control and to be effective should be able to prevent biofilm formation and expansion, disrupt existing films and/or kill biofilm organisms directly (16). With the development of solid *in vitro* oral biofilm models, the ability to test these compounds has greatly improved. To date, studies have shown that several widely used agents such as chlorhexidine, fluoride and triclosan are effective against oral biofilms, but more so in prevention than the clearance of preformed films (15). In addition, all of these compounds have drawbacks with respect to efficacy and host safety. For instance, chlorhexidine (CHL) is the most extensively studied agent in terms of controlling cariogenic bacteria and demonstrates potent antimicrobial activity against a broad spectrum of both Gram-positive and –negative organisms. At the same time, a systematic review of the literature concluded that CHL use must be closely monitored due to variable outcomes and host issues including tooth staining and taste alteration (17). Fluorides can interfere with various bacterial systems,

leading to reduced adhesion (15) and acid production (18), as well as cell death (19). However, although caries rates have declined since the introduction of fluoride consumption and its widespread use in dentistry, it is not a panacea as both caries and periodontal disease continue to persist among the population worldwide. Finally, triclosan is a broad-spectrum antimicrobial that has been shown to lessen both plaque and gingivitis scores via diminishing the total microbial load (15). Furthermore, it can inhibit numerous anaerobes and Actinomyces species (20), and exhibits potent anti-inflammatory properties on the host (21). However, it is feared that its current widespread use in a plethora of antibacterial soaps, scrubs, toothpastes, mouthwashes and the like may promote resistance to it and other antimicrobials. In addition to these agents, several antibiotics such as tetracycline, metronidazole and clindamycin can be extremely useful in some cases of periodontitis, especially in difficult cases involving virulent pathogens. Ultimately, while these and other chemical agents currently used by dental practitioners and the general public can be very effective at inhibiting microbial growth and reducing biofilm formation, no one agent can be considered a clinical triumph and other avenues to tackle these diseases should be pursued.

In this project, we proposed two novel strategies aimed at reducing caries and periodontitis: 1) the use of silicone-based compounds for

biofilm disruption, bacterial killing and antimicrobial delivery; and 2) the application of oral probiotics for their ability to inhibit pathogen growth. For this work, we utilized two well known organisms associated with caries and periodontal disease, namely *S. mutans* and *H. actinomycetemcomitans*. Our first project involved testing silicone oil (SO - polydimethylsiloxane) for its ability to disrupt existing SM and HA biofilms and kill cells within those films. A previous *in vivo* study examined the antibacterial properties of SO in the oral cavity, assessing *Streptococcus sobrinus* growth following surface pretreatment (10). Both plaque prevention following direct SO tooth application and gingivitis reduction using SO-supplemented toothpaste were noted, and when 0.3% triclosan (TCN) was added, superior antibacterial effects were further observed. Silicone compounds have since shown antimicrobial properties in other medical areas, imparting significant activity against *Staphylococcus epidermidis, Pseudomonas aeruginosa* and *Candida albicans* (22). It was therefore of interest to examine the effects of SO on oral bacterial biofilms.

The second project investigated the potential for polyvinyl siloxane impression material to be used as a delivery vehicle for antimicrobial agents. As this routinely-utilized material can be made into trays precisely fitting patients' mouths, it could potentially serve as a vehicle for the delivery of any oral health-promoting compounds.

Finally, alternative approaches to biofilm control and removal include the use of probiotics. Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host. They have been used successfully to control gastro-intestinal diseases and act through colonization resistance and/or immune modulation. Mechanisms of probiotic action in the oral cavity have been suggested where the introduction of microorganisms prevents and treats dental caries and periodontal disease (23). We specifically looked at two commercially-available oral probiotics for their ability to inhibit SM and HA growth.

The specific objectives of this pilot project were: 1) to assess biofilm removal and antibacterial activity of SO against SM and HA biofilms, compared to other oral antimicrobial compounds; 2) to profile SM and HA antibiotic susceptibility; 3) to examine the ability of antimicrobial compounds impregnated into polyvinyl siloxane impression material to elute out of the polymer and prevent SM and HA growth; and (4) to assess factors secreted by probiotic strains *Streptococcus salivarius* K12 and M18 for their ability to inhibit SM and HA growth using differential antagonism and well diffusion assays.

Materials and Methods

Bacterial Strains and Media

Streptococcus mutans 25175 (SM) and *Haemophilus actinomycetemcomitans* Y4 (HA) were purchased from the American Type Culture Collection (ATCC-Manassas, VA, USA). *Streptococcus salivarius* K12 (K12) and *S. salivarius* M18 (M18) were kindly provided by BLIS Technologies Ltd. (Dunedin, New Zealand). Bacterial media products were purchased from Difco (Mississauga, ON) and the remaining chemical reagents from Sigma Aldrich (Oakville, ON). Agar plates for all media were generated via the addition of 15 g/L Bacto agar. Routine culturing of all strains was conducted from -80°C stocks using Brain Heart Infusion broth and agar supplemented with 0.5% yeast extract (BHYE). For biofilm experiments, SM was cultured in Basal Mucin Media (BMM)(Table 1) containing 2% sucrose and HA in BMM:BHYE (1:1 ratio). Differential antagonism and well diffusion experiments were conducted using TS+ buffered media (Tryptic Soy media + 10% yeast extract + 2.5% calcium carbonate). Standard antibiotic and siloxane polymer disk diffusion assays were performed using Mueller Hinton II (SM) and BHYE (HA) agar plates. All culturing was performed in 2-5% CO_2 at 37°C using CO_2 gaspacks (VWR, Oakville, ON) and anaerobic jars.

Biofilm Experiments

To prepare biofilms, SM and HA overnight (O/N) cultures were diluted 100x in fresh media and plated in 96 well plates (100 uL/well). SM and HA biofilms were cultured 24 and 48 h, respectively, with the media in the HA wells replaced at 24 h. Biofilm treatment: At the appropriate timepoint, media was discarded and biofilms rinsed with 1x PBS (pH 7). One hundred microlitres of treatment solution H_20, CHL [0.12%], thymol [0.063%], SO [100% 5cSt polydimethylsiloxane], ethanol [20%] or a combination solution [Mix][0.12% CHL, 20% ethanol, 25% SO]) at room temperature (RT) or preheated to 50°C (HT) was added and plates sealed and vortexed for 1 or 2 min. Solutions were discarded and wells rinsed twice with PBS. For biofilm analysis, wells were stained for 15 min using 33% crystal violet, rinsed 3x with H_2O, dissolved in 10% acetic acid and quantified at 540 nm on an Ascent Multiskan spectrophometer. For viable counts, biofilms were serially diluted in PBS, plated on BHYE and enumerated.

Antibiotic Disk Diffusion Assays (DDA)

All media plates (MHII agar for SM; BHYE agar for HA) were divided into four quadrants using a permanent marker. Colonies of each strain were picked off of stock plates and transferred to 2 mL BHYE broth and

grown O/N (16-18 h) statically at 37°C. Cultures were diluted 5-fold in 1x phosphate-buffered saline (PBS; pH 7.0) and streaked twice (10 minutes apart) using a cotton-tipped swab on the appropriate plates. After all plates had air-dried, antimicrobial susceptibility test discs (VWR) were added, one disc to each quadrant per plate resulting in each plate housing four different antibiotics. This was continued until all eighteen antimicrobial discs utilized in the study were placed (Ciprofloxacin [CIP-5 μg], Norfloxacin [NOR-10 μg], Gentamicin [GM-10 μg], Amoxicillin/Clavulanic acid [AMC-20/10 μg], Erythromycin [E-15 μg], Trimethoprim [TMP-5 μg], Sulfamethoxazole [SSS-1 mg], Kanamycin [K-30 μg], Sulfamethoxazole/Trimethoprim [SXT-23.75 mg/1.25 mg], Tetracycline [TET-30 μg], Azithromycin [AZM-15 μg], Cefotaxime [CTX-30 μg], Ceftazidime [CAZ-30 μg], Vancomycin [VAN-], Piperacillin [PIP-100 μg], Imipenem [IMP-10 μg], Tobramycin [NN-10 μg] and Nitrofurantoin [F/M-300 μg])(VWR). Plates were incubated upside-down O/N and analyzed for zones of growth inhibition (ZOI) in millimetres around each disk. Only the diameters of zones of complete growth inhibition (completely clear) were measured. The ZOI for each strain was compared to referenced standards to determine the clinically-relevant category of susceptibility for each antibiotic (susceptible [S], intermediate [I] and resistant [R]). In addition, ZOI values falling on the border between two susceptibility categories were given an intermediate rating (i.e. IR or IS).

Siloxane Polymer Disk Preparation and Diffusion Assays

Circular disks (9mm diameter, 1.5mm thickness) of type 3, light-bodied polyvinyl siloxane impression material (Kettenbach Dental, Huntington Beach, CA, USA) were prepared as per the manufacturer's instructions. The material was supplied as two separate gels that were mixed in equal proportions, applied to small plastic eppendorf tube caps and cured at room temperature for 10 minutes. To create various antimicrobial-impregnated disks, selected antimicrobial solutions were mixed individually with one of the polymer gels prior to their combination and application to the tube caps. Seven disk groups were created, supplemented with the following final concentrations: 1) nothing (control); 2) 0.3% triclosan (TCN) in methanol (MeOH); 3) 2% MeOH alone (TCN control); 4) 0.12% CHL; 5) TCN + CHL; 6) 0.5% sodium fluoride (SF); and 7) 100 ug/mL lysozyme (LYZ). Disks were trimmed with scissors to remove any rough edges and stored aseptically in the dark in 50mL conical tubes.

For the polymer disk-diffusion assay, MH II and BHYE plates were streaked with either SM or HA, respectively, similar to the DDA described above. Prepared disks were applied to the surface, the plates incubated

O/N and assessed visually for zones of growth inhibition (ZOI) in millimetres.

Differential Antagonism, Spent Culture Supernatant (SCS) Preparation and Well Diffusion Assays

Bacterial strains (all 4 – SM, HA, K12 and M18) were grown as 2cm wide lawns on TS+ agar for 48 hours at 37°C under 2-5% CO_2 conditions. These lawns were then physically removed using a sterile microscope slide and any remaining organisms killed by incubating the plate in chloroform vapours for 20 min. Plates were then aired for an additional 20 minutes to remove any residual chloroform. Fresh overnight cultures of each strain were streaked as 1cm wide lawns perpendicular to the original 2cm streak and plates incubated O/N at 37°C. Growth inhibition was assessed by measuring zones of reduced growth in the secondary 1cm streaks, with a complete inhibition rating (++++) assigned if no growth occurred over the original 2cm lawn.

For SCS preparation, a colony of each strain was picked off a plate and cultured statically for 24 hours as described above in 3mL TS+ broth. This culture was then diluted 1000x into 10mL fresh TS+ broth (starting inoculum ~$1x10^5$ CFU/mL) and cultured for 24 hours. Bacterial cells were pelleted by centrifugation (10,000 X *g*, 20 min, 4 °C) and

supernatants collected and passed through 0.22μm filters. Supernatants were aliquoted and stored at -20°C until use.

In well diffusion assays, fresh O/N cultures of SM and HA were plated out twice on BHYE agar to generate lawns. Immediately following complete drying of the surface, 1cm diameter wells were cut out ~2.5cm apart (4 per plate) using a sterile pipette tip and the bottom of each sealed using 50μL of molten BHYE agar. Filter-sterile SCS from each strain were applied to the wells and the plates incubated at 37°C O/N. Growth inhibition was examined by measuring the zone of inhibition surrounding each well.

Statistical Analysis

Statistical analyses were performed using GraphPad prism 4 software (Carlsbad, CA, USA), employing one-way ANOVA and Dunnett's comparison post tests. Significance was assessed at a p-value < 0.05.

Results

Biofilm Challenge

As mentioned, previous work has shown that coating teeth with silicone oil (SO) can inhibit bacterial biofilm formation. However, it is unknown whether SO can also be used effectively to dislodge and/or kill bacterial cells within pre-existing biofilms. To assess this, we cultured biofilms of both SM and HA in 96-well polystyrene plates using an artificial saliva media termed basal mucin media (BMM). However, different additives and culture times needed to be used for the two strains to promote sufficient biofilm development for each. SM biofilms were cultured over 24 hours using BMM supplemented with 2% sucrose and HA biofilms were grown over 48 hours using a 50:50 ratio of BMM and Brain Heart Infusion (BHI) media. Following biofilm formation, SO as well as several other well-known oral antimicrobial compounds were used to challenge the biofilms for 1 and 2 minutes under constant vortexing (to simulate oral rinsing). The treatment solutions selected were 5 centistoke polydimethylsiloxane (Silicone oil-SO), 20% ethanol (Alcohol), 0.063% thymol (THY), 0.12% chlorhexidine (CHL) and a mixture containing 25% SO, 20% ethanol and 0.12% chlorhexidine (Mix). Double-distilled sterile water was used as a control. Furthermore, as heat above 37°C has been shown to induce both bacterial stress and death upon exposure, the same incubation protocol was repeated using

the identical set of solutions heated to 50°C. This temperature was selected as it is both antimicrobial to bacteria as well as tolerable to the human oral cavity. Following challenge, any remaining biofilm was rinsed, stained with crystal violet and quantified via spectrophotometry, where a decrease in optical density demonstrated biofilm removal. The initial experiments involved a one minute challenge and yielded no significant reductions in biofilm mass for either strain (Fig. 1). In fact, the optical density actually increased significantly for HA biofilms in both the CHL and Mix groups at 50°C (Fig. 1A). Specifically, OD_{540} increased by 57.8% in the CHL group and 60.8% in the Mix group over the RT H_2O control (both $p<0.01$). Following this, the focus was shifted to a 2 minute challenge to see if an increase in treatment time would have any effect. With respect to biofilm removal, striking differences were observed between the two organisms as well as between the various solutions. Firstly, SO, CHL and the Mix all induced significant changes in the HA biofilm readings (Fig. 2A). Of these, SO was the only treatment to actually reduce HA biofilm retention significantly, with decreases of 36.9 (RT) and 42.4% (50°C) (both $p<0.05$) compared to the control treatment. Similar to the results for the 1 minute incubation time, both CHL and the CHL-containing Mix actually showed increases in HA biofilm readings compared to controls (CHL[50°C]-↑43.2%, Mix-↑31.3% [both $p<0.05$]). For SM biofilms, no solution or heat-treatment had any statistically significant effect on biofilm levels (Fig. 2B).

To complement the above results assessing biofilm mass, the same panel of solutions was next examined for their antimicrobial activity against the preformed biofilms (Fig. 3). Since the only decreases in biofilm mass were shown to occur with 2 minutes of treatment, this time was selected for examination. Overall, several solutions as well as the heat treatment were found to be effective against both organisms. For HA biofilms, treatment at both temperatures with 20% ethanol (Alcohol), thymol (THY), CHL or the Mix caused significant reductions in bacterial survival (Fig. 3A). Firstly, incubation with CHL and the Mix at either RT or 50°C as well as THY at RT resulted in a complete loss of HA viability, determined via biofilm dissolution and dilution plating ($p<0.01$). Thymol treatment at 50°C, alcohol at 50°C and alcohol at RT resulted in reductions of approximately 3 logs, almost 2 logs (both $p<0.01$) and 75% ($p<0.01$), respectively. No effect was observed for SO or 50°C water. For SM biofilms, CHL at 50°C and the Mix at both temperatures were completely bacteriocidal ($p<0.01$) while RT CHL and THY at 50°C reduced viability by approximately 3 and 4 log fold, respectively (both $p<0.01$)(Fig. 3B). Alcohol at 50°C lead to a decrease of 71.3% ($p<0.01$). Alcohol at RT, SO and 50°C water had no effect on SM survival.

Antibiotic Susceptibility Profiling

To assist in the characterization of these oral pathogens, antibiotic susceptibility profiles were generated comprising 18 clinically-utilized antimicrobials or antimicrobial combinations (Table 2 and Fig. 4). With regards to SM, only 6 of these compounds (or combinations) are routinely utilized against streptococci. SM was found to be susceptible to all 6, namely azithromycin, cefotaxime, erythromycin, tetracycline, vancomycin and the combination of sulfamethoxazole and trimethoprim. Although no zone of inhibition (ZOI) measurements are provided for the remaining compounds tested with regards to streptococci, the SM ZOIs measured for all but 2 of these antimicrobials were in the susceptible range of all other organisms that are routinely tested. Only trimethoprim (SM-Intermediate) and sulfamethoxazole (SM-Resistant) when used alone had ZOIs smaller than the susceptible range cutoff. Representative examples of SM ZOIs are shown in Figs. 4A and B.

For HA, only 8 of the 18 antimicrobials are routinely tested against species of *Haemophilus*. HA was observed to be susceptible to all 8 of them, namely amoxicillin/clavulanic acid, azithromycin, cefotaxime, ceftazidime, ciprofloxacin, imipenem, tetracycline and the combination of sulfamethoxazole and trimethoprim. For all but one (vancomycin – HA resistant) of the remaining compounds, the ZOIs generated by HA were decidedly in the susceptible range upon considering all other organisms tested. Thus, neither one of the oral pathogens utilized in this work

demonstrated any appreciable level of antibiotic resistance, especially since sulfamethoxazole and trimethoprim are never utilized alone clinically for either of these organisms, and vancomycin is not customarily used against gram negative organisms like HA. Representative examples of HA ZOIs are shown in Figs. 4C and D.

Siloxane Polymer Disk Diffusion

Silicone-based materials are widely used in dentistry for numerous applications. Since cured silicone intra-oral trays are easy to generate and precisely molded to the teeth and gums, they can potentially be useful for delivering health-promoting compounds to the oral cavity. We created cured polyvinyl siloxane disks impregnated with a number of different antimicrobial factors to investigate their ability to elute from the material and inhibit HA and SM growth (Fig. 5). The antimicrobial agents tested were chlorhexidine (0.12%-CHL), triclosan solublized in 2% methanol (0.3%-TCN), CHL+TCN, sodium fluoride (0.5%) and lysozyme (100μg/mL). In addition, 2 control groups were implemented, the first containing blank disks and the second impregnated with 2% methanol (MeOH). For SM, only CHL and CHL+TCN showed any inhibitory effect on growth using this assay, as relatively small ZOIs formed around those disks (Fig. 5A). Specifically, the mean ZOI diameters for these two test

groups were 10.7mm (CHL) and 11.5mm (CHL+TCN). With a disk diameter of 9mm, this means that eluted CHL was only growth inhibitory up to 0.85mm (±0.17mm) from the disk and when combined with TCN up to 1.25mm (±0.29mm) from the disk. None of the other compounds tested had any effect. Against HA, TCN alone and in combination with CHL was appreciably more effective as mean ZOIs were 22.0±1.53mm and 26.67±1.76mm, respectively. Similar to SM, none of the other compounds tested had any effect against HA growth. Overall, the combination of TCN and CHL was the most effective at inhibiting the growth of both pathogens, albeit far more successful against HA than SM.

Oral Probiotics

As mentioned, several strains of *Streptococcus salivarius* have demonstrated benefits in the oral cavity, including pathogen exclusion and displacement. As a proof of principle, we investigated whether compounds secreted by two commercially-available oral probiotic strains of *Streptococcus salivarius* (namely K12 and M18) could inhibit the growth of HA and SM. Concurrently, we examined whether HA and/or SM secrete any antibacterial factors themselves. The two assays utilized to examine these hypotheses were differential antagonism (DA) and well

diffusion (WD). In DA, all four strains were grown separately as 2cm wide lawns on TS+ plates for 48 hours. These lawns were then removed and perpendicular streaks of the remaining three strains were plated across that original streak. Any observed growth inhibition of the indicator strains in the perpendicular streaks indicated the presence of a secreted antibacterial factor released from the original strain. The results are summarized in Table 3 and a representative plate for each test strain is shown in Figure 6. Firstly, SM and HA were affected very differently by both of the probiotic strains as well as each other. While the growth of SM was not affected at all when grown over the secreted factors of K12, M18 or HA (Fig. 6A, B, D, respectively), the growth of HA was completely inhibited by both probiotics and SM (Fig. 6A-C). Furthermore, SM was not only able to inhibit the growth of HA, but both of the probiotic strains as well. (Fig. 6C). Finally, factors secreted by HA into the TS+ media did not inhibit the growth of any of the three other strains (Fig. 6D). Overall, SM was able to inhibit all three of the other strains and was not affected by any of them. Conversely, HA was completely inhibited by all of the other strains but was unable to inhibit the growth of any of them.

While DA looks at factors secreted into a solid medium during surface-attached (biofilm) growth, WD looks at factors secreted in liquid medium during planktonic (free-swimming) growth. Following the DA results, all four strains were cultured in liquid media for 48 hours and

the spent culture supernatants (SCS) filter-sterilized and tested against the remaining strains (Fig. 7). Unlike the DA results, none of the SCS isolated from any of the four strains was able to affect the growth of any other. Thus, none of the four strains secreted any detectable factors during liquid culture growth that were inhibitory to any of the strains.

Table 1

BMM Composition (pH 6.8)	
Reagent	**Mass (per Litre)**
Pig Gastric Mucin (Type III)	2.5 g
Proteose Peptone	10.0 g
Tryptone	5.0 g
Yeast Extract	5.0 g
Potassium Chloride	2.5 g
Vitamin K	0.001 mg
Hemin	0.005 mg
Urea	0.06 mg
L-Arginine	0.2 mg

Table 2

	Zone of Growth Inhibition (mm) **Mean +/- SE** **N=3**	
Antibiotic	*S. mutans* 25175	*H. actinomycetemcomitans* Y4
Amoxicillin/Clavulanic Acid (AmC) (20/10μg)	47.3 ± 1.5 (NT-S)	41.0 ± 0.6 (S - ≥20)
Azithromycin (AZT) (15μg)	33.0 ± 2.1 (S - ≥18)	36.0 ± 1.5 (S - ≥12)
Cefotaxime (CTX) (30μg)	42.0 ± 2.3 (S - ≥24)	53.3 ± 0.7 (S - ≥26)
Ceftazidime (CAZ) (30μg)	38.0 ± 0 (NT-S)	44.0 ± 1.5 (S - ≥26)
Ciprofloxacin (CIP) (5μg)	22.3 ± 0.9 (NT-S)	51.7 ± 4.4 (S - ≥21)
Erythromycin (E) (15μg)	39.0 ± 0.6 (S - ≥21)	22.7 ± 0.7 (NT-S)
Gentamicin (GM) (10μg)	28.0 ± 1.2 (NT-S)	26.3 ± 1.2 (NT-S)
Imipenem (IMP) (10μg)	50.0 ± 2.3 (NT-S)	34.3 ± 1.9 (S - ≥16)
Kanamycin (K) (30μg)	19.3 ± 0.7 (NT-S)	31.3 ± 0.3 (NT-S)
Nitrofurantoin (F/M) (300μg)	35.7 ± 0.9 (NT-S)	52.3 ± 3.0 (NT-S)
Norfloxacin (NOR) (10μg)	17.0 ± 0.6 (NT-S)	51.0 ± 2.5 (NT-S)
Piperacillin (PIP) (100μg)	44.0 ± 1.2 (NT-S)	44.7 ± 1.8 (NT-S)
Sulfamethoxazole (SSS)(1mg)	0.0 ± 0 (NT-R)	30.0 ± 1.0 (NT-S)
Tetracycline (TE) (30μg)	28.7 ± 0.9 (S - ≥23)	44.0 ± 1.2 (S - ≥29)
Tobramycin (NN) (10μg)	22.3 ± 1.2 (NT-S)	28.0 ± 4.2 (NT-S)
Trimethoprim (TMP) (5μg)	14.3 ± 1.9 (NT-I)	50.3 ± 1.9 (NT-S)
Sulfamethoxazole/Trimethoprim (SXT) (23.75/1.25μg)	24.7 ± 1.7 (S - ≥19)	42.3 ± 1.9 (S - ≥16)
Vancomycin (VAN) (30μg)	28.0 ± 0.6 (S - ≥17)	0.0 ± 0 (NT-R)

Table 2. Antibiotic Disk Diffusion Assay. S – Organism considered susceptible to the antibiotic; I – Organism considered intermediate; R – Organism considered resistant; NT-S - Antibiotic not typically tested for strains of that species but ZOI considered susceptible based upon all other organisms routinely tested; NT-I - Similar to NT-S except ZOI considered intermediate; NT-R – Similar to NT-S except ZOI considered resistant. S - ≥20 – Organism considered susceptible to the antibiotic since all strains of that species or with ZOI greater than 20mm are considered susceptible.

Table 3

Test Strain- Primary Streak	Degree of Growth Inhibition			
	Indicator Strain – Secondary Streak			
	SM	HA	K12	M18
SM	N/A	++++	+++	++++
HA	-	N/A	-	-
K12	-	++++	N/A	+++
M18	-	++++	+++	N/A

Table 3. Differential Antagonism assay. - no inhibition; + slight inhibition (<25% reduction); ++ moderate inhibition (25-50% reduction); +++ strong inhibition (50-75% reduction); ++++ very strong to complete inhibition (>75%). N/A: not applicable as experiement not done. N=1.

Figure 1

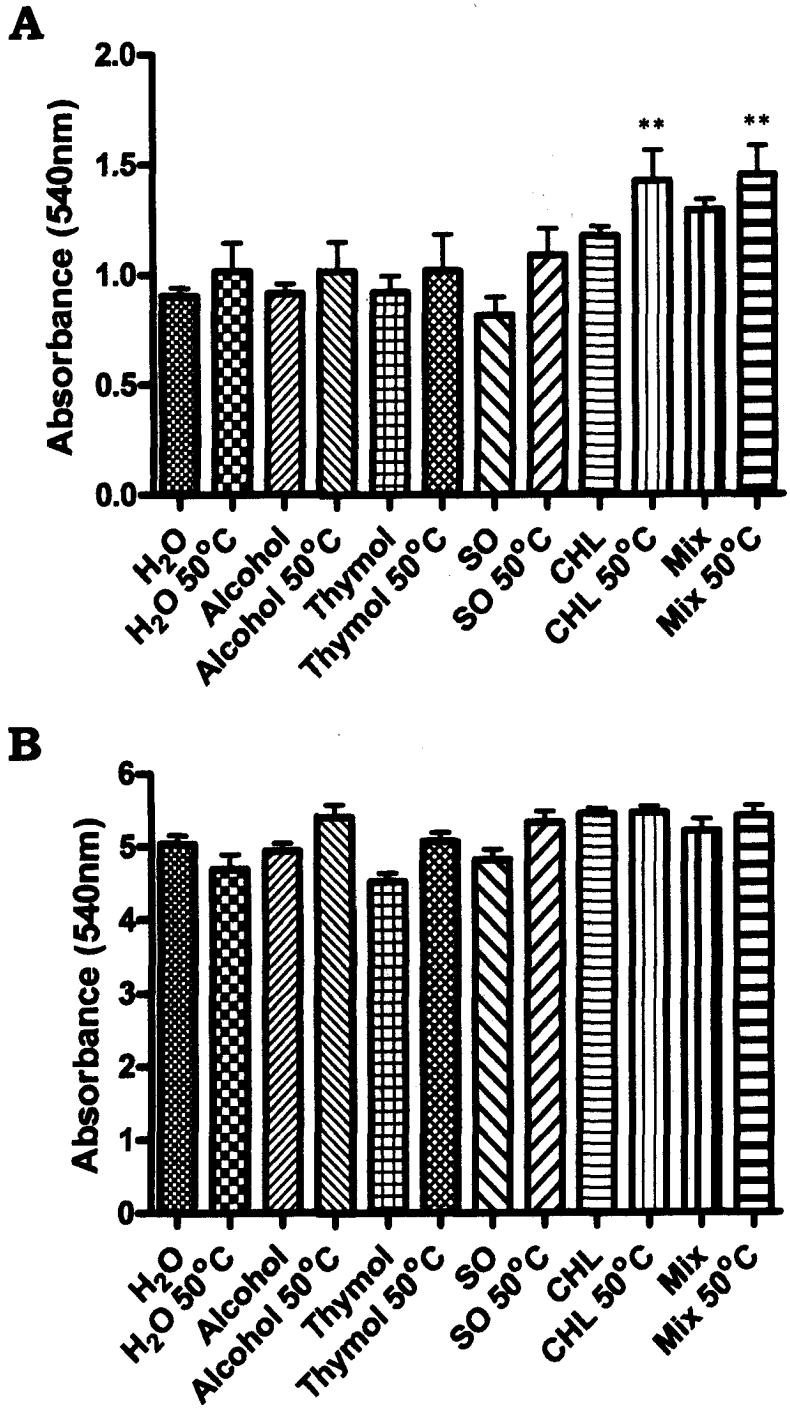

Figure 1. Crystal violet staining of *H. actinomycetem-comitans* Y4 (A) and *S. mutans* 25175 (B) biofilms remaining following 1 minute of treatment with numerous antimicrobial solutions. ** - $p<0.01$. Mean +/- SE; N=10

Figure 2

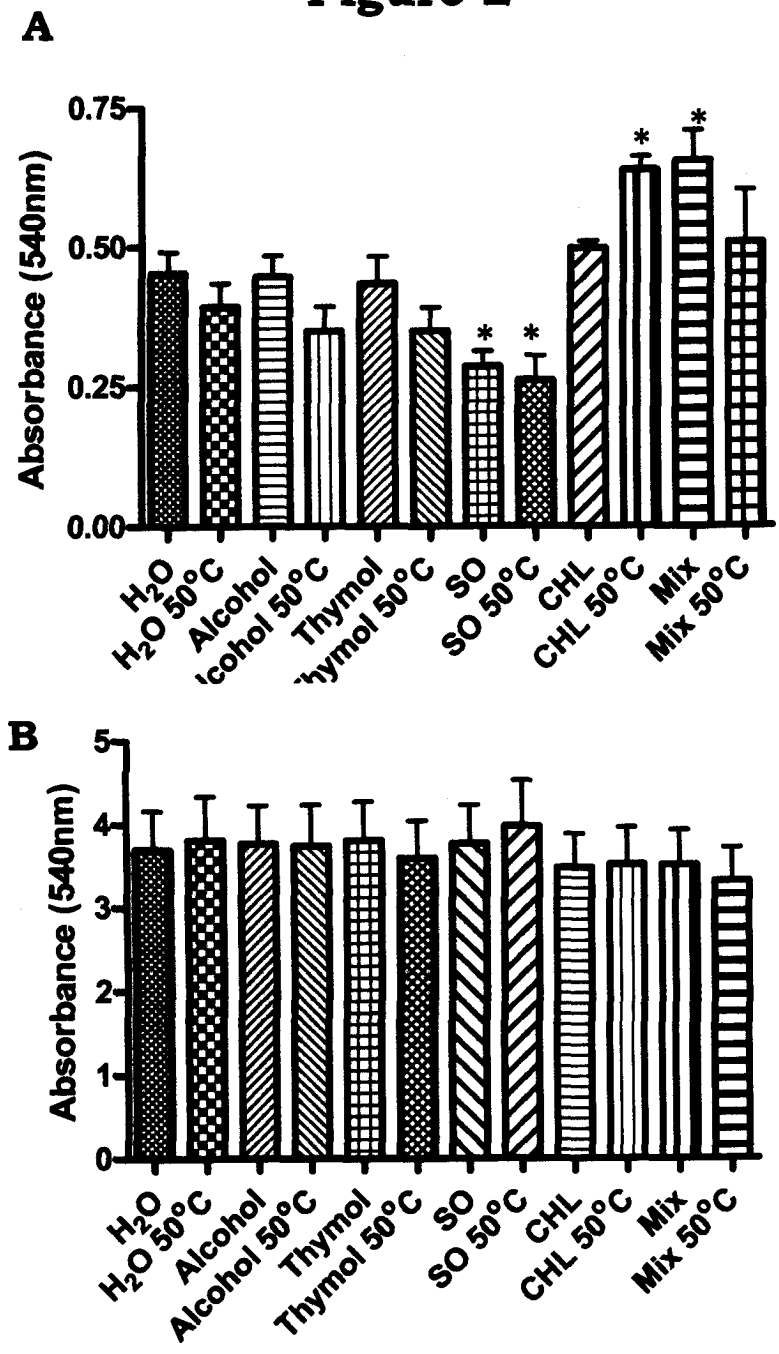

Figure 2. Crystal violet staining of *H. actinomycetem-comitans* Y4 (A) and *S. mutans* 25175 (B) biofilms remaining following 2 minutes of treatment with numerous antimicrobial solutions. *-$p<0.05$. Mean+/-SE;N=10.

A

Figure 3

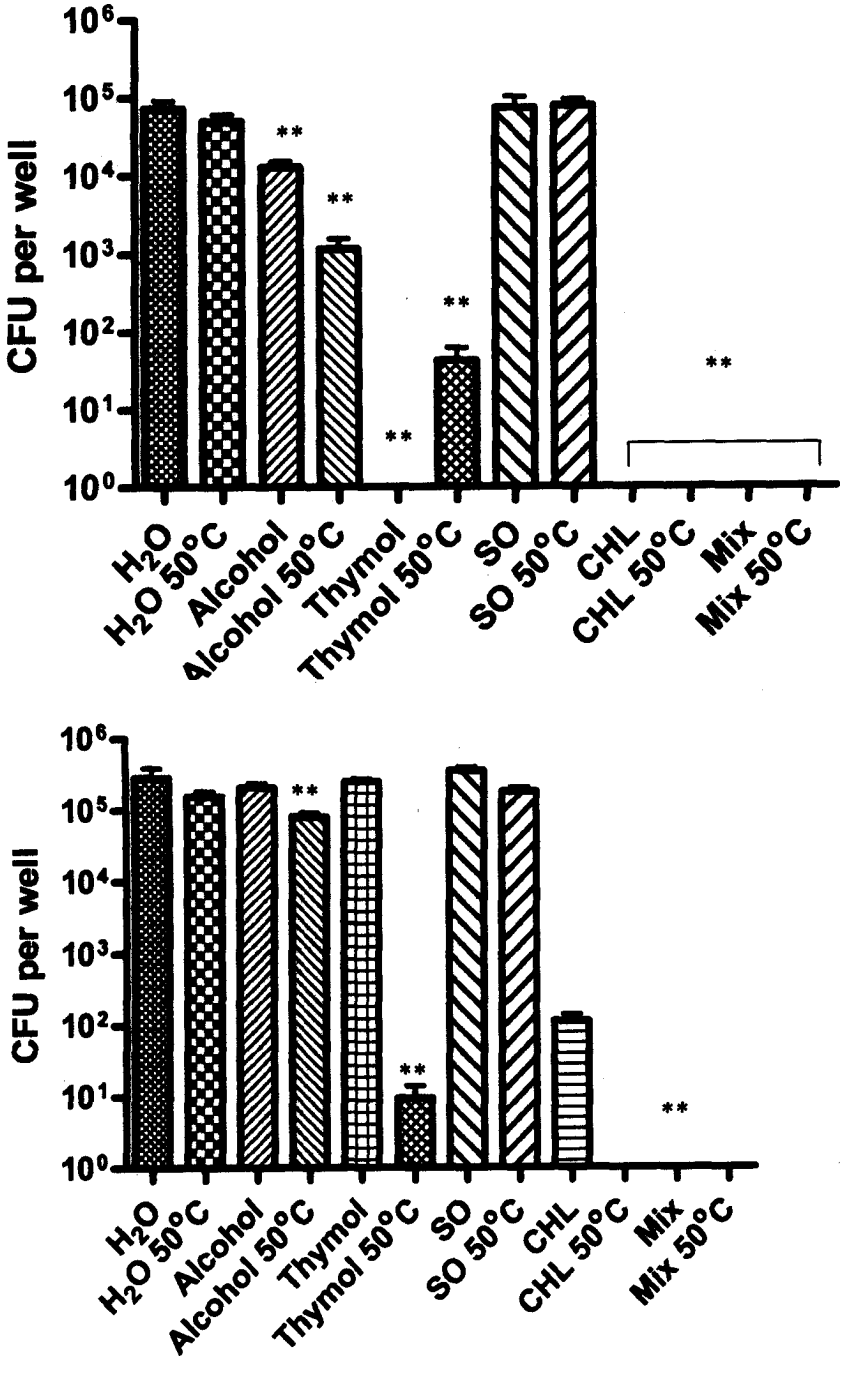

Figure 3. Viable organisms remaining for *H. actinomycetemcomitans* Y4 (A) and *S. mutans* 25175 (B) biofilms following 2 minutes of treatment with numerous antimicrobial solutions. *-p<0.05, **-p<0.01, ***-p<0.001. Mean+/-SE;N=5

Figure 4

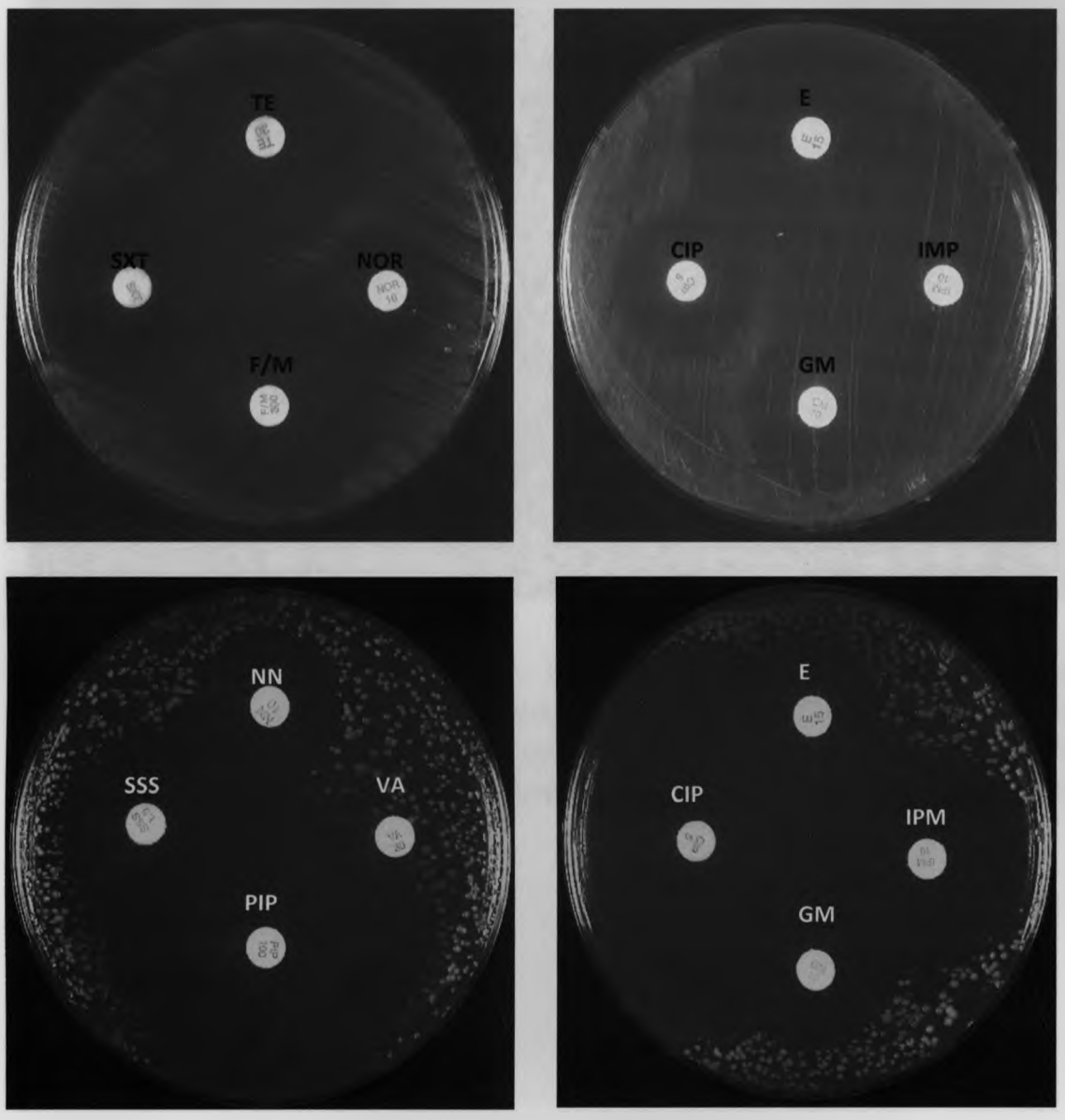

Figure 4. Representative plates from SM (A,B) and HA (C,D) antibiotic disk diffusion assays. The arrows in A demonstrate the complete diameter of the zone of inhibition (ZOI) for SM exposed to tetracycline (TE). SXT–sulfamethoxazole/Trimethoprim; F/M–nitrofurantoin; NOR–norfloxacin; E–erythromycin; CIP–ciprofloxacin; IMP–imipenem; GM–gentamicin; NN–tobramycin; SSS–sulfamethoxazole; VA-vancomycin; PIP–piperacillin. N=3.

Figure 5

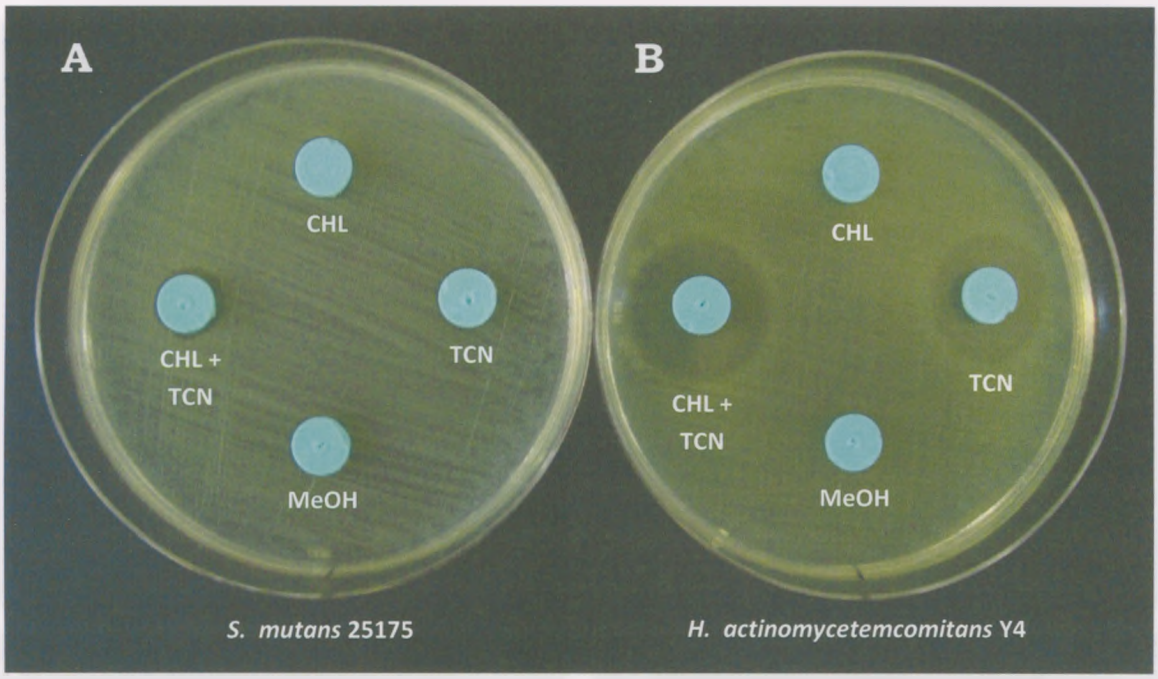

Figure 5. Disk diffusion assay using polyvinyl siloxane impression material impregnated various antimicrobials. Results are following 24 hours of growth on MHII (SM) and BHYE (HA) plates. CHL – 0.12% chlorhexidine; TCN – 0.3% triclosan; MeOH – 2% methanol (control). N=3.

Figure 6

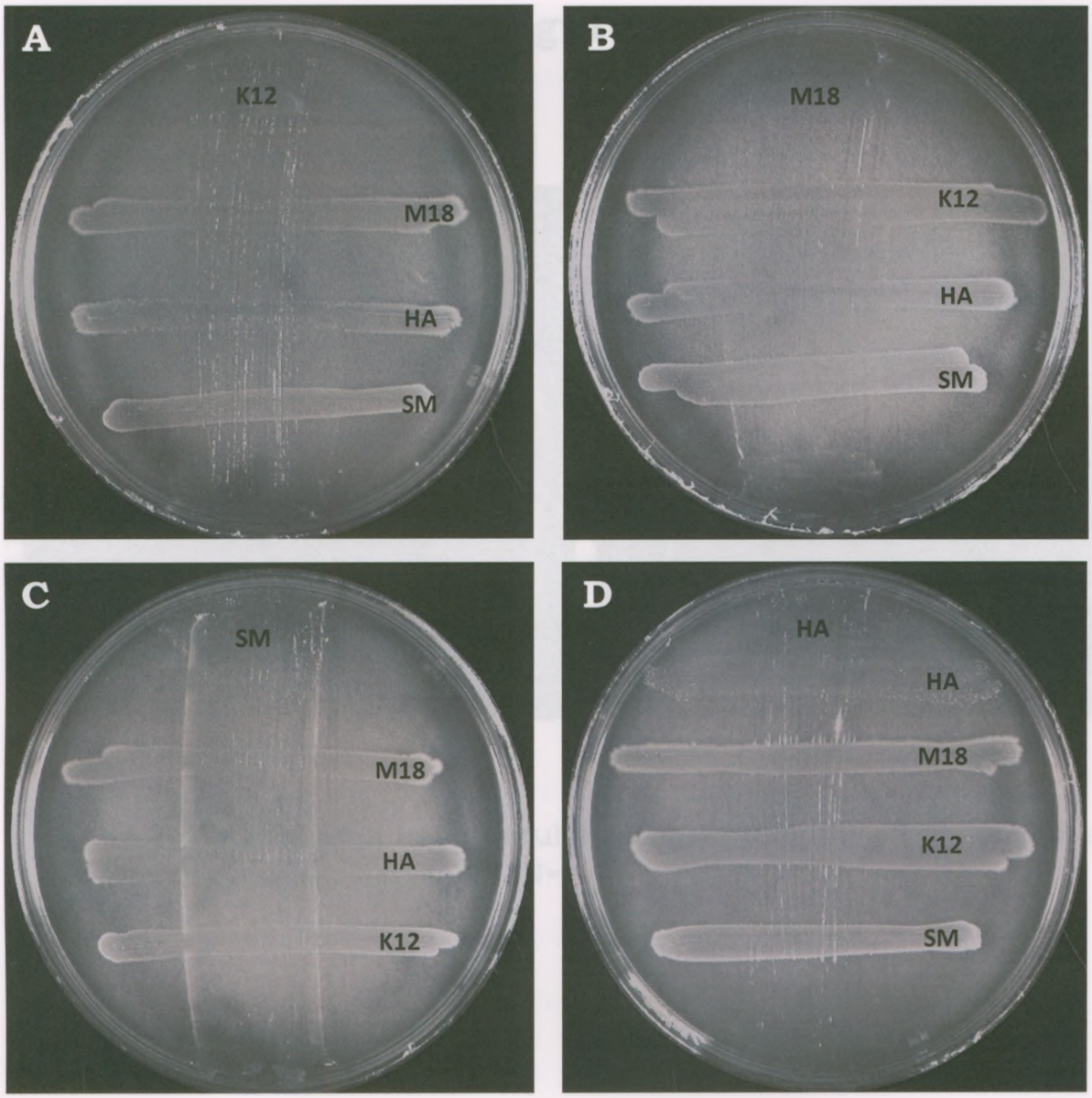

Figure 6. Representative plates from the differential antagonism assays. Top labels on each plate identify the original 2cm wide streak grown for 48hrs and removed. Side labels identify the secondary perpendicular streaks grown across the original streak. Growth inhibition was assessed qualitatively as a decrease in the thickness and degree of growth of the secondary streak. A) K12, B) M18, C) SM and D) HA. Results are summarized in Table 3. N=1.

Figure 7

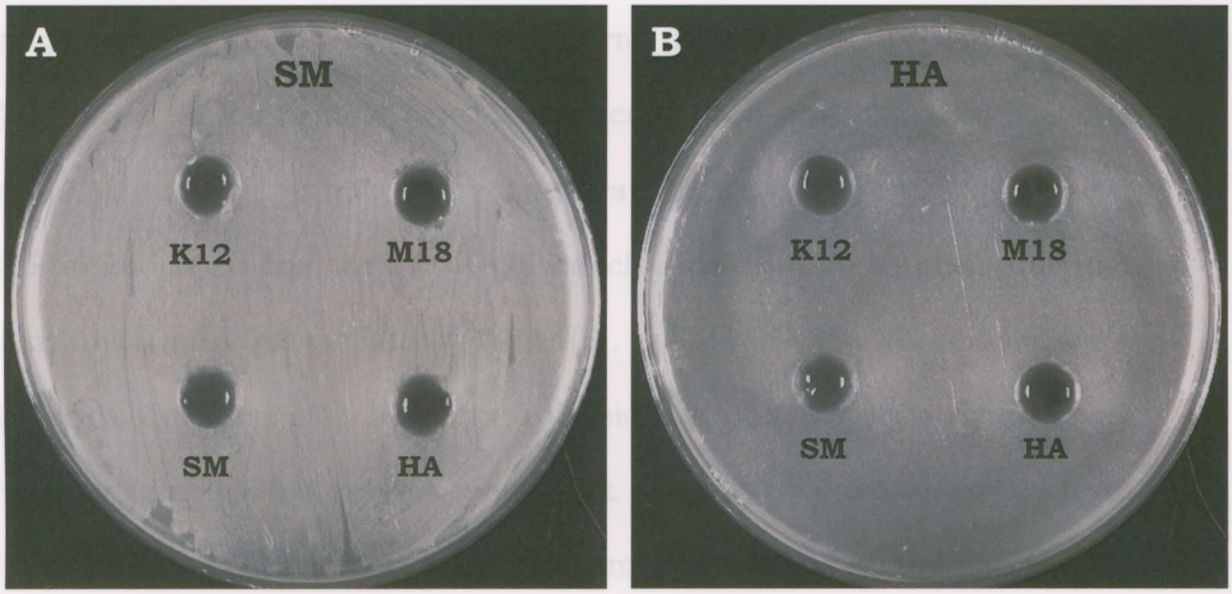

Figure 7. Well diffusion assay results demonstrating the effects of SCS on the growth of SM (A) and HA (B). N=1

DISCUSSION

Biofilm challenge

Microscopically, a biofilm is a highly hydrated and open structure, composed of noncellular material including water channels and exopolymeric substances (EPS) that form the extracellular matrix. The EPS forms the outermost layer and is composed of a hydrated, anionic mesh of bacterial exopolymers and trapped environmental molecules. EPS includes a wide variety of polysaccharides, proteins, glycoproteins, glycolipids and extracellular DNA (5). This matrix functions as a permeability barrier to limit the diffusion of beneficial nutrients away from the biofilm and inhibit movement of harmful substances such as antibiotics and predatory cells of the immune system into the film (5).

Chemotherapeutic agents for the prevention and treatment of biofilms remain an important adjunct to mechanical debridement of the oral cavity. The effects of these agents include interference with bacterial adhesion and co-aggregation, reduction of viability, the removal and/or disruption of existing biofilms and the enhancement of the host's inflammatory and immune responses against the film (15).

Although previous studies have already established that silicone-based compounds can inhibit bacterial biofilm formation, the current study examined silicone's potential for disrupting existing films. Firstly, it

should be noted that biofilm development varied greatly between HA and SM. SM generated biofilms far more robust and extensive than HA within 24 hours, even though the latter was cultured for 48 hrs. This may reflect an increased virulence of SM over HA and highlights the need for different environmental conditions and nutrients for optimal growth of the two strains. This may also explain why SM is an "early" colonizer and HA a "later" one. Once biofilms were established, they were challenged with SO and several other common oral antimicrobials for 1 and 2 minutes under constant vortexing to simulate oral rinsing. Total biofilm levels following treatment were quantified via crystal violet staining and spectrophotometry. The experiments were repeated and bacterial viability assessed via dilution plating. These methods were selected as they are both currently well accepted for bacterial biofilm research.

Although SO is available in a variety of viscosities, 5 centistoke (ct) polydimethylsiloxane was selected as it was the least viscous available and thus easiest to rinse with. The additional antimicrobials tested during this biofilm challenge were chlorhexidine (0.12%), ethanol (20%) and thymol (0.063%), all commonly found in commercially-available mouthwashes at the listed concentrations. In addition, a cocktail mix was made containing 25% SO, 20% ethanol and 0.12% chlorhexidine to investigate potential additive effects. Finally, the entire set of solutions

was also heated to 50°C and applied in a similar fashion, as this temperature should enhance antibacterial activity while remaining tolerable to the human oral cavity. For example, in one study a 100-fold increase in killing efficacy was observed between corresponding NaOCl (bleach) solutions at 20°C and 45°C (24). In the current study however, due to a high degree of variability across the results with respect to temperature, no clear differences were observed. This was most likely due to two key factors, namely the short treatment times and cooling of the solutions during vortexing. With respect to the various solution groups, the results were both time and compound dependent. Firstly, experiments involving a 1 minute challenge (Figure 1) did not yield any significant reductions in biofilm mass for either strain compared to controls. This is an important finding because currently, most dentists recommend a 1 minute rinse with chlorhexidine. Our data suggests that 1 minute may be an insufficient duration for application of the oral antimicrobial agents that were tested. A study by Pratton et al. supports this, as 1 minute biofilm exposure to even 0.2% CHL (maximum used in mouthwashes) had no significant effect on bacterial viability (1), further highlighting biofilm resilience. One interesting additional finding was that both the CHL and Mix groups at 50°C for HA actually had significantly increased crystal violet readings over controls. The likely reason for this result is CHL binding to bacterial cells and other matrix components within the biofilm and then to the crystal violet itself,

leading to increased staining. CHL has previously been shown to bind a wide variety of substrates including both lipopolysaccharide (LPS) and lipoteichoic acid (LTA) located on the surfaces of Gram-negative and –positive bacteria, respectively (25). An explanation for why only HA biofilms at 50°C were significantly affected could be the fact that HA produces far less robust films that may have been partially disrupted by the combination of the heat and solution treatments. This may have resulted in far more exposed HA biofilm and bacterial surface area for CHL to bind. In addition, the robust structure of SM biofilms may have made them less susceptible to disruption or buried the bacteria within the biofilm to a greater extent, thus minimizing CHL exposure

Following the overall poor results for the 1 minute challenge, focus shifted to increasing treatment length to two minutes (Figure 2). Interestingly, SO both at RT and 50°C was the only treatment to significantly reduce HA biofilm retention under these conditions. Again all four CHL containing solutions showed an increase in HA staining compared to the controls, two of which were statistically significant. Once more, binding of CHL to various cellular and biofilm structures may account for this finding. Similar to the 1 minute results, SM biofilms in the 2 minute challenge did not reveal any statistically significant differences. Perhaps longer challenge times or increased concentrations of solutions are required to penetrate these biofilms.

In this study, we demonstrated the ability of a silicone-based compound to inhibit HA biofilm surface retention. This effect could potentially have been due to its hydrophobic nature, which may have allowed it to solubilize certain biofilm components and interact with the lipid outer membrane of the Gram-negative HA. Furthermore, the solubility of SO may also have disrupted biofilm structure, thus elevating the relative CFU count. This latter point is an important consideration as biofilm dilution plating often underestimates the total viable number of organisms due to incomplete film dissolution. Overall, SO was found to be ineffective at killing either organism, a finding in sharp contrast to the remaining antimicrobials tested, all of which demonstrated at least some degree of antibacterial action (mostly on the Gram-negative HA). Future studies are ultimately warranted to determine the true clinical potential of SO and should involve additional SO formulations, organisms and application strategies.

Figure 3 clearly supports the use of ALC, THY, and CHL for clinical use. Most noteworthy was the finding that both CHL-containing solutions almost completely inhibited the growth and/or survival of HA and SM. CHL is a fairly broad-spectrum agent with both bacteriostatic and bacteriocidal effects (26), largely based upon concentration. In the current study, its effects were most likely due to a combination of biofilm penetration during challenge and its strong binding potential, which may

have prevented complete removal during post-treatment rinsing. This residual CHL may then have killed or slowed the growth of any organisms surviving the initial challenge. While this may be advantageous for CHL's antimicrobial effects, it may also increase the oral complications associated with its use as long-term use can lead to mucosal desquamation, discolouration, and taste impairment (6). Overall, it would appear that use of the compound is both organism and application dependent as a systematic review of the literature described variable results (17). While it revealed that rinsing with a mouthwash containing CHL (0.12% and 0.20%) did not produce any long-term effect on salivary SM, other formulations such as higher concentration gels could significantly decrease bacterial levels with repeated applications.

Antibiotic susceptibility profiling

In order to better characterize our study strains and establish a model for investigating the generation of antimicrobial-impregnated polyvinyl siloxane disks, we performed standard antibiotic susceptibility profiling of HA and SM. Both organisms were found to be susceptible (created the minimum necessary ZOI) to all 8 (HA) and 6 (SM) antibiotics clinically utilized against strains of their respective species (Table 2). Furthermore, due to the availability of other common antibiotics (albeit

those not routinely used against SM and HA), we expanded our profiling to incorporate an additional 10 compounds. As summarized in Table 2, neither one of these pathogens demonstrated any appreciable level of antibiotic resistance.

With respect to more serious cases of periodontal disease, several antibiotics often play a key role in any treatment strategy. Model compounds in this fight are the tetracyclines, which have shown utility in the management of both localized aggressive periodontitis (LAP) and refractory periodontitis (27). In LAP the main pathogen HA is very susceptible to tetracyclines (28) and a 3-6 week course of 1 g/day will often halt the progression of the disease (27). They are broad spectrum, bacteriostatic antimicrobials exhibiting a trifold mechanism of action. They also possess anti-collagenase activity, preventing the breakdown of collagen (29) and have been shown to enhance fibroblast attachment and colonization on pretreated root surfaces (30). While they are distributed widely in all tissues, they localize in both developing dental structures and bone. In addition, crevicular fluid concentrations can be 5-10 times greater than those in serum (31). Importantly, in this current study tetracycline was indeed effective against both SM and HA, supporting its use against these pathogens and application in periodontitis.

Siloxane Polymer Disk Diffusion

Antibiotics can be given both systemically or applied topically to the periodontal pocket (27). Techniques for local administration include subgingival irrigation, polymerization into acrylic strips and incorporation into biodegradable collagen. Although local application can be time consuming and penetration of the pocket is less predictable, this method can limit adverse reactions (27). Based upon this knowledge, we hypothesized that patient-molded polyvinyl siloxane trays impregnated with various antibiotics or other antimicrobial compounds could perhaps be a useful treatment strategy by increasing substantivity and reducing negative host reactions through controlled, local application. The successful impregnation and elution of antimicrobial compounds from the tray material in this study solidly demonstrates a proof of principle in using this polymer to deliver therapeutic compounds of interest. TCN may be a great candidate as it not only has a broad spectrum of activity as an antimicrobial, but also exhibits potent host anti-inflammatory and growth promoting properties. Thus, TCN could be applied to decrease bacterial load while simultaneously promoting healing directly at sites of interest. In this study, CHL and TCN both showed antimicrobial effects, although TCN alone was only effective against HA and CHL was only notably effective when combined with TCN (Figure 5). The most interesting finding was the additive effect of CHL

with TCN, supported by a 2007 study examining atopic dermatitis (32). This study concluded that low concentrations of TCN+CHL were suitable for pathogen reduction and reducing side effects. Although there were no established norms to determine effective ZOI's for the disk-diffusion experiments in this study, we can conclude that at least some antimicrobials can elute effectively and impact the growth of strains of oral pathogens, even if only at close proximity to the polymer. This is further supported by work showing that the efficacy of antimicrobial agents depends on many factors including vehicle of administration, concentration, treatment duration and substantivity (33). Future work in this area should involve a comprehensive comparison of all antimicrobials currently utilized in the oral cavity and include additional bacterial and fungal pathogens of relevance to this biological site.

Oral Probiotics

Studies have shown probiotics to confer anti-plaque action on the oral cavity by several mechanisms. Firstly, they can directly interact with host tissues and dental plaque, disrupting biofilm formation as the organisms compete for binding sites and nutrients. They also produce numerous antimicrobial compounds and modulate host immunity (23). In particular, the well-researched, safe and commercially-available oral strain *Streptococcus salivarius* K12 (K12)(34) has been shown to strongly

adhere to oral epithelial cells (35), downregulate host inflammation (36), attenuate pathogen adhesion and virulence (37), and produce the antimicrobial bacteriocins salivaricin A and salivaricin B (38) as well as the enzymes urease (neutralizes cariogenic acid) and dextranase (breaks down dextran, a major component of plaque). According to BLIS Technologies, the New Zealand biotech company which has researched and markets the strain, all of these factors can contribute to a healthy oral cavity. Based upon this potential, a collaboration is currently being set up to conduct additional research on this and other potential oral probiotic strains. As an introduction to this collaboration, we investigated the ability of K12 and another strain, *Streptococcus salivarius* M18, to inhibit the growth of SM and HA using two well-recognized methods of bacterial interaction. Our data reveals that when grown on solid media (but not in liquid), both K12 and M18 secrete factor(s) inhibitory to the growth of HA and each other, but not SM (Figure 6). Furthermore, SM was found on solid media to secrete an antimicrobial factor itself, one which was inhibitory to HA as well as both probiotics. However, HA did not produce anything inhibitory to any of the strains. The implications of these results are far reaching. Firstly, both K12 and M18 could potentially be used to treat localized aggressive periodontitis since HA is one of the disease's primary causative organisms. Secondly, according to BLIS Technologies, K12 and M18 have previously shown efficacy against strains of SM. Since we did not

find similar results in our study using SM 25175, future work is needed involving more SM strains to confirm the probiotic strains' true potential against this pathogen. If indeed most strains of SM can be inhibited by these probiotics, it would be a step towards eliminating caries, which would change the face of dentistry. Lastly, the production of a powerful antimicrobial factor by SM requires further investigation to identify and characterize it. Although it could very well be mutacin, a substance first isolated from SM in 1975, it should be confirmed (39). Like other bacteriocins, mutacin is used to reduce the levels of certain SM competitors within the oral cavity(39).

Conclusions

Due to the variety of viscosities available in silicone ranging from water-like to firm gel/matrix, there is promise for a wide range of application in the oral cavity. Since silicone is already widely used intraorally, it seems clinically feasible to think outside the box and widen its application. Additional testing of other antimicrobial and therapeutic agents will help determine the full utility of these compounds. The potential promise of controlling both caries and periodontal disease with this novel concept would be a breakthrough for dental research.

Further studies are needed, including investigations into the development of cost effective, target specific, safe methods to control

plaque-related diseases. They should reduce plaque levels without affecting the overall biological equilibrium within the oral cavity (23). The evaluation of the safety of chemical agents for antimicrobial use must be at the forefront of our research. We must demonstrate that the active agents are safe with no adverse effect on general health and that long term use still maintains the oral microbiota and doesn`t lead to the emergence of opportunistic pathogens (15).

APPENDICIES

Appendix 1: HA Biofilm Crystal Violet 1 minute with Stats 10 Expts Figure 1

	H2O	H2O 50oC	CHL	CHL 50oC	Alcohol	Alcohol 50oC	Thymol	Thymol 50oC	SO	SO 50oC	Mix	Mix 50oC
Expt 1	0.946	0.874	0.914	1.307	0.918	0.902	0.748	0.676	0.686	0.846	1.012	1.09
Expt 2	0.951	0.913	1.043	1.33	0.928	1.072	0.838	0.619	0.972	1.133	1.319	1.176
Expt 3	0.848	0.897	1.141	1.286	0.868	0.777	0.769	1.085	1.017	1.569	1.192	1.03
Expt 4	0.849	0.91	1.087	1.309	0.894	0.709	0.745	0.64	1.067	1.111	1.127	1.17
Expt 5	1.093	1.084	1.12	1.156	0.946	0.879	0.643	0.886	0.78	0.96	1.332	1.406
Expt 6	0.85	0.566	1.238	1.099	1.002	0.823	0.912	0.701	0.799	0.794	1.244	1.356
Expt 7	0.996	0.672	1.264	1.122	1.15	0.67	1.185	0.854	1.061	0.562	1.344	1.451
Expt 8	1.016	0.757	1.324	1.162	1.051	0.745	1.425	0.836	0.99	0.73	1.444	1.51
Expt 9	0.778	1.707	1.315	2.306	0.629	1.914	0.977	2.093	0.389	1.496	1.335	2.285
Expt 10	0.703	1.776	1.291	2.172	0.742	1.67	0.959	1.799	0.388	1.669	1.531	2.049

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	12
F	3.938
R squared	0.2863

Bartlett's test for equal variances	
Bartlett's statistic (corrected)	44.65
P value	P<0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	4.764	11	0.4331
Residual (within columns)	11.88	108	0.11
Total	16.64	119	

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
H2O vs H2O 50oC	-0.1126	0.7593	P > 0.05	-0.5275 to 0.3023
H2O vs CHL	-0.2707	1.825	P > 0.05	-0.6856 to 0.1442
H2O vs CHL 50oC	-0.5219	3.519	P < 0.01	-0.9368 to -0.1070
H2O vs Alcohol	-0.0098	0.06608	P > 0.05	-0.4247 to 0.4051
H2O vs Alcohol 50oC	-0.1131	0.7626	P > 0.05	-0.5280 to 0.3018
H2O vs Thymol	-0.0171	0.1153	P > 0.05	-0.4320 to 0.3978
H2O vs Thymol 50oC	-0.1159	0.7815	P > 0.05	-0.5308 to 0.2990
H2O vs SO	0.0881	0.5941	P > 0.05	-0.3268 to 0.5030
H2O vs SO 50oC	-0.184	1.241	P > 0.05	-0.5989 to 0.2309
H2O vs Mix	-0.385	2.596	P > 0.05	-0.7999 to 0.02994
H2O vs Mix 50oC	-0.5493	3.704	P < 0.01	-0.9642 to -0.1344

Appendix 2: SM Biofilm Crystal Violet 1 minute with Stats 10 Expts Figure 1

		H2O		CHL		Alcohol		Thymol		SO		Mix
	H2O	50oC	CHL	50oC	Alcohol	50oC	Thymol	50oC	SO	50oC	Mix	50oC
Expt 1	4.971	4.878	5.312	5.59	4.982	5.468	4.977	4.485	4.352	5.275	5.67	5.688
Expt 2	5.395	5.298	5.869	5.496	4.099	5.756	4.141	5.289	5.051	5.954	4.697	4.7
Expt 3	5.67	3.687	5.031	4.875	4.945	5.51	3.951	5.275	5.083	5.357	5.12	5.043
Expt 4	5.502	5.275	5.415	5.659	5.335	5.89	4.136	4.973	5.031	4.345	4.728	5.121
Expt 5	4.948	3.539	5.373	5.215	4.751	5.836	4.57	5.39	5.014	5.898	5.543	5.622
Expt 6	4.811	5.303	5.748	5.845	5.02	5.687	4.952	5.812	4.923	5.268	4.891	5.673
Expt 7	4.814	5.001	5.18	5.54	5.296	5.947	4.594	5.086	5.542	5.809	4.745	4.893
Expt 8	5.068	4.716	5.491	5.406	4.89	4.737	4.806	4.953	4.874	5.204	4.822	5.742
Expt 9	4.654	4.345	5.563	5.439	4.965	4.675	4.769	4.679	4.153	5.015	5.876	5.799
Expt 10	4.543	4.869	5.467	5.557	5.123	4.552	4.347	4.774	4.204	5.134	5.98	5.907

Data 1

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	12
F	5.387
R squared	0.3543
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	14.01
P value	0.2325
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
H2O vs H2O 50oC	0.3465	1.789	P > 0.05	-0.1954 to 0.8884
H2O vs CHL	-0.4073	2.103	P > 0.05	-0.9492 to 0.1346
H2O vs CHL 50oC	-0.4246	2.192	P > 0.05	-0.9665 to 0.1173
H2O vs Alcohol	0.097	0.5008	P > 0.05	-0.4449 to 0.6389
H2O vs Alcohol 50oC	-0.3682	1.901	P > 0.05	-0.9101 to 0.1737
H2O vs Thymol	0.5133	2.65	P > 0.05	-0.02865 to 1.055
H2O vs Thymol 50oC	-0.034	0.1755	P > 0.05	-0.5759 to 0.5079
H2O vs SO	0.2149	1.109	P > 0.05	-0.3270 to 0.7568
H2O vs SO 50oC	-0.2883	1.488	P > 0.05	-0.8302 to 0.2536
H2O vs Mix	-0.1696	0.8756	P > 0.05	-0.7115 to 0.3723
H2O vs Mix 50oC	-0.3812	1.968	P > 0.05	-0.9231 to 0.1607

ANOVA Table	SS	df	MS
Treatment (between columns)	11.12	11	1.01
Residual (within columns)	20.26	108	0.1876
Total	31.37	119	

Appendix 3: HA Biofilm Crystal Violet Data 10 Expts and Stats Figure 2a

	H2O	H2O 50oC	Alcohol	Alcohol 50oC	Thymol	Thymol 50oC	Silicone Oil	Silicone Oil 50oC	Mix	Mix 50oC	Chlorhexidine	Chlorhexidine 50oC
Expt 1	0.35	0.27	0.42	0.31	0.57	0.48	0.30	0.26	0.55	0.44	0.48	0.75
Expt 2	0.57	0.56	0.65	0.53	0.65	0.57	0.44	0.57	0.59	0.39	0.49	0.52
Expt 3	0.48	0.37	0.44	0.39	0.43	0.37	0.19	0.10	0.53	0.44	0.44	0.54
Expt 4	0.58	0.47	0.53	0.50	0.48	0.51	0.41	0.45	0.57	0.43	0.46	0.62
Expt 5	0.30	0.38	0.38	0.28	0.34	0.26	0.28	0.23	0.63	0.47	0.53	0.69
Expt 6	0.46	0.36	0.38	0.26	0.30	0.26	0.29	0.26	0.60	0.38	0.57	0.68
Expt 7	0.32	0.23	0.31	0.17	0.25	0.22	0.16	0.20	0.66	0.36	0.50	0.71
Expt 8	0.32	0.21	0.29	0.15	0.22	0.18	0.22	0.17	0.65	0.42	0.50	0.72
Expt 9	0.60	0.59	0.52	0.49	0.53	0.37	0.27	0.19	0.57	0.46	0.49	0.62
Expt 10	0.56	0.49	0.56	0.42	0.58	0.29	0.30	0.18	0.62	0.38	0.51	0.64
Average	0.45	0.39	0.45	0.35	0.44	0.35	0.29	0.26	0.60	0.42	0.50	0.65
STD Error	0.04	0.04	0.04	0.04	0.05	0.04	0.03	0.04	0.01	0.01	0.01	0.02

Table Analyzed
Data 1

One-way analysis of variance	
P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.0	Yes
Number of groups	12
F	10.98
R squared	0.5278
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	41.01
P value	P<0.0001
P value summary	***
Do the variances differ signif. (P < 0.	Yes

Dunnett's Multiple Compa	Mean Diff.	q	P value	95% CI of diff
H2O vs H2O 50oC	0.06026	1.234	P > 0.05	-0.07640 to 0.1969
H2O vs Alcohol	0.006012	0.1231	P > 0.05	-0.1307 to 0.1427
H2O vs Alcohol 50oC	0.1033	2.114	P > 0.05	-0.03339 to 0.2399
H2O vs Thymol	0.01803	0.369	P > 0.05	-0.1186 to 0.1547
H2O vs Thymol 50oC	0.1024	2.096	P > 0.05	-0.03429 to 0.2390
H2O vs Silicone Oil	0.1674	3.427	P < 0.01	0.03071 to 0.3040
H2O vs Silicone Oil 50o(	0.1922	3.936	P < 0.01	0.05557 to 0.3289
H2O vs Mix	-0.1418	2.904	P < 0.05	-0.2785 to -0.005161
H2O vs Mix 50oC	0.03637	0.7447	P > 0.05	-0.1003 to 0.1730
H2O vs Chlorhexidine	-0.04263	0.8727	P > 0.05	-0.1793 to 0.09404
H2O vs Chlorhexidine 5(	-0.196	4.013	P < 0.01	-0.3327 to -0.05936

ANOVA Table	SS	df	MS
Treatment (between columns)	1.44	11	0.1309
Residual (within columns)	1.288	108	0.01193
Total	2.728	119	

Appendix 4: SM Biofilm Crystal Violet Data 10 Expts and Stats Figure 2b

	H2O	H2O 50oC	CHL	CHL 50oC	Alcohol	Alcohol 50oC	Thymol	Thymol 50oC	SO	SO 50oC	Mix	Mix 50oC
Expt 1	4.32	4.95	4.21	4.92	5.12	4.87	5.02	3.72	5.10	5.32	4.92	4.65
Expt 2	4.81	4.85	4.97	4.75	4.82	5.27	5.02	4.47	5.10	5.42	4.71	4.72
Expt 3	4.48	5.00	4.49	4.85	4.88	5.12	4.96	4.63	5.22	5.79	4.63	4.97
Expt 4	4.58	4.61	4.75	4.59	4.68	4.29	4.65	4.13	4.75	4.78	4.67	4.77
Expt 5	1.52	1.33	1.49	1.41	1.53	1.31	1.26	1.43	1.50	1.46	1.26	1.44
Expt 6	1.66	1.49	1.60	1.53	1.65	1.49	1.66	1.61	1.63	1.46	1.61	1.56
Expt 7	1.50	1.37	1.55	1.42	1.68	1.57	1.78	1.55	1.63	1.51	1.81	1.64
Expt 8	1.49	1.36	1.74	1.49	1.74	1.44	1.83	1.56	1.72	1.40	1.58	1.51
Expt 9	5.39	5.37	4.35	4.41	5.07	4.91	4.83	4.87	4.53	5.11	4.36	3.83
Expt 10	4.79	5.31	4.16	4.42	4.84	4.96	4.98	4.97	4.78	5.13	4.33	3.64
Average	3.45	3.56	3.33	3.38	3.60	3.52	3.60	3.29	3.60	3.74	3.39	3.27
STD Error	0.53	0.60	0.48	0.52	0.53	0.57	0.54	0.49	0.54	0.63	0.50	0.49

Data 1

One-way analysis of variance	
P value	1
P value summary	ns
Are means signif. different? (P < 0.05)	No
Number of groups	12
F	0.07475
R squared	0.00756
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	1.295
P value	0.9998
P value summary	ns
Do the variances differ signif. (P < 0.05)	No

Dunnett's Multiple Comparison	Mean Diff.	q	P value	95% CI of diff
H2O vs H2O 50oC	-0.1091	0.1438	P > 0.05	-2.232 to 2.014
H2O vs CHL	0.1226	0.1616	P > 0.05	-2.000 to 2.246
H2O vs CHL 50oC	0.0771	0.1016	P > 0.05	-2.046 to 2.200
H2O vs Alcohol	-0.1454	0.1916	P > 0.05	-2.268 to 1.978
H2O vs Alcohol 50oC	-0.068	0.08962	P > 0.05	-2.191 to 2.055
H2O vs Thymol	-0.1459	0.1923	P > 0.05	-2.269 to 1.977
H2O vs Thymol 50oC	0.1606	0.2117	P > 0.05	-1.962 to 2.284
H2O vs SO	-0.1412	0.1861	P > 0.05	-2.264 to 1.982
H2O vs SO 50oC	-0.2831	0.3731	P > 0.05	-2.406 to 1.840
H2O vs Mix	0.0669	0.08817	P > 0.05	-2.056 to 2.190
H2O vs Mix 50oC	0.1819	0.2397	P > 0.05	-1.941 to 2.305

ANOVA Table	SS	df	MS
Treatment (between columns)	2.367	11	0.2152
Residual (within columns)	310.9	108	2.878
Total	313.2	119	

Appendix 5: Figure 3A - 2 min HA colony counts

	H2O	H2O 50oC	CHL	CHL 50oC	Alcohol	Alcohol 50oC	Thymol	Thymol 50oC	SO	SO 50oC	Mix	Mix 50oC
Expt 1	110000	30000	0	0	18000	500	0	20	120000	80000	0	0
Expt 2	100000	50000	0	0	15000	400	0	80	21000	50000	0	0
Expt 3	40000	80000	0	0	11000	1900	0	70	31000	120000	0	0
Expt 4	40000	40000	0	0	7000	1800	0	0	120000	60000	0	0
Expt 5	65000	70000	0	0	40000	1300	0	40	100000	110000	0	0
Average	71000	54000	0	0	18200	1180	0	42	78400	84000	0	0
STD Err	14697	9274	0	0	5757	315	0	15	21759	13638	0	0

Data 1

One-way analysis of variance

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	12
F	14.97
R squared	0.7743

Bartlett's test for equal variances

Bartlett's statistic (corrected)	
P value	
P value summary	ns
Do the variances differ signif. (P < 0.05	No

ANOVA Table	SS	df	MS
Treatment (between columns)	6.824E+10	11	6E+09
Residual (within columns)	1.989E+10	48	4E+08
Total	8.813E+10	59	

Appendix 5 (continued): Figure 3A 2 min HA colony counts:

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
H2O vs H2O 50oC	17000	1.32	P > 0.05	-19750 to 53750
H2O vs CHL	71000	5.514	P < 0.01	34250 to 107700
H2O vs CHL 50oC	71000	5.514	P < 0.01	34250 to 107700
H2O vs Alcohol	52800	4.101	P < 0.01	16050 to 89550
H2O vs Alcohol 50oC	69820	5.423	P < 0.01	33070 to 106600
H2O vs Thymol	71000	5.514	P < 0.01	34250 to 107700
H2O vs Thymol 50oC	70960	5.511	P < 0.01	34210 to 107700
H2O vs SO	-7400	0.5747	P > 0.05	-44150 to 29350
H2O vs SO 50oC	-13000	1.01	P > 0.05	-49750 to 23750
H2O vs Mix	71000	5.514	P < 0.01	34250 to 107700
H2O vs Mix 50oC	71000	5.514	P < 0.01	34250 to 107700

Appendix 6: SM Colony Counts Final 5 Expts with Stats Figure 3b

Exp/ H2O	H2O 50oC	CHL	CHL 50oC	Alcohol	Alcohol 50oC	Thymol	Thymol 50oC	SO	SO 50oC	Mix	Mix 50oC
1/370 000	110000	60	0	220000	50000	270000	20	330000	150000	0	0
2/530 000	140000	70	0	250000	90000	210000	14	400000	230000	0	0
3/ 110 000	130000	140	0	110000	100000	210000	2	430000	220000	0	0
4/120 000	240000	180	0	240000	80000	310000	2	240000	110000	0	0
5/ 280 000	160000	50	0	170000	85000	260000	65	40000	100000	0	0

Data 1

One-way analysis of variance

P value	P<0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	12
F	11.79
R squared	0.734

ANOVA Table	SS	df	MS
Treatment (between columns)	7.46E+11	11	6.781E+10
Residual (within columns)	2.7E+11	47	5.752E+09
Total	1.02E+12	58	

Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
H2O vs H2O 50oC	126500	2.486	P > 0.05	-18800 to 271800
H2O vs CHL	282400	5.551	P < 0.01	137100 to 427700
H2O vs CHL 50oC	282500	5.553	P < 0.01	137200 to 427800
H2O vs Alcohol	84500	1.661	P > 0.05	-60800 to 229800
H2O vs Alcohol 50oC	201500	3.961	P < 0.01	56200 to 346800
H2O vs Thymol	30500	0.5995	P > 0.05	-114800 to 175800
H2O vs Thymol 50oC	282500	5.552	P < 0.01	137200 to 427800
H2O vs SO	-5500	0.1081	P > 0.05	-150800 to 139800
H2O vs SO 50oC	120500	2.369	P > 0.05	-24800 to 265800
H2O vs Mix	282500	5.553	P < 0.01	137200 to 427800
H2O vs Mix 50oC	282500	5.553	P < 0.01	137200 to 427800

Appendix 7 Antibiotic	Zones of Inhibition (mm) S. mutans 25175			Mean	SE	H. actinomycetemcomitans Y4			Mean	SE
Amoxicillin / Clavulanic Acid	50	45	47	47.3	1.5	42	41	40	41.0	0.6
Azithromycin	34	36	29	33.0	2.1	39	35	34	36.0	1.5
Cefotaxime	46	42	38	42.0	2.3	54	52	54	53.3	0.7
Ceftazidime	38	38	38	38.0	0.0	47	42	43	44.0	1.5
Ciprofloxacin	24	21	22	22.3	0.9	60	45	50	51.7	4.4
Erythromycin	40	38	39	39.0	0.6	22	24	22	22.7	0.7
Gentamicin	30	26	28	28.0	1.2	24	28	27	26.3	1.2
Imipenem	54	50	46	50.0	2.3	32	38	33	34.3	1.9
Kanamycin	20	18	20	19.3	0.7	32	31	31	31.3	0.3
Nitrofurantoin	36	34	37	35.7	0.9	58	48	51	52.3	3.0
Norfloxacin	16	18	17	17.0	0.6	56	49	48	51.0	2.5
Piperacillin	46	44	42	44.0	1.2	48	44	42	44.7	1.8
Sulfamethoxazole	0	0	0	0.0	0.0	28	31	31	30.0	1.0
Tetracycline	30	29	27	28.7	0.9	46	42	44	44.0	1.2
Tobramycin	23	20	24	22.3	1.2	22	36	26	28.0	4.2
Trimethoprim	13	12	18	14.3	1.9	54	48	49	50.3	1.9
Sulfamethoxazole / Trimethoprim	23	28	23	24.7	1.7	46	40	41	42.3	1.9
Vancomycin	29	28	27	28.0	0.6	0	0	0	0.0	0.0

Appendix 8

	Zone of Growth Inhibition (mm)				
S. mutans 25175	Expt 1	Expt 2	Expt 3	Mean	SE
Chlorhexidine (0.12%)	11	10.5	10.5	10.66667	0.166667
Chlrohexidine (0.12%) + Triclosan (0.3%)	11.5	11	12	11.5	0.288675
All other zones are 0mm					

H. actinomycetemcomitans Y4	Expt 1	Expt 2	Expt 3		
Triclosan (0.3%)	21	20	25	22	1.527525
Chlorhexidine (0.12%) + Triclosan (0.3%)	26	24	30	26.66667	1.763834
All other zones are 0mm					

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