Effect of Commonly Prescribed Liquid Medications on *Streptococcus mutans* Biofilm, an in-vitro study

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ABSTRACT

Objective: This study addressed the effect of pediatric liquid antibiotic medications on *Streptococcus mutans* UA159. These suspensions commonly contain sugars such as sucrose to make them more palatable for children. The study was designed to evaluate the effects of oral liquid antibiotics on *Streptococcus mutans* growth and biofilm formation.

Study Design: A 24 hour culture of *S. mutans* was treated with various concentrations of liquid medications commonly prescribed to children for odontogenic or fungal infections - amoxicillin, penicillin VK, clindamycin, and nystatin. The study was conducted in sterile 96-well flat bottom microtiter plates. The minimum inhibitory and biofilm inhibitory concentrations (MIC/MBIC) of *S. mutans* were determined for each medication. *S. mutans* was cultured with and without the test drugs, the amount of total growth measured, the biofilms washed, fixed, and stained with crystal violet. The absorbance was determined to evaluate biofilm formation.

Results: Higher concentrations of amoxicillin, penicillin VK and clindamycin had decreased biofilm and overall growth than the control. The MICs were 1:2,560 (1.95 ug/ml), 1:2,560 (1.95 ug/ml) and 1:40 (9.375 ug/ml), while the MBIC were 1:640 (7.8 ug/ml), 1:1,280 (3.9 ug/ml) and 1:20 (18.75 ug/ml), respectively. Lower concentrations provided increased biofilm and overall growth. Nystatin induced significantly more biofilm and overall growth than the control at all concentrations.

Conclusion: At high concentrations, approximately at the levels expected to be present in the oral cavity of children, amoxicillin, penicillin, and clindamycin inhibited *S. mutans* biofilm and overall growth due to their antibiotic activity, while at lower concentrations the three antibiotics demonstrated an increase in biofilm and growth. The increase in *S. mutans* biofilm and overall growth is most likely attributed to the sugar content in the medications. Nystatin provided an increase in biofilm and growth at each concentration tested.

Key Words: pediatric medications, early childhood caries (ECC), *S. mutans*, biofilm,

INTRODUCTION

Getting children to take medications can be a struggle due to the naturally aversive taste. Oral administration of liquid suspensions is popular and accepted by parents and children due to the ease of administration. Pharmaceutical companies have found that adding sucrose to flavor medications can mask the unpleasant taste.[1] However, the sucrose in these medications can have a detrimental effect on the dentition. Some medications have been found to have as much as 70 gm of sucrose/100 ml.[2] In addition to the cariogenic potential of sucrose, many medications are also acidic.[3] It has been shown that sugared medications can elicit a longer and lower drop in pH following administration than non-sugared medications.[1] This presents a double insult of acidity to teeth. Medications are usually given in small doses throughout the day coating the teeth with fermentable carbohydrates more frequently than a normal dietary intake.[3] The frequency of consuming carbohydrates has been shown to have an effect on the etiology of dental caries with higher frequency having a correlation with increased caries.[4]

Sucrose is commonly used to flavor medications because it is cheap, non-hygroscopic, and easy to process.[4] Companies have also used fructose and glucose, which are equally detrimental to dental health.[4] Sucrose is one of the most cariogenic carbohydrates because it is easily fermentable and it serves as a substrate for formation of extracellular (EPS) and intracellular (IPS) bacterial polysaccharides of dental biofilms.[5,6] Development of caries is dependent on the formation of the oral biofilm which contains elevated levels of *S. mutans.*[7] Caries development is dependent on early colonization of these bacteria, followed by biofilm formation. They are recognized as determinants for the caries process[7]. Biofilms are orientated groups of microorganisms that attach to each other or to a surface. They are enclosed in an EPS, which promotes bacterial accumulation to the tooth surface.[5] EPS also influence the physical and biochemical properties of biofilms. IPS serve as an endogenous source of carbohydrates. Biofilms have been found to form faster in the presence of sucrose.[5] Biofilms become more complex physiologic structures in the presence of fermentable carbohydrates.[8] Biofilms with sucrose also demonstrated lower pH levels, higher mutans streptococci and lactobacilli counts, and enhanced cariogenicity than did those formed in the absence of sugar.[5] Multiple strains of *S. mutans* have been isolated and recognized from carious lesions.[9] Carbohydrates in biofilm are metabolized by bacteria, producing lactic acid which causes dissolution of minerals, calcium and phosphate from teeth.[9,10,5] A fall in plaque pH caused by bacterial fermentation of carbohydrates

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causes a shift in the equilibrium concentration, leading to enamel erosion.[3] Acid production is considered one of the most important events in the pathogenesis of dental caries.[3] The acid produced from the bacteria weaken the teeth. This makes the teeth more susceptible when acidic products are put on the teeth.[3] Being one of the most cariogenic bacteria in the oral cavity, *S. mutans* multiplies and competes for nutrients in the dental biofilm.[5] *S. mutans* metabolizes carbohydrates in the biofilm into large amounts of lactic acid.[5,9] Since *S. mutans* is acid tolerant and able to grow at a pH of 4.0, this increased acidic environment inhibits growth of other bacterial species and favors the growth of *S. mutans*.[5,9]

Although dental caries is a multi-factorial disease, it has been shown that fermentable carbohydrate intake and frequency of intake are two major factors in the etiology of dental caries.[4] Pediatric medications may not contact the teeth for an extended period of time when consumed, but many of them are given before bed and/or multiple times throughout the day.[1] Tooth brushing and rinsing is minimal after administration due to parents concern of disrupting the child's sleep and/or interfering with the medication just given.[1] There is also a lack of saliva production and mastication movements during the night, which increases the cariogenic potential of medications.[1,11] Liquid medications also take longer to clear the mouth than capsules or tablets due to binding to hard and soft tissues.[1] Some studies have shown that approximately 75% of medications analyzed have endogenous pHs lower than the critical pH of 5.5, ranging from pH 3.6-5.3. Antibiotics were found to have a mean pH of 5.5.[1,3] Many drug labels omit the type of sugar and concentration, but simply state the presence or absence of a sugar. Neiva et al, (2001) found that sucrose was present in 70% of different brands of pediatric antibiotic suspensions.[1] Pediatric medications have been shown to have a mean sugar content of approximately 50%.[1,3]

The American Academy of Pediatric Dentists (AAPD) defines early childhood caries (ECC) as the presence of one or more decayed, missing or filled tooth surfaces in any primary tooth.[12] ECC is a tremendous health concern worldwide and affects 28% of children in the United States.[13] In 1996 Ramos-Gomez et al. estimated the cost to treat a child with 2 to 5 lesions or 16 to 20 lesions was $408 and $1725, respectively.[14] Restorative care for severe early childhood caries is the primary cause of childhood hospitalization for treatment under general anesthesia.[13] While ECC may lead to clear immediate problems such as pain, fever, malnutrition, and infections, it can also have long term effects such as malocclusion, phonetic

issues, low self-esteem, and possible systemic issues.[15] The link between caries and fermentable carbohydrate frequency of consumption has been clearly described.[3] While parents aim to monitor and limit the amount of sugar intake, most are unaware of hidden added sugars found in fruit juices, sports drinks, and liquid medications.

Determining the effect of commonly prescribed oral medications on *S. mutans* biofilm formation could demonstrate the cariogenicity of these medicines. This would allow for further education of healthcare providers when prescribing medications. It would also be beneficial in demonstrating that sugar-free medications should be used whenever possible. These sugar-free medications could be artificially sweetened with xylitol. In order to help discern this association between medications containing sucrose and caries, the purpose of this study was to evaluate the ability of commonly prescribed medications containing sucrose to affect *S. mutans* biofilm formation.

MATERIALS AND METHODS

Bacterial Strain and Growth and Antibiotic Suspensions

S. mutans strain UA159 (ATCC 700610) was used due to its completely sequenced genome.

[9,10,16] The strain was stored at -80°C in tryptic soy broth (TSB, Acumedia, Baltimore, MA) with

20% glycerol until used. Mitis Salivarius Sucrose Bacitracin (MSSB, Anaerobe Systems,

Morgan Hill, CA) agar plates were used to initially grow the strains. Unless otherwise stated,

TSB was used and the growth conditions were 5% CO_2 at 37°C.[12] All medications were

obtained from Sigma Chemical Co., St. Louis, MO. The starting concentrations were 500 ug/ml,

500 ug/ml and 75 ug/ml for amoxicillin, penicillin VK and clindamycin, respectively. Table 1

demonstrates the sugar contents and supplemental ingredients of the medications tested.

Sugar Content of Medications

Drug	Contents	Sucrose Content (g/5 ml)
Amoxicillin	anhydrous citric acid, colloidal silicon dioxide, refrachessement, FD&C Red 4, sodium benzoate, sodium citrate, sucrose, xantham gum[17]	4.024[17, 18]
Penicillin VK	cherry flavor, FD&C Red #40, saccharin sodium, sodium benzoate, sucrose[18,20]	2.4[18]
Clindamycin	artificial cherry flavor, dextrin, ethylparaben, pluronic F68, simethicone, sucrose[18,19]	1.85[18]
Nystatin	Alcohol, artificial wild cherry flavor, banana flavor, D&C yellow #10, FD&C red #40, glycerin, USP, Magnesium, Aluminum, Silicate, Methylparaben, NF, Potassium, Phosphate dibasic, propylene glycol,	3[18]

	propylparaben, purified water, sucrose 33.5%, citric acid	

Table 1. The ingredients and sucrose content (g/5 mL) found in penicillin, amoxicillin, clindamycin, and nystatin. For example, amoxicillin has a sucrose content of 4.024 g/5 mL with other ingredients listed.

Biofilm Formation

To determine the amount of biofilm formation, an overnight *S. mutans* culture (10^6 CFU/ml) in TSB was treated with various concentrations of the medication suspensions. The medications were diluted in TSB (190 ul total volume) and 10 ul of the overnight culture of *S. mutans* was added and incubated for 16 h in 5% CO_2 at 37°C in sterile 96-well microtiter plates.[10,21] The total absorbance at 595 nm was determined for MIC calculations. Biofilm in the original plate was washed twice with saline, fixed with 10% formaldehyde (Sigma) for 30 min, washed twice again with saline, and stained with 0.5% crystal violet for 30 min.[10] After washing biofilm three times with saline, crystal violet was extracted from biofilm cells by addition of 200 ul of 2-propanol (Fisher Scientific, Co., Fair Lawn, NJ) for 30 minutes.[10] The extract was diluted 1:5 with 2-propanol and the amount of crystal violet released was determined by measurement at 490 nm using 2-propanol as a blank control.[10]   Controls included TSB with and without bacteria.

Statistical Analyses

Comparisons against the TSB control biofilm will be made by determining if the treated biofilm to TSB control biofilm was significantly different. Comparisons among groups were made using one-way ANOVA, followed by pair-wise comparisons if the overall test indicated a significant difference among groups.

RESULTS

Nystatin induced significantly more biofilm and overall growth than the control at all concentrations (Figs. 1 and 2). Higher concentrations of amoxicillin had significantly decreased biofilm and overall growth than the control. At a dilution of 1:640 of amoxicillin there was a shift in growth from inhibition to increased biofilm, demonstrating the MIC at the 1:2560 dilution and the MBIC at the 1:640 dilution with respective concentrations of 1.95 and 7.8 ug/mL (Table 2). At lower concentrations, amoxicillin had significantly increased biofilm and overall growth. At higher concentrations, penicillin had significantly decreased biofilm and overall growth than the control. At a dilution of 1:640 of penicillin there was a shift in growth from inhibition to increased biofilm, allowing the detection of the MIC and MBIC (Figs. 1 and 2). The MIC was observed at the 1:2560 dilution with a concentration of 1.95 ug/mL. The MBIC was at the 1:640 dilution with a concentration of 7.8 ug/mL (Table 2). At lower concentrations, penicillin provided significantly increased biofilm and overall growth than the control. At higher concentrations, clindamycin provided significantly decreased biofilm and overall growth (Figs. 1 and 2). There was a shift from inhibition to increased growth at the 1:40 dilution, signifying the MIC at the 1:40 dilution with a concentration of 9.375 ug/mL and the MBIC at the 1:20 dilution with a concentration of 18.75 ug/mL (Table 2).

DISCUSSION

Nystatin exhibited significant consistent positive overall and biofilm growth as compared to the control. This is can be attributed to the fact that nystatin is not an antibiotic, but an anti-fungal. There is a large concentration of sucrose in the nystatin suspension and the increase in biofilm can be attributed to the large amount of sucrose (Table 1).

Amoxicillin biofilm and overall growth was significantly lower than the control at the higher concentrations. This is consistent with the antibacterial properties amoxicillin has. There is a shift from inhibition of biofilm and overall growth to increased biofilm and overall growth beginning at the 1:640 dilution. This indicates that the MIC is at the 1: 2560 dilution with a concentration of 1.95 ug/mL. At this point biofilm growth with amoxicillin becomes significantly increased. The MBIC was at the 1:640 dilution with a concentration of 7.8 ug/mL (Table 2). With the lower concentrations of medication the overall and biofilm growths are significantly increased, most likely due to the amount of sucrose present.

Compared to the control, a significant decrease in penicillin biofilm and overall growth was seen at higher concentrations. This is most likely attributable to the antibacterial properties of the medication. There is a shift from inhibition of biofilm and overall growth to increased biofilm and overall growth at dilution 1:640. This indicates that the MIC is at the 1: 2560 dilution with a concentration of 1.95 ug/mL. The MBIC was at the 1:640 dilution with a concentration of 7.8 ug/mL (Table 2). At this point the biofilm growth with penicillin becomes significantly increased. With the lower concentrations of medication the overall and biofilm growths are significantly increased. This can most likely be attributed to the amount of sucrose in the suspension.

Clindamycin biofilm and overall growth, as compared to the control, was significantly lower at higher concentrations. The cause for the inhibition of biofilm growth is due to the strong antibacterial properties of the clindamycin. There is a shift from biofilm and overall growth being inhibited to positive growth at the 1:40 dilution, indicating the MIC and MBIC. The MIC was at the 1:40 dilution with a concentration of 9.375 ug/mL (Table 2). The MBIC was found to be at the 1:20 dilution with a concentration of 18.75 ug/mL (Table 2). At lower concentrations of the medication there is significantly more overall and biofilm growth.

There is a delicate relationship between increasing and decreasing biofilm formation with these medications. Although penicillin, amoxicillin, and clindamycin are antibiotics with

activity against *S. mutans*, there is a point in the dilution scheme where the sugar content has a greater effect than the antibacterial effect, causing an increase in biofilm development. This could be due to the differing anti-bacterial mechanisms of each medication. If used at high enough concentrations, there seems to be a preventative effect with biofilm formation. A preliminary study in this laboratory demonstrated that *S. mutans* had significantly increased biofilm at sucrose concentrations from 1.5-192 mg/ml with an optimum amount of biofilm at 12 mg/ml of sucrose (L. Hinds, manuscript in preparation). We demonstrated MBIC and the associated sucrose concentrations for amoxicillin, penicillin VK and clindamycin were 1:640 (1.26 mg/ml), 1:1,280 (0.375 mg/ml) and 1:20 (18.5 mg/ml), respectively (Table 2). Nystatin at the highest concentration tested had a sucrose concentration of 60 mg/ml (Table 1). There are many factors that make it difficult to discern if these pediatric medications directly contribute to the initiation of caries. One of them may be the variability among humans with respect to salivary flow. Salivary rate decreases during sleep, slowing the clearance of the liquid from the oral cavity.[11] Since there are individual differences in salivary flow, it is hard to know an exact concentration the medications would be present in the mouth at any one point in time after administration. A range of concentrations was tested in the present study with the amount of biofilm formation varying among concentrations. These medications are also prescribed in varying concentrations, such as 250 or 400 mg/5 mL. These different concentrations will have a different balance between sucrose and antibiotic, potentially making one more cariogenic than the other.

A study conducted by Jarvinen, et al found the MIC for pure amoxicillin and pure penicillin when used in the presence of *S. mutans* to be 0.063 and 0.031 ug/ml, respectively.[23] Teng, et al found the MIC for pure clindamycin when used in the presence of *S. mutans* to be ≤ 0.12 ug/ml.[24] From this experiment in the presence of antibiotic suspensions containing sucrose the MIC were 1.95, 1.95 and 9.4 ug/ml for amoxicillin, penicillin, and clindamycin, respectively (Table 2). When comparing the difference in MIC it can be shown that approximately 30x more amoxicillin must be used, when given in the presence of sucrose, to obtain inhibition of *S. mutans* growth. There is approximately a 60 fold increase in the amount of penicillin that needs to be given in the presence of sucrose to obtain the same inhibition of *S. mutans*. Comparing the MIC of clindamycin from the two studies, it can be deduced that approximately 77 times more

clindamycin must be given (when given in the presence of sucrose) to obtain inhibition of *S. mutans* growth. The balance between antibiotic and sucrose content in these medications appears to have an important role. Although the role is not completely clear it appears that with increase sucrose content there must be an increased antibiotic content. This increase in antibiotic isn't completely medically necessary to treat the infections at hand, but necessary to allow the suspension to achieve functionality. This could increase GI upset commonly seen with antibiotics and also be contributing to antibiotic resistance with medically unnecessary amounts of antibiotics being given.

It appears that these liquid medications containing sucrose increase *S. mutans* biofilm formation. The AAPD classifies children with visible plaque in a category of "high caries risk". Thus it can be deduced with an increased biofilm the chance for caries increases as well. Parents of children with special health care needs that are on medications unremittingly or any child chronically taking a medication, should be informed and educated on the sucrose content and potential carious effect.

CONCLUSION

The results of this study demonstrated an increase in *S. mutans* biofilm formation by commonly prescribed liquid antibiotic medications. Although it cannot be determined whether these medications cause caries, it can be said they increase biofilm formation, which in turn increases the chance for caries and increased *S. mutans* development.
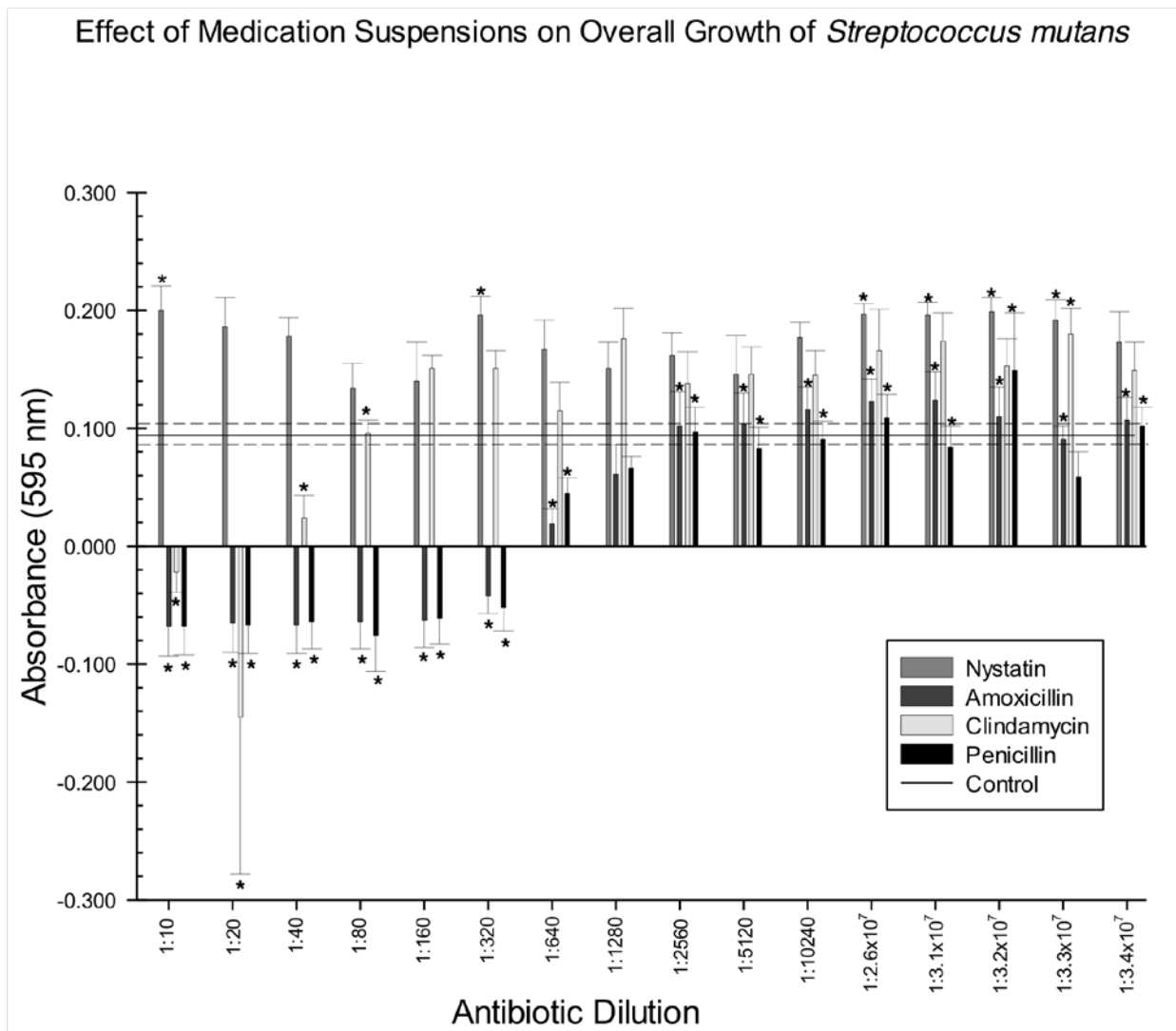
Figure 1. Effect of medication suspensions on overall growth of *Streptococcus mutans*. Each bar represents the ratio of biofilm formation for a specific dilution of the indicated medicine compared to the TSB control. For example, nystatin at its first concentration significantly increased biofilm formation compared to the control. Asterisks indicate statistically significant differences compared to control values ($p<0.05$). Starting concentrations were 500, 500 and 75 ug/ml for amoxicillin, penicillin, and clindamycin, respectively.
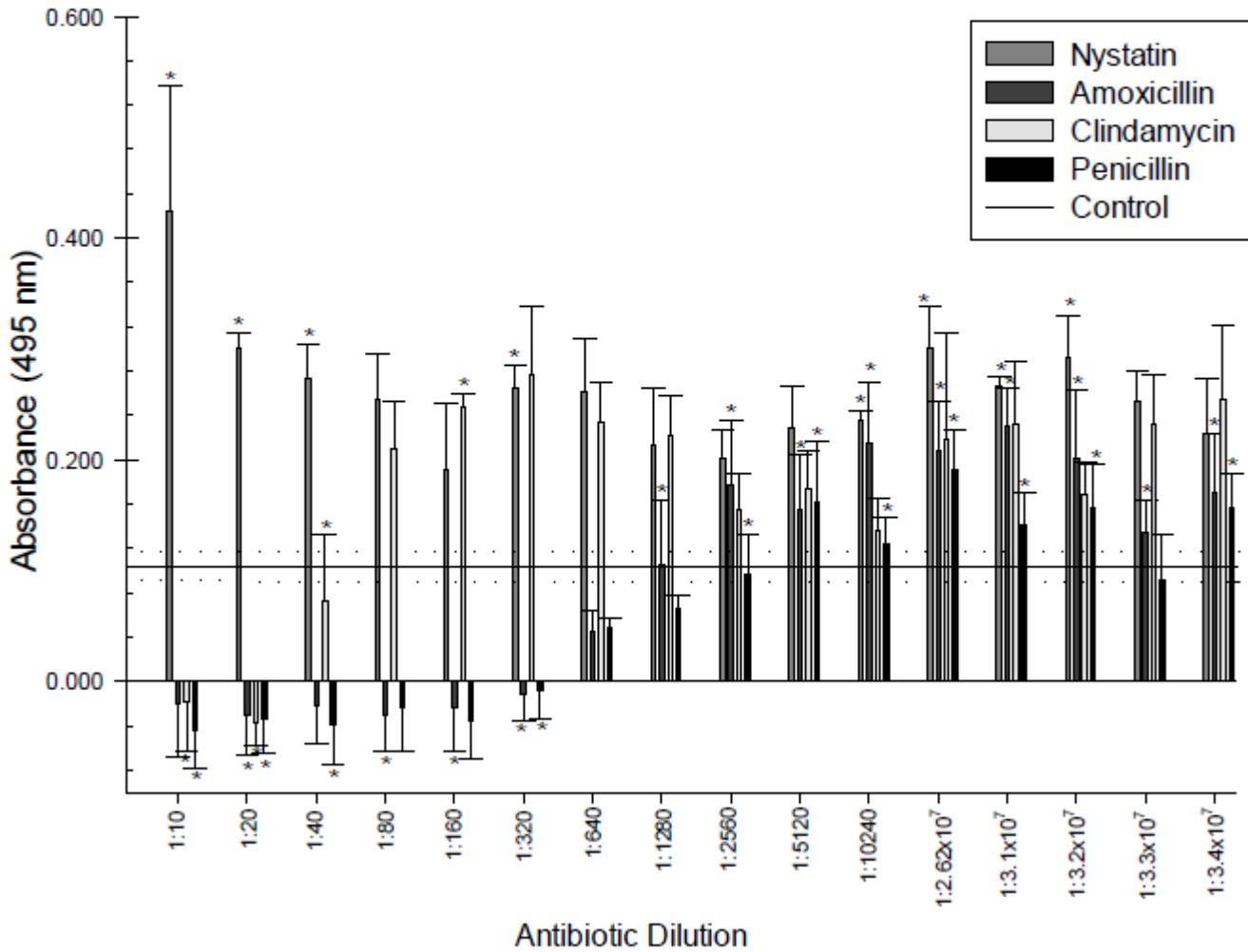
Figure 2. Effect of medication suspensions on biofilm growth of *Streptococcus mutans*. Each bar represents the ratio of biofilm formation for a specific dilution of the indicated medicine compared to the TSB control. For example, nystatin at its first concentration significantly increased biofilm formation compared to the control. Asterisks indicate statistically significant differences compared to control values (p<0.05). Starting concentrations were 500, 500 and 75 ug/ml for amoxicillin, penicillin, and clindamycin, respectively.

Medication	MIC	MBIC
Amoxicillin	1:2,560; 1.95 ug/mL	1:640; 7.8 ug/mL
Penicillin	1:2,560; 1.95 ug/mL	1:1,280; 3.9 ug/mL
Clindamycin	1:40; 9.375 ug/mL	1:20; 18.75 ug/mL

Table 2. Minimal inhibitory concentrations (MIC) and minimal biofilm inhibitory concentrations (MBIC) of clindamycin, amoxicillin, and penicillin on overall growth and biofilm growth of *Streptococcus mutans*. Dilutions for each concentration are included as well. For example, the MIC of Amoxicillin was found at the 1:2,560 dilution with a concentration of 1.95 ug/mL. The MBIC of amoxicillin was found at the 1:640 dilution with a concentration of 7.8 ug/mL.

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