COMBINED EFFECTS OF SOFT DRINKS AND NICOTINE ON *STREPTOCOCCUS MUTANS* METABOLIC ACTIVITY AND BIOFILM FORMATION

by

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Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, December 2019.

Thesis accepted by the faculty of the Department of Operative Dentistry, Indiana University, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

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DEDICATION

I dedicate this work to the God Almighty for his blessings, graces, and virtues. Thanks, God, for guidance, strength, power of mind, protection, and skills, as well as for giving me a healthy life.

In addition, I dedicate this work to my beloved parents Sami and Jehan, whose affection, unconditional love, and prayers of days and night make me able to get such success and honor. My awesome husband Ahmad, who has been a constant source of support and encouragement during the challenges of graduate school and life, I'm truly thankful for having him in my life. My lovely siblings who continually provide their moral, spiritual, and emotional support.

ACKNOWLEDGMENTS

My appreciation and thanks go to my research committee – Dr. Richard L. Gregory, Dr. N. Blaine Cook, and L. Jack Windsor for their guidance and encouragement throughout my research. Without their knowledge and thoughtful advice, this research project would never have been completed. I would also like to extend thanks to Mr. George Eckert for his help in the biostatistics section.

I am grateful to my amazing and loving parents for their endless support and encouragement. I would like to thank my wonderful husband and sisters for their love and encouragement. Many thanks go to all my supportive friends and fellow residents.

Finally, I extend my profound thanks to the government of the Kingdom of Saudi Arabia and to the Ministry of Education for sponsoring me and for providing all that I needed during this program.

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INTRODUCTION

For decades, dental caries has been one of the most widespread diseases. It affects 60 percent to 90 percent of school children and about 100 percent of grown-ups around the world.[1] It is known to be a chronic infectious disease that can lead to tooth loss. Dental caries is deemed to be a microbial multifactorial disease. Some risk factors may include host factors such as tooth structure, pellicle, and salivary flow.[1] Also, it requires the presence of dental plaque, sufficient time and a fermentable diet, where acids are generated from cariogenic bacteria that dissolve the hard enamel surface of the tooth and infiltrate into enamel inner layer when it is not controlled.[2]

The most commonly studied bacterium for dental caries is *Streptococcus mutans*. It is identified as gram-positive with acidic metabolic products that have strong abilities in changing the etiology of dental plaque.[3] These bacteria are able to adhere to the teeth and propagate, causing the formation of a mature cariogenic biofilm.[4] *S. mutans* is an important colonizer in oral biofilm during the final maturation process.[5]

S. mutans is recognized by its main cariogenic characteristics. The first is adhesion, where it is able to attach to the initial stages of plaque formation. The second is acidogenicity, which is the capability of acid production. The third is aciduricity, where they are able to resist and proliferate in the acidic environment.[3] It has been found that prolonged periods of acidic food or drink consumption can cause the pH in the dental plaque to fall rapidly to 5.5 or below.

One of the *S. mutans* adhesion phases is sucrose-dependent virulence. It is based on glucosyltransferase (GTF) activity, along with glucans and glucan-binding proteins

(GBP). The presence of sucrose will lead to glucan formation that will later be aided by GBP that bind glucans and cell-free and cell-associated GTFs existing in the oral biofilm.[6]

Oral biofilm is a collection of polymers, bacterial cells, and food debris formed in layers with slime consistency.[7] Biofilm formation starts when acquired salivary pellicle glycoproteins attach to the enamel surface through electrostatic interactions that are bacteria-free. This is followed by bacterial colonization as a result of reversible binding to salivary proteins. During maturation, bacteria will bind to colonizers through protein receptors.[5]

The relation between high-fructose corn syrup (HFCS) and increasing the activity of GTF has been demonstrated in recent studies.[8] HFCS is a refined corn starch, composed of 55 percent fructose, 42 percent glucose, and 3 percent variable saccharides.[8,9] It has convenient properties compared with other sweeteners, a factor which has led to its use in drinks, bread, dairy products, and canned foods.[8,9] It has been found that 70 percent of soft drinks in the US contain HFCS. This large percentage has directed attention to more investigations about its various effects. An inquiry study demonstrated that HFCS accelerated the speed and extent of *S. mutans* acid production compared with sucrose.[10]

Soft drinks are known for their unfavorable effects on multiple organs, and their regular consumption is linked to abundant health problems including diabetes, heart diseases, obesity and dental decay.[11] In the US, soft drinks are recognized as the dominant drink among teenagers, and the average consumption is increasing rapidly among the population.[12,13] It has been shown that soft drinks increase caries development

and progression as they decrease the pH through fermentation, leading to lactic acid production.[13] The presence of caries-promoting sugar and HFCS in non-diet soft drinks is considered one of the most cariogenic factors.[14,15] Moreover, soda drinks cause erosion and enamel weakness due to high acidity according to multiple studies.[16] A review has indicated that regular soft drinks are more erosive compared with diet drinks due to *S. mutans* activity causing the enamel surface to be weaker.[17] As a result, tooth surfaces are more susceptible environments for caries.[14]

Another important ingredient in most soft drinks is caffeine. It is the most consumed psychoactive substance around the world.[18] Focusing on its effects on teeth, studies demonstrate that caffeine reduces adherence of *S. mutans* to enamel as evidenced in interference with *S. mutans* adsorption to saliva-coated hydroxyapatite beads.[19] Further study has indicated that it has no cariogenic effects.[20] On the other hand, a recent study concluded that caffeine in caffeine-containing drinks inhibits *S. mutans* in comparison with caffeine-free drinks.[21] In addition, there is preliminary unpublished data from our laboratory by DuBois and Gregory supporting the inhibitory effect of caffeine on *S. mutans.* This effect must be taken into consideration when studying *S. mutans* activation factors.

In 2017 a study was done in this laboratory to evaluate the effects of the most prominent components of soft drinks, HFCS and caffeine. HFCS, a fermentable carbohydrate, was found to boost biofilm formation and metabolic activity, which potentially may increase caries activity in teeth. Further studies are needed to measure caries activity. On the other hand, caffeine's impact on biofilm formation and metabolic activity is not as obvious and is less significant compared with HFCS's effect.[22]

Another causative factor in dental caries is smoking. Studies indicate that in the US, around 42.1 million adults are smokers.[23] It is already known that smoking has adverse effects on cardiovascular and pulmonary systems in addition to its high prevalence in causing oral cancer.[24,25] It increases periodontal diseases as well.[26] Further research has exhibited its relation to causing an imbalance in oral microflora, which leads to dental caries.[27]

Research from this laboratory has indicated that nicotine increases *S. mutans* biofilm growth and the expression of several virulence factors such as antigen I/II, GTF and GBP, as well as the metabolic activity of biofilm cells.[28,29] Its concentration in saliva of smokers ranges between 0 mg/ml to 2.27 mg/ml.[30,31] Nicotine is an alkaloid that approximately represents 0.6 percent to 3 percent of the dry tobacco weight.[23] It binds to nicotine acetylcholine receptors, which inhibits fibroblasts and facilitates the invasion of *Porphyromonas gingivalis* into epithelial cells and human gingival fibroblasts.[26] Several research projects have related smoking to higher scores for decayed, missing, and filled teeth (DMFT). Recent studies have concluded that nicotine accelerates the growth of *S. mutans*, while others report a greater association between the presence of Lactobacilli and smoking than between smoking and *S. mutans*.[29,32,33]

OBJECTIVES

According to previous studies, soft drinks and cigarettes are greatly consumed in the US. It is assumed that a large percentage of smokers will drink soft drinks while smoking. Regular soft drinks have a large percentage of HFCS sugars that increase the activity of *S. mutans*, which makes these HFCS-containing drinks a high caries risk compared with sugar-free soft drinks. In addition, nicotine demonstrated a strong

correlation to developing caries. In the present study, we aimed to investigate the effects of different soft drinks (regular, sugar-free, and caffeine-free) and the presence of nicotine on *S. mutans* activity, and on the formation of biofilm to provide accurate information to patients.

HYPOTHESES

Alternative hypotheses:

1. The addition of sugar/HFCS-containing cola soft drinks to *S. mutans* cultures in the presence of nicotine will increase bacterial and biofilm growth as well as increase *S. mutans* metabolic activity.
2. The addition of caffeine-containing cola soft drinks to *S. mutans* cultures will inhibit biofilm formation and growth.

Null hypotheses:

1. The addition of sugar/HFCS-containing cola soft drinks to *S. mutans* cultures treated with nicotine will not increase the biofilm, growth, and metabolic activities of *S. mutans*.
2. The addition of caffeine-containing cola soft drinks to *S. mutans* cultures will not inhibit biofilm formation and growth.

REVIEW OF LITERATURE

Dental caries is considered a prime issue for health service providers worldwide.[34] It is known to be a multifactorial disease resulting in numerous cycles of demineralization and remineralization.[35] Simply, caries occurs as a result of a dissolution of minerals in enamel and dentin by acids that are produced during metabolism of food residues by microorganisms colonizing the tooth surfaces.[36, 37]

Causes of dental caries include the main factors of the epidemiologist's triad: agent (oral microorganisms), host (teeth, salivary flow), and environment (food).[36] Other factors that are contributing to the process of dental caries are: socioeconomic status, education, and behavior.[38]

MICROBIAL AGENTS

The oral cavity assists in the growth of diverse communities of microorganisms[39] due to its ideal humidity and temperature, the presence of nutrients needed by many microbial species, and the availability of ecological housing.[36] Over multiple studies, around 700 different types of bacterial species were found in the oral cavity.[40] These organisms persist on all surfaces as multispecies biofilms and form the resident oral microbiome, which generally exists in harmony with the host and delivers benefits that contribute to overall health and well-being. The oral environment has a major influence on the structure of the microbiome.[41] Typically a diverse number of oral microbes are harmless, although under certain circumstances they can cause oral infections such as dental caries or periodontal diseases.[42]

The aggregation of microorganisms that are attached to each other or to a surface and enclosed in extracellular polymeric substance (EPS) and associated with moisture is known as biofilm.[43-45] This oral biofilm passes through different stages of formation. The first stage is known as the acquired pellicle formation. In this stage, a thin film contains protein derived from the salivary glycoproteins attached to the surface, known as acquired pellicle. The second stage is the initial adhesion, where some of the planktonic bacteria may bind to the pellicle through the available binding proteins, such as α-amylase and proline-rich glycoproteins. After the attachment takes place, the pellicle starts producing EPS, which aids in binding the bacteria together. Third, maturation starts when the colonized bacteria provide specific binding sites to other pioneer organisms for subsequent bacterial colonization, promoting the development of biofilm. After that, the colonizing bacteria recognize polysaccharide or protein receptors on the pioneer bacterial surface and attach to them.[46-48] Polysaccharide recognition between bacteria is renowned as the fundamental mechanism of aggregation. Each recognition site varies between one bacterial pair and the other. This variation is a result of the specific sites on bacterial species in addition to the different types of proteins directing the adhesion process.[27] These aggregations can be inhibited by lactose, but not by sucrose.[5] Lastly, dispersion of biofilm cells occurs when the bacteria leave the biofilm by either single-cell or cluster-of-cells detachment. The cause of detachment could be explained by multiple reasons: the first is limited nutrients, causing bacteria to have to find a new site with more nutrients for growth. The second reason may be the presence of host defense, which tries to limit the biofilm development.[49,50] In addition, mechanical disturbance could result from brushing or rubbing forces.[51]

S. mutans has been recognized as a major causative agent of dental caries. It has an important role in biofilm formation – or what is known as dental plaque – on tooth surfaces. To clarify, *S. mutans* produces multiple GBP that promote adhesion. In addition, one of the major surface proteins of *S. mutans* is known as surface protein antigen-c (PAc). The virulence of *S. mutans* is correlated to its ability to facilitate interaction between the salivary pellicle and bacteria causing adherence.[52,53] Moreover, *S. mutans* is a strong acid producer, causing an acidic environment and increasing caries risk.[34]

HOST FACTORS

The most valuable oral fluid is saliva, which is a clear, slightly acidic mucoserous exocrine secretion. It is a mixture of fluids from major and minor salivary glands. The average daily flow of saliva varies between 1 liter and 1.5 liters.[54,55] The function of saliva includes cleansing, lubrication, buffering, remineralization, taste, digestion, and posing anti-microbial properties.[54] The amount and composition of saliva significantly influence carcinogenesis.[56] Saliva is composed of high levels of calcium and phosphate and low levels of fluoride.[57] In addition, it contains immunoglobulins, proteins, enzymes, mucins, and nitrogenous products such as urea and ammonia.[54] Mucins are known for their important role in maintaining the oral cavity in optimum health. Mucin is considered one of the prime lubricants in the oral cavity, facilitating several functions such as swallowing, eating, and talking. An important feature is that mucins decrease the colonization of bacterial species. Mucins are available in two forms, MG1 (highly glycosylated with high molecular mass) and MG2 (single glycosylated with lower molecular mass). MG1 is strongly attached to the tooth surface, aiding in the formation of

dental pellicle that in turn increases the MG1 rate in caries-susceptible subjects. On the other hand, MG2 is easily removed from the tooth surface, enhancing the clearance of the bacteria.[58,59]

Previous studies have shown that oral pH levels usually decrease within 5 min to 6 min after eating and return to normal in about 15 min.[60] This buffering and neutralizing effect is mediated by the bicarbonate, phosphate, and urea present in saliva. This process inhibits the demineralization process to protect the tooth from acid attacks.[61,62] Any dysfunction in the salivary glands can result in a considerable decrease in buffering capacity, increasing the risk of demineralization of tooth structure.[63,64]

Other salivary components are antimicrobial agents such as IgA, lactoferrin, and lysozyme, which interfere with the cariogenicity of bacteria. IgA binds to bacterial antigens that interpose the bacterial attachment. Lactoferrin binds to ferric iron, which is a main nutrient for cariogenic bacteria that in turn will decrease nutrient availability. Lysozyme plays a role in limiting bacterial growth in host tissues because it destroys the cell wall.[56, 65-67]

DIET

Nutrition has an important role in tooth development. For instance, periodontal and oral infectious diseases could occur due to malnutrition, (overnutrition/undernutrition).[68] In many studies, high-sugar food has shown its cariogenicity, and a direct correlation was found between the amount of available sugar and the average amount of dental caries in the community.[36]

Once teeth are erupted, their susceptibility to dental caries is increased. Dental caries occurs whenever the demineralization process exceeds remineralization. When

sugars (sucrose) or other fermentable carbohydrates (starches) are digested, the organic acids produced by bacteria like *S. mutans* increase the calcium hydroxyapatite solubility in dental hard tissues, causing loss of calcium followed by demineralization. The results of this sequenced process will cause a drop below the critical pH where the demineralization occurs at approximately 5.5 for enamel and 6.5 for dentin.[68,69]

Different epidemiological studies have shown the association between the amount of sugars consumed and the presence of dental caries.[68] The amount and frequency of sugar intake in addition to sugar-rich foods and drinks are strongly related to dental caries.[70]

One of the most commonly cited studies in this era was in Vipeholm, Sweden, at a mental institution where they fed adult patients severe amounts of sugars. They found that dental caries is highly influenced by the frequency of sucrose consumption rather than the amount. Moreover, the solid form of sugar that was easily retained in teeth was more cariogenic than the liquid form.[71] Another study was done to measure the cariogenicity of three different sweeteners: sucrose, fructose, and xylitol. Researchers concluded that sucrose causes the highest carious rate while fructose and xylitol had rates lower than sucrose, by 32 percent and 85 percent, respectively.[72]

In addition, a significant factor in caries development is time. Carious lesions intermittently progress as demineralization occurs. The demineralization-remineralization cycle in the oral cavity starts after food consumption: first, by acid formation during microbial metabolism, followed by inorganic ions formation from saliva. As a result, the microorganisms are calm between meals or snacks. Due to meals distribution through the

day, then, the available time for remineralization is longer than for demineralization, so that lesions do not progress.[36]

FACTORS ASSOCIATED WITH HIGH-CARIES-RISK SOFT DRINKS

According to the National Dietary Intake Surveys, soft drink consumption among children and adolescents during the recent decade has been high and is increasing dramatically.[73] In another study, soft drinks were deemed to be a major beverage consumed by teenagers in the US, and the average amount of soft drinks consumed per person per year is approximately 500 12-oz. cans.[13]

High consumption of soft drinks is one of the factors that negatively affects general and oral health.[22,74,75] Different studies were done to investigate the correlation between soft drink consumption and the risk of caries,[75] one of which concluded that the cariogenicity of cola is higher than sucrose and dairy products.[15] They attributed this finding to the presence of high fructose corn syrup (HFCS) sugar, which is a caries-promoting factor found in almost all non-diet soft drinks in the US.[15]

HFCS is considered an added sugar and was developed during the past three decades.[76] It has been used recently to replace other sweeteners in beverages and different processed and packaged foods.[15,76,77] That is because the HFCS is sweeter and less expensive than other caloric sweeteners.[78,76] Recent data showed that HFCS represents almost 40 percent of all added caloric sweeteners in the US. Additionally, it has the ability to be converted into glucose and fructose.[79] HFCS-55 is composed of 55 percent fructose, 42 percent glucose, and 3 percent of various saccharides.[9,80] This type of sugar is implicated in different studies to cause cardiovascular diseases, diabetes, obesity, and

dental caries.[9] In studies done by Raben et al. and Ludwig et al., they concluded that the rapid rise in intake of calorically sweetened soft drinks is considered a contributing factor to the weight gain epidemic.[8] In a prospective study among children, it was found that a single serving of soft drinks per day raises the risk of being overweight by 60 percent in one year.[81]

In addition, HFCS-55 has been found to affect biofilm-associated genes – specifically GTF-B, a protein that catalyzes the structure of water-insoluble glucan. When GTF-B is stimulated and the subsequent glucan is formed, in turn, this will reinforce *S. mutans* biofilm formation.[9,10,82]

In addition, soft drinks are acidic in nature, which increases the potential of dental erosion. Dental erosion is the mineral loss of tooth structure due to chemical solutions, either extrinsic such as diet, or intrinsic like gastroesophageal discharges.[17] Studies show that classic soft drinks have higher potential to cause dental erosion compared with diet soft drinks.[83]

CAFFEINE

Caffeine is known to be the most consumed psychoactive substance around the world.[18] Citizens in the UK and Nordic countries consume between 2 cups to 3 cups of caffeine-containing beverages per day that contain about 300 mg of caffeine.[84] According to several experiments on caffeine's effects, it has been found that caffeine ameliorates mood and promotes psychomotor and cognitive performance in healthy adults.[85,86] Results also showed that it sustains attention, memory, and logical reasoning, as well as the focus to drive a car.[86] Normal human consumption of caffeine causes inhibition of adenosine receptors that affect neurons and glial cells of all brain areas. Further

investigations on its biological effect found that caffeine is antagonizing all types of adenosine receptors (ARs): A1, A2A, A3 and A2B.[87] Caffeine, when acting as an AR antagonist, is helping to remove endogenous adenosinergic tonus. Caffeine metabolism occurs in the liver via the cytochrome P450 system when it is oxidized into paraxanthine, theobromine and theophylline.[88] In cola drinks, a 12-fl-oz can contains between 30 mg to 50 mg (0.11 mg/ml) caffeine.[89] Polyphenols are found in cranberry, propolis, coffee, wine, cocoa, tea and some dairy products.[90] Literature has confirmed that polyphenols have a lineal effect versus *S. mutans* as it interacts with microbial membrane proteins inhibiting adherence of bacterial cells to the tooth surface.[21] It is believed that caffeine disturbs bacterial cell walls and membranes leading to cell lysis. A recent report by DuBois and Gregory (unpublished data), demonstrate that the minimum biofilm inhibitory concentration (MBIC) of caffeine-treated *S. mutans* was 8 mg/ml.

SMOKING

In the US around 42.1 million adults are smokers, and the percentage is similar worldwide.[23] To illustrate the fact that smoking is one of the more serious health issues globally, the World Health Organization (WHO) found in 2012 that about 23 percent of the population older than age 15 years were smokers. In addition, the estimated yearly death rate as a result of tobacco use, either smoking or smokeless, is around 8 million yearly, according to the WHO report published in 2019.[91,92]

Smoking causes many harmful diseases as it affects almost every organ in the body. In different studies, smoking was established to be the cause of 30 percent of cancer-related deaths.[93,94] Moreover, there is a strong association between smoking and lung cancer, which has been shown to be a prime reason for cancer-related deaths in the

US as well as the main cause of chronic obstructive pulmonary disease (COPD).[95,96] In addition, smoking debilitates the immune system, particularly the oral cavity, leading to dental decay and periodontal diseases.[26,97,98] Another study showed that it increases the risk of peri-implantitis and implant failure.[99]

Although tobacco consists of around 7,357 different chemical materials, nicotine is the most plentiful alkaloid chemical.[100] Tobacco addiction is attributed primarily to the presence of nicotine, which is considered as a biobehavioral chemical compound.[101] Many studies investigated the effects of nicotine on several diseases, one of which was dental caries. They found that nicotine has a direct influence on *S. mutans* and concluded that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for nicotine were 16 mg/dl and 32 mg/ml, respectively.[28]

In addition, nicotine fosters the cariogenicity of *S. mutans* and *S. sanguinis* by increasing biofilm formation and biofilm metabolic activity.[27] Different studies have demonstrated that nicotine changes the microbial composition of the oral cavity and raises the pathogenicity of many oral microorganisms.[102] Moreover, a recent study found that nicotine causes a two-fold increase in lactic acid production.[27,103]

MATERIALS AND METHODS

BACTERIAL GROWTH AND PREPARATION

In this study *S. mutans* strain UA159 (ATCC 700610) was used and was initially grown on Mitis Salivarius Sucrose Bacitracin (MSSB, Anaerobe Systems, Morgan Hill, CA) agar plates (Figure 1). The strain was stored at -80°C in tryptic soy broth (TSB, Acumedia, Baltimore, MA) with 20-percent glycerol before use (Figure 2). Unless otherwise stated, TSB was used, and the growth condition was at 5.0-percent carbon dioxide at 37°C.[28]

PRELIMINARY EXPERIMENT

The aim of the preliminary experiment was to identify the MBIC of nicotine on *S. mutans* growth. *S. mutans* was grown in TSB overnight. Different nicotine concentrations of 0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 4.0 mg/ml, 8.0 mg/ml, 16.0 mg/ml, and 32.0 mg/ml nicotine (Sigma-Aldrich Chemical Co., St. Louis, MO) (Figure 3) were prepared in serial dilutions of TSB supplemented with 1.0-percent sucrose (TSBS). Next, 190-µl aliquots of TSBS containing each nicotine concentration were pipetted into wells of a sterile 96-well flat bottom microtiter plate. Ten µl of the fresh overnight TSB culture of *S. mutans* was added to each well (containing approximately 106 bacteria). The microtiter plate was incubated in 5.0-percent CO2 at 37°C for 24 hours. The next day, total absorbance (biofilm and planktonic growth) was measured in a spectrophotometer (SpectraMax 190; Molecular Devices Inc., Sunnyvale, CA) (Figure 4) at 595 nm. Then, 120 µl from each well were transferred to a corresponding well of a new microtiter plate. The absorbance of each well was read at

595 nm to measure planktonic growth. The remaining planktonic cells were removed from the biofilm microtiter plate wells (leaving attached biofilm), and 200 μl of 10-percent formaldehyde were added to each well for 30 min to fix the cell. After 30 min, the formaldehyde was removed, and the biofilm cells were washed three times with deionized water. Two hundred μl of 0.5-percent crystal violet dye were added to each well and the cells were stained for 30 min. The wells were then rinsed three times and 200 μl of 2-isopropanol were placed into each well for 1 h to lyse the cells and extract the crystal violet. The plates then were read in a spectrophotometer at 490 nm to measure biofilm formation. The MBIC was determined as described earlier.[28] It was defined as the lowest concentration producing a change in the optical density at 490 nm of less than 0.050 from the negative control.

EFFECTS OF SOFT DRINKS ON NICOTINE-TREATED *S. MUTANS* ESTABLISHED BIOFILM

Cola drinks were purchased from a local supermarket (Figure 5) and their detailed ingredients adapted from a previous work done in our lab are indicated in Table I.[22] Types of cola drinks were chosen according to caffeine and sugar content; three types contained caffeine, and three did not; two contained sugar, while four did not. The cola drinks were opened for 24 hours to remove carbonation.

Based on the dilution effects of saliva on the consumed beverages, a dilution of 1:3 was used (Figure 6). Then, to measure the effects of cola drinks on the established nicotine-treated *S. mutans* biofilm formation, a concentration of 8 mg/ml of nicotine was used with soft drinks at 1:3 dilution in TSBS and added to the biofilm.

Briefly, a TSB culture of *S. mutans* was grown overnight. The next day, 8 mg/ml of nicotine (Sigma-Aldrich Chemical Co., St. Louis, MO) was diluted in TSBS based on the previous preliminary experiment to treat nicotine-treated *S. mutans*. Then a dilution of 1:3 of soft drinks and nicotine-TSBS was prepared for each type of soft drink (cola, diet cola, cola zero, caffeine-free cola, caffeine-free diet cola, and caffeine-free cola zero). Soft drinks used in the study are shown in Table II. Next, 190 µl of the 8 mg/ml of nicotine in TSBS with 1:3 dilutions of soft drinks were aliquoted into wells of a sterile 96-well flat bottom microtiter plate. Ten µl of the fresh overnight TSB culture of *S. mutans* were added to each well. The microtiter plate was incubated for 24 h (Figure 7).

The next day, total absorbance (biofilm and planktonic growth) was measured in a spectrophotometer (SpectraMax 190; Molecular Devices Inc., Sunnyvale, CA) at 595 nm (Figure 8). Later, the remaining planktonic cells were removed from the biofilm microtiter plate wells (leaving attached biofilm), and 200 µl of 10-percent formaldehyde was added to each well for 30 minutes to fix the cells. After the 30 min, the formaldehyde was removed, and the biofilm cells were washed three times with deionized water. Two hundred µl of 0.5-percent crystal violet dye was added to each well and the cells were stained for 30 min (Figure 9). After that, the wells were rinsed three times and 200 µl of 2-isopropanol was placed into each well for 1 hour to lyse the cells and to extract the crystal violet. Finally, the plate was read in a spectrophotometer at 490 nm to measure biofilm formation.

BIOFILM METABOLIC ACTIVITY

Metabolic activity of *S. mutans* biofilm was measured by a method initially described by Pierce et al.[104] for *Candida albicans* but adapted by our lab for *S. mutans*.[28] The method is based on biofilm cells reducing 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) to a water-soluble organic compound in the presence of menadione.[28] Twenty-four-hour-established *S. mutans* biofilm was prepared as mentioned above and then treated with nicotine and 1:3 dilutions of soft drinks in TSBS. Fresh XTT menadione reagent was prepared according to Pierce et al.[104] (Figure 10), and the biofilm was washed twice with saline. Two hundred μl of XTT reagent were added (Figure 11) and the plate was kept in the dark without 5.0-percent CO2 for 2 h. Following the incubation period, the XTT reagent was transferred to another 96-well plate to detect the color change through the spectrophotometer at 490 nm (Figure 12). The ratio of specific biofilm metabolic activity/mass, which is indicative of relative biofilm cell metabolic activity, was calculated.[28] This provides a method to compare the residual effect of an antimicrobial agent on the surviving bacteria, because some agents (e.g., nicotine[26]) kill many, but not all bacteria, and the surviving microbes could remain extremely metabolically active.

CONTROLS

The control groups included biofilms of *S. mutans* treated with and without nicotine without cola drinks, and media without *S. mutans* was used as a sterility control. Control groups are clearly described in (Table III).

STATISTICAL ANALYSIS

Each experiment was repeated three times. One-way ANOVA was used to compare the effects of cola exposure (no cola drink, Coca-Cola-based drink, Diet Coke-based drink, Coke Zero-based drink), caffeine (yes or no), and nicotine (yes or no) and their interactions on *S. mutans* total growth, biofilm formation, and metabolic activity. Pair-wise comparisons were made between different groups for all three outcomes using the Sidak method. Analyses were performed using the ranks of the data. A 5.0-percent significance level was used for all the tests.

SAMPLE SIZE CALCULATIONS

Based on prior studies, the coefficient of variation was expected to be approximately 0.6. With a sample size of 5 per group, the study had 80-percent power to detect a 3.5x difference in bacterial growth, biofilm growth, and metabolic activity between two groups, assuming two-sided tests were each conducted at a 5.0-percent significance level.

RESULTS

RESULTS OF THE PRELIMINARY EXPERIMENT

The preliminary experiment results showed that nicotine was able to inhibit the biofilm formation significantly ($p < 0.05$). The significant effects observed in biofilm formation were between 4 mg/ml and 32 mg/ml. At 32 mg/ml of nicotine, it was almost bactericidal.

The results confirmed that the MBIC is 16 mg/ml, as found in previous studies. On the other hand, we did not aim to totally inhibit the biofilm formation, but still enhance its formation, so that we used the concentration of 8 mg/ml of nicotine. This information was used to establish the effects of different soft drinks on the *S. mutans* nicotine-treated biofilm. In Figure 13, the results of the preliminary experiment are illustrated.

RESULTS OF THE MAIN EXPERIMENT

In general, there were considerable effects for HFCS and caffeine, in the presence of nicotine, and their interaction in all measures: total growth (Table IV), biofilm formation (Table V), and metabolic activity (Table VI). One-way ANOVA was used for comparison (Table VII). Results were categorized in the following sections.

EFFECTS OF SOFT DRINKS AND NICOTINE ON *S. MUTANS* TOTAL GROWTH

Bacterial total absorbance of each well was measured before removing the planktonic bacterial cells at 595 nm. All groups that contained TSBS and *S. mutans* in the presence of nicotine and soft drinks exhibited a significant increase in comparison to the

control group (TSBS + *S. mutans*). In addition, Caffeine-free Coke demonstrated higher bacterial growth in comparison with all other types of soft drinks. On the other hand, Diet Coke demonstrated a significant decrease in total growth compared with Coke (Figure 14).

EFFECTS OF SOFT DRINKS AND NICOTINE ON *S. MUTANS* BIOFILM FORMATION

The results of the experimental groups in the crystal violet assay demonstrated a significant enhancement of the biofilm formation in all groups compared with the negative bacterial control. The presence of nicotine formed the highest biofilm in contrast with other groups that included soft drinks. When comparing specific beverages, Diet Coke formed less biofilm compared with Coke. In terms of caffeine and biofilm formation, Caffeine-Free Coke displayed a significant increase in biofilm formation in comparison with Coke, Diet Coke, and Caffeine-Free Diet Coke. Moreover, Caffeine-Free Diet Coke enhanced the biofilm formation parallel to Diet Coke. It was also similar to the relation between Caffeine-Free Coke Zero and Coke Zero (Figure 15).

EFFECTS OF SOFT DRINKS AND NICOTINE ON *S. MUTANS* METABOLIC ACTIVITY

The metabolic activity of *S. mutans* without nicotine or soft drinks was obviously lower than the metabolic activity of *S. mutans* in the presence of soft drinks and nicotine. The caffeinated soft drinks (Coke, Diet Coke, and Coke Zero) were more metabolically active in comparison with their paired caffeine-free sodas. In term of HFCS and *S. mutans* metabolic activity, Diet Coke and Coke Zero were less active than Coke. Also, the Caffeine-Free Diet Coke and Caffeine-Free Coke Zero were less active than Caffeine-

Free Coke. The highest metabolic activity of *S. mutans* in the presence of nicotine and soft drinks was noticed in Coke (Figure 16).

FIGURES AND TABLES

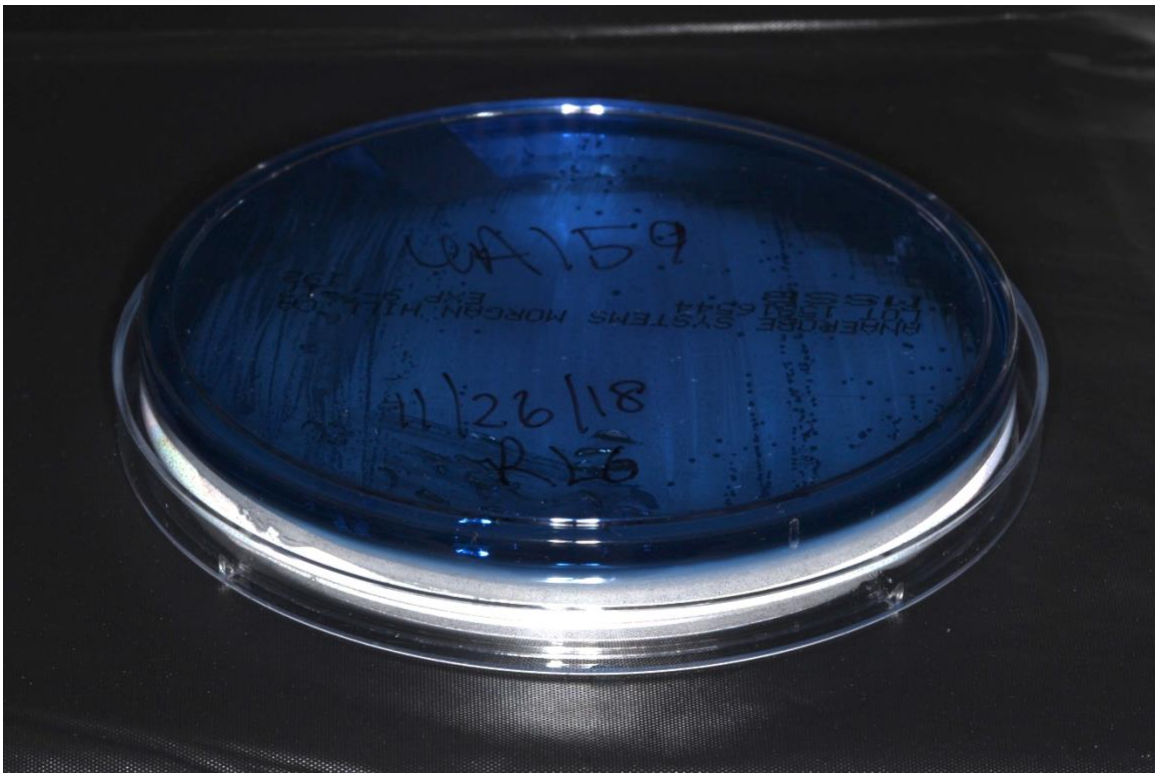

FIGURE 1. *S. mutans* strain UA159 was initially grown on Mitis Salivarius Sucrose Bacitracin agar plates.

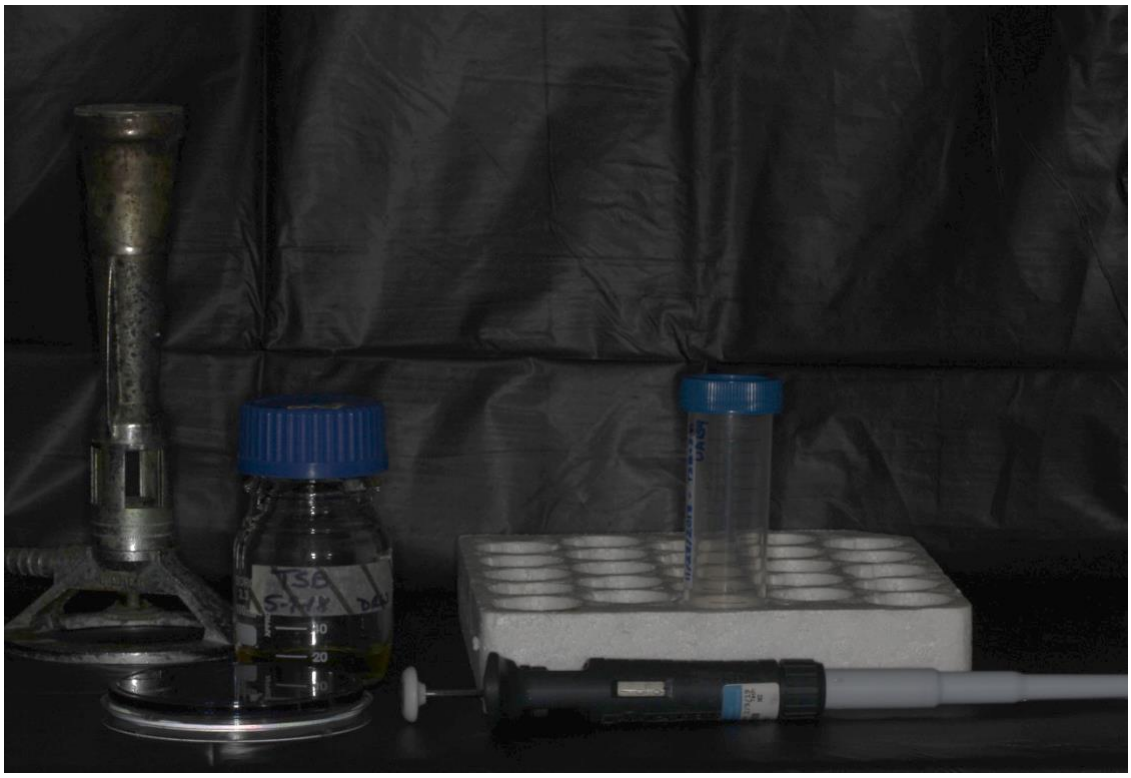

FIGURE 2. *S. mutans* UA159 was grown in TSB at 37°C in 5.0-percent CO2 for 24 hours and stored with 10.0-percent glycerol at -80°C.

FIGURE 3. Nicotine (Sigma-Aldrich Chemical Co., St. Louis, MO).

FIGURE 4. Spectrophotometer (SpectraMax 190; Molecular Devices Inc., Sunnyvale, CA).

FIGURE 5. Cola drinks were purchased from a local supermarket.

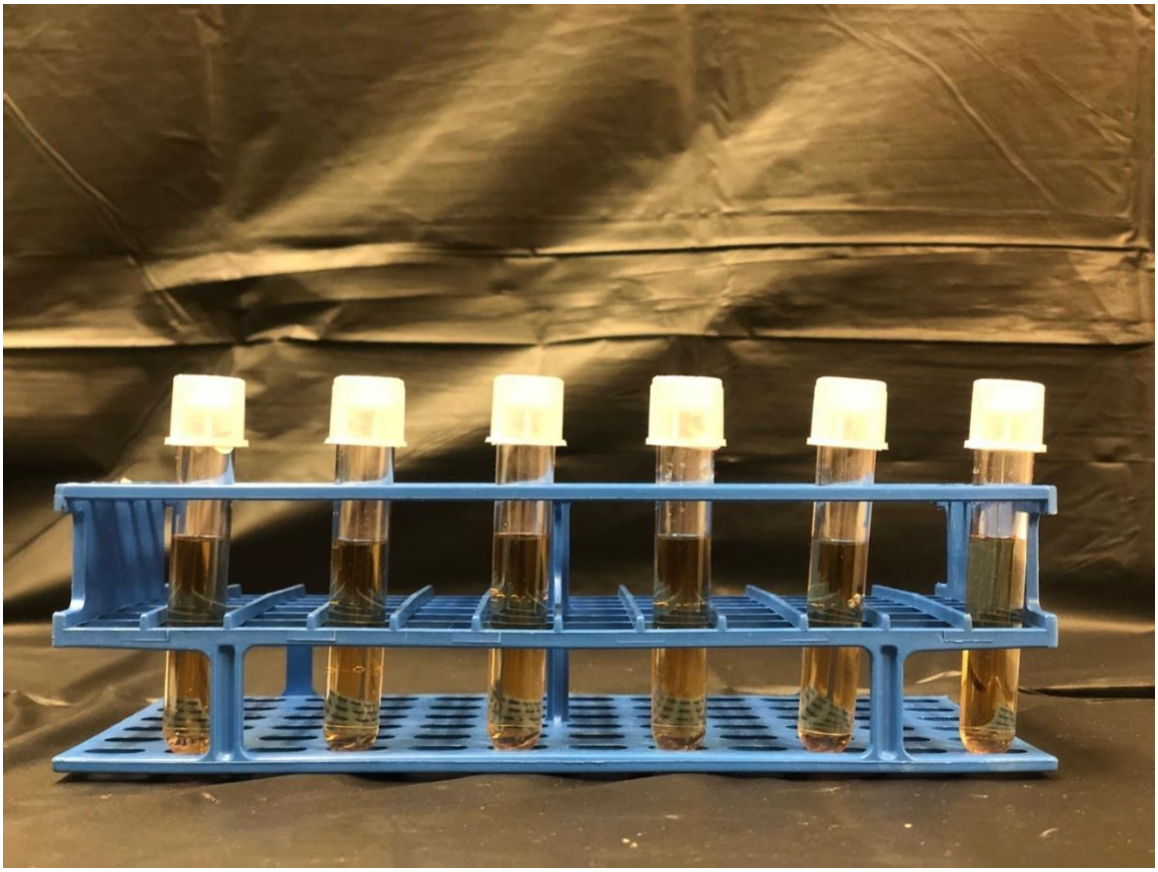

FIGURE 6. Soft drinks in 1:3 dilutions in TSBS with 8 mg/ml of nicotine.

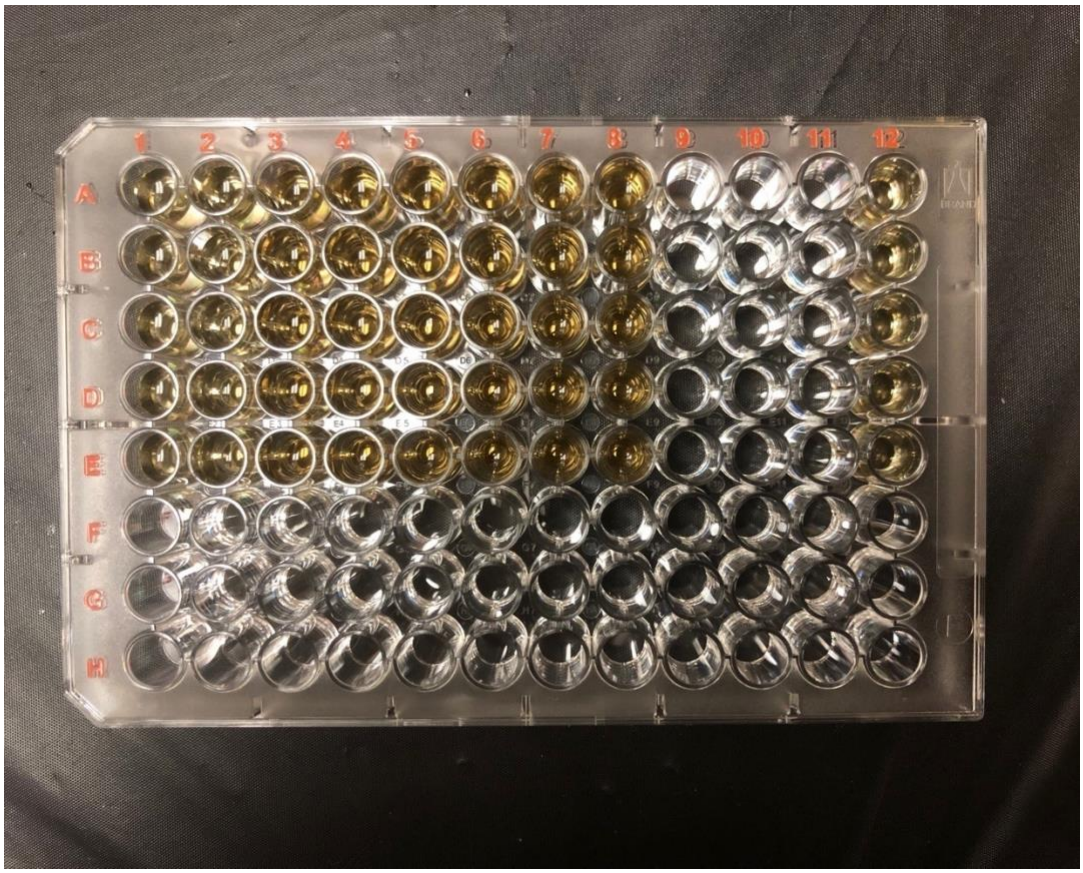

FIGURE 7. Sterile 96-well flat bottom microtiter plate; contents of each column:

1. *S. mutans* + TSBS.
2. *S. mutans* + TSBS + 8 mg/ml nicotine.
3. *S. mutans* + 1:3 dilutions of Coke in TSBS + 8 mg/ml nicotine.
4. *S. mutans* + 1:3 dilutions of Diet Coke in TSBS + 8 mg/ml nicotine.
5. *S. mutans* + 1:3 dilutions of Coke Zero in TSBS + 8 mg/ml nicotine
6. *S. mutans* + 1:3 dilutions of Caffeine free Coke in TSBS + 8 mg/ml nicotine
7. *S. mutans* + 1:3 dilutions of Caffeine free Diet Coke in TSBS + 8 mg/ml nicotine
8. *S. mutans* + 1:3 dilutions of Caffeine free Coke Zero in TSBS + 8 mg/ml nicotine
9. blank
10. blank
11. blank
12. TSBS (Sterility control).

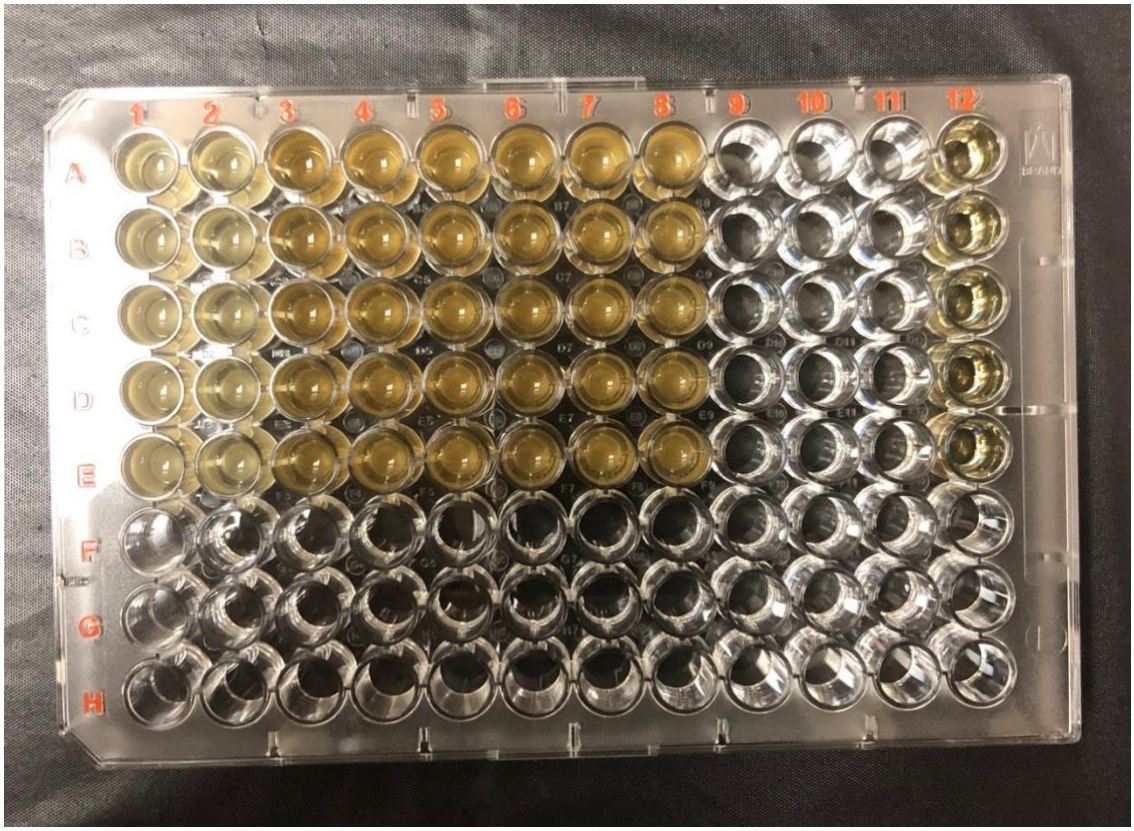

FIGURE 8. Bacterial planktonic growth was measured after 24 h of incubation using a spectrophotometer at 595 nm.

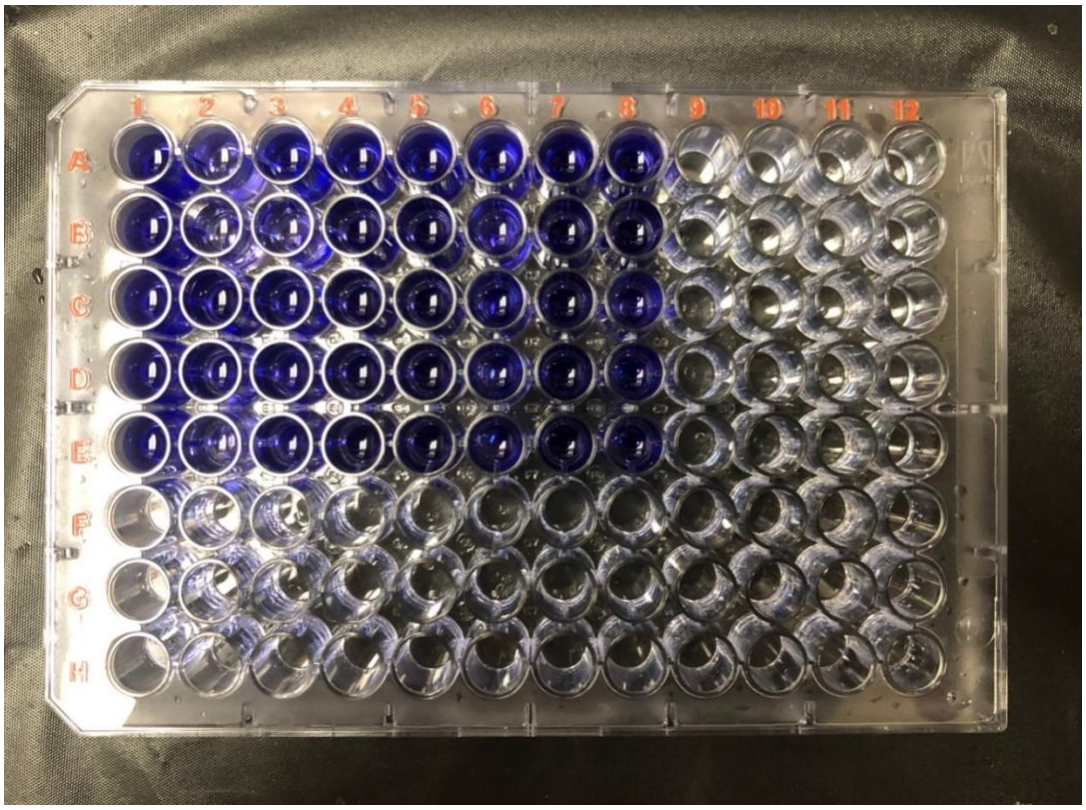

FIGURE 9. Crystal violet dye were added to each well for 30 min.

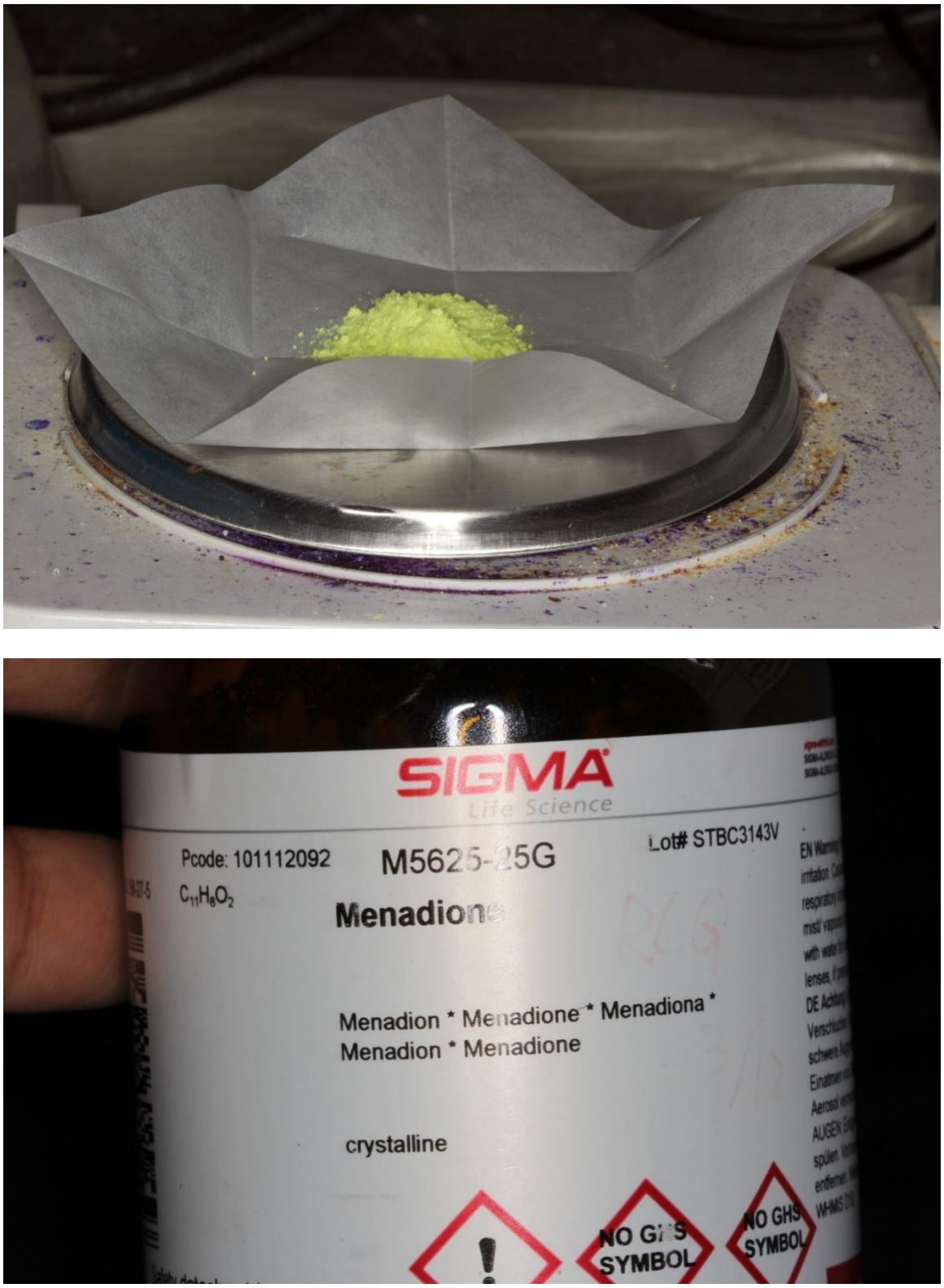

FIGURE 10. Preparation of XTT menadione reagent according to Pierce et al.[104]

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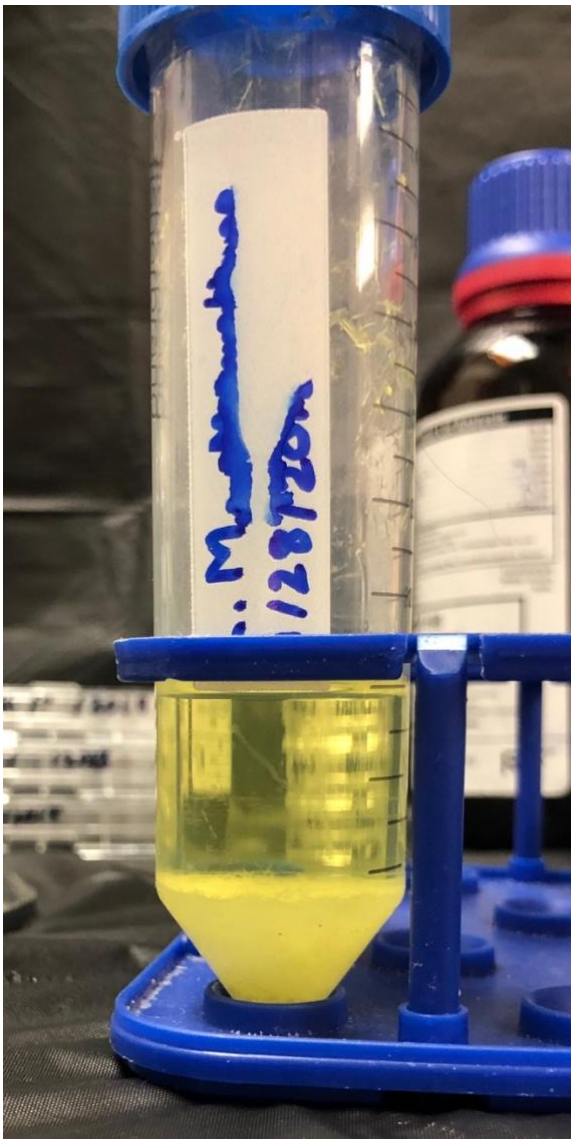


FIGURE 11. XTT reagent.

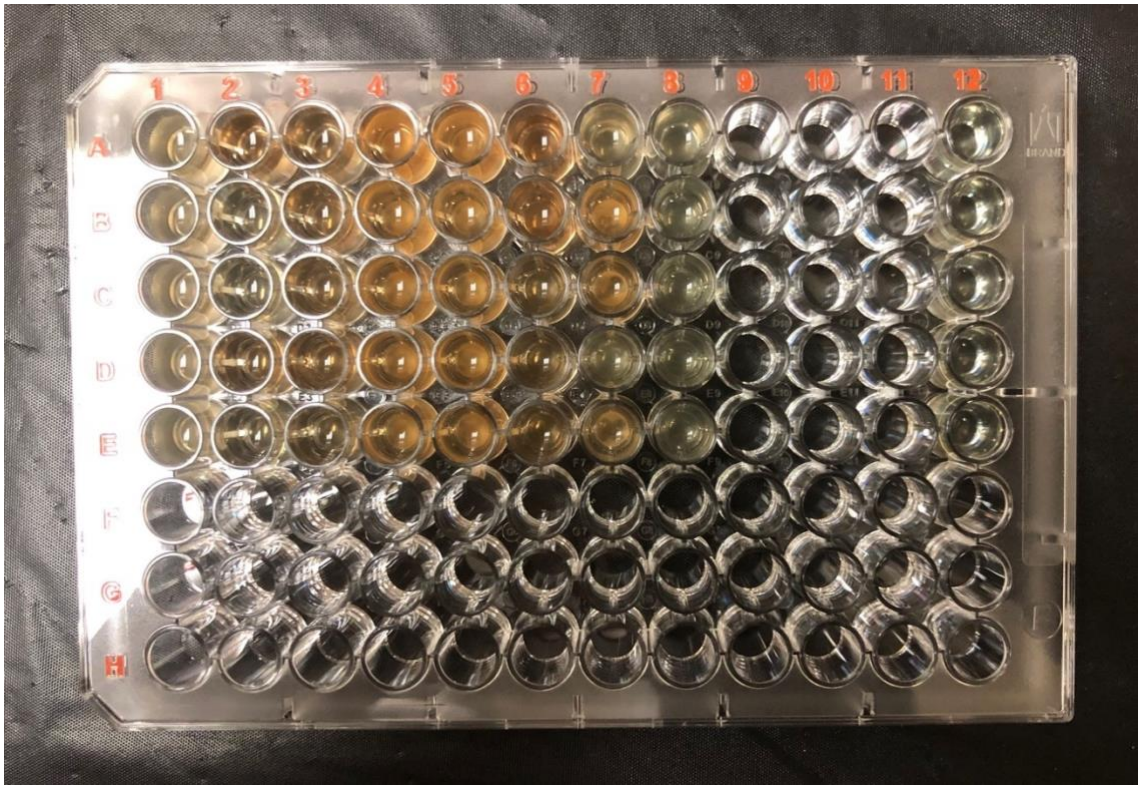


FIGURE 12. XTT reagent after 2-h incubation with control and experimental groups.

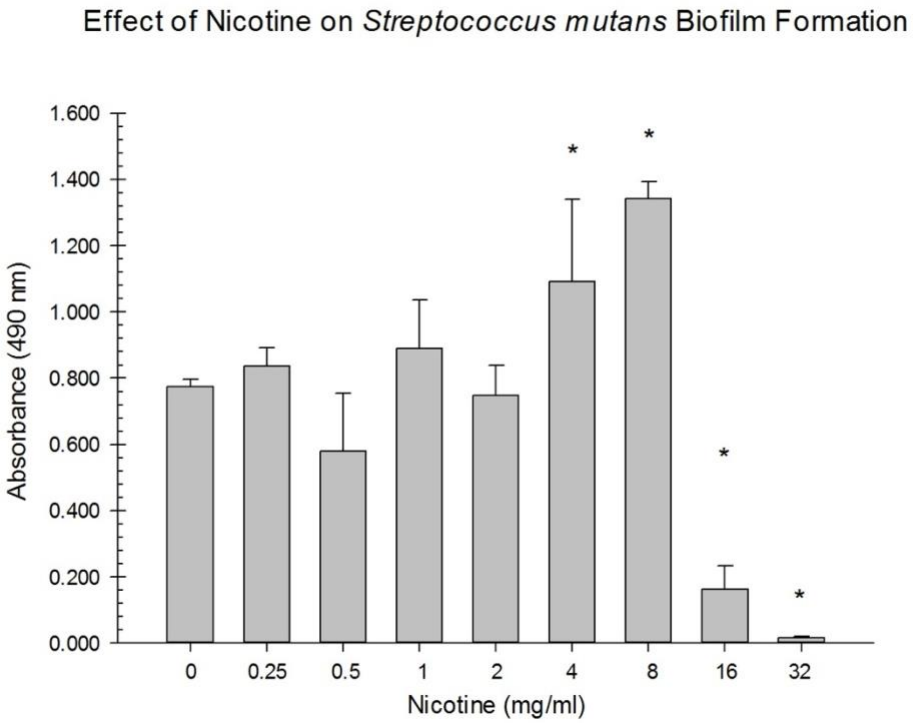
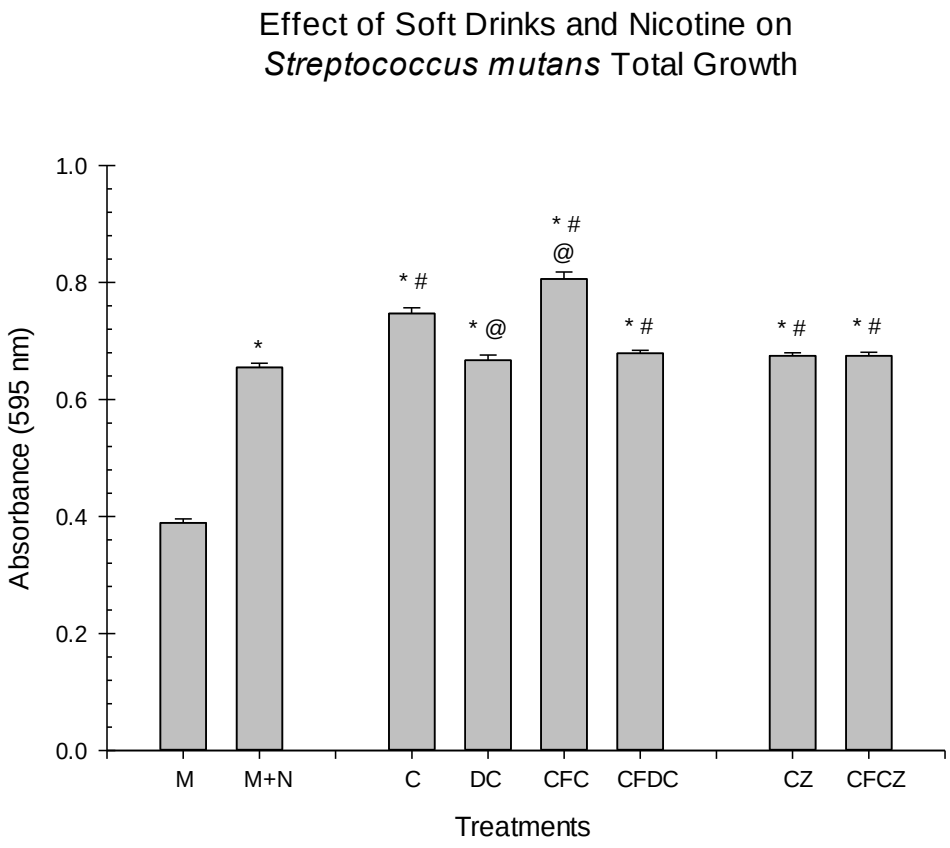
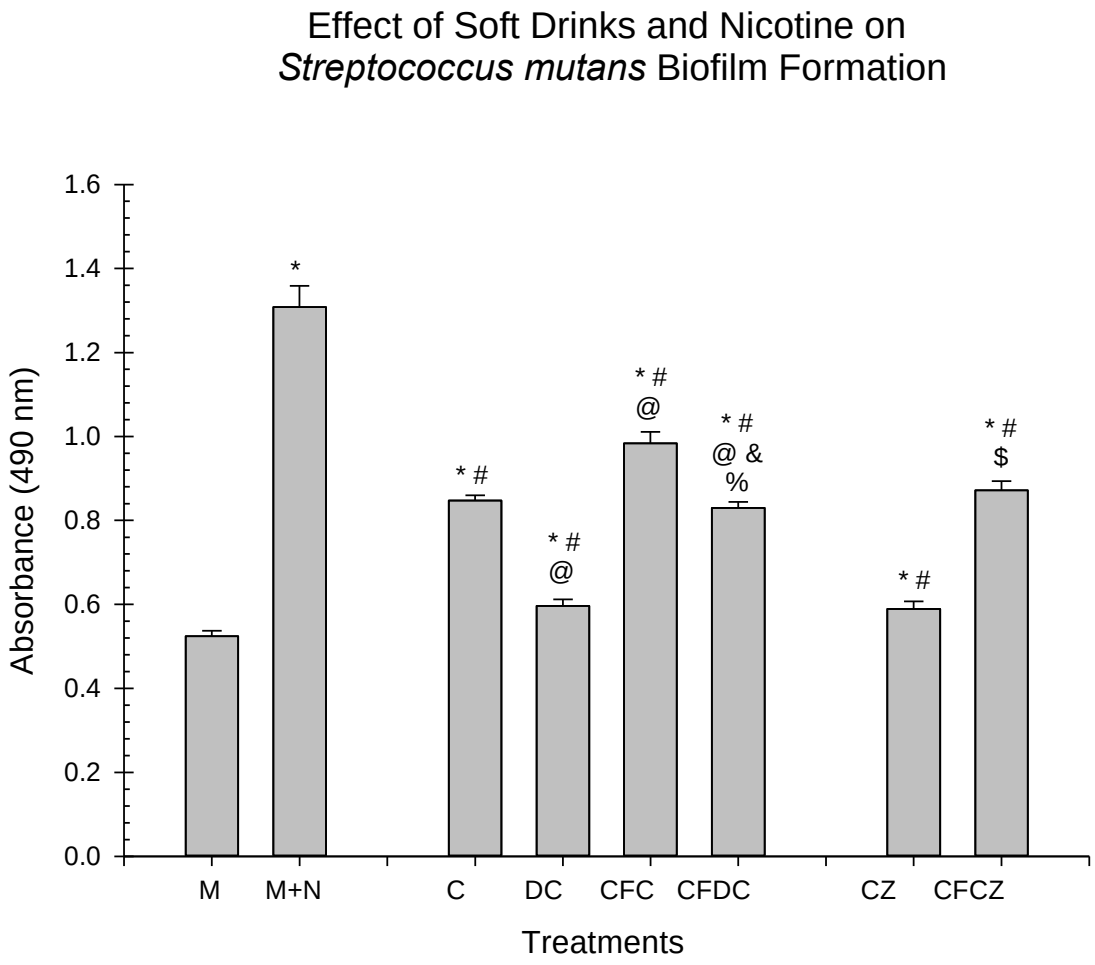


FIGURE 13. Preliminary experiment results.



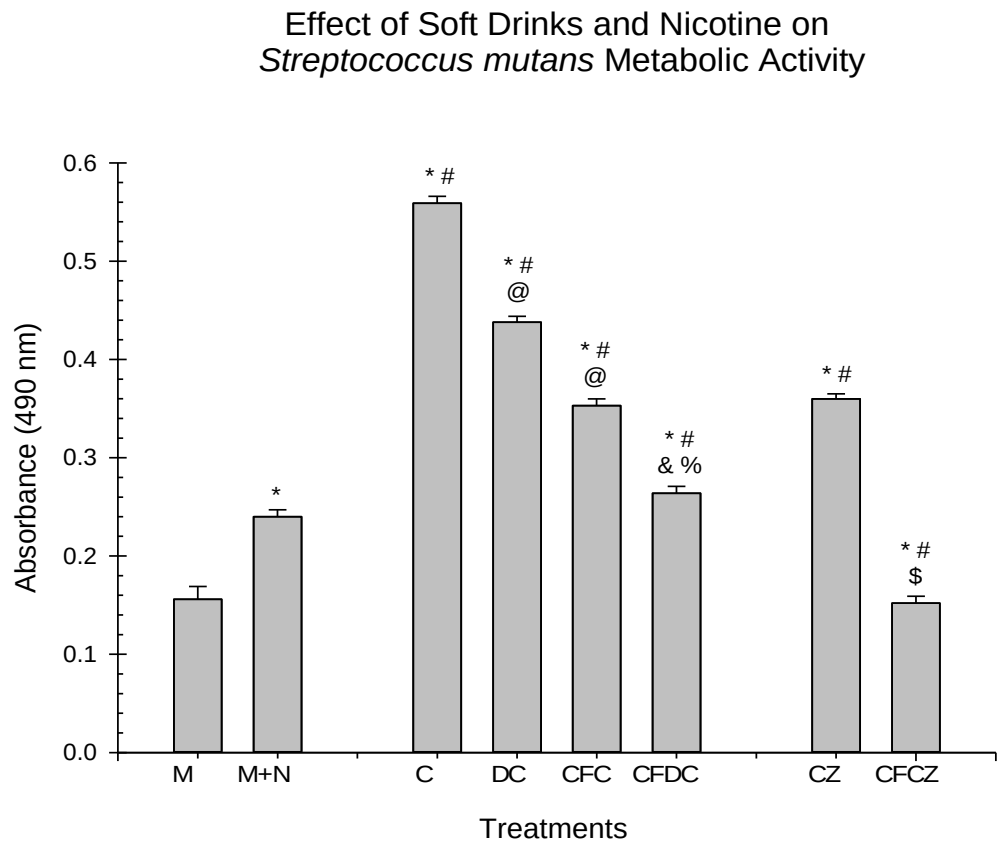
M: *S. mutans* + TSBS
M+N: *S. mutans* + TSBS + 8 mg/ml nicotine
C: *S. mutans* + 1:3 dilutions of Coke in TSBS + 8 mg/ml nicotine
DC: *S. mutans* + 1:3 dilutions of Diet Coke in TSBS + 8 mg/ml nicotine
CFC: *S. mutans* + 1:3 dilutions of Caffeine free Coke in TSBS + 8 mg/ml nicotine
CFDC: *S. mutans* + 1:3 dilutions of Caffeine free Diet Coke in TSBS + 8 mg/ml nicotine
CZ: *S. mutans* + 1:3 dilutions of Coke Zero in TSBS + 8 mg/ml nicotine
CFCZ: *S. mutans* + 1:3 dilutions of Caffeine free Coke Zero in TSBS + 8 mg/ml nicotine

FIGURE 14. Results of the effects of soft drinks and nicotine on *S. mutans* total growth. Asterisks indicate significant differences between the experimental groups compared with the control group (TSBS + *S. mutans*); # indicates significant differences with *S. mutans* in TSBS and nicotine, @ indicates significant differences between the groups compared with Coke.



M: *S. mutans* + TSBS
M+N: *S. mutans* + TSBS + 8 mg/ml nicotine
C: *S. mutans* + 1:3 dilutions of Coke in TSBS + 8 mg/ml nicotine
DC: *S. mutans* + 1:3 dilutions of Diet Coke in TSBS + 8 mg/ml nicotine
CFC: *S. mutans* + 1:3 dilutions of Caffeine free Coke in TSBS + 8 mg/ml nicotine
CFDC: *S. mutans* + 1:3 dilutions of Caffeine free Diet Coke in TSBS + 8 mg/ml nicotine
CZ: *S. mutans* + 1:3 dilutions of Coke Zero in TSBS + 8 mg/ml nicotine
CFCZ: *S. mutans* + 1:3 dilutions of Caffeine free Coke Zero in TSBS + 8 mg/ml nicotine

FIGURE 15. Results of the effects of soft drinks and nicotine on *S. mutans* biofilm formation. Asterisks indicate significant differences between the experimental groups and the control group (*S. mutans* + TSBS); # indicate significant differences between the groups and the *S. mutans* with TSBS and nicotine; @ indicates significant differences compared with Coke, and indicates significant differences compared with Diet Coke, and $ indicates significant differences compared with Coke Zero; and % indicate significant differences compared with caffeine free Coke.



M: *S. mutans* + TSBS
M+N: *S. mutans* + TSBS + 8 mg/ml nicotine
C: *S. mutans* + 1:3 dilutions of Coke in TSBS + 8 mg/ml nicotine
DC: *S. mutans* + 1:3 dilutions of Diet Coke in TSBS + 8 mg/ml nicotine
CFC: *S. mutans* + 1:3 dilutions of Caffeine free Coke in TSBS + 8 mg/ml nicotine
CFDC: *S. mutans* + 1:3 dilutions of Caffeine free Diet Coke in TSBS + 8 mg/ml nicotine
CZ: *S. mutans* + 1:3 dilutions of Coke Zero in TSBS + 8 mg/ml nicotine
CFCZ: *S. mutans* + 1:3 dilutions of Caffeine free Coke Zero in TSBS + 8 mg/ml nicotine

FIGURE 16. Results of the effects of soft drinks and nicotine on *S. mutans* metabolic activity. Asterisks indicate significant differences between the experimental groups and the control group (*S. mutans* + TSBS), # indicate significant differences between the groups and the *S. mutans* with TSBS and nicotine; @ indicates significant difference compared with Coke, and indicate significant differences compared with Diet Coke, and $ indicates significant differences compared with Coke Zero.

TABLE I

Detailed ingredients present in the various cola-flavored beverages studied[22]

Beverage (12 oz)	Ingredients	Sugars (amt/12 oz)	Caffeine (amt/12 oz)
Coca-Cola Classic	Carbonated water, high fructose corn syrup, caramel color, phosphoric acid, natural flavors, caffeine	39 g 0.11 g/mL	32 mg 0.09 mg/mL
Diet Coke	Carbonated water, caramel color, aspartame, phosphoric acid, potassium benzoate, natural flavors, citric acid, caffeine	0	42 mg 0.12 mg/mL
Coke Zero	Carbonated water, caramel color, phosphoric acid, aspartame, potassium benzoate, natural flavors, potassium citrate, acesulfame potassium, caffeine	0	32 mg 0.09 mg/mL
Caffeine Free Coca-Cola	Carbonated water, high fructose corn syrup, caramel color, phosphoric acid, natural flavors	39 g 0.11 g/mL	0
Caffeine Free Diet Coke	Carbonated water, caramel color, aspartame, phosphoric acid, potassium benzoate, natural flavors, citric acid	0	0
Caffeine Free Coke Zero	Carbonated water, caramel color, phosphoric acid, aspartame, potassium benzoate, natural flavors, potassium citrate, acesulfame potassium	0	0

TABLE II

Serial numbers of the soft drinks used in the study

Coke	2018-00721 206283062A
Diet Coke	2017-00776 209283011A
Coke Zero	2018-00722 209283131B
Caffeine free Coke	2014-02077 206282981B
Caffeine free Diet Coke	2017-00774 206282592B
Caffeine free Coke Zero	2014-02082 170283142A

TABLE III

Detailed description of the experimental groups

Description	Concentration
TSBS (Sterility control)	200 μl
TSBS + *S. mutans*	190 μl + 10 μl
S. mutans + TSBS containing nicotine	10 μl + 190 μl TSBS contains 8 mg/ml nicotine
S. mutans + 1:3 dilution of soft drinks + TSBS containing nicotine (Experimental)	10 μl + 190 μl of 1:3 dilutions of each soft drink and TSBS containing 8 mg/ml nicotine

TABLE IV

Overall descriptive statistics for *S. mutans* total absorbance by 1:3 dilution of soft drinks in the presence of 8 mg/ml nicotine

Variable	**N**	**Mean**	**SD**	**SE**	**Min**	**Max**
TSBS + *S. mutans*	15	0.389	0.026	0.007	0.328	0.418
TSBS + *S. mutans* + Nicotine	15	0.655	0.027	0.007	0.611	0.695
Coke	15	0.747	0.039	0.01	0.668	0.801
Diet Coke	15	0.667	0.034	0.009	0.603	0.708
Coke Zero	15	0.675	0.019	0.005	0.639	0.698
Caff. Free Coke	15	0.806	0.048	0.012	0.742	0.898
Caff. Free Diet Coke	15	0.679	0.021	0.005	0.643	0.711
Caff. Free Coke Zero	15	0.675	0.022	0.006	0.647	0.723

TABLE V

Overall descriptive statistics for *S. mutans* biofilm formation by 1:3 dilution of soft drinks in the presence of 8 mg/ml nicotine

Variable	**N**	**Mean**	**SD**	**SE**	**Min**	**Max**
TSBS + *S. mutans*	15	0.524	0.05	0.013	0.432	0.584
TSBS + *S. mutans* + Nicotine	15	1.308	0.198	0.051	0.987	1.619
Coke	15	0.848	0.048	0.012	0.757	0.918
Diet Coke	15	0.596	0.062	0.016	0.491	0.678
Coke Zero	15	0.589	0.068	0.018	0.434	0.669
Caff. Free Coke	15	0.984	0.103	0.027	0.819	1.183
Caff. Free Diet Coke	15	0.83	0.055	0.014	0.761	0.975
Caff. Free Coke Zero	15	0.872	0.084	0.022	0.72	1.012

TABLE VI

Overall descriptive statistics for *S. mutans* metabolic activity by 1:3 dilution of soft drinks in the presence of 8 mg/ml nicotine

Variable	**N**	**Mean**	**SD**	**SE**	**Min**	**Max**
TSBS + *S. mutans*	15	0.156	0.05	0.013	0.115	0.32
TSBS + *S. mutans* + Nicotine	15	0.24	0.026	0.007	0.207	0.296
Coke	15	0.559	0.029	0.007	0.513	0.593
Diet Coke	15	0.438	0.024	0.006	0.401	0.481
Coke Zero	15	0.36	0.021	0.005	0.331	0.392
Caff. Free Coke	15	0.353	0.026	0.007	0.301	0.395
Caff. Free Diet Coke	15	0.264	0.027	0.007	0.21	0.296
Caff. Free Coke Zero	15	0.152	0.027	0.007	0.109	0.196

TABLE VII

One-way ANOVA comparing the effect of soft drinks and nicotine on *S. mutans* metabolic activity and biofilm formation

Total Absorbance			
Comparison	Difference	SE	P-value
(Coke, Diet Coke, Coke Zero) vs (Caff. free Coke, Caff. free Diet Coke, Caff. free Coke Zero)	10.39	1.31	< .0001
(Coke, Caff. free Coke) VS (Diet Coke, Caff. free Diet Coke, Caff. free Coke Zero)	-9.94	1.39	< .0001

Biofilm Formation			
Comparison	Difference	SE	P-value
(Coke, Diet Coke, Coke Zero) vs (Caff. free Coke, Caff. free Diet Coke, Caff. free Coke Zero)	2.06	0.92	0.028
(Coke, Caff. free Coke) vs (Diet Coke, Caff. free Diet Coke, Caff. free Coke Zero)	-4.52	0.98	< .0001

Metabolic Activity			
Comparison	Difference	SE	P-value
(Coke, Diet Coke, Coke Zero) VS (Caff. free Coke, Caff. free Diet Coke, Caff. free Coke Zero)	10.32	0.63	< .0001
(Coke, Caff. free Coke) VS (Diet Coke, Caff. free Diet Coke, Caff. free Coke Zero)	-21.79	0.66	< .0001

DISCUSSION

Numerous studies have investigated the correlation between smoking and caries risk, and all concluded that nicotine enhances *S. mutan*s biofilm formation and biofilm metabolic activity.[27,28,103] Furthermore, in a study done by Huang et al., the results suggested that smoking can raise caries progression through promoting *S. mutans* biofilm formation on the tooth surface.[28] *S. mutans* is a normal flora species in the oral cavity that becomes harmful under certain conditions in the presence of fermentable carbohydrates.[7,40,105] In addition, previous studies have indicated the effects of sweetened drinks, particularly soda, on caries risk and progression.[106-108] Although soft drink consumption is widespread across the US, there has been a lack of evidence regarding its direct effects in the presence of nicotine on *S. mutans* biofilm formation and metabolic activity. In this study we aimed to investigate the effects of different soft drinks (regular, sugar-free, and caffeine-free) in the presence of nicotine on *S. mutans* activity on the formation of biofilm, which will help in patient education and disease prevention.

In this experiment we focused on exploring the impact of HFCS and caffeine: the two main components of soft drinks. Our hypotheses that the addition of sugar-containing cola-flavored products to *S. mutans* cultures in the presence of nicotine will increase bacterial biofilm growth, as well as increase *S. mutans* metabolic activity and that the addition of caffeinated soft drinks to *S. mutans* cultures will inhibit biofilm formation and growth were accepted based on the results.

Each single cigarette contains almost 1 mg of nicotine. While a slight amount will be absorbed into the bloodstream through the mucosal lining in the mouth, the remaining

nicotine will accumulate in the oral cavity. Research was performed to measure the amount of nicotine present in smokers' and non-smokers' saliva. The results showed that light-to-medium smokers' saliva contained 0 mg/ml to 1.33 mg/ml of nicotine while heavy smokers' saliva had 0 mg/ml to 2.27 mg/ml.[31] These studies were done *in vitro* and more *in-vivo* studies are necessary to define the amount of nicotine present in saliva and that absorbed in biofilm. If we assume that 1 mg/ml of nicotine is present in human saliva, then the caffeine concentration in soft drinks will be significant in inhibiting the formation of *S. mutans*.

Microtiter plates were used to conduct the experiment. Each column had a separate group, including a sterility group to assure the absence of contamination. The experiment was repeated three times with five samples per group per trial, and a random effect of multiple trials was used. Due to non-normality, a rank transformation was used prior to analysis. A one-way ANOVA and pairwise tests were used to compare the effect of different soft drinks in the presence of nicotine on *S. mutans* biofilm growth and metabolism.

Bacterial growth has two phases, planktonic and biofilm, where the second one is considered more important and the most favorable phase for oral bacteria to grow *in vivo* and cause disease. In addition, biofilm protein expression is not similar to planktonic protein expression.[109]

The results of this experiment demonstrated that HFCS increases both biofilm formation and metabolic activity, which supports previous findings that the presence of fermentable carbohydrates will elevate caries activity.[22,78,110] On the other hand, caffeine's effect on biofilm formation was not significantly greater than the control

group. However, its effect was considerably noticeable on metabolic activity. In both biofilm growth and metabolism as illustrated in Figure 15 and Figure 16, caffeine plays a role in increasing their rates, even though it is not as impactful as the role of HFCS. In each case, the results do not negate the hypothesis that caffeine has an inhibitory effect.

For soft drink selection, consumers should be conscious of the risks of each type of beverage and how they can cause systemic and dental problems. The most important conclusion from our point of view is the effects of soft drinks on dental biofilm establishment through the growth and metabolism of *S. mutans*. Caffeine inhibits biofilm formation and metabolism in addition to its psychoactive effect. Thus, if a smoker is consuming a soft drink, it is best to choose a caffeinated and sugar-free soda. This conclusion is based on our findings, where HFCS increased the biofilm formation and the metabolism of *S. mutans*, and caffeine inhibited them in the absence of HFCS.

The limitations of the present study can be summarized by noting: 1) A single strain of *S. mutans* was used where other strains could have different effects; 2) The study lacked a multi-species model; 3) The experiment lacked salivary components; and 4) The study used a fixed nicotine concentration.

SUMMARY AND CONCLUSION

In our study we concluded that sugar-free caffeinated soft drinks have shown strong effects in inhibiting *S. mutans* biofilm formation, as well as metabolic activity in the presence of nicotine. On the other hand, nicotine-induced *S. mutans* demonstrated increased biofilm formation and metabolic activity in the presence of HFCS and caffeine in soft drinks. Therefore, smokers with a high consumption of soft drinks should consider shifting to sugar-free and caffeinated versions in order to minimize their chance of developing dental caries by reducing biofilm formation.

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ABSTRACT

COMBINED EFFECTS OF SOFT DRINKS AND NICOTINE ON *STREPTOCOCCUS MUTANS* METABOLIC ACTIVITY AND BIOFILM FORMATION

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Objective: This study aimed to explore the combined effect of different types of soft drinks (regular, sugar-free and caffeine-free) and nicotine on the activity of *Streptococcus mutans*. Regular soft drinks have a large percentage of high-fructose corn syrup, which increases the activity of *S. mutans*. In turn, this is a high-caries risk compared with the effect of sugar-free soft drinks. In addition, nicotine exhibited a strong correlation to increased *S. mutans* biofilm formation.

Materials and methods: A tryptic soy broth (TSB) culture of *S. mutans* was grown overnight. The next day, 8 mg/ml of nicotine were diluted in TSB supplemented with 1.0-percent sucrose (TSBS). Then, a dilution of 1:3 of soft drinks in the nicotine-TSBS was prepared for each type of soft drink examined (cola, diet cola, cola zero, caffeine-free cola, caffeine-free diet cola, and caffeine-free cola zero). One hundred ninety µl of each dilution was pipetted into wells of a sterile 96-well flat bottom microtiter plate with 10 µl of the fresh overnight TSB culture of *S. mutans*. The microtiter plate was incubated for 24 h. A spectrophotometer was used to determine total growth absorbance and biofilm growth. The microtiter plate wells were washed, fixed and stained with crystal violet dye and the absorbance measured to determine biofilm formation. Metabolic activity was measured based on the ability of biofilm in reducing XTT to a water-soluble orange compound.

Results: There was a considerable effect for HFCS and caffeine, in the presence of nicotine, and their interaction in all measures: total growth, biofilm formation, and metabolic activity. One-way ANOVA was used for comparison.

Conclusion: Sugar-free caffeinated soft drinks demonstrated a strong effect in inhibiting *S. mutans* biofilm formation as well as metabolic activity in the presence of nicotine. On the other hand, nicotine-induced *S. mutans* exhibited increased biofilm formation and metabolic activity in the presence of HFCS and caffeine in soft drinks. Therefore, smokers with a high consumption of soft drinks should consider shifting to sugar-free and caffeine versions in order to minimize their chance of developing dental caries by reducing biofilm formation.

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