

NICOTINE: ITS STIMULATING AND INHIBITORY EFFECTS ON ORAL MICROORGANISMS

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ABSTRACT

Tobacco users are much more susceptible to dental caries and periodontal diseases than non-tobacco users. Research suggests that this increased susceptibility may be due in part to nicotine, a primary active component of tobacco. Five bacterial species and one yeast species commonly found in the human oral cavity, *Lactobacillus casei*, *Actinomyces viscosus*, *Actinomyces naeslundii*, *Rothia dentocariosa*, *Enterococcus faecalis*, and *Candida albicans* respectively, were utilized to investigate if any correlation existed between exposure to various concentrations of nicotine ranging from 0 to 32 mg/ml and the growth of each microorganism. The minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC), and planktonic growth were measured. The MIC was determined to be 16 mg/ml for all organisms except *E. faecalis*, which had an MIC of 32 mg/ml. Nicotine had a varying effect on planktonic growth across the different species. A distinct upward trend in biofilm formation was found in *A. viscosus*, *L. casei*, *E. faecalis*, and *C. albicans* through 8 mg/ml. Nicotine also enhanced *R. dentocariosa* biofilm formation in all concentrations through 8 mg/ml but was most enhanced at 1 mg/ml. Alternatively, *A. naeslundii* exhibited a complete downward trend through 32 mg/ml. The MBIC was found to be 16 mg/ml in all organisms studied. These findings further support research suggesting that the increased susceptibility to oral health diseases experienced by tobacco users may be caused in part by an upregulation in biofilm formation of these oral pathogens.

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KEYWORDS

- dental caries
- periodontal disease
- biofilm
- nicotine
- tobacco
- *Lactobacillus casei*
- *Actinomyces viscosus*
- *Actinomyces naeslundii*
- *Rothia dentocariosa*
- *Enterococcus faecalis*
- *Candida albicans*

INTRODUCTION

Periodontal disease is a condition that causes inflammation of the gums including diseases such as gingivitis, in which gums become swollen and bleed easily. Periodontitis, another form of periodontal disease, is an advanced form of gingivitis

in which the gums pull away from the teeth and form pockets where dental plaque begins to grow. This infection can lead to both severe tissue and bone damage due to the increased exposure to previously protected areas. Periodontal disease is also

closely linked with dental caries. The occurrence of both periodontal disease and dental caries can be associated with several factors. Some of the most common risk factors include smoking, diabetes, autoimmune disorders, genetics, and obesity (14). The use of tobacco products remains the largest risk factor for periodontal disease. Environmental tobacco smoke has been associated with the increased likelihood of caries formation in children (2). Recent studies in this laboratory demonstrated increased biofilm formation of *S. mutans* with increasing nicotine concentrations (20). The average nicotine content in a single cigarette has been found in amounts up to 2 mg (12). A recent study tested over 40 smokeless tobacco products, such as snuff and chew, and it was discovered that they contained anywhere from 3.6–25.3 mg of nicotine per gram of smokeless tobacco (33). Nicotine contents of cigars have been found to vary greatly, ranging from 5.9 mg per cigar to 335.2 mg per cigar (18). Studies have demonstrated that when smoking cigarettes, an average of approximately 1 mg of nicotine per cigarette is absorbed by the body, however, the amount of nicotine within oral biofilm of smokers has not yet been explored (5). This study used the following opportunistic pathogens, commonly found in the human oral cavity, to investigate possible effects on growth and biofilm formation.

LACTOBACILLUS CASEI

Lactobacillus casei is a Gram-positive, facultative anaerobic bacterium. They are nonmotile and have a rod shape. Like other strains of lactobacilli, *L. casei* produces a significant amount of lactic acid, allowing it to remain viable under various pH levels. Poor diet, weak tooth enamel, previous plaque buildup, and acidic conditions contribute to an optimal environment for *L. casei* to thrive (22). *L. casei* alone

lacks adequate strength to adhere to tooth enamel, however, when co-cultured with *Streptococcus mutans*, *L. casei* gains the ability to synthesize glucans thus improving tooth enamel adhesion. Because of this, *L. casei* along with *S. mutans* have been found to be the most prevalent bacteria leading to dental caries (19).

ACTINOMYCES VISCOSUS

Actinomyces viscosus is a facultative anaerobic, Gram-positive bacterium that is often isolated from the oral cavity, especially from subgingival plaque and along the teeth and gums. *A. viscosus* is rod-shaped and filamentous. Distinct characteristics of the filamentous shape include branching with swollen, rounded, or clavate ends (7). *A. viscosus* is a severely cariogenic bacterium that acts as an agent in promoting and initializing carious lesions (15). Studies have shown that the bacterium has adhesins which allow it to bind to complementary receptors on a substrate. *A. viscosus* binds to salivary acidic proline-rich proteins (PRPs) adsorbed onto the apatitic surfaces of the tooth. Cryptic segments exposed in adsorbed molecules are identified by the adhesins, which allow *A. viscosus* to efficiently attach to teeth while planktonically suspended in saliva (1).

ACTINOMYCES NAESLUNDII

Actinomyces naeslundii is a Gram-positive bacterium characterized by its rod shape and prominent fimbriae that allow it to bind to the tooth surface. *A. naeslundii* is facultatively anaerobic, nonmotile, non-spore forming, and commonly grows in the oral cavity. (6) *A. naeslundii* is also a major component of dental plaque and it is known to cause dental caries, root canal infections, and periodontal disease (27). It is one of the specific species of the genus *Actinomyces* capable of causing the chronic bacterial infection, actinomycosis,

in humans (8). *A. naeslundii* plays a role in actinomycosis by initiating infections in carious teeth, as well as causing infections in places where the mucosal integrity of the tooth is compromised (32). These infections are the precursor of the actinomycosis, however, the bacterium itself exhibits very low pathogenic properties. These properties are enhanced by the presence of microorganisms such as species of *Prevotella*, *Staphylococcus*, and *Streptococcus* (35).

ROTHIA DENTOCARIOSA

Rothia dentocariosa is a facultative anaerobic, Gram-positive, pleomorphic bacterium that is commonly found in either filamentous or coccoid forms (24). It is common in the oral cavity, and has been associated with many types of bacterial infections. Although *R. dentocariosa* was first isolated in dental caries, endocarditis is the most common infection caused by the organism. Cases have also been reported associating *R. dentocariosa* with intrauterine fetal death (16,25,28). *R. dentocariosa* is commonly associated with dental caries and periodontal disease. Studies have demonstrated the ability of *R. dentocariosa* to induce the release of TNF- α by macrophages, thereby eliciting inflammatory responses within the gingiva (36).

ENTEROCOCCUS FAECALIS

Enterococcus faecalis is a facultative anaerobic, Gram-positive cocci bacterium that is typically found in pairs or short chains. The most common locations for this microorganism in humans are the gastrointestinal tract, the vagina, and the oral cavity. *E. faecalis* is considered to be the most prevalent bacterium found in root canal infections (23). It is able to resist nutritional deprivation in part due to its ability to utilize a number of energy sources including malate, citrate, lactate,

carbohydrates, and a number of amino acids. It is also able to withstand extremely basic environments, high salt concentrations, desiccation, and the presence of many antibiotics (17). These attributes allow *E. faecalis* to thrive in deep root canals. Additionally, scientists recently discovered *E. faecalis* has the ability to suppress lymphocyte function as well as produce lytic enzymes which aid in destruction of gingival tissues (9).

CANDIDA ALBICANS

Candida albicans is a ubiquitous pleomorphic fungus that colonizes the gastrointestinal, epithelial, and mucosal tissues of over 70% of the human population (26). Whether *C. albicans* takes on hyphal, pseudohyphal, or yeast form is dependent upon environmental factors such as temperature, pH, and presence of serum. *C. albicans* is an opportunistic pathogen that is often the cause of infection in immunocompromised patients, such as those with HIV/AIDS or indwelling medical devices. Imbalances in normal body flora due to antibiotic and steroid use have also been shown to provoke the overgrowth of *Candida*, causing candidiasis (21). Oral candidiasis, commonly known as thrush, can exacerbate already existing oral diseases. *C. albicans* has also been found within dental caries and is a contributor to periodontal disease (26). Many proteins within saliva have been found to promote adherence of *C. albicans* to gingival tissues (10). Additionally, tissues already colonized by streptococcal strains greatly enhance the ability of *C. albicans* to colonize the oral cavity (10).

MATERIALS AND METHODS

MICROBIAL STRAINS AND MEDIA

One yeast and five bacterial strains were utilized for this investigation. All microorganisms were obtained from the American Type Culture Collection (ATCC), Manassas, VA. The bacterial strains used were: *Lactobacillus casei* (ATCC 393), *Rothia dentocariosa* (ATCC 17931), *Enterococcus faecalis* (ATCC 29212), *Actinomyces naeslundii* (ATCC 12104) and *Actinomyces viscosus* (ATCC 43146). The yeast strain used was *Candida albicans* (ATCC 10231). Four of the bacterial strains were grown in tryptic soy broth + 1% sucrose (TSBS; Difco Laboratories, Detroit, MI), while *Actinomyces naeslundii* was grown in brain heart infusion broth (BHI; Difco). All bacteria were incubated at 37°C in 5% CO₂ for 24 or 48 hours. *C. albicans* was grown in yeast peptone dextrose broth (YPD; Difco) at 30°C in 5% CO₂ for 48 hours. The design of the experiment is consistent with other studies that have been both published and conducted in this laboratory (20).

MINIMUM INHIBITORY CONCENTRATION AND PLANKTONIC GROWTH QUANTIFICATION

Nicotine dilutions were prepared using a 1 g/ml nicotine stock solution (Sigma Chemical Co., St. Louis, MO) in TSBS or BHI for the bacteria and in YPD for the yeast. An initial dilution of 32 mg/ml of nicotine was prepared and a 1:2 serial dilution series was made through 0.25 mg/ml. Additionally, a control was prepared containing only 200 µl of media with no nicotine. Diluted aliquots of 190 µl were transferred into a sterile 96-well microtiter

plate along with 10 µl of an overnight culture of the respective organism. The bacterial microtiter plates, excluding *A. naeslundii*, were incubated at 37°C in 5% CO₂ for 24 hours and *C. albicans* and *A. naeslundii* were incubated under the same conditions for 48 hours. After incubation, the absorbance of each well in the plates was measured in a spectrophotometer (SpectraMax 190; Molecular Devices, Inc., Sunnyvale, CA) at an optical absorbance of 595 nm (OD₅₉₅) to determine the minimum inhibitory concentration (MIC). (20) To quantify the planktonic growth, 120 µl aliquots of planktonic culture fluid was removed from each well into corresponding wells in a fresh 96-well microtiter plate and the absorbances determined in a spectrophotometer at 595 nm. Absorbance values greater than .05 units of the control would be considered a significant change. Anything equal or less to .05 absorbance units would be considered insignificant.

MINIMUM BIOFILM INHIBITORY CONCENTRATION

Ninety six-well plates of the organisms in varying concentrations of nicotine were prepared as described above. Following incubation, the remaining planktonic culture fluid was disposed of and 200 µl of 10% formaldehyde (Fisher Scientific Co., Pittsburg, PA) was added to each well for 30 minutes at room temperature. The plate was then washed in deionized (DI) water before adding 200 µl of 0.3% crystal violet (Sigma) to each well. The plate was allowed to incubate for an additional 30 minutes and then washed in DI water. 200 µL of isopropanol (Fisher) was then added to each well to allow extraction of the crystal violet from the biofilm cells. Following a one-hour incubation in isopropanol at

room temperature, the optical density of the biofilm was recorded at 490 nm. Using this method, both *R. dentocariosa* and *A. naeslundii* were unable to form tight biofilms in their 96-well plates. Therefore, instead of utilizing formaldehyde, crystal violet and isopropanol, the biofilm of *R. dentocariosa* and *A. naeslundii* were resuspended in 200 μ l of a 0.9% saline solution and the optical density was measured at 595 nm.

STATISTICAL ANALYSES

All experiments were carried out in quadruplicate wells at least three times. Means, standard deviations, and statistical significance were calculated using Excel. Student t-tests were used to determine p values, which were considered significant if $P < 0.05$. Data was transferred to SigmaPlot 12.0 to graph and further analyze results.

Table 1 Minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MBIC) of nicotine on microorganisms

Organisms	MIC (mg/ml)	MBIC (mg/ml)
<i>L. casei</i>	16	16
<i>A. viscosus</i>	16	16
<i>A. naeslundii</i>	16	16
<i>R. dentocariosa</i>	16	16
<i>E. faecalis</i>	32	16
<i>C. albicans</i>	16	16

RESULTS

Table 1 indicates the MIC and MBIC of each microorganism tested. All organisms had an MIC of 16 mg/ml of nicotine except *E. faecalis*, which had an MIC of 32 mg/ml of nicotine. Similarly, the MBIC of all organisms tested was 16 mg/ml of nicotine.

The total growth of the organisms in each nicotine concentration is presented in Fig. 1. This data represents the total of biofilm and planktonic growth. *L. casei*, *A. viscosus*, *R. dentocariosa*, and *C. albicans* had an increase in growth through 8 mg/ml of nicotine. *E. faecalis* growth increased through 16 mg/ml but growth of all other organisms was

decreased at this concentration of nicotine. *A. naeslundii* growth decreased through 8 mg/ml and demonstrated complete inhibition beginning at 16 mg/ml. Additionally, all organisms displayed a decrease in total growth at 32 mg/ml of nicotine.

Fig. 2 denotes the planktonic growth of each organism in nicotine. Decreased growth occurred at 32 mg/ml of nicotine in all organisms tested. Additionally, *L. casei*, *A. viscosus*, and *R. dentocariosa* demonstrated statistically significant inhibition of planktonic growth at 16 mg/ml. An increase in growth occurred at 8 mg/ml of

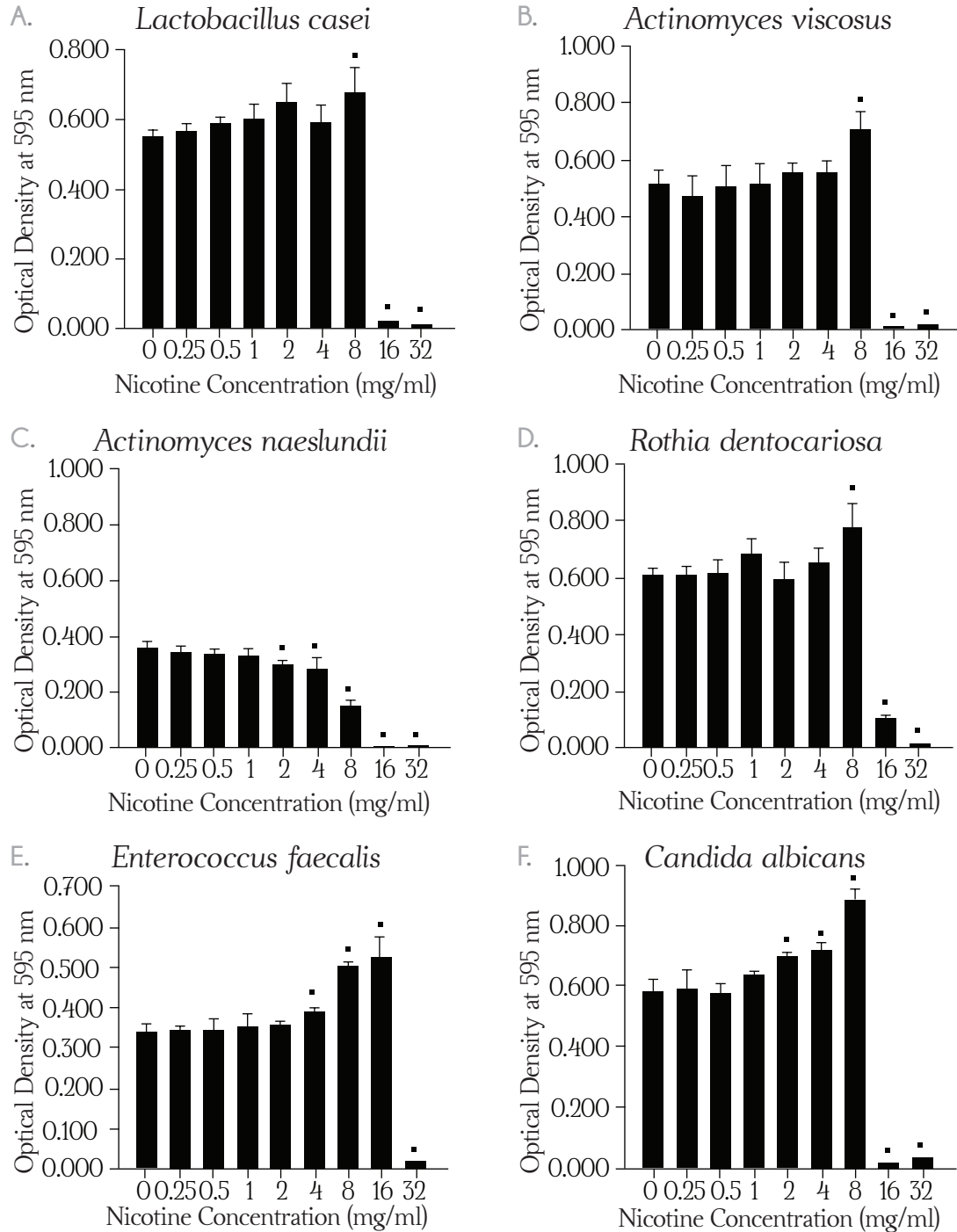


Fig. 1: Total growth of nicotine-treated microorganisms in quadruplicate wells. After incubating with various concentrations of nicotine the OD was measured at 595 nm. The OD value is represented as (mean + SD). Each experiment was repeated at least three times. Asterisks indicate statistical significance ($P < 0.05$) compared to the 0 mg/ml nicotine control.

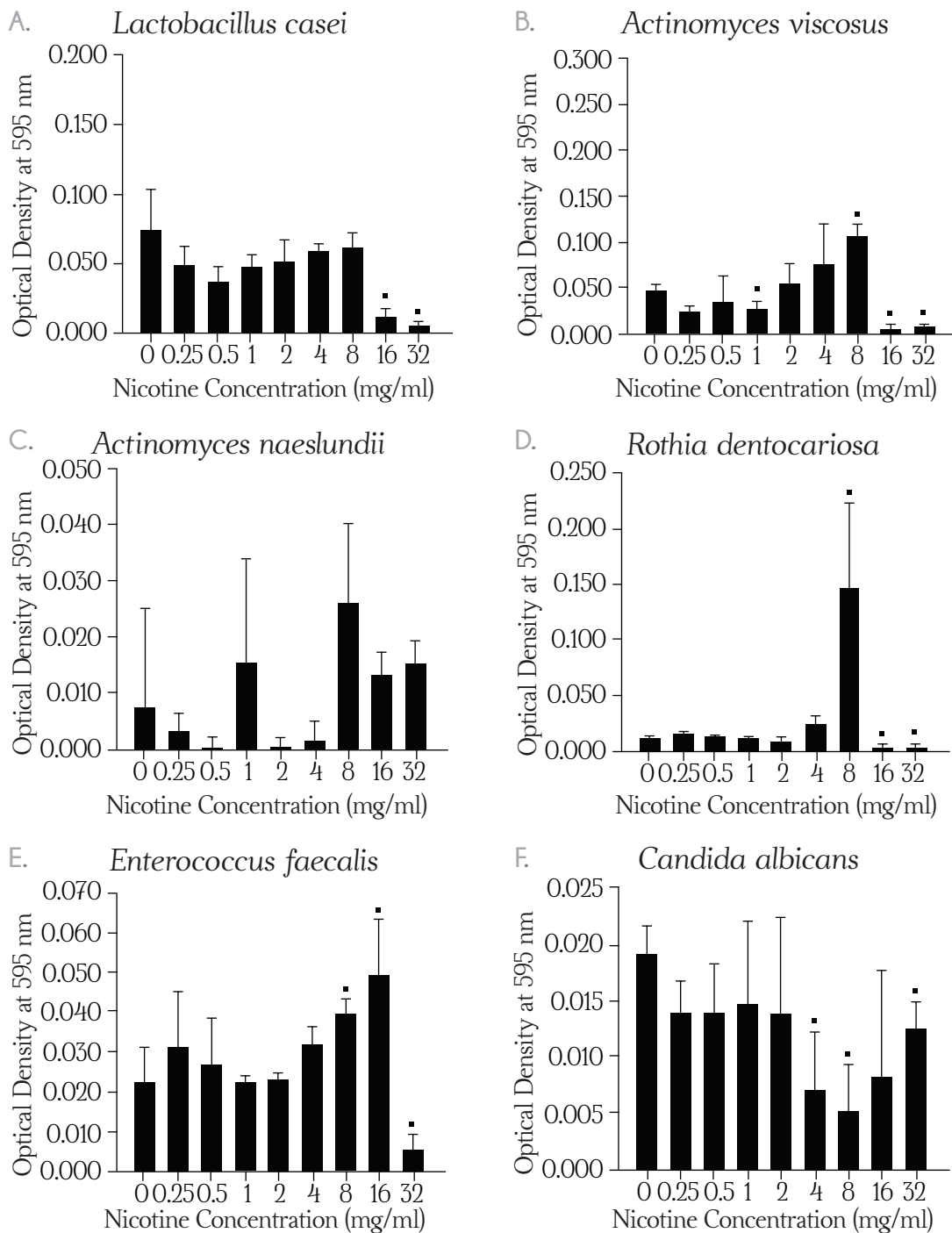


Fig. 2: Planktonic growth of nicotine-treated microorganisms in quadruplicate wells. After incubating with various concentrations of nicotine for 48 hours, the supernatant was removed and the OD of the supernatant was measured at 595 nm. The OD value is represented as (mean + SD). Each experiment was repeated at least three times. Asterisks indicate statistical significance ($P < 0.05$) compared to the 0 mg/ml nicotine control.

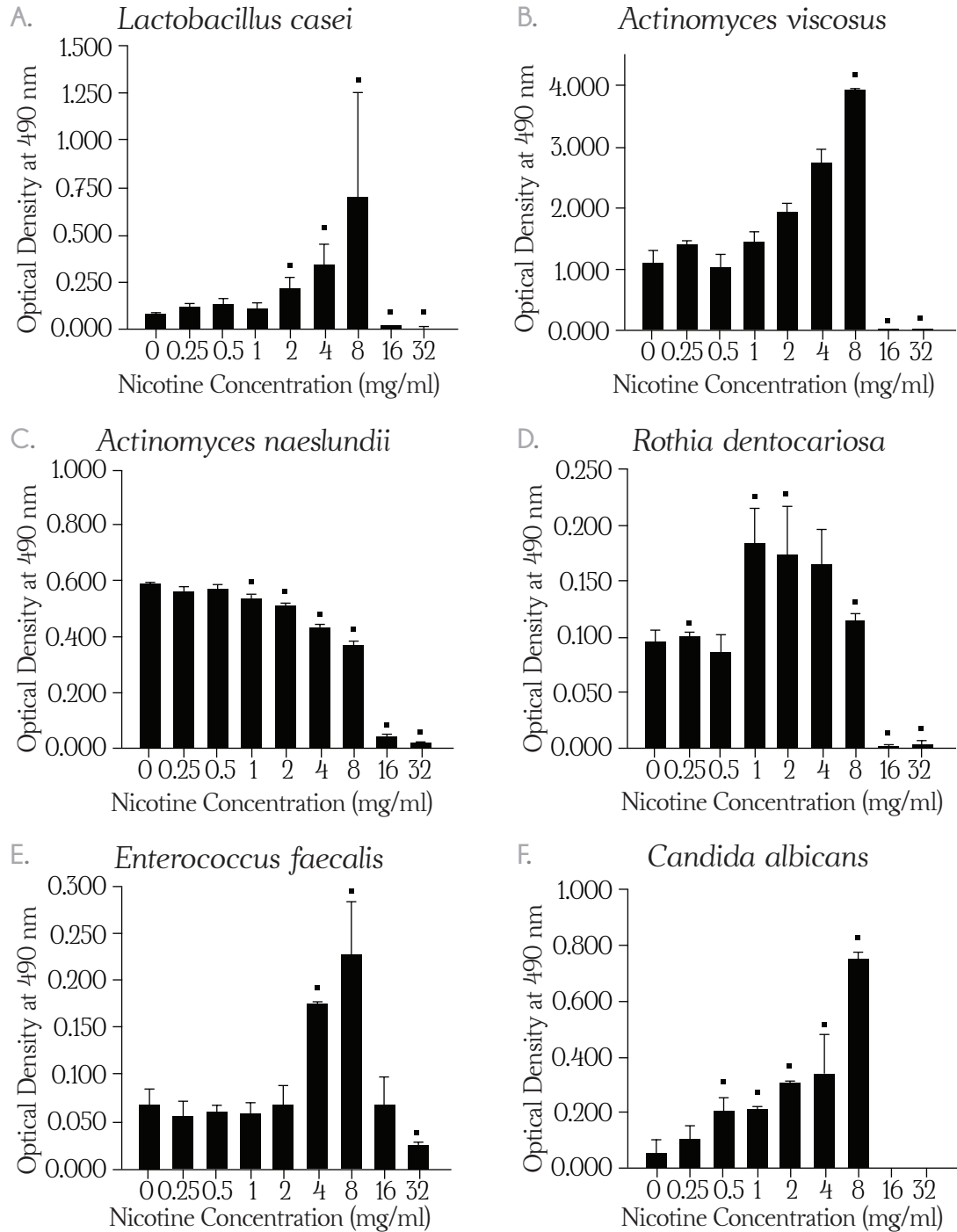


Fig. 3: Biofilm formation of nicotine-treated microorganisms in quadruplicate wells. After incubating with various concentrations of nicotine for 24 or 48 hours, the biofilm was either stained with crystal violet or suspended in saline and the OD was assessed at 490 nm or 595 nm, respectively. The OD value is represented as (mean + SD). Each experiment was repeated at least three times. Asterisks indicate statistical significance ($P < 0.05$) compared to the 0 mg/ml nicotine control.

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increase in growth occurred at 8 mg/ml of nicotine for *A. viscosus* and *R. dentocariosa*. Moreover, *E. faecalis* planktonic growth was enhanced at 8 mg/ml and 16 mg/ml of nicotine. Significant inhibition was observed in 1 mg/ml of nicotine for *A. viscosus* and for 4, 8, and 32 mg/ml of nicotine in *C. albicans*. *A. naeslundii* along with other bacteria in this study depict a high sensitivity to nicotine. The planktonic readings varied between trials, which made interpretations of planktonic response to nicotine inconsistent at times and difficult to interpret.

Biofilm formation of the organisms in each nicotine concentration is indicated in Fig. 3. An upward trend in biofilm growth through 8 mg/ml of nicotine was observed for *L. casei*, *A. viscosus*, *E. faecalis*, and *C. albicans*. *R. dentocariosa* exhibited the most growth at 1 mg/ml of nicotine but also had increased growth in 0.25, 2, and 8 mg/ml. Inhibition of biofilm formation for all organisms was indicated at 16 and 32 mg/ml of nicotine. Additionally, *A. naeslundii* demonstrated statistically significant inhibition beginning at 1 mg/ml and continuing through 32 mg/ml of nicotine.

DISCUSSION

Significant inhibition occurred with all tested organisms in nicotine concentrations of 16 and 32 mg/ml except *E. faecalis*, providing a MIC of 32 mg/ml for *E. faecalis* and a MIC of 16 mg/ml for the remaining organisms. Similar studies demonstrate other oral microorganisms, most notably *S. mutans*, also have a MIC of 16 mg/ml. (7)(20) It is possible that the MIC exists at nicotine concentrations between 8 and 16 mg/ml but further studies are required to determine the validity of this supposition. Research by Huang *et al.* (2014) also

demonstrated stimulatory effects by nicotine on *Streptococcus gordonii* (34). Another study investigating nicotine effects on oral microorganisms like *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *S. gordonii* demonstrated no effect could be observed in concentrations less than 1 mg/ml (13). The research conducted with these microorganisms is consistent with this study and demonstrates significant variations in activity at higher concentrations of nicotine. Alternatively, Pavia *et al.* (2000) were able

to demonstrate that nicotine concentrations between 100 and 250 µg/ml reduced growth of *Escherichia coli*, *Listeria monocytogenes*, *Candida albicans*, *Klebsiella pneumoniae* and *Cryptococcus neoformans* (27).

All concentrations of nicotine exerted some inhibition of planktonic growth, particularly at 16 and 32 mg/ml, when compared to the non-nicotine-treated controls for both *L. casei* and *C. albicans*. Previous research suggests that planktonic bacteria are more susceptible to various chemicals and antibiotics and so it is possible that the presence of nicotine induced this effect on the microorganisms studied (4). No reports were found on planktonic yeasts relating to chemical susceptibility, but it is plausible that similar effects as those observed in planktonic bacteria might also exist in yeast. All species displayed planktonic inhibition at 32 mg/ml. Furthermore, at concentrations of 16 mg/ml both *R. dentocariosa* and *L. casei* were significantly inhibited. *C. albicans* growth was also inhibited in nicotine concentrations of 4 and 8 mg/ml. *L. casei*, *A. viscosus*, *R. dentocariosa*, *E. faecalis*, and *C. albicans* demonstrated no statistically significant differences in non-nicotine treated planktonic organisms compared to those treated with nicotine concentrations up to 2 mg/ml. *A. viscosus*, *R. dentocariosa* and *E. faecalis* had planktonic growth enhancement in concentrations of 16, 8, and 8 through 16 mg/ml, respectively. All OD values measured from the planktonic growth were much lower than OD values recorded from biofilm growth. Visual analysis of the microtiter plates also signified greater biofilm growth when compared to planktonic growth. It is known that bacteria prefer existing in biofilms rather than as unicellular planktonic cells and it has been suggested that nearly 90% of bacteria exist as biofilms in nature. (31) The present study further supports this theory as well as extends the observation to *C. albicans* due to

its perceived preference to grow as a biofilm rather than as a planktonic yeast phase. As Fig. 3 indicates, a notable upward trend in the biofilm formation of *L. casei*, *A. viscosus*, *A. naeslundii*, *E. faecalis* and *C. albicans* was demonstrated through 8 mg/ml of nicotine. Biofilm growth was most notably enhanced in 8 mg/ml of nicotine in the aforementioned organisms. *R. dentocariosa* presents a unique demonstration of enhancement in concentrations of nicotine ranging from 0.25 to 8 mg/ml but was most prominently enhanced at 1 mg/ml. In addition, studies have shown that an increase in adhesion to surfaces occurs over time when *C. albicans*, *S. mutans*, and *S. pneumoniae* are exposed to cigarette smoke condensate (CSC). (4)(29) CSC is a crude aqueous extract of tobacco and contains approximately 2.4% nicotine by weight along with many other chemical components of tobacco. It is plausible that one of the attributing factors to the increase in adhesion is due to the nicotine contained within the CSC, which would thus account for the results observed in this study. Additionally, Antunes *et al.* 2012 (3) found that oxidative stress caused increased biofilm formation of *P. aeruginosa*. This study identified hydrogen peroxide as one causative agent of oxidative stress in cigarette smoke. Other research has indicated nicotine can also act as an oxidative stressor, which might account for the increased biofilm formation seen in many of the nicotine concentrations used in the current study (11). The MBIC of nicotine for all organisms studied was determined to be 16 mg/ml as indicated in Fig. 3. As with the MIC, it is possible the MBIC also exists at some concentration between 8 and 16 mg/ml of nicotine, but further studies are needed to explore this probability.

In this present study, concentrations at which enhanced growth was demonstrated correspond to average nicotine concentrations found in many of the tobacco

products discussed previously. It is believed that the findings of this study support the hypothesis that the increased risk of oral health issues faced by many tobacco users is caused in part by the stimulation of oral microorganism biofilm formation by nicotine and other tobacco components.

This study can be used to provide confirmation that further research on the relationship between oral microorganisms and nicotine is needed. Under similar growth conditions, the organisms could be exposed to nicotine intermittently throughout a specified length of time, much like how a smoker smokes periodically throughout the day. This study emulates the nicotine exposure which occurs to the

oral flora of a smoker. Additionally, because microbes often exhibit different behaviors when interacting with multiple species, it would be beneficial to conduct a study which assesses the biofilm formation of multiple bacterial species in the mouth. This would more closely simulate an in vivo environment in an attempt to simulate dental caries, oral biofilm, periodontal disease and other diseases present in the mouth. Furthermore, investigating the types of interactions which occur between nicotine, proline-rich proteins, and bacteria would allow for a more thorough understanding of the specific mechanisms which cause biofilm enhancement in the presence of nicotine.

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