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EFFECTS OF LOW-TEMPERATURE ATMOSPHERIC PRESSURE

PLASMA ON *STREPTOCOCCUS MUTANS*

by

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ABSTRACT

EFFECTS OF LOW TEMPERATURE ATMOSPHERIC PRESSURE PLASMA ON *STREPTOCOCCUS MUTANS*

The recent emergence of low temperature atmospheric pressure plasma (LTAPP) technology has led researchers to explore biomedical applications. The present study set out to determine if LTAPP has the ability to inactivate dental caries causing bacteria, specifically *Streptococcus mutans*. **Methods**: A total of 90 samples were processed. Seventy-two samples of *S. mutans* at 1:100 dilution in BHI broth were exposed to LTAPP for various time intervals (60, 120, 180, 300 seconds). Eighteen control samples were plated but not exposed to LTAPP. Samples were plated on Mitis salivarius (MS) agar and incubated 48 hours at 37° C. Colony forming units (CFU) were counted and inactivation factor (IF) were determined. Data were analyzed using repeated measures ANOVA at 0.05α significance. **Results**: Analysis revealed a statistically significant bactericidal effect of LTAPP on *S. mutans* at time exposures of 60, 120, 180, 300 seconds (p=0.272, p=0.0001, p=0.0001, p=0.0001, respectively). **Conclusion**: LTAPP had a statistically significant bactericidal effect on *S. mutans* at all time exposures, as measured by CFU/mL counts and inactivation factors that ranged from 53-95%.

Key words: *Streptococcus mutans*, low-temperature atmospheric pressure plasma, plasma pencil, cold plasma

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CHAPTER I

INTRODUCTION

Found in the mouth, oral biofilm is a complex community of over 500 species of bacteria that are embedded in an intermicrobial matrix of bacterial and salivary origin.[1, 2] Through the metabolism of carbohydrates within these biofilms, the formation of acid by-products occurs as the principle mechanism of caries initiation and progression.[3] *Streptococcus mutans*, the primary cariogenic organism present in plaque biofilm, rapidly decompose carbohydrates and create low pH environments on the tooth.[5, 6] The fundamental process of dental caries involves initial tooth structure demineralization by high organic acid concentrations produced by *S. mutans*.[4]

The current prevention and treatment modalities for dental caries include the use of fluoride and antimicrobial agents in conjunction with mechanical biofilm removal; however, a concern regarding with the use of antimicrobials is increasing.[7] A more advantageous proposal for the management of dental caries may be to destroy the causative agent, *S. mutans*.[8]

Recent studies in the inactivation methods of biological matter using non-thermal plasma technology have emerged. Plasma is a distinct state of matter, separate from solids, liquids and gases. By combining charged particles and free radicals, plasma has the potential to destroy bacterial cells. Atmospheric plasmas produce ozone and short-living reactive species like oxygen O· and hydroxyl ·OH radicals which have powerful antibacterial properties.[9, 10] Low-temperature atmospheric pressure plasmas (LTAPP) can be created in different forms including dielectric barrier discharge,[11-13] resistive discharge[14-16] and atmospheric pressure plasma jets sustained by microwave or radio frequency.[17-18] Researchers have investigated the effects of non-thermal

plasma on various pathogenic bacteria such as *Escherichia coli*, *Geobacillus stearothermophilus*, *Bacillus cereus*, *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Vibrio cholera*, and *Streptococcus sanguis* with varying results.[9, 19, 20]

Statement of the Problem

Given the current knowledge on the bactericidal effects of LTAPP, this investigation attempted to answer the following question:

- What effect does LTAPP exposure have on the inactivation of *Streptococcus mutans*?

Significance of the Problem

The present study explored the effectiveness of LTAPP as a potential method to destroy *S. mutans*, the predominant caries causing microorganisms present in oral biofilm. Determining if LTAPP has a bactericidal effect on *S. mutans* may represent an alternative to conventional preventive interventions that preserves tooth structure.

Definition of Terms

Bacteria - a microorganism that is unicellular, prokaryotic and contains both DNA and RNA. Bacteria that are capable of growth and reproduction, and contain a rigid cell wall.[21]

Colony forming units (CFU) - the measure of viable bacterial numbers. CFU/mL represents one of the dependent variables of the present study.

D-value - decimal reduction time. The time required at a certain temperature to kill 90% of the organisms being studied. After an organism is reduced by 1 D, only 10% of the original organisms remain and the population number has been reduced by one decimal place in the counting scheme. For example, (1/D) means that 90% of the microorganisms from the original concentration were killed and 10% remain viable. [22]

Inactivation – decreased microorganisms as a result of plasma exposure.

Inactivation factor (IF) - percentage of microorganisms that were destroyed due to LTAPP exposure. The experimental CFU/mL is subtracted from the control CFU, divided by the control CFU and multiplied by 100. Compares the proportion of the original count of bacteria to the final amount; the resulting number is an index of the effectiveness of inactivation.

Ionized - "partially ionized," "weakly ionized" and "highly ionized" indicate qualitatively what fraction of the gas atoms are charged.

Ionized Gases - adding energy to a gas to break the internal bonds of some or all of its individual atoms, ionizing those atoms and freeing electrons.

Lactobacillus acidophilus - a gram-positive aerobic rod-shaped bacterium that produces lactic acid. Production of organic acids by lactobacilli causes decalcification of the dentinal matrix once the caries process has been initiated by *S. mutans*.

Low-temperature atmospheric pressure plasma (LTAPP) - non-thermal atmospheric pressure plasma that involves producing electrically charged particles of gas such as helium with the use of a low power source to produce an ionized gas-like substance. This is the independent variable of the present study.

Plasma - a distinct fourth state of matter separate from solid, liquid and gaseous states; and contains several electrically charged particles sufficient enough to affect its electrical properties and behavior. Ions and electrons move independently forming an ionized gas.

Plasma pencil - a hand held device used for precise application of plasma plume generated by helium or other gas and an electrical power source.

Plasma plume - a narrow, 5 cm-long bluish flame of low-temperature plasma gas

Streptococcus mutans - gram-positive, facultative, acidogenic, aciduric, anaerobic bacteria responsible for the initiation of the dental caries process. [21, 23]

Too numerous to count (TNTC) - bacteria remaining on a culture plate which exceeds visual countability of 100 CFU.

Assumption

The following assumptions are made regarding the application of LTAPP to *S. mutans:*

- LTAPP has the potential to be an effective method of inactivating *S. mutans.*

Limitations

Factors that may have threatened the validity of the results are:

- Contamination of the polystyrene cell culture cluster may have occurred if the researchers did not use proper precautions to prevent contamination between the control and the experimental test tubes once the experimental samples were exposed to LTAPP. This threat was minimized by having the control and experimental groups separated following plasma exposure and by using sterilized cell culture plates.
- Accurate CFU readings are necessary for the results to be valid. The CFU represents the number of microorganisms that remain following the inoculation of the bacteria samples onto the agar medium and after 48-hour incubation time. Then, IF calculation requires the ratio of the number of microorganisms before and after the exposure to LTAPP. Therefore, a discrepancy in the CFU count and the IF ratios may occur, threatening the validity of the results. This discrepancy was minimized by having a single experienced biology researcher count CFU. In addition, prior to the study, the researchers conducted a calibration and training session to determine intra-rater reliability for CFU counting to augment validity and reliability.

Hypothesis

The following hypothesis was tested at a .05 α level:

HO: There is no statistically significant difference in the bactericidal effect of the LTAPP exposure on *S. mutans* at 60, 120, 180 and 300 seconds compared to those bacteria not treated, as measured by CFU/mL.

CHAPTER II

REVIEW OF THE LITERATURE

Streptococcus mutans

Bacteria Overview

Bacteria are a group of single-cell microorganisms with a procaryotic cellular design. There is no nuclear membrane to encase genetic material in the cytoplasm of the cells. This feature is the definitive difference between more highly evolved eukaryotic cells present in plant and mammal life.[24]

Bacteria are comprised of protein, lipid, polysaccharide, nucleic acid and peptidoglycan, all which must be continually manufactured for growth and reproduction. Peptidoglycan, a protein-sugar polysaccharide, forms the rigid cell wall of bacteria. The main function of peptidoglycan is to regulate internal cell pressure, maintain bacterial shape and anchor proteins.[25]

Bacteria reproduce by binary fission, whereby the parent cell divides to form a duplicate genetic offspring. A logarithmic growth rate tracks the amount of time a bacterium can continue to replicate itself. Using a nutrient medium in the laboratory, a growing bacterial population doubles at regular intervals. Exponential growth is a growth by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3...........2^n (n = the number of generations). When a fresh medium is inoculated with a given number of bacterial cells, and the growth is monitored over a period of time, plotting the data will yield a typical bacterial growth curve (Fig 1).[21, 24]

There are four phases of bacterial growth: lag phase, log phase, stationary phase and the death phase.[26] In the lag phase, bacteria are inoculated into fresh medium and the population remains unchanged. Although bacteria do not divide immediately, they undergo vigorous metabolic activity. Rapid cell division occurs during the log phase. The number of new bacteria

emerging per unit of time is proportional to the current population. In the stationary phase, growth rate slows as a result of exhaustion of available nutrients, accumulation of by-products or exhaustion of "biological space." In this phase, the bacteria have met their maximum cell yield. It is during the stationary phase that spore-forming bacteria have to induce the activity of the genetic material that may be involved in the sporulation process. During the death phase, nutrients are depleted and bacteria die.

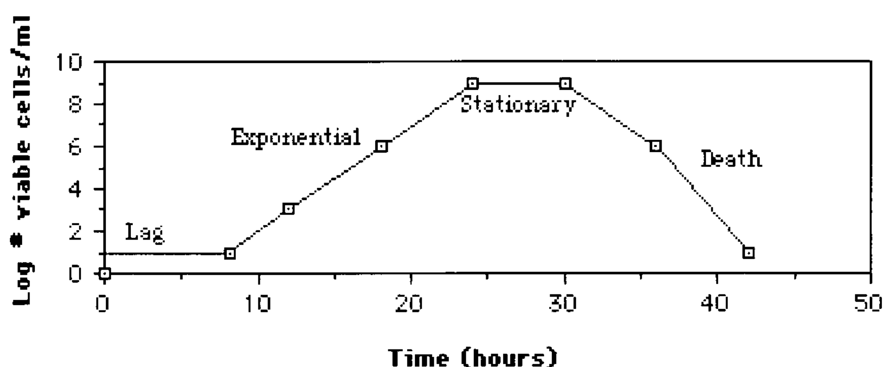

Figure 1. Typical bacterial growth curve[16]

Bacterial Metabolism

Respiration of bacteria includes essential metabolic processes within the cell to convert energy from nutrients into adenosine triphosphate (ATP) and secrete waste products. In aerobic cellular respiration, the pyruvate produced in the second step of glycolysis moves into the cell where it is then oxidized to Acetyl-CoA. The Acetyl-CoA combines with oxaloacetate in order to enter the citric acid cycle. The products of the citric acid cycle are four NADH molecules, one FADH molecule and one guanine triphosphate (GTP), which is the energy equivalent of ATP.

The NADH and FADH molecules function to donate electrons to the electron transport chain, with oxygen as the final electron acceptor.[28]

Anaerobic organisms use other inorganic compounds as final electron acceptors. The energy released from the movement of electrons down the electron transport chain is used to pump hydrogen ions across the cytoplasmic membrane. The hydrogen ions then flow back across the membrane through ATP synthase, the protein responsible for converting the energy to molecules of ATP. Facultative organisms produce ATP using aerobic respiration if oxygen is present; however, these organisms are also capable of performing fermentation.[28]

Fermentation is the process of acquiring energy from oxidizing organic compounds, such as carbohydrates. Fermentation, however, does not require an anaerobic environment.[27] Glucose is the most common sugar that enzymes react with in the fermentation process. Glucose is converted to glucose-6-P by the enzyme hexokinase using the energy from 1 ATP. The glucose-6-P is then converted to fructose-6-P by the enzyme isomerase. Another ATP is broken down to fuel the conversion of fructose-6-P to P-fructose-1,6-P by the enzyme phosphofructokinase. Aldoase then converts the P-fructose-1,6-P to two glyceraldehyde-3-phophates (G-3-P). The two G-3-Ps are converted to two pyruvate molecules. In the process, electrons are donated to NAD^+, converting it to NADH. Four molecules of ATP are also produced in this step through substrate-level phosphorylation. The net gain of the first two steps of glycolysis is two molecules of pyruvate, two molecules of ATP and two molecules of NADH. [28]

Streptococcus mutans

S. mutans are facultative, anaerobic groups of streptococci bacteria (Fig. 2). Cells are spherical, stain Gram-positive and appear in pairs or chains. Nutritionally rich media is required for optimum growth. Mitis salivarius is the media of choice for *S. mutans* isolation and

identification by differentiating characteristic colonial morphology from other oral streptococci. Temperature for streptococcal growth is limited to 25-45° C. with optimum growth of 37° C. [29] Streptococci form the majority of bacteria that thrive in the oral cavity. Once colonies form, *S. mutans* can be detected in saliva, on the tongue and oral mucous membranes.[30]

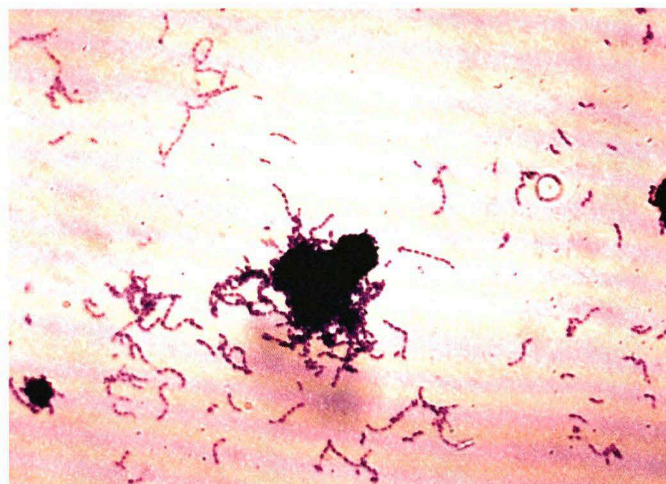

Figure 2. Gram stain of *S. mutans* [26]

Caries

S. mutans are the dominant organisms responsible for initiating dental caries. *S. mutans* metabolize fermentable carbohydrates to form lactic acid and maintain a low pH necessary for demineralization of enamel more rapidly than any other plaque biofilm bacteria.[31] Sucrose, in particular, is the primary factor in the pathogenicity of *S. mutans*. In the presence of this fermentable carbohydrate, *S. mutans* adheres to, colonizes, and accumulates on tooth surfaces, thereby forming adhesive microbial deposits. The bacterium also coheres into macroscopically visible biofilm masses in the presence of sucrose and extracellular sucrose by-products, mainly

glucans. The ability of *S. mutans* to synthesize glucans from sucrose allows *S. mutans* to adhere and cohere to tooth surfaces. [32]

Caries development in a susceptible tooth involves the tooth surface in a carrier state (Fig 3). An incipient lesion occurs when fermentable carbohydrates such as sucrose, glucose or fructose are ingested. Acids produced during the metabolic process includes lactic acid. Secondary cariogens, such as the lactobacilli, emerge as a result of the increased aciduric environment. Together, these two organisms succeed as the predominant flora in the development and progression of a carious lesion.[21]

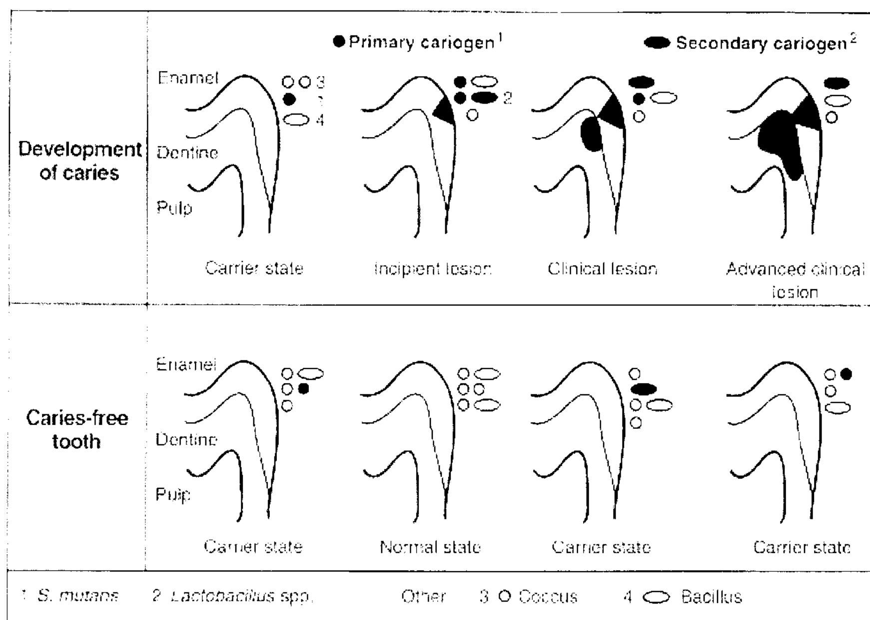

Figure 3. Relationship between bacteria and dental caries development.[30]

If demineralization continues, the disease process continues to an irreversible stage of a clinical lesion. Tissue morbidity occurs, tooth surface dissolves and an expanding void develops within the tooth structure. This expanding lesion is referred to as an advanced clinical lesion.[24]

[30, 31] In a caries-free tooth, *S. mutans* is confined to saliva. Caries-free surfaces may acquire and lose *S. mutans* over time, thus fluxing between carrier state and normal state.[24, 30, 31, 33]

Epidemiologically, the prevalence of *S. mutans* is evident in nearly all populations world-wide.[34] Dental caries remains one of the most common chronic, infectious, transmittable, destructive yet preventable diseases known to humankind. Dental caries is the most common chronic disease of children aged 5 to 17 years and is 5 times more common than childhood asthma. Many adults have untreated dental caries, of those 27% between the ages of 35 to 44 years old and 30% of those 65 years and older.[35]

Saliva

Saliva is a complex oral fluid which consists of approximately 99% water, electrolytes (calcium, chloride, magnesium, bicarbonate, phosphate) and proteins (enzymes, antimicrobial factors, mucosal glycoproteins and other trace components).[32] Saliva buffers the acids produced by bacteria thus preventing enamel demineralization and maintains the physical and chemical integrity of enamel by fluctuating between demineralization and remineralization.[36] High concentrations of calcium and phosphate in saliva assure ionic exchange targeted toward tooth surfaces. Remineralization of a carious tooth before the clinical lesion stage occurs is possible, mainly due to the availability of calcium and phosphate ions in saliva.[37]

Plasma

Plasma is a substance often referred to as the fourth state of matter where electrical charges flow freely among atoms or groups of atoms. Plasma is a distinct state of matter separate from solid, liquid and gaseous states; and contains several electrically charged particles sufficient enough to affect its electrical properties and behavior (Fig 4). Lightening is the most

common form of natural plasma on Earth. The sun (a superheated plasma), neon lights, fluorescent lights and plasma television sets are other familiar forms of plasma.[38]

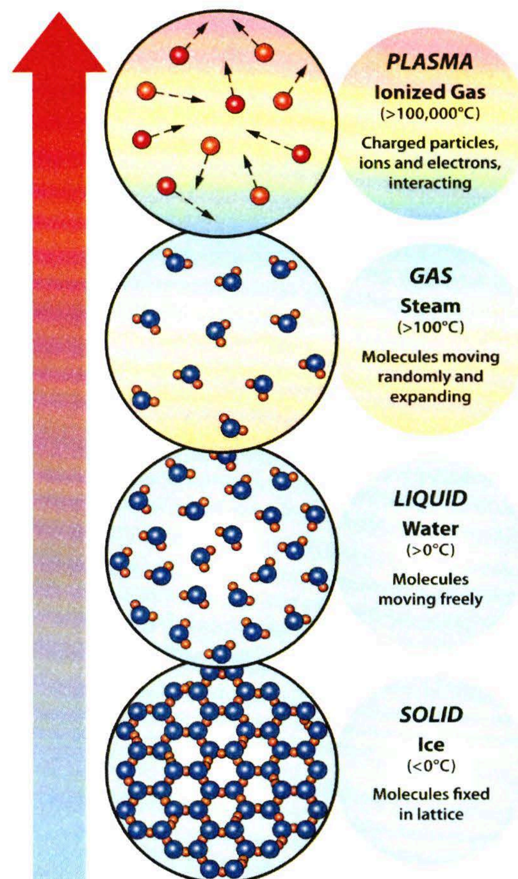

Figure 4. The four states of matter
(Diagram courtesy of G. McCombs and D. Emminger, Old Dominion University)

Gases, another form of matter, contain atoms with an equal number of positive and negative charges. Positively charged protons in the nucleus are surrounded by an equal number of negatively charged electrons, making each atom electrically neutral. Adding energy to the gas causes atoms to release electrons and the gas becomes plasma. The separated negative electrons

move freely resulting in an ionized electrically charged gas. When enough atoms are ionized to significantly affect the electrical characteristics of the gas, plasma emerges.[39, 40]

There are two categories of plasmas: thermal and non-thermal. Thermal plasmas are obtained at high pressure and require substantial power such as a plasma torch. Non-thermal plasmas are obtained at lower pressures and use less power such as electric discharges. A subcategory of non-thermal plasmas are formed at atmospheric pressure and ambient temperature such as low-temperature atmospheric pressure plasma, and are of particular interest in biomedical applications.[41]

Low-Temperature Atmospheric Pressure Plasma (LTAPP)

LTAPP is artificially generated plasma that applies an electric field and is generated in a laboratory setting for industrial use. It can be categorized by type of power used, ionization type, temperature and applied use.[38] LTAPP can be generated using methods such as dielectric barrier discharge,[42] resistive barrier discharge[43] and atmospheric pressure plasma jet.[44] These plasma devices generate large volumes of low-temperature plasma at atmospheric pressure and may use various gas mixtures.

Extensive studies in the inactivation methods of biological matter using non-thermal plasma technology have emerged. Combining charged particles and free radicals, LTAPP has the potential to destroy bacterial cells. Available literature contains studies on the effectiveness of LTAPP to destroy certain microbes such as *E. coli,* [45] *G. stearothermophilus* and *B. cereus.* [9, 10, 17, 41, 45-64]

LTAPP Pencil

The LTAPP "pencil" developed by Laroussi [24] uses a low power source and helium as a main component of the operating gas (Fig. 5). Resembling a miniature light saber, the plasma

pencil is approximately 10 cm in length, 2.5cm in diameter and is comprised of two copper-ringed disk electrodes with a space of 5mm between them. Sub-microsecond square high voltage pulses are applied between the two electrodes while a gas or gas mixture is piped through holes in the electrodes.

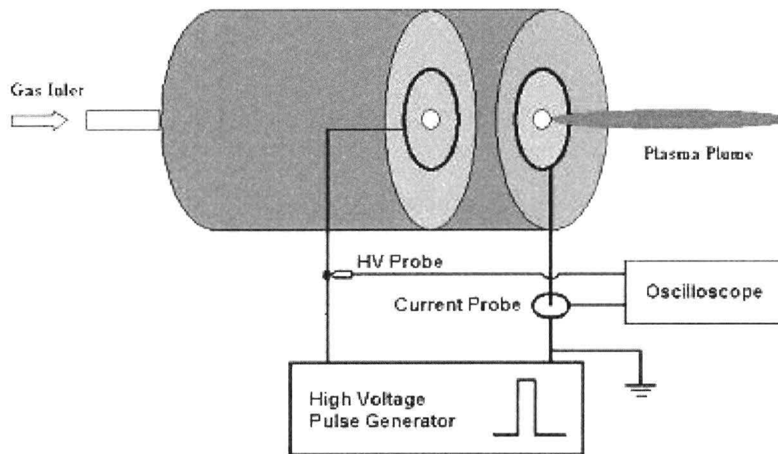

Figure 5. Schematic example of the plasma pencil [43]
(Diagram courtesy of M. Laroussi, Old Dominion University)

The plasma plume from the pencil can reach a length of up to 5 cm through a hole in the outer electrode when the discharge is ignited between the two electrodes. Plume length is controlled by the gas flow rate and by magnitude of applied voltage pulses and frequency. The plasma is ignited when 1000 nanoseconds-wide high voltage pulses at 9 kV repetition rate are applied between the two electrodes and gas is flowing through the holes of the electrodes.

A removable plastic "pipe" or tubing, which measures approximately 4 mm in length, can be added to the base of the pencil to provide a more focused plume. The gas mixture content can also be adjusted allowing the LTAPP plume to remain stable for several hours.[56]

Generated by the plasma pencil, the plume, or cold flame, is bluish in color and poses no immediate threat to live human tissue (Fig. 6). Researchers speculate that LTAPP destroys bacteria, not living epithelial tissue, due to plasma interference with the integrity of the bacterial cell wall. Further research is necessary to explore this complex issue.

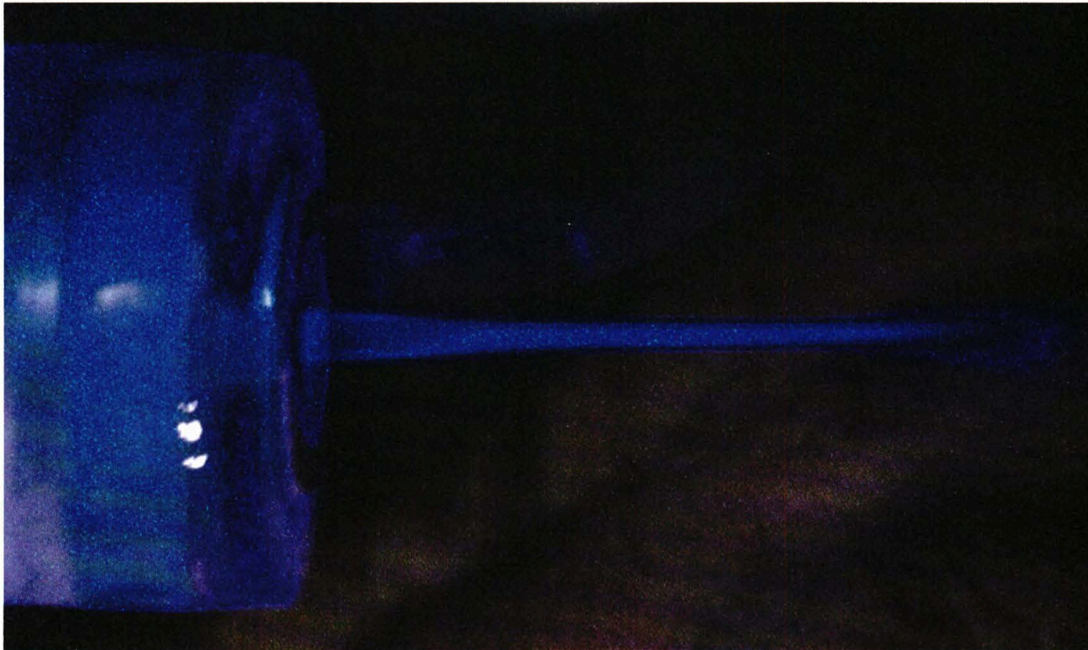

Figure 6. Plasma plume applied to human skin [56]
(Photo courtesy of M. Laroussi, Old Dominion University)

Effect of Low Temperature Atmospheric Pressure Plasma on *S. mutans*

Goree and colleagues tested LTAPP to destroy *S. mutans* using a low-power atmospheric pressure glow discharge millimeter size plasma needle.[51] Low power, low-pressure plasma was applied to agar nutrient in a Petri dish containing *S. mutans*. Over several trials, *S. mutans* were destroyed within a 5-mm diameter site on agar with a 10-second exposure time. This finding led researchers to suggest that low-power atmospheric pressure plasma may offer site-specific dental applications for destroying *S. mutans in vivo.*

Sladek et al., investigated the antimicrobial effects of a non-thermal plasma radio frequency driven plasma needle on *S. mutans* biofilm for 60 seconds. Samples were inoculated in either BHI (Brain Heart Infusion) or BHI with a 0.15% sucrose solution and anaerobically incubated.[57] A 0.2% chlorhexidine diglucate rinse (a known antimicrobial agent) was used on the samples and compared with plasma treatment of the *S. mutans* biofilm samples. Although researchers did not specify the level of bacterial reduction, results indicated no regrowth of *S. mutans* biofilm without sucrose after plasma treatment; the sucrose treated *S. mutans* biofilm was reduced, but destroyed. Researchers concluded that sucrose might be a factor in oral biofilm survival after non-thermal atmospheric plasma exposure. Therefore, the nutrient source must also be considered when plasma testing is conducted.

Effect of Low Temperature Atmospheric Pressure Plasma on Other Microbes

The survival of bacteria exposed to LTAPP is directly related to the type of bacteria, growth medium and plasma involved.[9] Gram-negative bacteria, such as *E. coli*, have an outer lipopolysaccharide layer, which is easily penetrated and destroyed during LTAPP exposure. However, gram-positive bacterial spores state have a much thicker, protective rigid layer. Although plasma failed to compromise metabolic function of the spores, biochemical changes, such as enzyme functions, were apparent.[10, 20] Researchers noted that due to the complexity of cellular chemistry, long term effects and permanent inactivation require further investigation.[10, 20]

Morris et al., exposed *G. stearothermophilus* and *B. cereus* vegetative cells and spores to cold plasma using indirect and direct plasma. Bacteria samples were exposed to either direct or indirect cold plasma for various times and incubated for 12 to 16 hours. CFU were counted; percent kill and log concentration reductions were calculated. Results indicated that there was a statistically significant difference in the inactivation of *G. stearothermophilus* and *B. cereus*

vegetative cells, as well as *B. cereus* spores receiving indirect and direct exposure to cold plasma. There was no statistically significant difference in inactivation of *G. stearothermophilus* spores exposed to direct and indirect cold plasma. Results were favorable for inactivating *G. stearothermophilus* and *B. cereus* vegetative cells, as well as *B. cereus* spores; however, *G. stearothermophilus* spores showed no conclusive inactivation.[10]

Fridman and colleagues used direct exposure of non-thermal atmospheric pressure plasma on staphylococcus, streptococcus and candida species taken from human cadaver skin samples and showed marked inactivation as compared to indirect exposure.[65] A microplasma reactor demonstrated to be effective in inactivating *B. subtilis* spores, *B. sterothermophilus* spores and *Chromobacteruim violacteum*-based biofilms;[66] however, this plasma delivery system uses high pressure and is not as economical or as user-friendly as LTAPP.

In relation to bio-weaponry and bio-terrorism, several pathogens pose significant threats to public health. Spore-forming bacteria such as anthrax, vegetative bacteria such as *E. coli*, and viruses such as smallpox may be inactivated with LTAPP. *Bacillus anthracis* is highly resilient to extreme temperatures, low nutrient environments and chemical sterilizing agents; however, *B. globigii* is relatively close in composition to *B. anthracis* and safe for testing. *B. globigii* was inactivated using an atmospheric pressure plasma jet[30] concluding that LTAPP could successfully inactivate anthrax.

Kelly-Wintenberg and colleagues[52] used one atmosphere uniform glow discharge plasma chamber (OAUGDPC) to reduce log concentration numbers of *Staphylococcus aureus* and *E. coli* on seeded solid surfaces, fabrics, filter paper, and powdered culture media. *S. aureus* was grown on tryptic soy agar and *E. coli* was grown on Luria-Bertani agar. CFUs were counted and a dose-response kill curve was prepared. The results indicated that no colonies of *S. aureus* were

detected after a 30-second exposure in the OAUGDP chamber and no colonies of *E. coli* were detected after 15-second exposure of OAUGDP, however, 90% of *E. coli* colonies were inactivated after a 5-second exposure. Similar testing was conducted with packaged and unpackaged polypropylene, a common material used in the medical industry, with similar results.

Researchers testing nitric oxide plasma in dentistry have investigated its effects on surgical sites after periodontal surgery. Results indicated that nitric oxide plasma exposure decreased secondary post-operative infection and accelerated periodontal healing in 46% of the test subjects.[67] Additionally, medical researchers are investigating ways to utilize plasma applications in medicine which include tissue sterilization, skin regeneration, blood coagulation, wound healing and melanoma treatment. Preliminary results suggest that plasma has the potential for treating certain medical and dental conditions such as dental caries and periodontal disease.[49, 68-70]

In summary, LTAPP has the potential to inactivate many opportunistic pathogens, such as *G. stearothermophilus, B. cereus, E. coli, B. subtilis,* as well as streptococcus and candida species. The site-specific bacterial destruction characteristics of the LTAPP pencil are specifically attractive to dentistry given the requirements needed for the target delivery of a treatment in a relatively small area. For targeted delivery, researchers envision a plasma device designed with portability and low cost, which has potential to transform prevention and treatment for caries and periodontal diseases. Given that dental caries and periodontal disease continues to affect all populations, future LTAPP research is promising.[71] Moreover; LTAPP holds promise for use as a surface decontaminant and sterilization in both medicine and dentistry.

CHAPTER III

METHODS AND MATERIALS

Research Design

Samples of *S. mutans* in sterile 24-well polystyrene cell culture clusters were exposed to non-thermal atmospheric pressure plasma for various time intervals (60, 120, 180, 300 seconds). A total of 90 samples were used. The experimental group consisted of 72 *S. mutans* samples exposed to LTAPP for various times (60, 120, 180, 300 seconds) and then inoculated onto MS agar. The control group contained 18 *S. mutans* samples not exposed.

The Department of Biological Sciences was utilized to conduct all microbiology procedures. LTAPP exposure occurred at the Laser and Plasma Engineering Institute Department of Electrical and Computer Engineering, both at Old Dominion University.

Table 1. Research Design

Group	LTAPP Exposure Times in seconds (Independent Variables)	Dilution	Exposure Sessions	N=90 Samples	*S. mutans* (Dependent Variable)
Treatment	**60, 120, 180, 300**	10^{-2}	**5**	**n=72**	***S. mutans*** **as measured by CFU/mL**
Control	**0**	10^{-2}	**5**	**n=18**	***S. mutans*** **as measured by CFU/mL**

Procedures and Materials

Pilot Testing

The LTAPP pencil is a new technological device; therefore, extensive pilot testing was conducted to determine specific laboratory procedure combinations resulting in successful *S. mutans* inactivation. Three strains of *S. mutans* (K2, G3, and G33) were tested to determine which strain responded to LTAPP exposure.

Bacteria were cultured in BHI broth overnight. Glass slides were individually wrapped in aluminum foil, secured with temperature sensitive tape and autoclaved for 30 minutes at 121^{0} C. at 15 psi. Dilutions were prepared with BHI broth at 1:10, 1:100 and 1:1000 of the overnight culture for each strain (K2, G3, and G33). A small circle measuring approximately 10 mm was drawn on the underside of the slide to assist with placement of the sample. Each slide was then inoculated with 20 μL of the corresponding strain/dilution and placed in a fume hood for 30 minutes to dry. Slides were loosely rewrapped in aluminum foil, placed in a disinfected sealable plastic container and transported to the plasma lab for LTAPP exposure.

Sterile test tubes were labeled with bacterial strain; dilution and LTAPP exposure times (60, 120, 180, 300 seconds) to correspond with each inoculated and treated slide, as well as all control samples. Using a sterile pipette, each slide was rinsed with 1 mL of BHI broth into the corresponding test tube, vortexed, plated on BHI and Todd Hewitt (TH) agar using the Autoplate 4000, a microprocessor-controlled spiral plater used for bacterial enumeration.[72] The automated spiral plater uses a dispensing stylus, which deposits a regulated sample volume onto a rotating agar plate. The stylus moves from the center of the agar outward while the plate rotates (Fig. 20). Numbers of viable bacteria were determined by sector plate counts according to standard spiral-plating methodology by the manufacturer (Fig. 21).

Biology Protocol Revisions

Several tests were conducted using the initial protocol. Due to contamination issues, adjustments were made to the procedures. First, we eliminated placing the glass slides inoculated with samples in the fume hood, but contamination continued. Next, glass slides were replaced with autoclavable acrylic 24-well trays, with each well measuring approximately 10 mm in diameter and 2 mm in depth. Acrylic well trays were wrapped in aluminum foil, secured with temperature sensitive tape and autoclaved for 30 minutes at 121^{0} C at 15 psi. Bacterial dilutions from overnight cultures were prepared in BHI broth at 1:10, 1:100 and 1:1000 for each strain. Twenty µL of each strain of bacteria and each dilution were pipetted into separate wells of an acrylic tray. Samples were exposed to LTAPP for 60, 120, 180 or 300 seconds.

After exposure to LTAPP; trays were rewrapped, placed in a disinfected sealable plastic container and transported back to the biology laboratory. Sterile test tubes were labeled with bacterial strain, dilution and LTAPP exposure time to correspond with each inoculated and treated well, as well as all control samples.

Using a sterile pipette, 1 mL BHI broth was placed in the wells to reconstitute the treated samples. The samples were then removed with a pipette and placed into a sterile test tube, vortexed, plated on BHI agar and incubated inverted for 48 hours at. Several tests were conducted using this protocol and results continued to exhibit excessive contamination and with unknown bacteria and TNTC *S. mutans*.

In an effort to further reduce contamination and TNTC, BHI agar was changed. The same protocol was followed substituting Mitis salivarius (MS), a selective media agar, for BHI and TH agar. To eliminate bacterial contamination, potassium tellurite was added to the agar to inhibit Gram-negative bacilli and non-streptococci Gram-positive bacteria. These modifications greatly

reduced contamination; however, overgrowth of *S. mutans* remained apparent and results were inconclusive. *S. mutans* strain G3 1:100 appeared marginally sensitive to LTAPP time exposures; therefore, K2 and G33 strains, as well as 1:10 and 1:1000 dilutions were eliminated from further testing protocols (See Appendices B, C and D).

In further effort to validate laboratory procedures, another *S. mutans* strain, ATTC #27351, was added. In addition, another LTAPP time exposure of 120 seconds was added to the time exposure protocol. These additional results demonstrated less contamination and colonies that were TNTC, yet more refined adjustments continued to be made to reflect a more consistent progressive bacterial reduction.

To further reduce contamination and TNTC, reusable autoclavable acrylic wells were replaced with disposable sterile 24-well polystyrene cell culture clusters. Additional testing of G3 and #27351 strains indicated a more consistent bacterial reduction; however, *S. mutans* strain #27351 was more constant than G3, thus G3 was eliminated from further testing (See Appendices B, C, D, E).

Plasma Laboratory Protocol Revisions

In an effort to reduce TNTC after biology laboratory modifications were made, plasma laboratory protocol was modified. During pilot testing, *S. mutans* samples were previously dried at least 30 minutes prior to LTAPP exposure. Subsequently, a plastic "pipe" or tubing was attached to the plasma pencil to allow plume to insure accurate contact with wet samples, thus we were able to compare results between wet and dry exposed samples. Wet samples demonstrated a more graduated decrease in countable bacteria.

Pilot Testing Results

It was determined final testing would include using *S. mutans* strain #27351 cultured overnight in BHI broth then diluted 1:100. Wet samples were placed within the disposable sterile 24-well polystyrene cell culture clusters and would be exposed to LTAPP for 60, 120, 180 and 300-second increments. Samples were plated on MS agar using a spiral plater, incubated for 48 hours; colonies counted and recorded. Data from plates with significant test results, which yielded no contamination or TNTC were documented (See Appendices B, C, D).

Final Testing

Sample Description

A total of 90 samples were processed. Seventy-two samples of *S. mutans* at 1:100 dilution in BHI broth were exposed to LTAPP for various times (60, 120, 180, 300 seconds). Eighteen control samples were plated but not exposed to LTAPP. Samples were plated on MS agar and incubated 48 hours at 37^{0} C. CFU and IF were determined. Each sample consisted of *S. mutans* 27351 (N1063-74) acquired from American Type Culture Collection (ATCC).[73]

Experimental and control samples of *S. mutans* were pipetted into sterile wells and exposed to LTAPP for various time intervals (60, 120, 180, 300 seconds). Adding 1 mL of BHI broth to each exposed sample yielded 4 additional samples which were plated on MS agar using the Autoplate 4000 spiral plater. Plates were inverted and incubated for 48 hours at 37° C. Bacterial colonies were counted and recorded.

Pre-Plasma Exposure Laboratory Procedures

- Wash hands thoroughly with antimicrobial soap
- Spray all working surfaces with 5.25% sodium hypochlorite and allowed contact for 10 minutes then wiped dry

- Subculture *S. mutans* (Fig. 7)
- Inoculate *S. mutans* into 5.0 ml of BHI broth
- Flame a bacterial loop
- Swipe the loop across a T-streak of the microorganisms
- Dispense the inoculated loop into the broth

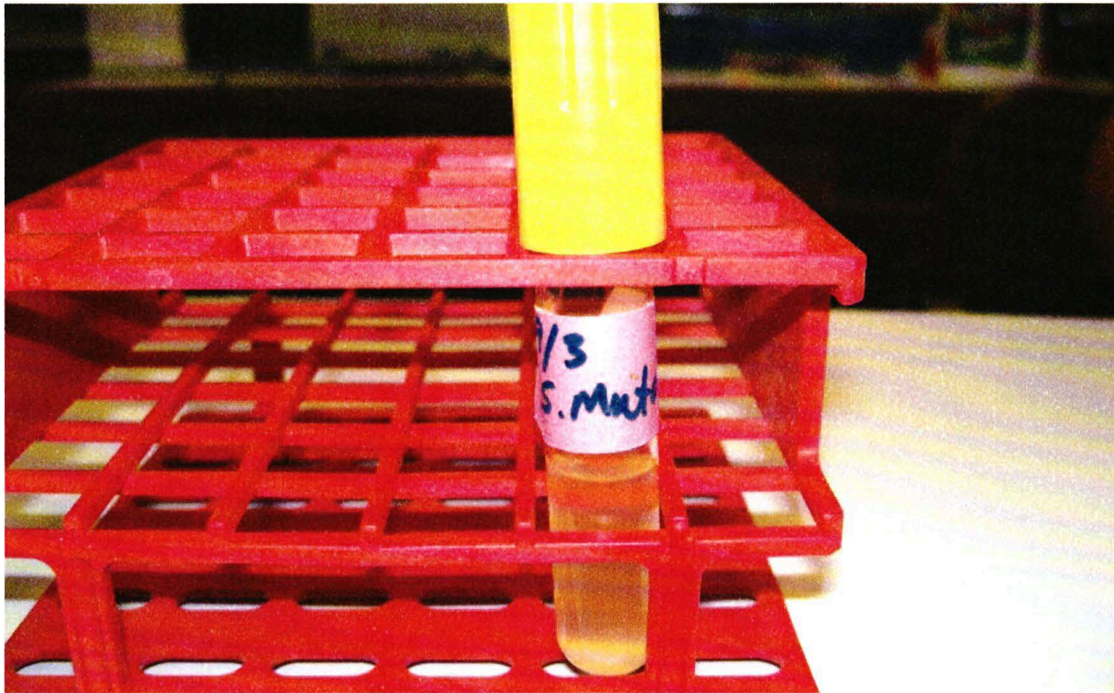

Figure 7. Subculture of *S. mutans*

- Label and place inoculated broth in CO_2 incubator 24 hours
- Open sterile 24-well polystyrene cell culture cluster (Fig. 8)

Polystyrene cell culture cluster characteristics:

Well circumference is 9.4 mm

Well depth is 7.2 mm

Maximum sample size is 1.0 ml

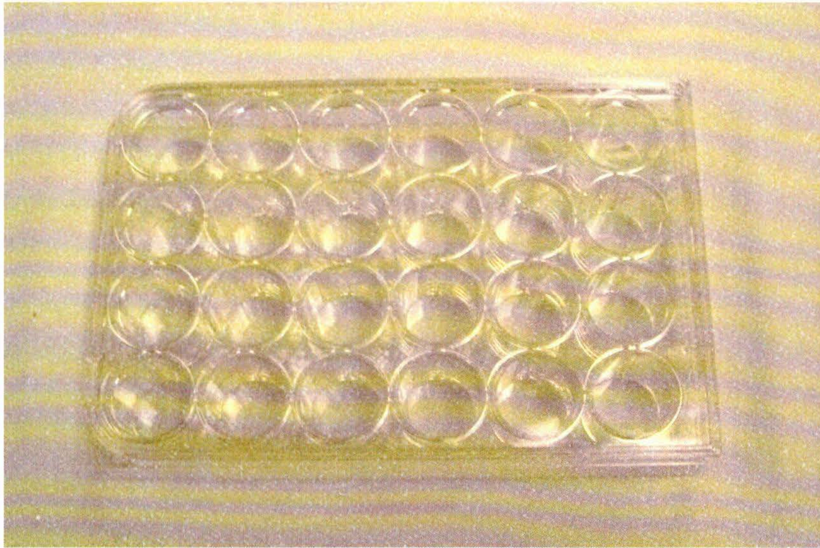

Figure 8. Sterile polystyrene cell culture cluster

- Prepare MS agar plates (Fig. 9)
- Label sample: date of plating, *S. mutans* strain, dilution and time exposure

Figure 9. Mitis salivarius media used in culturing *S. mutans*

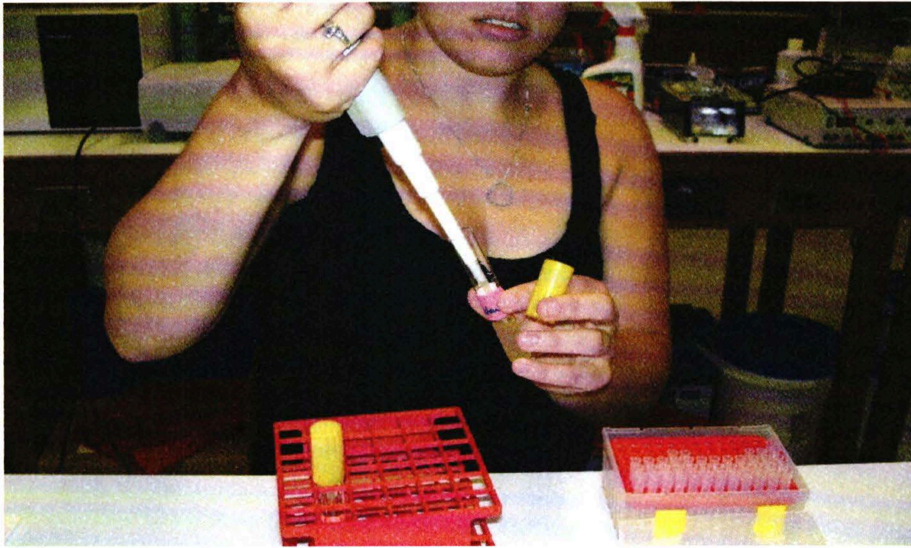

Figure 10. Pipetting 0.1 mL *S. mutans* subculture

- Prepare 1:100 dilution using 9.9 mL BHI and 0.1 mL of *S. mutans* subculture (Fig. 10)
- Vortex *S. mutans* dilution 5 seconds
- Label the appropriate time exposure on the outside and bottom of each polystyrene cell culture cluster (Fig.11)

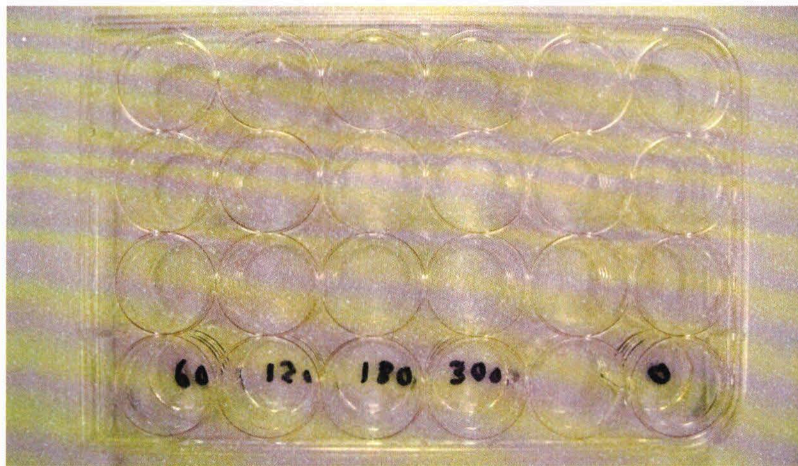

Figure 11. Polystyrene cell culture cluster labeled with time exposures.

- Open sterile polystyrene cell culture cluster
- Pipette 20 μL of dilution into 5 of the wells
- Replace lid on polystyrene cell culture cluster
- Place polystyrene cell culture cluster into disinfected sealed plastic container
- Transport to plasma lab in sealable disinfected plastic container

Low-Temperature Atmospheric Pressure Plasma Exposure Procedures.

- Prepare plasma pencil equipment (Fig. 12)
- Test plasma pencil before treatment (Fig. 13)
- Adjust gas flow
- Set direct current to 9kV and He 6.6L/minute gas flow
- Adjust and measure pencil/ plume length from sample

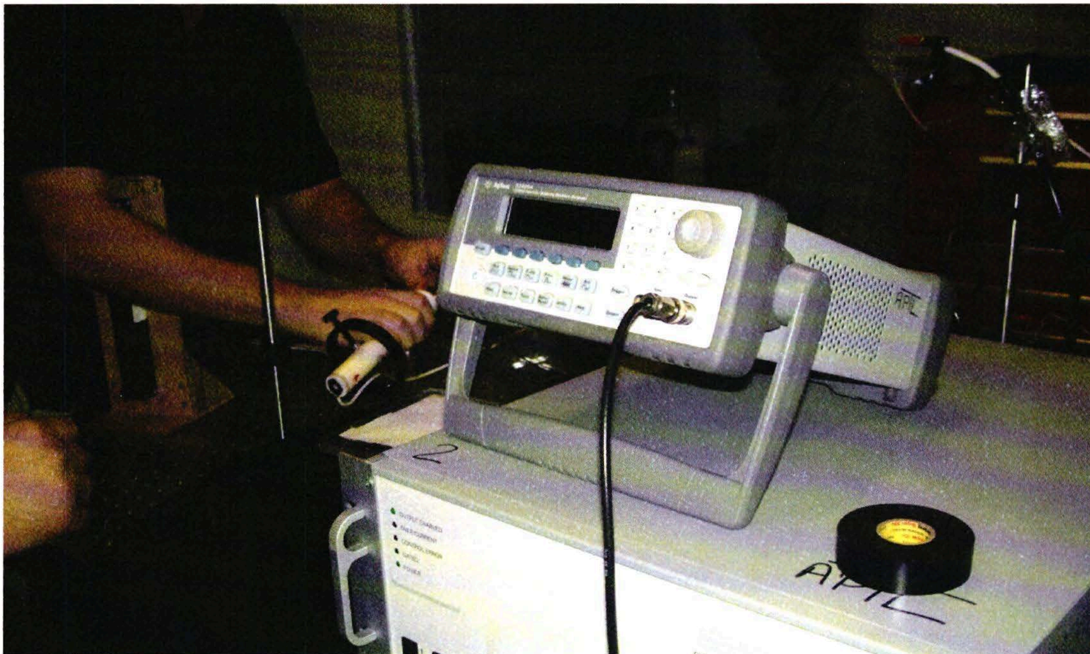

Figure 12. Plasma pencil set-up for treatment in laboratory

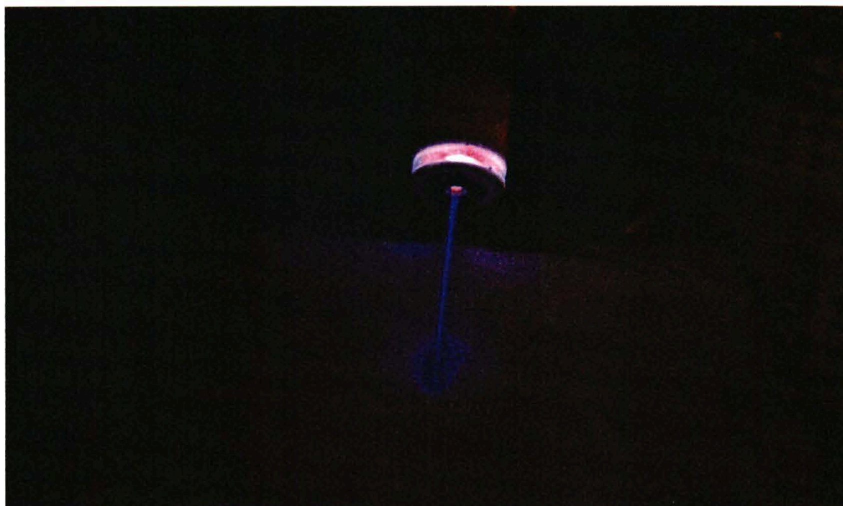

Figure 13. Testing LTAPP pencil prior to treatment of samples

Figure 14. Adjusting polystyrene cell culture cluster for treatment

- Adjust polystyrene cell culture cluster for LTAPP exposure (Fig. 14, 15)
- Carefully place plume on sample to prevent sparking if plume touches sides of acrylic well

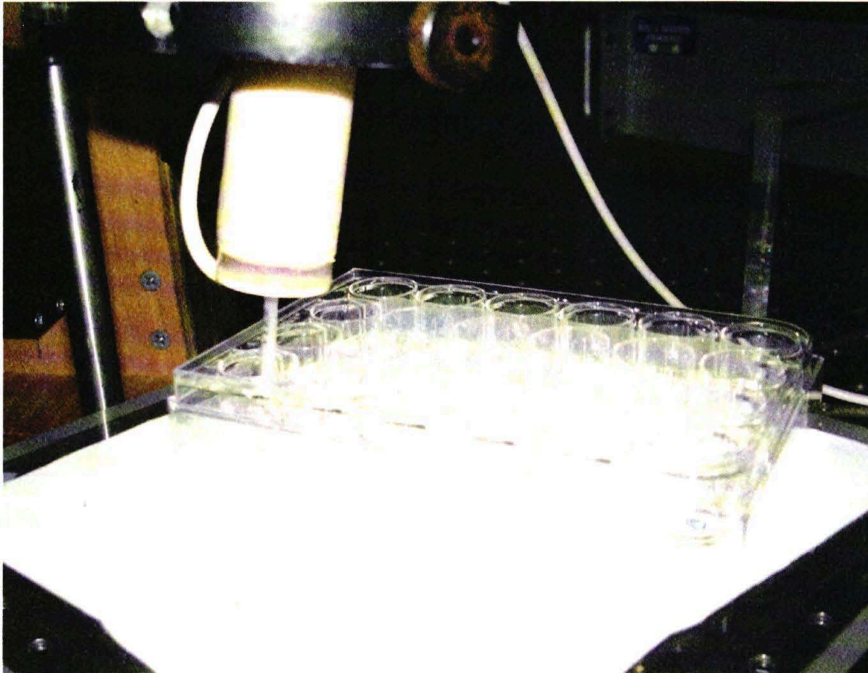

Figure 15. Polystyrene cell culture cluster with samples ready for treatment

- Plasma pencil characteristics:

    Circumference is 2.5 cm

    Distance from top of plume to sample is 4 cm

    Plume measures 5 mm circumference at base of pen and tapers to 2.5 mm

    Plume length measures 35.9 mm

    Plastic "pipe" or tubing measures 4.1 mm from base of plume to sample

    Helium gas flow 4.4 liters per minute at 6 kvolts

    Plume angles 80 to 90 degree angle to sample

- Expose samples to plasma (Fig.16)
- Place the lid, return entire polystyrene cell culture cluster to plastic container
- Seal container to transport back to biology lab

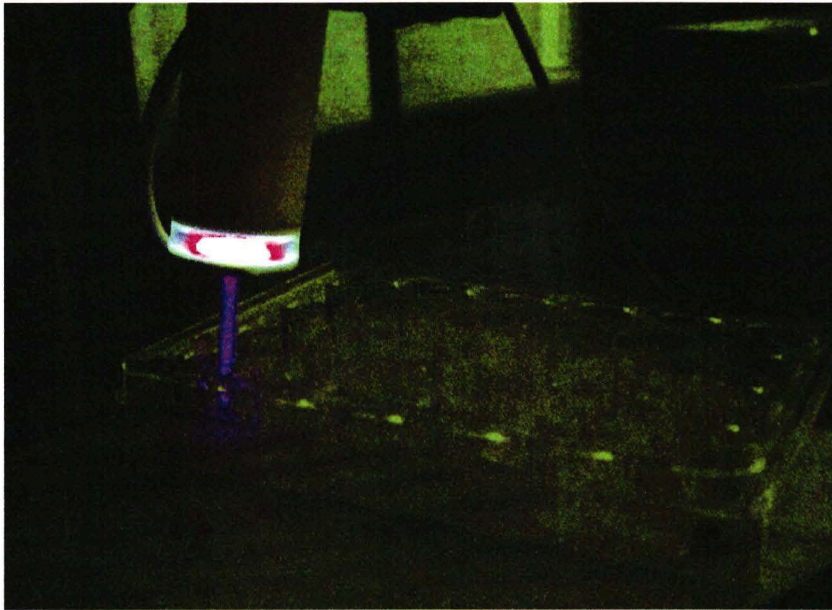

Figure 16. Exposing sample to plasma pencil

Post-Plasma Exposure Laboratory Procedures.

- Open polystyrene cell culture cluster and added 1 mL of BHI to each treated sample
- Aspirate the samples from each of the five wells and place in the corresponding sterile test tubes marked with bacteria, dilution and time exposure (Fig. 17)

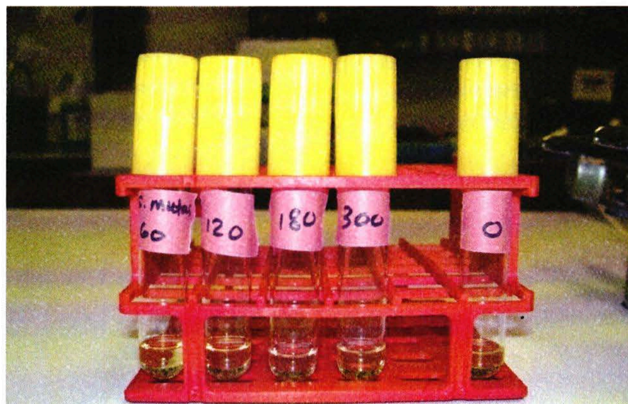

Figure 17. Pipetted samples

- Vortex each test tube 10 to 15 seconds
- Engage Autoplater 4000, inoculating 3 or 4 MS plates per time exposure (Fig. 18, 19, 20)

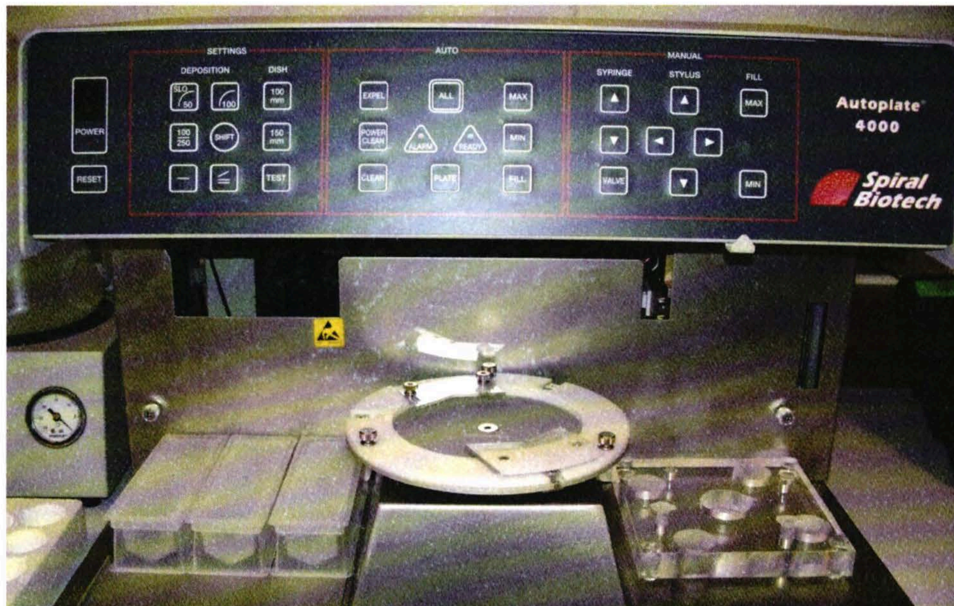

Figure 18. Autoplate 4000 automated spiral plater

Figure 19. Spiral plater stylus obtaining sample from test tube

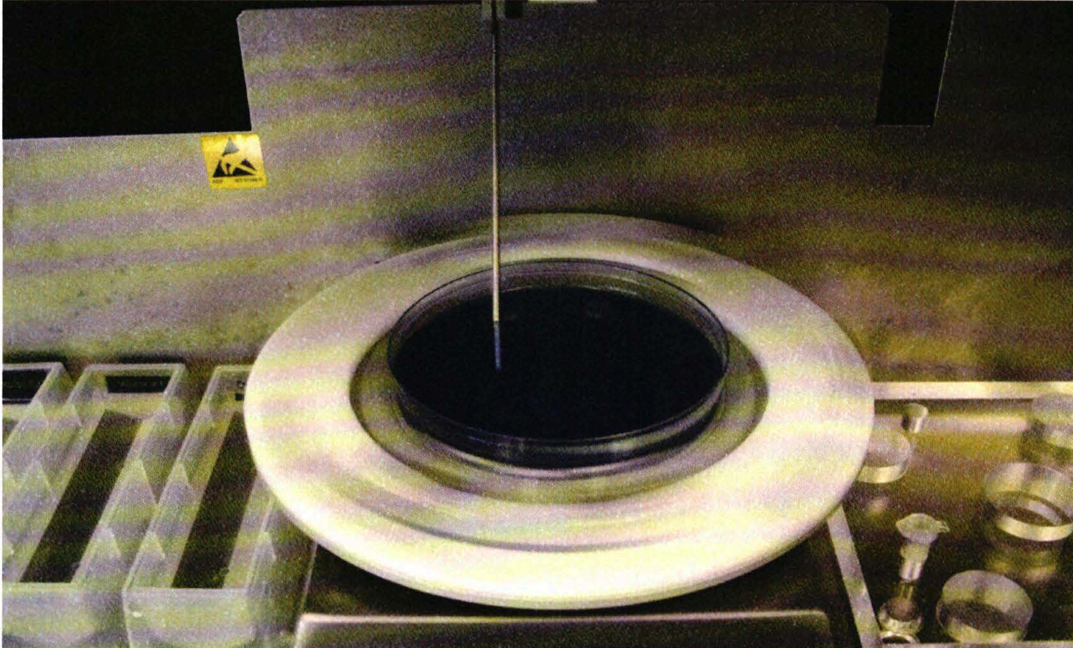

Figure 20. Stylus dispensing sample of *S. mutans* on to Mitis salivarius agar

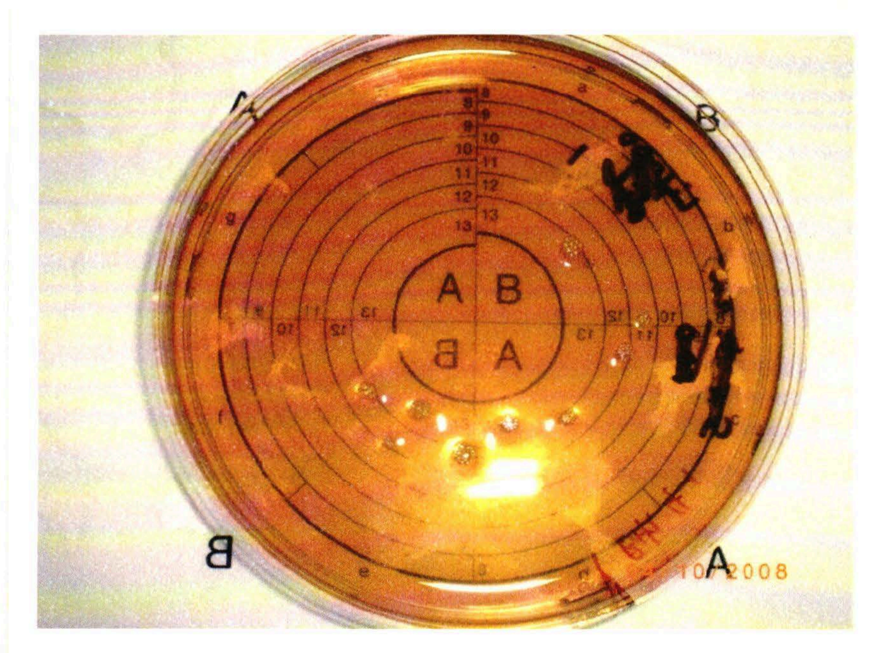

Figure 21. Sample sector plate count

- Invert plates and incubate in the CO_2 incubator for 48 hours
- Read and record results (Fig. 21)
- Dispose of contaminated polystyrene cell culture cluster in appropriate biohazard container
- Spray all working surfaces with 5.25% sodium hypochlorite and allow contact for 10 minutes then wipe dry
- Wash hands thoroughly with antimicrobial soap

Data Collection Instruments

S. mutans was inoculated onto MS agar Petri dishes and colonies were counted to determine the concentration of the bacteria (CFU/mL) (Figure 22). CFU were also used to calculate the inactivation factor (IF), which is the proportion of colonies that were killed by LTAPP exposure (experimental group) compared to the number of colonies in the control group.

Statistical Analysis

Data analysis was conducted using SAS® software program, version 9.1.[60] Mixed model repeated measures analysis of variance (ANOVA) test was conducted to determine means and standard deviations for CFU and IF data that was normally distributed and to test the hypotheses for statistically significant results. General linear model (GLM) was used to provide contrast for random effects and to provide adjusted means for covariates.

CHAPTER IV

RESULTS AND DISCUSSION

This study was conducted to determine the bactericidal effects of LTAPP on *S. mutans*. A total of 90 samples were processed. Seventy-two samples of *S. mutans* at 1:100 dilution in BHI broth were exposed to LTAPP at various time intervals (60, 120, 180, 300 seconds). Eighteen control samples were plated but not exposed to LTAPP. All samples were plated on MS agar and incubated. CFU and IF were obtained with their corresponding means and standard deviations.

Results

There is a statistically significant difference in the bactericidal effect of the LTAPP exposure on *S. mutans at* 60, 120, 180 and 300 seconds compared to those bacteria not treated, as measured by CFU/mL. This null hypothesis was rejected. Analysis revealed a statistically significant bactericidal effect of LTAPP on *S. mutans* at time exposures of 60, 120, 180, 300 seconds (p=0.272, p=0.0001, p=0.0001, p=0.0001, respectively) (Table 2). Figure 22 reflects mean log concentration CFU/mL over time after LTAPP exposure to bacterial samples.

Table 2. Summary statistics

Exposure Time In Seconds	Exposure Sessions	N=90 Agar Plate Samples	*p*-value	Standard Deviation	Mean Log Concentration (CFU/mL)
0 (control)	5	18	-	-	TNTC
60	5	18	0.0272*	0.604	6.05
120	5	18	<.0001*	0.919	6.10
180	5	18	<.0001*	1.13	4.70
300	5	18	<.0001*	0.459	4.59

*indicates statistically significant mean log concentration CFU/mL reduction compared to the control (0 seconds)

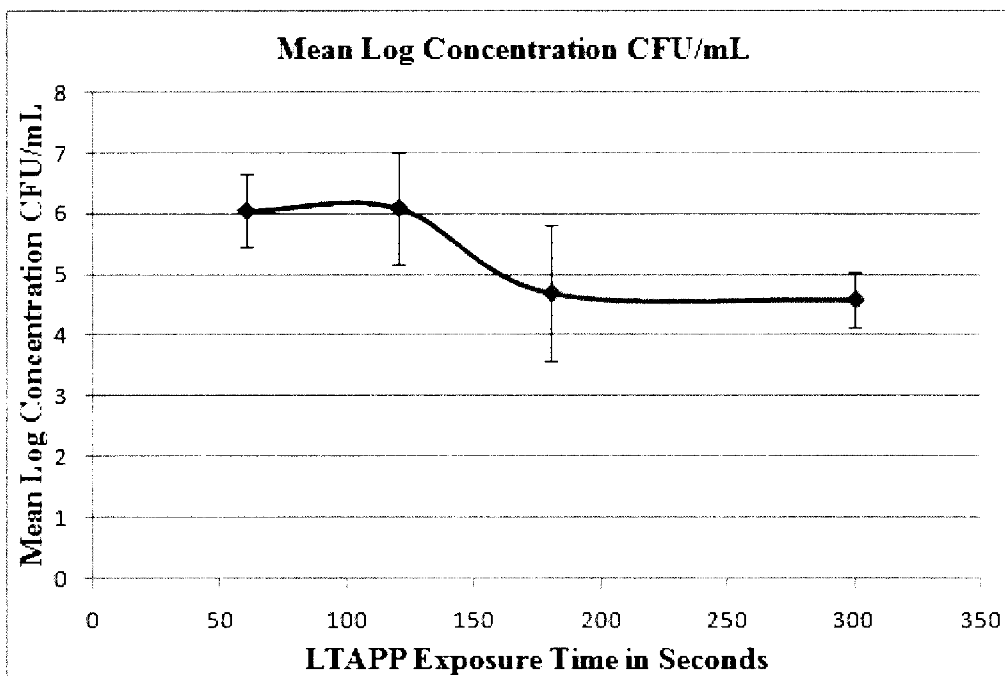

Fig. 22. Mean log concentration CFU/mL effect over time

Table 3. Percent Inactivation

Inactivation Factor						
Exposure Time in Seconds	**Exposure Sessions**	**N=90 Agar Plate Samples**	**Mean**	**Standard Deviation**	**Minimum**	**Maximum**
0 (Control)	5	18	0	0	0	0
60	5	18	0.53	0.15	0.29	0.71
120	5	18	0.76	0.14	0.53	1.00
180	5	18	0.92	0.07	0.78	1.00
300	5	18	0.95	0.03	0.89	1.00

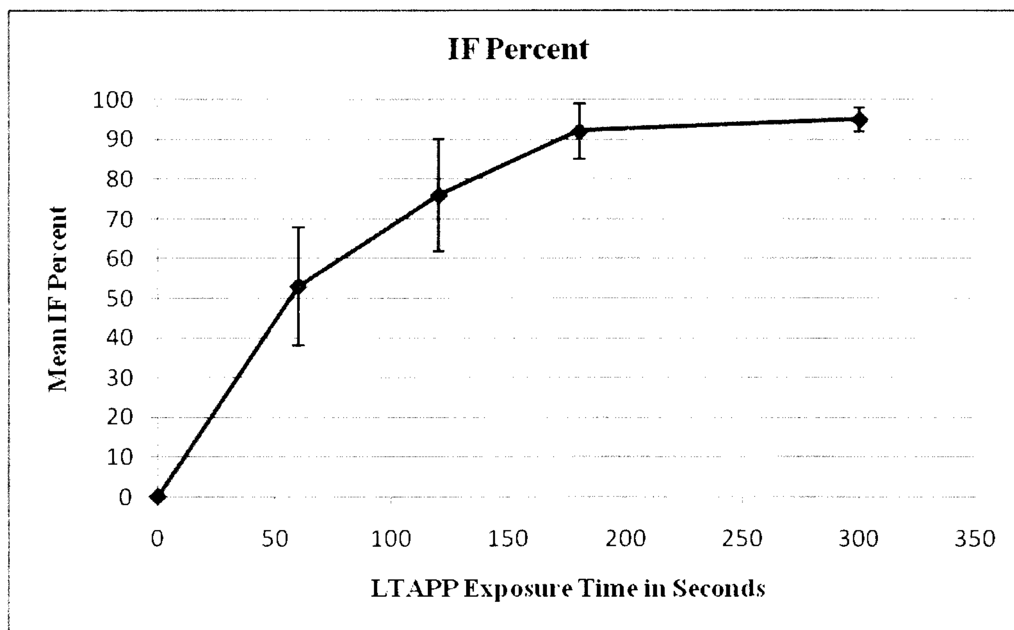

Fig. 23. Mean IF percent change that occurred following various LTAPP exposure times

Table 3 illustrates mean percent activation and standard deviations of *S. mutans* resulting from LTAPP exposure. There was a mean IF of LTAPP on *S. mutans* for 60, 120, 180, 300 seconds (53%, 76%, 92% and 95%, respectively). Figure 23 depicts multiple mean IF changes for each LTAPP exposure time.

Discussion

There were statistically significant differences between the control and exposed samples in terms of countability of bacteria regardless of LTAPP exposure times; as LTAPP exposure time increased, bacteria numbers decreased as shown in Table 2. Increasing the LTAPP exposures times had a direct effect on the decrease in bacteria. As exposure time increased from 60 to 300 seconds, mean log concentration CFU/mL decreased from 6.05 to 4.09.

There were statistically significant decreases in mean log concentration CFU/mL when *S. mutans* received greater time exposure to LTAPP. The percent inactivation for *S. mutans* was

95% after 300-second LTAPP exposure, whereas a 60-second LTAPP exposure revealed a 53% IF. As time exposure of LTAPP increased, the mean IF significantly increased suggesting a dose-response attributed to LTAPP exposure. To date no other studies have evaluated IF in relation to the bactericidal effects of LTAPP to *S. mutans*.

The results of the present study support the findings of Goree et al.,[6] who exposed *S. mutans* to a form of non-thermal plasma using a plasma needle for various time increments. Goree and colleagues concluded that *S. mutans* was inactivated using a plasma needle; however, the study did not compare time increments or bactericidal effect of treatments, but rather compared the image patterns of bacterial growth after treatment using spatial photography.

Results of the present study support the work conducted by Sladek et al.,[7] which concluded that exposing *S. mutans* to a plasma needle rendered a partial bactericidal effect but *S. mutans* was not completely destroyed. The current study found that although *S. mutans* was not completely destroyed, LTAPP may have a bactericidal effect by inhibiting S. mutans growth. However, these results may only be generalized to ATCC 27351 *S. mutans*. Researchers concluded that LTAPP may be a useful treatment for inactivating *S. mutans* thus decreasing dental caries risk.

Recommendations for Future Studies

Replication of the present study is needed to further explore the relationship between LTAPP and the inactivation of *S. mutans*, and the morphological changes that might occur in the bacterial cell. In this study, structural changes caused by LTAPP exposure were not assessed, but research conducted by Laroussi[15,54] utilized scanning electron microscopy to determine structural changes of bacteria. The present investigation employed CFU/mL as a gauge for bactericidal effect. No attempts were made to assess morphological cell damage caused by LTAPP. Because

the main focus of this study was to analyze the bactericidal effect of *S. mutans*, more research is needed to evaluate the effects of LTAPP on other pathogens. Therefore, a recommendation for future research is to use transmission electron microscopy, scanning electron microscopy or other instruments of measurement to identify the morphological changes that occur to microorganisms after receiving LTAPP exposure.

As facultative bacteria, *S. mutans* can survive both anaerobic and aerobic conditions; however, *S. mutans* prefers the anaerobic environment. Incubating samples anaerobically may determine if LTAPP has the ability to inactivate *S. mutans* at the clinical lesion phase of caries development.[30]

Utilizing a smaller acrylic well size may allow for complete exposure of LTAPP on *S. mutans* samples. Samples pipetted into the acrylic wells may not have been completely exposed to the plasma plume. A smaller well size will further focus and contain the plasma plume for a more complete LTAPP exposure.

Using other gases or gas mixes, such as oxygen and argon, may be more effective in inactivating *S. mutans*. Studies indicate that various plasma gases such as oxygen and argon are capable of inactivating certain microbes.[9, 11-13, 16-19, 45, 46, 51, 53, 57, 58, 64, 75, 76]

Kinetics may determine how complete microbial destruction occurs. Microorganisms vary in their survival characteristics and it is difficult to determine at what phase of bacterial growth inactivation occurs. It is also difficult to predict ideal conditions that inactivate all bacteria. Thus, exploring the kinetics of death for of a multitude of organisms will assist in further research on optimizing plasma treatment conditions resulting in complete pathogenic microorganism destruction.

Investigating specific periodontal pathogens, such as *Tannerella forsythensis, Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia,* and *Treponema denticola* is recommended to understand the potential of LTAPP as a treatment for full mouth contamination. In addition, LTAPP research should be conducted on other oral conditions such as oral cancer, herpetic lesions, apthous ulcers, lichen planus and *Candida albicans* infections since it is postulated that LTAPP may affect the healing rate of various oral lesions.

CHAPTER V

SUMMARY AND CONCLUSIONS

S. mutans is a Gram-positive, facultative species of aerobic bacteria that is commonly found in the human oral cavity and is a significant indicator of dental caries. *S. mutans* plays a critical role in metabolizing sucrose to lactic acid, which leads to demineralization of the tooth structure.

The current study evaluated the bactericidal effect of the LTAPP pencil as a method of inactivating *S. mutans*. *S. mutans* samples were diluted in BHI broth and exposed to LTAPP via the plasma pencil at various times. Bacteria were inoculated on MS agar and incubated. CFU were counted and results were recorded. Plates which yielded no growth were recorded as zero and others that revealed a colony count over 100 were recorded as TNTC.

The results of this study indicate that there is a statistically significant reduction of *S. mutans* after receiving LTAPP pencil exposure compared to those that were not exposed. As LTAPP exposure time increased, the CFU/mL decreased. LTAPP has a statistically significant bactericidal effect at all time exposures, as measured by CFU/mL and a mean inactivation effect on *S. mutans* that ranged from 53-95%.

Although LTAPP had statistically significant bactericidal effects on *S. mutans* with mean 95% inactivation, complete sterilization did not occur.

Based on data analysis, the following conclusions are made:

- As LTAPP exposure time increased, bactericidal effects on *S. mutans* increased as evidenced by a decrease in mean CFU/mL.
- As LTAPP exposure time increased, the percent inactivation of *S. mutans* increased as evidenced by the increase in IF.

- LTAPP has a dose-response effect on *S. mutans*. As LTAPP exposure time increased, the bactericidal effects also increased.

In dentistry alone, the plasma pencil offers the possibility of a precise focused delivery system that may effectively treat dental caries-causing pathogens without the side effects associated with antibacterial chemotherapeutic agents. Further collaborative multi-disciplinary research efforts utilizing plasma technology will go well beyond inactivating caries causing pathogenic bacteria and may influence the future of healthcare.

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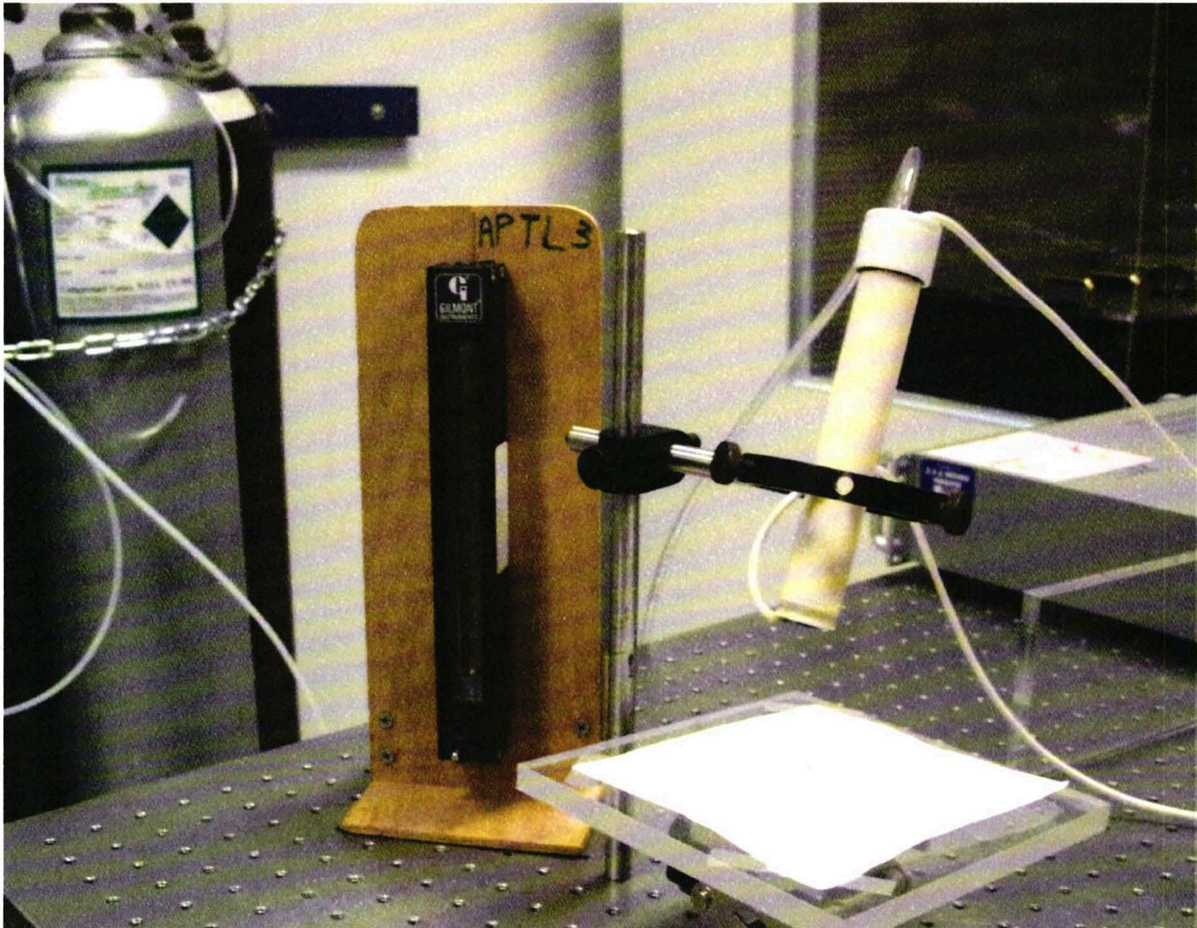
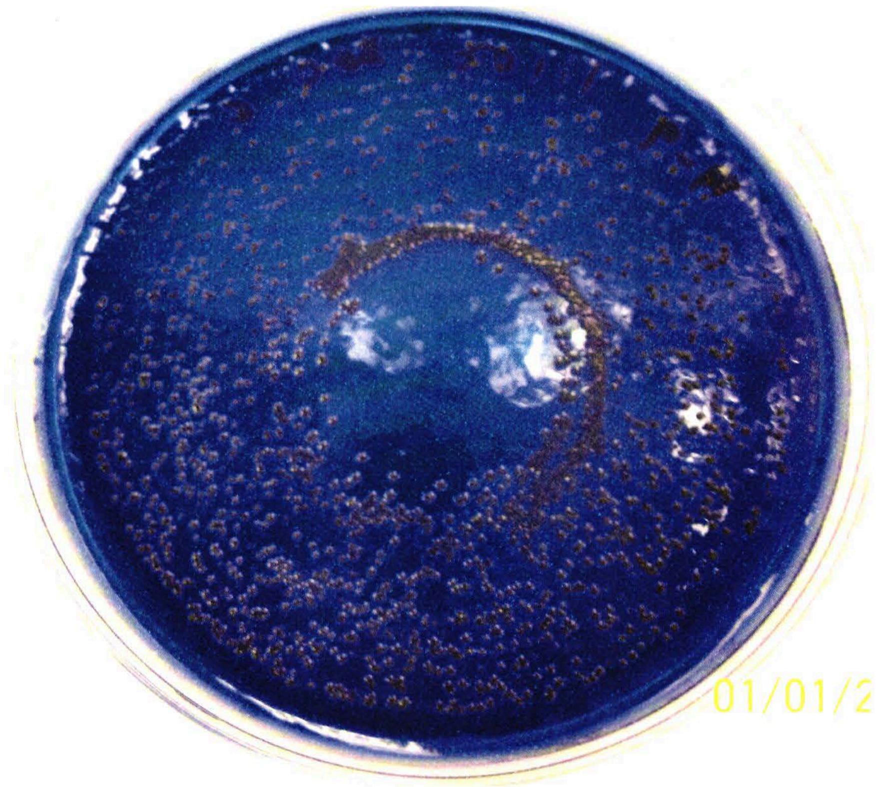
APPENDIX A

Plasma Pencil Set-up



APPENDIX B

Control plate with *Streptococcus mutans* growth after 48 hour incubation



APPENDIX C

Significant Pilot Data: *S. mutans* Strain K2

LTAPP Exposure Time in Seconds			CFU/mL Characteristics				
Control	120	300	Undiluted	1:10	1:100	Count Type	CFU/mL
x			31			TP	6.2×10^2
	x		2			TP	4.0×10^1
		x	12			TP	2.4×10^2
x				158		TP	3.2×10^4
	x			5		TP	1.0×10^3
		x		10		TP	2.0×10^3
	x				5	TP	1.0×10^4
		x			0	TP	0
	x		150			TP	3.0×10^3
	x			11		TP	2.2×10^3
		x		57		TP	1.1×10^4
	x				18	TP	3.6×10^4
		x			6	TP	1.2×10^4
x			150			TP	3.0×10^3
	x		33			HC-A	2.6E+05
		x	40			SC	3.1×10^5
x				53		TP	1.1×10^4
	x			56		SC	4.4×10^6
		x		6		TP	1.2×10^3
x					1	TP	2.0×10^3
	x				3	TP	6.0×10^3
		x			14	TP	2.8×10^4
x			24			TP	0
	x		1			TP	0
		x	4			TP	0
	x				0	TP	0
		x			0	TP	0

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor
CFU/mL for High Plate Count (HC) = #/volume constant for single segment *1000* Dilution Factor

APPENDIX D

Significant Pilot Data: *S. mutans* Strain G33

LTAPP Exposure Time in Seconds			CFU/mL Characteristics				
Control	120	300	Undiluted	1:10	1:100	Count Type	CFU/mL
	x		33			TP	6.6×10^2
		x	4			TP	8.0×10^1
x				48		TP	9.6×10^3
	x			10		TP	2.0×10^3
		x		0		TP	0
x					11	TP	2.2×10^4
	x				0	TP	0
		x			0	TP	0
		x	163			TP	3.3×10^3
	x				31	TP	6.2×10^4
		x			34	TP	6.8×10^4
x			300			TP	6.0×10^3
	x		26			TP	5.2×10^2
		x	84			TP	1.7×10^3
x				47		TP	9.4×10^3
	x			0		TP	0
		x		0		TP	0
x					1	TP	2.0×10^3
	x				0	TP	0
		x			0	TP	0
x			40			SC-8	3.3×10^4
x				21		TP	4.2×10^3
	x			87		SC	1.7×10^4
x					7	TP	1.4×10^4
	x				1	TP	2.0×10^3
		x			0	TP	0

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor
CFU/mL for High Plate Count (HC) = #/volume constant for single segment *1000* Dilution Factor

APPENDIX E

Significant Pilot Data: *S. mutans* Strain G3

LTAPP Exposure Time in Seconds					CFU/mL Characteristics				
Control	60	120	180	300	Undiluted	1:10	1:100	Count Type	CFU/mL
		x			4			TP	8.0×10^{1}
				x	1			TP	2.0×10^{1}
x						36		TP	7.2×10^{3}
		x				13		TP	2.6×10^{3}
				x		3		TP	6.0×10^{2}
x							2	TP	4.0×10^{4}
		x					8	TP	1.6×10^{5}
				x			3	TP	6.0×10^{4}
x					100			TP	2.0×10^{3}
		x			76			TP	1.5×10^{3}
				x			95	TP	1.9×10^{6}
x					300			TP	6.0×10^{3}
		x			26			TP	5.2×10^{2}
				x	19			TP	3.8×10^{2}
x						3		TP	6.0×10^{2}
		x				0		TP	0
				x		2		TP	4.0×10^{2}
x							0	TP	0
		x					0	TP	0
				x			1	TP	2.0×10^{1}
x					75			TP	1.5×10^{3}
		x			35			SC	2.9×10^{4}
				x	28			TP	5.6×10^{2}
x						93		TP	1.9×10^{4}
		x				13		TP	2.6×10^{3}
				x		3		TP	6.0×10^{2}
x							0	TP	0
		x		x			0	TP	0
							1	TP	2.0×10^{1}
x							26	HC	2.0×10^{7}
x							34	HC	1.3×10^{7}

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor
CFU/mL for High Plate Count (HC) = #/volume constant for single segment *1000* Dilution Factor

LTAPP Exposure Time in Seconds					CFU/mL Characteristics				
	x						62	TP	1.2×10^4
	x						24	SC	1.7×10^5
	x						41	SC	2.8×10^5
		x					30	SC	5.5×10^5
		x					36	SC	6.5×10^5
		x					76	SC	1.4E+06
		x					50	TP	1.0×10^3
x							39	HC	3.1×10^7
x							28	HC	1.1×10^7
x							27	HC	2.1×10^7
	x						28	HC	1.1×10^7
	x						30	HC	1.2×10^7
	x						28	HC	1.1×10^7
		x					39	HC	1.0×10^7
		x					39	HC	1.5×10^7
		x					20	HC	7.8×10^6
				x			29	HC	7.4×10^6
				x			21	HC	4.0×10^5
				x			26	HC	6.7×10^6
x							51	HC	4.0×10^7
		x					39	HC	3.1×10^7
	x						95	TP	1.9×10^5
		x					48	SC	4.0×10^6
		x					54	SC	4.4×10^6
		x					47	SC	3.9×10^6
			x				25	SC	2.1×10^6
			x				29	SC	2.4×10^6
				x			63	SC	5.2×10^6
				x			81	SC	6.7×10^6
x							145	TP	2.9×10^4
	x						77	TP	1.5×10^4
	x						87	TP	1.7×10^4
	x						0	TP	0
		x					12	TP	2.4×10^3
		x					6	TP	1.2×10^3
		x					3	TP	6.0×10^2
			x				17	TP	3.4×10^3
			x				11	TP	2.2×10^3
			x				12	TP	2.4×10^3

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor
CFU/mL for High Plate Count (HC) = #/volume constant for single segment *1000* Dilution Factor

LTAPP Exposure Time in Seconds					**CFU/mL Characteristics**				
				x			20	TP	4.0×10^3
				x			14	TP	2.8×10^3
				x			19	TP	3.8×10^3
x							67	SC-8	5.5×10^6
x							63	SC-8	5.2×10^6
	x						76	SC-9	2.6×10^6
	x						52	SC-9	1.8×10^6
		x					45	SC-9	1.5×10^6
		x					47	SC-9	1.6×10^6
			x				92	SC-9	3.1×10^6
			x				42	SC-8	3.5×10^6
				x			36	SC-9	1.2×10^6
				x			60	SC-10	1.1×10^6

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

APPENDIX F

Significant Pilot Data: *S. mutans* Strain 27351

LTAPP Exposure Time in Seconds					CFU/mL Characteristics		
Control	60	120	180	300	1:100	Count Type	CFU/mL
x					10	TP	2.0×10^4
x					4	TP	8.0×10^3
	x				0	TP	0
	x				3	TP	6.0×10^3
	x				3	TP	6.0×10^3
		x			13	TP	2.6×10^4
		x			11	TP	2.2×10^4
		x			22	TP	4.4×10^4
			x		16	TP	3.2×10^4
			x		4	TP	8.0×10^3
			x		3	TP	6.0×10^3
				x	5	TP	1.0×10^4
				x	11	TP	2.2×10^4
				x	7	TP	1.4×10^4
x					29	HC	1.1×10^7
	x				25	TP	5.0×10^4
	x				62	TP	1.2×10^5
	x				29	SC	2.4×10^6
		x			0		0
		x			56	SC	1.9×10^6
		x			54	SC	1.8×10^6
			x		1	TP	2.0×10^3
			x		0	TP	0
			x		0	TP	0
				x	1	TP	2.0×10^3
				x	0	TP	0
				x	0	TP	0
x					69	TP	1.4×10^5
x					68	TP	1.4×10^5
	x				108	TP	2.2×10^5
	x				93	TP	1.9×10^5

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor
CFU/mL for High Plate Count (HC) = #/volume constant for single segment *1000* Dilution Factor

LTAPP Exposure Time in Seconds					CFU/mL Characteristics		
		x			39	TP	7.8 x10^4
		x			53	TP	1.1 x10^5
			x		41	TP	8.2 x10^4
			x		56	TP	1.1 x10^5
				x	145	TP	2.9 x10^5
				x	126	TP	2.5 x10^5
x					60	TP	1.2 x10^5
x					57	TP	1.1 x10^5
	x				9	TP	1.8 x10^4
	x				65	TP	1.3 x10^5
	x				75	TP	1.5 x10^5
		x			77	TP	1.5 x10^5
		x			65	TP	1.3 x10^5
		x			79	TP	1.6 x10^5
			x		61	TP	1.2 x10^5
			x		76	TP	1.5 x10^5
			x		71	TP	1.4 x10^5
				x	52	TP	1.0 x10^5
				x	63	TP	1.3 x10^5
				x	68	TP	1.4 x10^5

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

APPENDIX G

Raw Data

Exposure Number	Count Type (TP, SP)	Exposure Time in Seconds	Dilution	Plate Number	CFU	Concentration (CFU/mL)	Mean Concentration (CFU/ mL)
1	TP	0	-1	1	TNTC	TNTC	TNTC
1	TP	0	-1	2	TNTC	TNTC	
1	TP	0	-1	3	TNTC	TNTC	
1	TP	60	-1	1	106	$2.12x10^5$	$2.39x10^5$
1	TP	60	-1	2	136	$2.72x10^5$	
1	TP	60	-1	3	117	$2.34x10^5$	
1	TP	120	-1	1	51	$1.02x10^5$	$3.60x10^4$
1	TP	120	-1	2	1	$2.00x10^3$	
1	TP	120	-1	3	2	$4.00x10^3$	
1	TP	180	-1	1	13	$2.60x10^4$	$2.53x10^4$
1	TP	180	-1	2	13	$2.60x10^4$	
1	TP	180	-1	3	12	$2.40x10^4$	
1	TP	300	-1	1	10	$2.00x10^4$	$1.33x10^4$
1	TP	300	-1	2	5	$9.99x10^3$	
1	TP	300	-1	3	5	$9.99x10^3$	

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

Exposure Number	Count Type (TP, SP)	Exposure Time in Seconds	Dilution	Plate Number	CFU	Concentration (CFU/mL)	Mean Concentration (CFU/ mL)
2	SP	0	-1	1	TNTC	TNTC	TNTC
2	SP	0	-1	2	TNTC	TNTC	
2	SP	0	-1	3	TNTC	TNTC	
2	SP	0	-1	4	TNTC	TNTC	
2	SP	60	-1	1	177	$3.22x10^6$	$3.47x10^6$
2	SP	60	-1	2	166	$3.02x10^6$	
2	SP	60	-1	3	205	$3.73x10^6$	
2	SP	60	-1	4	214	$3.89x10^6$	
2	SP	120	-1	1	131	$2.38x10^6$	$2.27x10^6$
2	SP	120	-1	2	127	$2.31x10^6$	
2	SP	120	-1	3	119	$2.16x10^6$	
2	SP	120	-1	4	123	$2.24x10^6$	
2	SP	180	-1	1	25	$4.55x10^5$	$3.87x10^5$
2	SP	180	-1	2	24	$4.36x10^5$	
2	SP	180	-1	3	21	$3.82x10^5$	
2	SP	180	-1	4	15	$2.73x10^5$	
2	TP	300	-1	1	12	$2.18x10^5$	$2.05x10^5$
2	TP	300	-1	2	11	$2.00x10^5$	
2	TP	300	-1	3	10	$1.82x10^5$	
2	TP	300	-1	4	12	$2.18x10^5$	

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

Exposure Number	Count Type (TP, SP)	Exposure Time in Seconds	Dilution	Plate Number	CFU	Concentration (CFU/mL)	Mean Concentration (CFU/ mL)
3	SP	0	-1	1	TNTC	TNTC	TNTC
3	SP	0	-1	2	TNTC	TNTC	
3	SP	0	-1	3	TNTC	TNTC	
3	SP	60	-1	1	87	$1.58x10^6$	$1.77x10^6$
3	SP	60	-1	2	98	$1.78x10^6$	
3	SP	60	-1	3	107	$1.95x10^6$	
3	SP	120	-1	1	48	$8.73x10^5$	$1.09x10^6$
3	SP	120	-1	2	54	$9.82x10^5$	
3	SP	120	-1	3	77	$1.40x10^6$	
3	SP	180	-1	1	41	$7.45x10^5$	$7.39x10^5$
3	SP	180	-1	2	38	$6.91x10^5$	
3	SP	180	-1	3	43	$7.82x10^5$	
3	TP	300	-1	1	10	$2.00x10^4$	$2.13x10^4$
3	TP	300	-1	2	12	$2.40x10^4$	
3	TP	300	-1	3	10	$2.00x10^4$	

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

Exposure Number	Count Type (TP, SP)	Exposure Time in Seconds	Dilution	Plate Number	CFU	Concentration (CFU/mL)	Mean Concentration (CFU/ mL)
4	SP	0	-1	1	TNTC	TNTC	TNTC
4	SP	0	-1	2	TNTC	TNTC	
4	SP	0	-1	3	TNTC	TNTC	
4	SP	0	-1	4	TNTC	TNTC	
4	SP	60	-1	1	TNTC	TNTC	TNTC
4	SP	60	-1	2	TNTC	TNTC	
4	SP	60	-1	3	TNTC	TNTC	
4	SP	60	-1	4	TNTC	TNTC	
4	SP	120	-1	1	72	5.93×10^6	5.93×10^6
4	SP	120	-1	2	79	6.51×10^6	
4	SP	120	-1	3	69	5.68×10^6	
4	SP	120	-1	4	68	5.60×10^6	
4	SP	180	-1	1	65	5.35×10^6	4.30×10^6
4	SP	180	-1	2	42	3.46×10^6	
4	SP	180	-1	3	46	3.79×10^6	
4	SP	180	-1	4	56	4.61×10^6	
4	TP	300	-1	1	25	5.00×10^4	5.30×10^4
4	TP	300	-1	2	26	5.20×10^4	
4	TP	300	-1	3	23	4.60×10^4	
4	TP	300	-1	4	32	6.40×10^4	

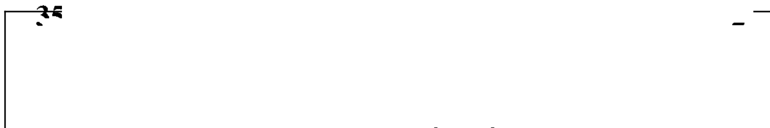
CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

Exposure Number	Count Type (TP, SP)	Exposure Time in Seconds	Dilution	Plate Number	CFU	Concentration (CFU/mL)	Mean Concentration (CFU/ mL)
5	SP	0	-1	1	TNTC	TNTC	TNTC
5	SP	0	-1	2	TNTC	TNTC	
5	SP	0	-1	3	TNTC	TNTC	
5	SP	0	-1	4	TNTC	TNTC	
5	SP	60	-1	1	TNTC	TNTC	TNTC
5	SP	60	-1	2	TNTC	TNTC	
5	SP	60	-1	3	TNTC	TNTC	
5	SP	60	-1	4	TNTC	TNTC	
5	SP	120	-1	1	140	1.15×10^{7}	6.11×10^{6}
5	SP	120	-1	2	63	5.19×10^{6}	
5	SP	120	-1	3	46	3.79×10^{6}	
5	SP	120	-1	4	48	3.95×10^{6}	
5	TP	180	-1	1	0	0	---------
5	TP	180	-1	2	0	0	
5	TP	180	-1	3	0	0	
5	TP	180	-1	4	0	0	
5	TP	300	-1	1	0	0	2.85×10^{4}
5	TP	300	-1	2	0	0	
5	TP	300	-1	3	24	4.80×10^{4}	
5	TP	300	-1	4	33	6.60×10^{4}	

CFU/mL for Total Plate Count (TP) = #/volume constant (50.030) *1000* Dilution Factor
CFU/mL for Segment Plate Count (SC) = #/volume constant for segment *1000* Dilution Factor

APPENDIX H

Margaret F. Lemaster



EDUCATION:

Master of Science in Dental Hygiene August 2009
Old Dominion University, Norfolk, Virginia

Bachelor of Science in Dental Hygiene May 1992
Old Dominion University, Norfolk, Virginia

EXPERIENCE:

- Private Practice, 1992-present (detailed experience available upon request)
- Adjunct Faculty, Clinical Instructor, Old Dominion University, 2003-present
- Internship, Old Dominion University, Accreditation Self- Study, 2006-2007
- Peer Reviewer, *Dimensions of Dental Hygiene*, 2009

TEACHING EXPERIENCE:

- Clinical Instructor, Old Dominion University, 2003-present
- Full-Time Dental Hygiene Faculty, Spring 2009

PUBLICATIONS:

Lemaster M. Tooth Wear and Dentinal Hypersensitivity: A Review of Etiology, Management and Prevention. *Dimensions of Dental Hygiene*, June 2009.

MANUSCRIPTS IN PROGRESS:

Lemaster M. Ultrasonic Scalers: A Review for the Dental Professional. Part One. *Dimensions of Dental Hygiene.*

Lemaster M. Ultrasonic Scalers: A Review for the Dental Professional. Part Two. *Dimensions of Dental Hygiene.*

Lemaster M. Ultrasonic Scalers: A Review for the Dental Professional. Part Three. *Dimensions of Dental Hygiene.*

Lemaster M, McCombs G, Darby M, Laroussi M, Hynes W. Effects of Low-Temperature Atmospheric Pressure Plasma on *Streptococcus mutans. Journal of Contemporary Dental Practice.*

Lemaster M, Seifert, K. Vitamin D: What Dental Professionals Should Know. *Dimensions of Dental Hygiene.*

HONORS, AWARDS AND PRIZES:

- Gene W. Hirschfeld Outstanding Masters Degree Student 2009
- Alpha Eta Honor Society, Student President 2007
- Virginia Dental Hygienists' Association Student Award 1992
- John J. Donahue Memorial Award 1992
- Who's Who Among American Colleges and Universities 1991-1992
- Who's Who Among American Colleges and Universities 1990-1991
- Gene W. Hirschfeld Scholarship Award 1989-1992

UNIVERSITY SERVICE

- Student Ambassador 1991-1992
- Student Senate 1990-1992

PROFESSIONAL SERVICE:

- President, Student American Dental Hygienists' Association 1991-1992
- Vice President, Student American Dental Hygienists' Association 1990-1991

COMMUNITY SERVICE:

- Annual Sealant Project, National Children's Dental Access Day at Old Dominion University
  2004 - present
- Mission of Mercy Faculty Supervisor
  2004 - present

PROFESSIONAL GOALS AND INTERESTS:

- Advance dental hygiene and related allied health fields through evidence-based research
- Teach undergraduate and/or graduate courses in dental hygiene, other related medical, and health areas
- Design, implement and evaluate teaching methods that include the use of new technologies and other innovation approaches to learning
- Mentor both traditional and nontraditional students to accomplish educational goals in effective and meaningful ways

PRESENTATIONS:

- Ethics, Jurisprudence, and Practice Management in Dental Hygiene,
  Junior Dental Hygiene Students, Spring 2006
- Care and Maintenance of Restorations,
  Junior Dental Hygiene Students, Spring 2006

POSTER PRESENTATIONS:

- **Margaret Lemaster, MS,** Koren Goodman, MSEd, PhD Student, John Hudson, PhD Student, Joyce Downs, PhD student, Mohammad Alzaharani, PhD student and Stacey B. Plichta, ScD.
  Predictors of Psychiatric Length of Stay and Emergency Room Department Utilization
  Poster presentation at the American Public Health Association 137th Annual Meeting, Pittsburg, PA (November 2009)

- **Margaret Lemaster, MS Candidate,** Gayle McCombs, MS, Michele Darby, MS, Wayne Hynes, PhD, Mounir Laroussi, PhD. **Effects of Low-Temperature Atmospheric Pressure Plasma on *Streptococcus mutans.***
  Poster presentation at American Dental Hygienists' Association Annual Session, Washington, DC (June 2009)

- **Margaret Lemaster, MS Candidate,** Koren Goodman, MSEd, PhD Student, John Hudson, PhD Student, Nakia Howard, MS Candidate, Grace Kogi, MS Candidate, and Stacey B. Plichta, ScD. **Hospital Emergency Departments: Men and Their Characteristic Utilization**
  Poster presentation at Old Dominion University's Annual Research Day, Norfolk, Virginia (March 2008)

- **Margaret Lemaster, MS Candidate,** Koren Goodman, MSEd, PhD Student, John Hudson, PhD Student, Joyce Downs, PhD student, Mohammad Alzaharani, PhD student and Stacey B. Plichta, ScD. **Emergency Department use Among Men**
  Poster presentation at the Virginia Public Health Association Regional Conference, Fairfax, Virginia (March 2008)

- **Margaret Lemaster, MS Candidate,** Gayle McCombs, MS, Michele Darby, MS, Wayne Hynes, PhD, Mounir Laroussi, PhD. **Effects of Low-Temperature Atmospheric Pressure Plasma on *Streptococcus mutans.***
  Poster presentation at Virginia Council of Graduate Schools Research Forum, Richmond, Virginia, (February 2008)

PPROFESSIONAL AFFILIATIONS AND MEMBERSHIPS:

Member, American Dental Hygienists' Association	1989-present
Virginia Dental Hygienists' Association	1989-present
Tidewater Dental Hygienists' Association	1989-present