THE EFFECTS OF NICOTINE ON THE PROTEOLYTIC ACTIVITY OF PERIODONTAL PATHOGENS

by

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INTRODUCTION

Periodontal disease is the leading cause of tooth loss in adults. Bacterial biofilm on tooth surfaces is the primary initiator of periodontal disease. Various factors contribute to the severity of periodontal disease including the different virulence factors of the bacteria within the biofilm. In the progression of periodontal disease, the microflora evolves from a predominantly Gram positive microbial population to a mainly Gram negative population. Specific gram negative bacteria with pronounced virulence factors have been implicated in the etiology and pathogenesis of periodontal disease, namely *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* which form the *red complex* of bacteria. The *orange* complex bacteria become more dominant in the maturation process of dental plaque and act to bridge the early colonizers of plaque with the later more dominant *red* complex bacterial and consists of such bacteria as *Campylobacter showae, Campylobacter rectus, Fusobacterium nucleatum* and *Prevotella intermedia*. Perhaps the most investigated contributing factor is the relationship between smoking and periodontal disease. When examining the association between cigarette smoking and interproximal bone loss, greater bone loss is associated with higher cigarette consumption, longer duration (i.e., pack year history) and higher lifetime exposure. The presence of various virulence factors such as the production of a capsular material, as well as the proteolytic activity of the various periopathodontic bacteria has been associated with the pathogenesis of periodontitis. Even though many different enzymes are produced in large quantities by these periodontal bacteria, trypsin-like enzymes, chymotrypsin-like enzymes and elastase-like enzymes, as well as dipeptidyl peptidase-like enzymes, have been thought to increase the destructive potential of the bacterium and mediate destruction of the periodontal apparatus.

PURPOSE OF THE STUDY

The objective of this study was to determine the effects of nicotine on the secreted enzymatic activity of *P. intermedia, F. nucleatum* and *Porphyromonas assacharolyticus*.

HYPOTHESES

Hypothesis

We hypothesize that the secreted enzymatic activity of *P. intermedia, F. nucleatum* and *P. assacharolyticus* will be increased in the presence of nicotine versus the absence of nicotine.

Null Hypothesis

The secreted enzymatic activity of *P. intermedia, F. nucleatum* and *P. assacharolyticus* will be unchanged in the presence of nicotine.

REVIEW OF LITERATURE

Periodontal diseases are the leading cause of tooth loss in adults. These diseases affect the tooth-supporting tissues resulting in progressive destruction of the attachment apparatus and is characterized by gingival bleeding, alveolar bone destruction, progressive tooth mobility and subsequent tooth loss. Albandar et al. analyzed National Health and Nutrition Examination Survey III - (NHANES-III) data from 1988 to 1994 and estimated that approximately 35% of the dentate U.S. adults aged 30 to 90 have periodontitis, 21.8% having a mild form and 12.6% having a moderate or severe form[1]. Bacterial biofilm on tooth surfaces is the primary initiator of periodontal disease. This is further propagated by the host responses to the biofilm. Various factors contribute to the severity of periodontal disease including the presence of different virulence factors of the bacteria within the biofilm.

Commensal microflora of the oral cavity are responsible for most forms of periodontitis[2, 3]. In the progression of periodontal disease, the microflora progresses from a Gram positive microbial population to a predominantly Gram negative population. Specific gram negative bacteria with pronounced virulence factors have been implicated in periodontal disease, namely *Porphyromonas gingivalis* and *Tannerella forsythia* [3]. According to Socransky et al., the *red complex* of periodontopathic bacteria has been found to be associated with chronic periodontitis and includes *P. gingivalis*, *T. forsythia* and *Treponema denticola* [4]. The *orange complex* bacteria, also described by Socransky et al., are thought to become more dominant in the maturation process of dental plaque and act to bridge the early colonizers of plaque with the later more dominant *red complex* bacteria. The *orange complex* consists of such bacteria as *Campylobacter showae*, *Campylobacter rectus*, *Fusobacterium nucleatum* and *Prevotella intermedia* (Figure 1).

Associated with the late colonizers of the *red complex, P. gingivalis* is an opportunistic, anaerobic, non-motile, Gram negative bacillus. Subgingival colonization by *P. gingivalis* is facilitated by the ability of the bacterium to adhere to the available substrates such as absorbed salivary molecules, matrix proteins, epithelial cells and other bacteria which have established a biofilm on the tooth and epithelial surfaces[5]. *P. gingivalis* can be a difficult bacterium to culture and subsequently grow in laboratory conditions. Thus a suitable alternative, even though it is not a periodontal pathogen per se, is *Porphyromonas assacharolyticus*. Shah and Collins in 1988 proposed a reclassification of the genus *Bacteroides* to the genus *Porphyromonas* and stated that *Bacteroides assacharolyticus, Bacteroides gingivalis*, and *Bacteroides endodontalis* are well-defined species and on the basis of biochemical and chemical properties form a relatively homogeneous group quite unrelated to the type species of the genus *Bacteroides* and should be reclassified as *Porphyromonas assacharolyticus, Porphyromonas gingivalis* and *Porphyromonas endodontalis* [6]. They noted that the proteolytic activity of this genus in general is variable but is exhibited in all three of the species. Thus, based on the evidence provided by Shah and Collins, *P. assacharolyticus* can be used as a viable substitute to *P. gingivalis*, which can sometimes prove difficult to grow under laboratory conditions, to determine the relative changes in proteolytic activity.

Despite the knowledge that periodontal disease is the result of specific microorganisms, various risk factors can modify susceptibility to the disease. These risk factors include, but are by no means limited to, smoking, obesity and diabetes mellitus[7-9]. Perhaps the most investigated is the relationship between smoking and periodontal

disease. According to the American Heart Association, an estimated 24.8 million men (23.1%) and 21.1 million women (18.3%) are smokers in the United States. Sheiham found that smokers had more periodontal disease, as well as increased amounts of plaque and calculus, when compared to their nonsmoking counterparts [10]. When examining the association between cigarette smoking and interproximal bone loss, greater bone loss is associated with higher cigarette consumption, longer duration and higher lifetime exposure [11].

Numerous *in vitro* studies have been conducted to show the various effects of nicotine on the oral cavity. Teughels et al. (2005) examined whether nicotine and its metabolite, cotinine, could make epithelial cells more prone to colonization by periopathogens and found the susceptibility of these cells to *P. gingivalis* could only be moderately altered in non-physiological circumstances (high nicotine/cotinine concentrations, cigarette smoke condensate (CSC)) [12]. Further to the results found by Teughels et al., Cogo et al. (2008, 2009) found that nicotine and cotinine did not affect the growth of *P. gingivalis* when incubated with various concentrations of nicotine and cotinine [13]; however they did report that cotinine at higher concentrations may improve colonization of *P. gingivalis* to epithelial cells only after a previous exposure [14]. Cogo et al., (2009) also stated that nicotine had no statistically significant effect on the ability of *P. gingivalis* to invade soft tissues [14]. Several studies have examined the effects of varying concentrations of nicotine on human fibroblasts and have found that nicotine increased the collagen-degrading ability of fibroblasts in a dose-dependent manner[15-19]. Bagaitkar et al. (2009) hypothesized and subsequently showed that CSC posed an environmental stress to *P. gingivalis* which results in adaptation by alteration in its

pattern of gene expression[20]. In adapting to the environmental stress posed by CSC, Bagaitkar et al. found that cysteine proteinases were upregulated approximately 14 fold based on their microarray results[20]. *In vivo* bacteriologic studies have not shown conclusively that there is a difference in the rate of plaque formation, proportion of anaerobes or the number of gram negative or positive bacteria in smokers versus nonsmokers [21]. However, *in vitro* studies have indicated that bacteria, particularly the *red complex* bacteria, are affected by cigarette smoke and that a higher mean proportion of anaerobes are present in the plaque of smokers[21-23]. Therefore, evidence suggests that smoking exerts a negative effect on periodontal health and pathogens and can thus be a major contributing factor in the pathogenesis and ultimate progression of the disease. But the question still remains -- how? Studies have shown that smoking increases the levels of other oral bacteria including *Streptococcus mutans*, which is the primary microorganism involved in dental caries[24-26]. Preliminary unpublished data from Zheng and Gregory (2008, 2009), as well as from Gregory et al. (2009) and Morgan and Gregory (2010), have shown that CSC and nicotine increase gene expression of adhesion factors along with virulence factors of *S. mutans*. Evidence indicates that physiological concentrations of nicotine increase *S. mutans* growth, hydrophobicity, biofilm formation, acid production, glucosyltransferase, fructosyltransferase, lactate dehydrogenase, and glucose-phosphotransferase enzyme activities. From this, it is evident that these effects work in concert to increase the virulence of *S. mutans* and thereby possibly increase the cariogenicity of the bacterium.

Virulence factors have been implicated in the pathogenicity of periopathogens and are one possible explanation as they can be responsible for the inhibition of certain host

defense mechanisms thereby resulting in progression of disease. The presence of fimbriae, lipopolysaccharide, toxic by-products and production of a capsular material, as well as the proteolytic activity of the various periodontopathic bacteria, have been associated with the pathogenesis of periodontitis[27-31]. Many different enzymes, including such proteolytic enzymes as trypsin-like enzymes, chymotrypsin-like enzymes, elastase-like enzymes and dipeptidyl peptidase-like enzymes, are produced in large quantities by these bacteria and have been implicated in the pathogenicity of the bacteria subsequently increasing the destructive potential of the different periodontal pathogenic bacteria and thereby mediating the destruction of the periodontal apparatus [32]. For example, gingipains, which are cysteine proteases unique to the C25 family of cysteine proteases, consist of arginine-specific proteases (Arg-gingipain, Rgp; 95 and 50 kDa) which have multiple functions and are essential for bacterial growth [33]. In addition, gingipains play a role in complement and immunoglobulin degradation [34], inactivation of cytokines and their receptors, platelet aggregation, attenuation of neutrophil antibacterial activities, increasing vascular permeability, prevention of blood clotting, disrupting cell-cell and cell-matrix adhesion, and induction of apoptosis in several cell types [35]. Gingipains are associated with *P. gingivalis*; however, interpain A, another cysteine protease, is associated with *P. intermedia* but further studies are required to better understand its specific targets and function[36]. Other than the ability of *P. intermedia* to obtain haem from hemoglobin it is unknown how this is actually elucidated and what it means in terms of the pathogenesis of periodontal disease[37]. Ogawa et al. (2006) studied and attempted to characterize the proteolytic activity of *F. nucleatum* and found that metallic, serine and cysteine as well as aspartic proteases were present [38].

Studies have shown that periodontopathic bacteria are active against various synthetic substrates such as N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) and thus BAPNA can be used to determine enzymatic activity [32]. BAPNA is a simple colorless ester which is recognized by trypsin as a substrate (chromogenic substrate). Trypsin cleaves the bond between the arginine and the *p*-nitroaniline to release free *p*-nitroaniline causing a measurable color change. Therefore, it follows, that the proteolytic activity of periodontal pathogens in conjunction with various chromogenic synthetic substrates, such as BAPNA, can be used to determine trypsin-like proteolytic activity of *P. intermedia, F. nucleatum* and *P. assacharolyticus*.

Based on the evidence that smoking has a deleterious effect on periodontal health, it can be speculated that smoking has an effect on the pathogenicity of the major periopathogens. More specifically, it can be hypothesized that the proteolytic activity of periodontopathic bacteria such as, *P. intermedia* and *F. nucleatum*, is altered in the presence of nicotine. Thus, the purpose of this study was to determine the effects of nicotine on the secreted trypsin-like cysteine proteolytic activity of *P. intermedia, F. nucleatum* and *P. assacharolyticus*.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

P. intermedia, *F. nucleatum* and *P. assacharolyticus (*American Type Culture Collection (ATCC) 25611, 10953 and 25260 respectively) were maintained on anaerobic blood agar plates containing 3% sheep blood. Cultures were incubated in an anaerobic GasPak jar at 37°C with an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide and allowed to grow for 7 days prior to use for the enzyme assay. *P. intermedia, F. nucleatum* and *P. assacharolyticus* cells were incubated, along with the chromogenic substrates with 1 mg/ml of nicotine (Sigma-Aldrich Products, St. Louis, MO, USA) for 60 minutes during the enzyme assay. Bacterial cells not incubated with nicotine were used as positive controls and the substrates alone were used as negative controls [39]. Each experimental condition was assessed at least three times.

ENZYME ASSAYS

The enzyme assay was carried out based on the procedure described by Cookson et al. [40] and is described as follows. Secreted enzyme activity was measured using the synthetic chromogenic substrates glycyl-L-proline-*p*-nitroanilide (GPPNA), N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA), N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAAPPPNA) and BAPNA (Sigma). The enzymatic hydrolysis was assessed in a total reaction volume of 200 µl in 50 mM-Tris base, pH 7.4, containing 100 µl of the washed cells and 100 µl each of the synthetic chromogenic substrates at a final concentration of 1.2 mM at 37°C in 96 well microtiter plates.

HARVESTING OF THE BACTERIAL CELLS AND PREPARATION FOR ENZYME ASSAYS

The bacteria were swabbed from the anaerobic blood agar plates using sterile cotton swabs. The bacteria were resuspended in 5 ml of the Tris base and centrifuged at 3300 rpm for 10 minutes. The resultant supernatant was removed carefully so as not to disturb the pellet; the bacterial cells were resuspended in Tris base to a final volume of 5 ml. The bacteria were centrifuged at 3300 rpm for an additional 10 minutes. The resultant supernatant was removed and the bacteria were resuspended in Tris base so as to have an optical density of 2.0 at 600 nm as measured by an UV-visible split-beam scanning spectrophotometer (Milton Roy Spectronic 1201, New York, USA). A total volume of 100 µl of bacteria was added to the wells of the microtiter plate. One hundred microliters (100 µl) of each of the substrates (either with nicotine of 1 mg/ml final concentration or without) was added to the appropriate wells. The microtiter plate was then incubated at 37°C for 1 hour and read every 5 minutes at 660 nm (absorbance used for proteolytic activity). Each substrate and bacteria combination was run in triplicate. The bacteria and substrate/nicotine combinations were also incubated at 37°C for 1 hour and read every 5 minutes at 660 nm. The absorbance of *p*-nitroaniline released from the substrate was measured for 1 hour at 660 nm using a Molecular Devices microplate reader (Molecular Devices Corp, Spectra MAX 190, Sunnyvale, CA) [41]. Appropriate controls (without bacterial cells) were included to account for spontaneous hydrolysis of the substrate. One unit (U) of enzyme activity corresponds to 1 nmol *p*-nitroaniline released min^{-1} at 37°C.

STATISTICAL ANALYSIS

For each experiment, the secreted enzymatic activity was calculated by examining the absorbance at 660 nm at a time of 60 minutes. Appropriate means and standard deviations were determined for each of the enzymatic activities measured and analysis of variance (ANOVA) was used to compare all the groups. A 5% significance level was used for all comparisons.

RESULTS

The mean absorbance at 660 nm in the presence of and absence of nicotine was measured for *P. intermedia, F. nucleatum* and *P. assacharolyticus* using each of the substrates, GPPNA, SAAAPNA, SAAPPPNA and BAPNA and are presented in Tables 1-15. The absorbance at 660 nm of the chromogenic substrates alone were as follows: GPPNA 0.01; SAAAPNA 0.03; SAAPPPNA 0.02; and BAPNA 0.04. All experiments were carried out three times in triplicate and a two-way repeated measures analysis of variance was performed to compare each of the bacteria and respective substrates in the presence and absence of nicotine.

Porphyromonas assacharolyticus

Tables 1 through 4 show the mean absorbance at 660 nm of *P. assacharolyticus* in the presence and absence of nicotine. Table 1 illustrates the absorbance with the substrate GPPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.59 ± 0.14, whereas in the presence of nicotine at 60 minutes the absorbance was 0.67 ± 0.14. This demonstrates an increase in absorbance at 60 minutes, albeit not statistically significant ($p = 0.860$). Table 2 displays the absorbance with the substrate SAAAPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.62 ± 0.26 and 0.68 ± 0.26 in the presence of nicotine. Once again this increase in absorbance is not statistically significant at 60 minutes ($p = 0.710$). Table 3 represents the absorbance with the substrate SAAPPPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.71 ± 0.23, whereas in the presence of nicotine at 60 minutes the absorbance was 0.72 ± 0.23, a non-statistically significant increase once again ($p =$

0.851). Finally, Table 4 demonstrates the absorbance with the substrate BAPNA, the mean absorbance ± standard errors of the means in the absence and presence of nicotine at 60 minutes is 0.55 ± 0.24 and 0.74 ± 0.25, respectively. This indicates an increase in the absorbance, however, not statistically significant ($p = 0.141$). Table 5 summarizes the overall change in absorbance after 60 minutes and the respective *p* values. Figure 2 depicts this data graphically. Figures 3 and 4 demonstrate the representative proteolytic activity against BAPNA in the presence of nicotine and in the absence of nicotine, respectively, over a period of 60 min in 5 min intervals.

Fusobacterium nucleatum

Tables 6 through 9 depict the mean absorbance at 660 nm of *F. nucleatum* in the presence and absence of nicotine. Table 6 illustrates the absorbance with the substrate GPPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.68 ± 0.10 whereas in the presence of nicotine at 60 minutes the absorbance was 0.81 ± 0.10. This demonstrates a statistically significant increase in absorbance at 60 minutes ($p = 0.012$). Table 7 displays the absorbance with the substrate SAAAPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.64 ± 0.10 and 0.79 ± 0.10 in the presence of nicotine. Once again this increase in absorbance is statistically significant at 60 minutes ($p = 0.002$). Table 8 represents the absorbance with the substrate SAAPPPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.73 ± 0.15 whereas in the presence of nicotine at 60 minutes the absorbance was 0.79 ± 0.15, a statistically insignificant increase ($p = 0.451$). Finally, Table 9 demonstrates the

absorbance with the substrate BAPNA, the mean absorbance ± standard errors of the means in the absence and presence of nicotine at 60 minutes was 0.69 ± 0.08 and 0.79 ± 0.08, respectively. This represents a statistically significant increase in the absorbance (p = 0.022). Table 10 illustrates the overall change in absorbance after 60 minutes and the respective p values. Figure 5 depicts this data graphically. Figures 6 and 7 demonstrate the representative proteolytic activity against SAAPPPNA in the presence of nicotine and in the absence of nicotine, respectively, over a period of 60 min in 5 min intervals.

Prevotella intermedia

Tables 11 through 14 display the mean absorbance at 660 nm of *F. nucleatum* in the presence and absence of nicotine. Table 11 represents the absorbance with the substrate GPPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.65 ± 0.09 whereas in the presence of nicotine at 60 minutes the absorbance was 0.60 ± 0.09. This demonstrates a statistically insignificant decrease in absorbance at 60 minutes (p = 0.139). Table 12 indicates the absorbance with the substrate SAAAPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.65 ± 0.11 and 0.61 ± 0.11 in the presence of nicotine. Once again this decrease in absorbance is not statistically significant at 60 minutes (p = 0.173). Table 13 establishes the absorbance with the substrate SAAPPPNA, the mean absorbance ± standard errors of the means in the absence of nicotine at 60 minutes was 0.68 ± 0.12 whereas in the presence of nicotine at 60 minutes the absorbance was 0.82 ± 0.13, an increase, however, not statistically significant once again (p = 0.097). Finally, Table 14 illustrates the absorbance with the substrate BAPNA the mean absorbance ±

standard errors of the means in the absence and presence of nicotine at 60 minutes was 0.58 ± 0.08 and 0.63 ± 0.09, respectively. This represents a statistically insignificant increase in the absorbance ($p = 0.835$). Table 15 demonstrates the overall change in absorbance after 60 minutes and the respective *p* values. Figure 8 depicts this data graphically. Figures 9 and 10 demonstrate the representative proteolytic activity against SAAPPPPNA in the presence of nicotine and in the absence of nicotine, respectively, over a period of 60 min in 5 min intervals.

FIGURES AND TABLES

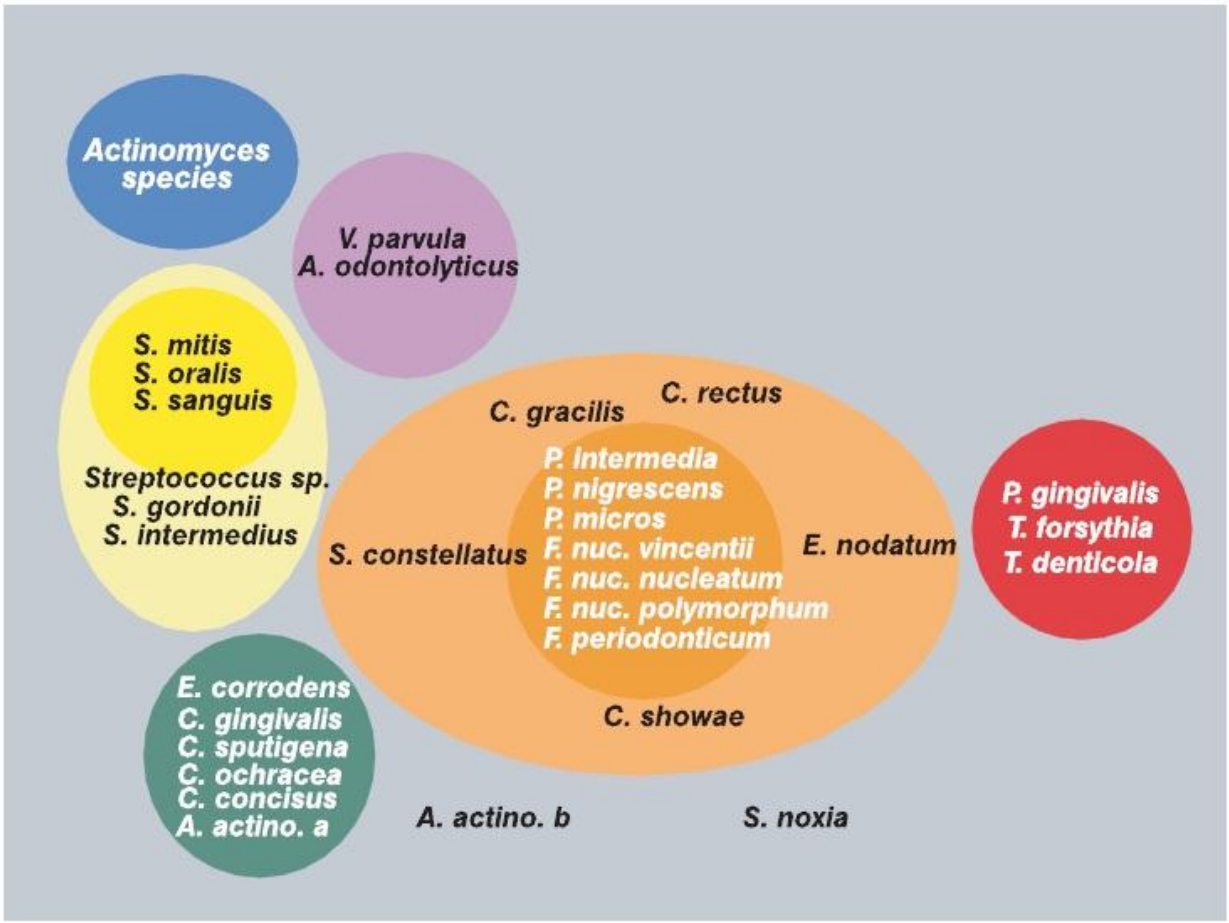

FIGURE 1: Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes of the major periopathogen complexes. Adapted from Socransky and Haffajee 2005. [1]

1. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000 2005;38:135-87.

TABLE I: Absorbance at 660 nm for *Porphyromonas assacharolyticus* cells with the chromogenic substrate glycyl-L-proline-*p*-nitroanilide (GPPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.604	0.144	0.676	0.150
5	0.585	0.144	0.669	0.150
10	0.569	0.143	0.656	0.150
15	0.560	0.143	0.651	0.149
20	0.557	0.142	0.653	0.148
25	0.555	0.142	0.642	0.148
30	0.559	0.141	0.638	0.147
35	0.562	0.141	0.634	0.147
40	0.566	0.140	0.637	0.146
45	0.574	0.139	0.644	0.145
50	0.580	0.140	0.649	0.145
55	0.587	0.139	0.662	0.144
60	0.592	0.138	0.671	0.143

TABLE II: Absorbance at 660 nm for *Porphyromonas assacharolyticus* cells with the chromogenic substrate N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.668	0.260	0.686	0.268
5	0.654	0.261	0.677	0.269
10	0.640	0.261	0.664	0.270
15	0.626	0.261	0.662	0.269
20	0.621	0.260	0.659	0.268
25	0.613	0.260	0.653	0.268
30	0.612	0.259	0.647	0.267
35	0.615	0.259	0.645	0.267
40	0.618	0.258	0.646	0.266
45	0.620	0.257	0.651	0.265
50	0.623	0.257	0.655	0.264
55	0.625	0.256	0.665	0.263
60	0.619	0.255	0.675	0.262

TABLE III: Absorbance at 660 nm for *Porphyromonas assacharolyticus* cells with the chromogenic substrate N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAAPPPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.691	0.213	0.693	0.217
5	0.681	0.212	0.682	0.216
10	0.672	0.212	0.677	0.216
15	0.668	0.214	0.689	0.219
20	0.670	0.216	0.688	0.221
25	0.674	0.217	0.689	0.223
30	0.684	0.220	0.687	0.226
35	0.689	0.221	0.688	0.228
40	0.695	0.223	0.692	0.230
45	0.696	0.224	0.697	0.231
50	0.696	0.224	0.705	0.231
55	0.708	0.225	0.714	0.233
60	0.711	0.226	0.723	0.234

TABLE IV: Absorbance at 660 nm for *Porphyromonas assacharolyticus* cells with the chromogenic substrate N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=13)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=8)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.531	0.248	0.730	0.261
5	0.517	0.248	0.718	0.261
10	0.500	0.247	0.705	0.261
15	0.490	0.247	0.697	0.260
20	0.492	0.246	0.690	0.259
25	0.517	0.244	0.691	0.256
30	0.524	0.243	0.687	0.255
35	0.526	0.243	0.689	0.254
40	0.536	0.242	0.693	0.253
45	0.536	0.242	0.703	0.252
50	0.539	0.241	0.712	0.252
55	0.546	0.241	0.723	0.251
60	0.551	0.240	0.737	0.250

TABLE V: Overall changes in absorbance at 660 nm after 60 minutes of incubation of *Porphyromonas assacharolyticus* cells with the four synthetic substrates.[a]

	Porphyromonas assacharolyticus	*Porphyromonas assacharolyticus with Nicotine*	Mean Change	*P value*
GPPNA	0.592 ± 0.138	0.671 ± 0.143	+ 0.079	0.8599
SAAAPNA	0.619 ± 0.255	0.675 ± 0.262	+ 0.057	0.7098
SAAPPPNA	0.711 ± 0.226	0.723 ± 0.234	+ 0.012	0.8511
BAPNA	0.551 ± 0.240	0.737 ± 0.250	+ 0.186	0.1409

[a] *P* value <0.05 was considered statistically significant as obtained from a two factor analysis of variance with replication between the two groups. GPPNA: glycyl-L-proline-*p*-nitroanilide; SAAAPNA: N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; SAAPPPNA: N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; BAPNA: N-α-benzoyl-L-arginine-*p*-nitroanilide.

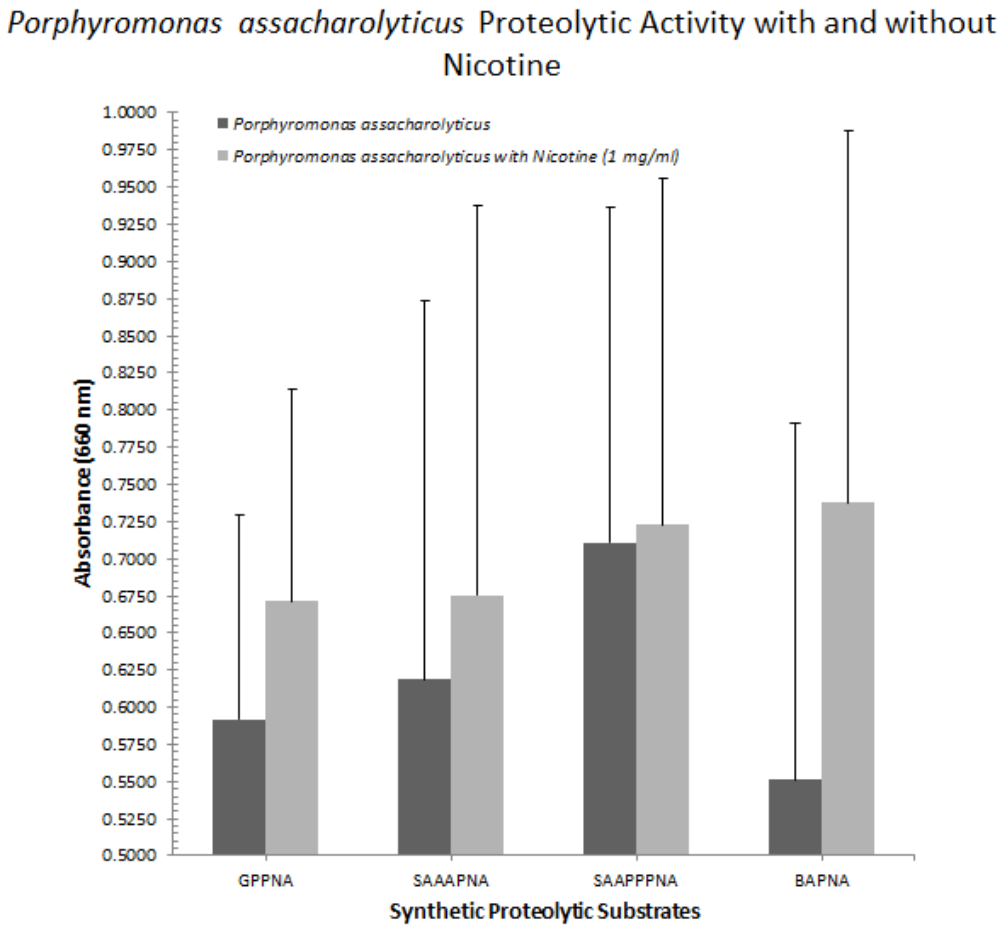

FIGURE 2: Proteolytic activity (mean ± standard errors of the means) of *Porphyromonas assacharolyticus* cells after 60 minutes of incubation with and without nicotine (1 mg/ml) at an absorbance of 660 nm. Asterisks indicate significant difference ($p<0.05$) between the control and test group. GPPNA: glycyl-L-proline-*p*-nitroanilide; SAAAPNA: N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; SAAPPPNA: N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; BAPNA: N-α-benzoyl-L-arginine-*p*-nitroanilide.

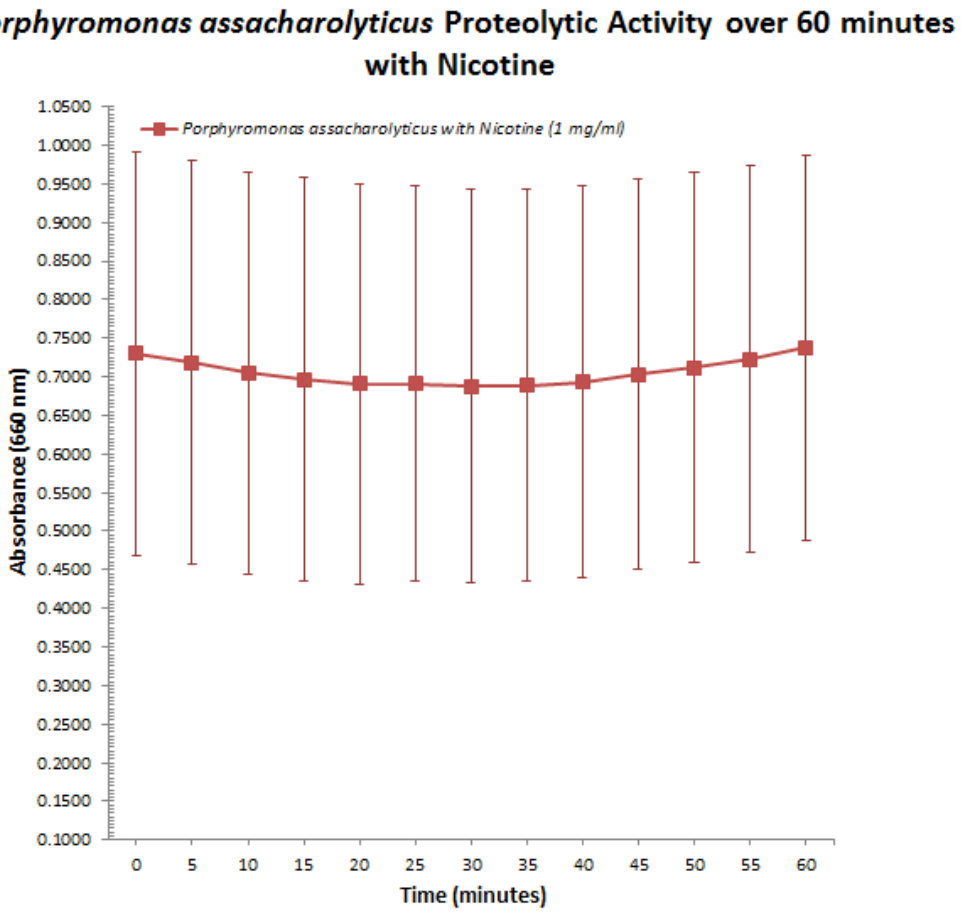

FIGURE 3: Representative proteolytic activity (mean ± standard errors of the means) of *Porphyromonas assacharolyticus* cells over 60 minutes of incubation with nicotine (1 mg/ml) and N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) at an absorbance of 660 nm.

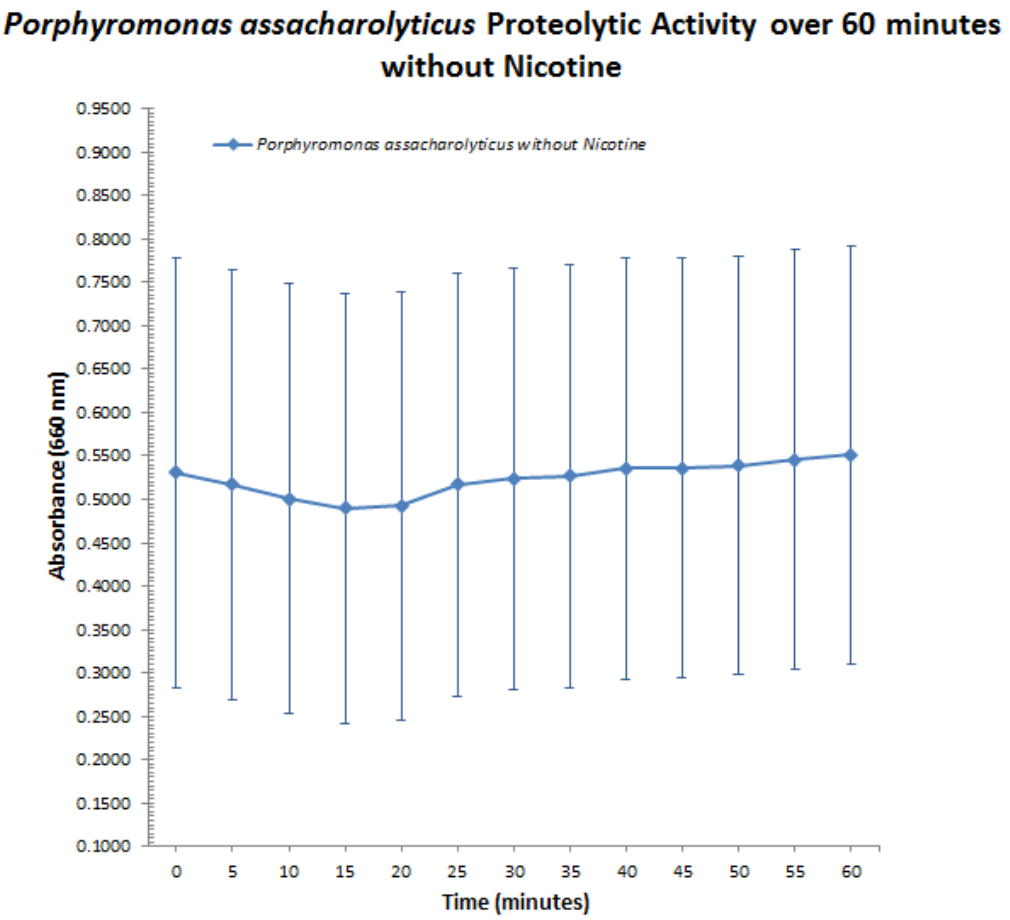

FIGURE 4: Representative proteolytic activity (mean ± standard errors of the means) of Porphyromonas assacharolyticus cells over 60 minutes of incubation without nicotine and N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) at an absorbance of 660 nm.

TABLE VI: Absorbance at 660 nm for *Fusobacterium nucleatum* cells with the chromogenic substrate glycyl-L-proline-*p*-nitroanilide (GPPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.691	0.098	0.741	0.099
5	0.672	0.097	0.727	0.098
10	0.660	0.097	0.715	0.098
15	0.643	0.097	0.710	0.098
20	0.635	0.098	0.703	0.098
25	0.639	0.098	0.708	0.099
30	0.640	0.099	0.706	0.100
35	0.645	0.099	0.712	0.101
40	0.646	0.099	0.731	0.100
45	0.658	0.100	0.753	0.101
50	0.661	0.100	0.772	0.102
55	0.670	0.100	0.791	0.102
60	0.676	0.100	0.810	0.102

TABLE VII: Absorbance at 660 nm for *Fusobacterium nucleatum* cells with the chromogenic substrate N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.663	0.094	0.739	0.094
5	0.646	0.094	0.727	0.094
10	0.629	0.093	0.713	0.094
15	0.613	0.093	0.701	0.094
20	0.604	0.094	0.695	0.094
25	0.606	0.094	0.692	0.095
30	0.604	0.094	0.693	0.095
35	0.606	0.095	0.697	0.096
40	0.610	0.095	0.711	0.096
45	0.619	0.095	0.734	0.097
50	0.626	0.096	0.751	0.097
55	0.634	0.096	0.771	0.097
60	0.641	0.095	0.787	0.097

TABLE VIII: Absorbance at 660 nm for *Fusobacterium nucleatum* cells with the chromogenic substrate N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAAPPPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITHOUT NICOTINE	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.690	0.134	0.746	0.135
5	0.686	0.134	0.741	0.135
10	0.677	0.134	0.731	0.135
15	0.668	0.134	0.725	0.135
20	0.658	0.135	0.722	0.135
25	0.658	0.136	0.720	0.137
30	0.661	0.137	0.718	0.138
35	0.681	0.139	0.716	0.142
40	0.700	0.142	0.729	0.145
45	0.713	0.145	0.743	0.149
50	0.720	0.147	0.758	0.151
55	0.722	0.147	0.776	0.152
60	0.734	0.149	0.787	0.154

TABLE IX: Absorbance at 660 nm for *Fusobacterium nucleatum* cells with the chromogenic substrate N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.691	0.077	0.747	0.078
5	0.675	0.077	0.733	0.077
10	0.655	0.076	0.717	0.077
15	0.638	0.076	0.705	0.077
20	0.631	0.076	0.703	0.077
25	0.632	0.077	0.704	0.077
30	0.631	0.077	0.704	0.078
35	0.641	0.077	0.716	0.079
40	0.646	0.078	0.731	0.079
45	0.656	0.078	0.749	0.080
50	0.666	0.079	0.764	0.080
55	0.677	0.079	0.780	0.081
60	0.689	0.080	0.793	0.081

TABLE X: Overall changes in absorbance at 660 nm after 60 minutes of incubation of *Fusobacterium nucleatum* cells with the four synthetic substrates.[b]

	Fusobacterium nucleatum	*Fusobacterium nucleatum with Nicotine*	Mean Change	*P value*
GPPNA	0.676 ± 0.100	0.810 ± 0.102	+ 0.133	0.0118
SAAAPNA	0.641 ± 0.095	0.787 ± 0.097	+ 0.146	0.0024
SAAPPPNA	0.734 ± 0.149	0.787 ± 0.154	+ 0.053	0.4506
BAPNA	0.689 ± 0.080	0.793 ± 0.081	+ 0.104	0.0218

[b] *P* value <0.05 was considered statistically significant as obtained from a two factor analysis of variance with replication between the two groups. GPPNA: glycyl-L-proline-*p*-nitroanilide; SAAAPNA: N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; SAAPPPNA: N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; BAPNA: N-α-benzoyl-L-arginine-*p*-nitroanilide.

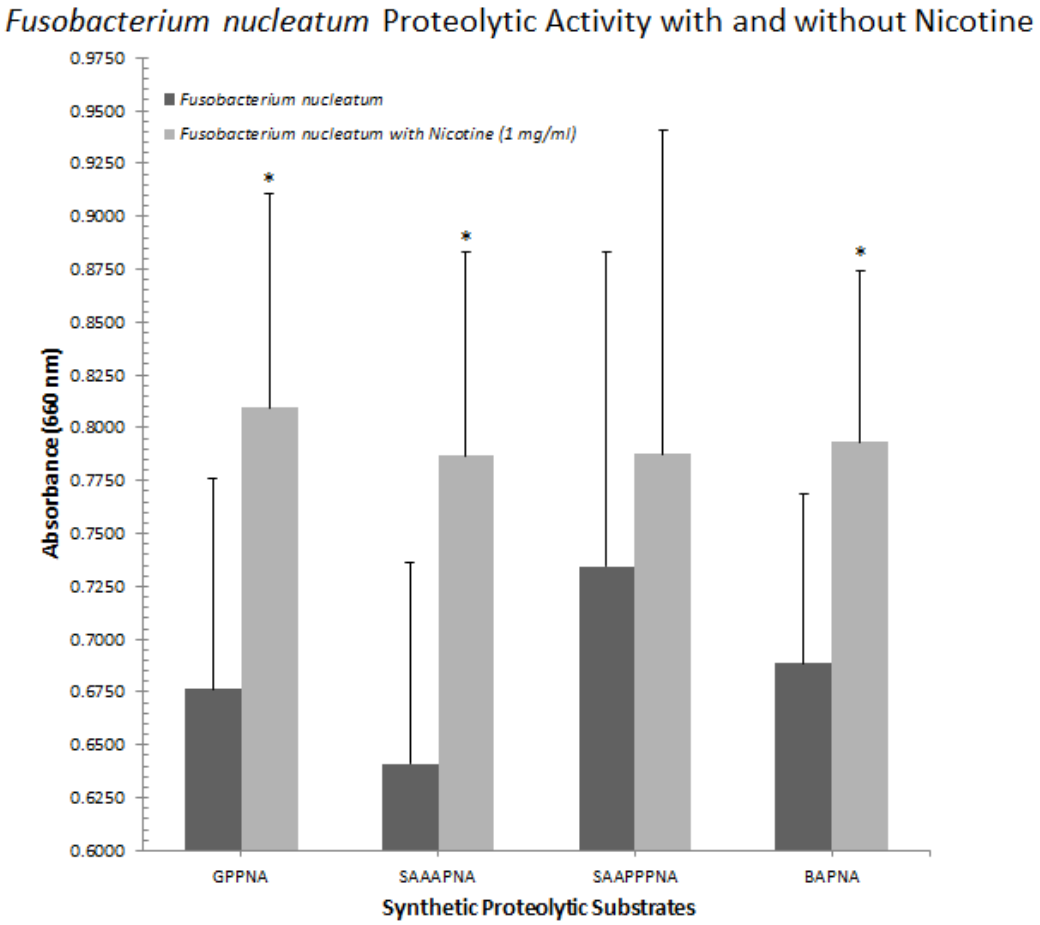

FIGURE 5: Proteolytic activity (mean ± standard errors of the means) of *Fusobacterium nucleatum* cells after 60 minutes of incubation with and without nicotine (1 mg/ml) at an absorbance of 660 nm. Asterisks indicate significant difference ($p<0.05$) between the control and test group. GPPNA: glycyl-L-proline-*p*-nitroanilide; SAAAPNA: N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; SAAPPPNA: N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; BAPNA: N-α-benzoyl-L-arginine-*p*-nitroanilide.

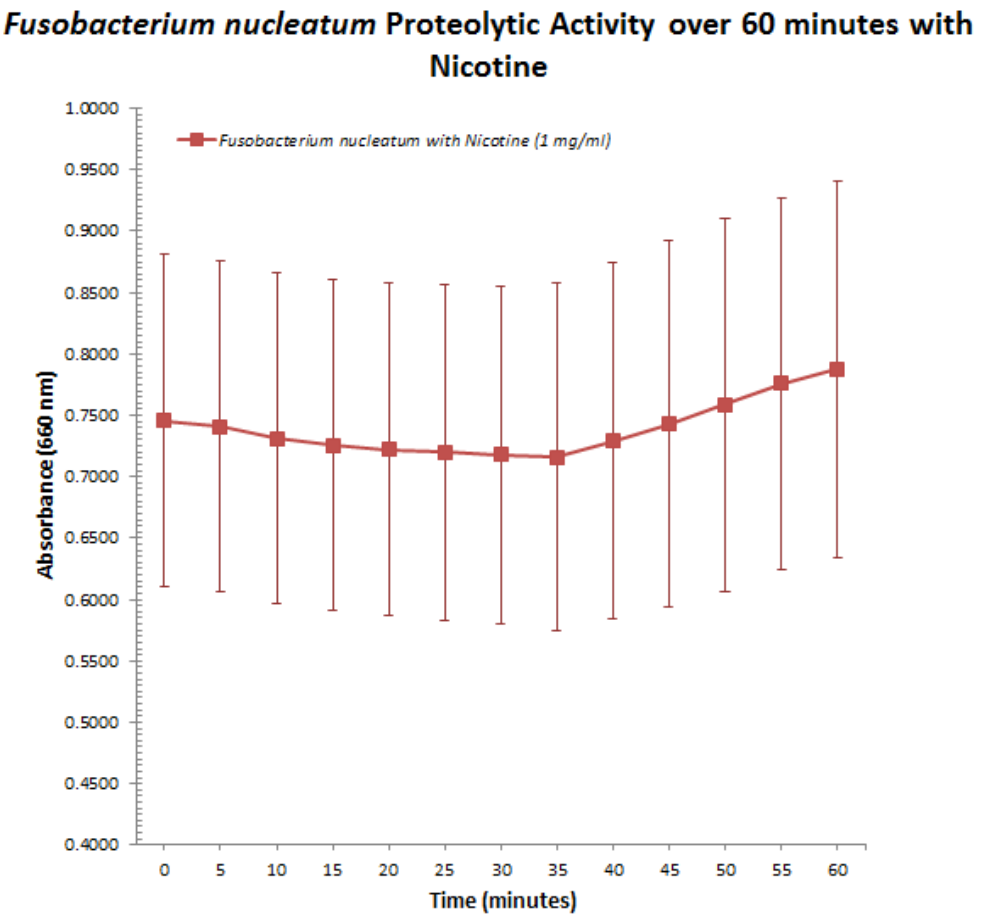

FIGURE 6: Representative proteolytic activity (mean ± standard errors of the means) of *Fusobacterium nucleatum* cells over 60 minutes of incubation with nicotine (1 mg/ml) and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPPPNA) at an absorbance of 660 nm.

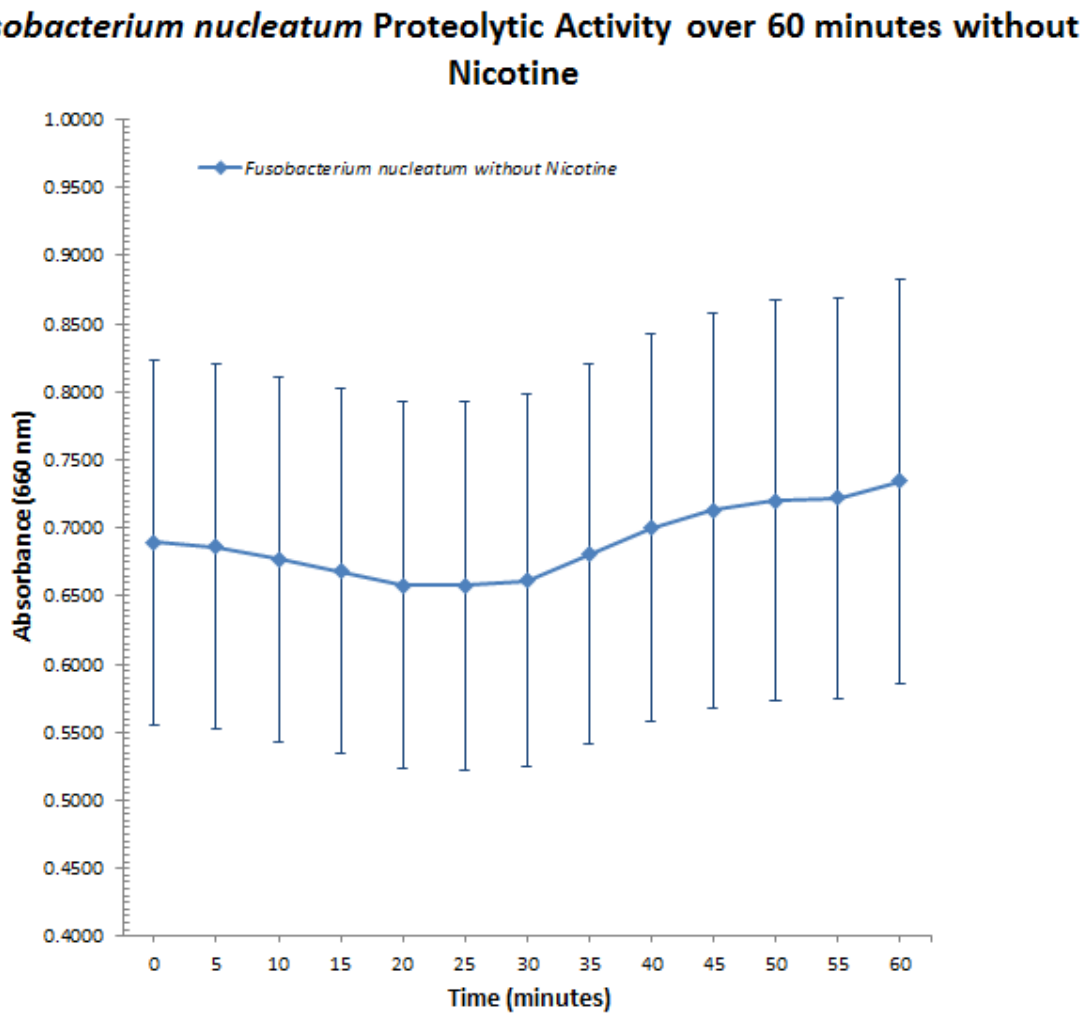

FIGURE 7: Representative proteolytic activity (mean ± standard errors of the means) of *Fusobacterium nucleatum* cells over 60 minutes of incubation without nicotine and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPPPNA) at an absorbance of 660 nm.

TABLE XI: Absorbance at 660 nm for *Prevotella intermedia* cells with the chromogenic substrate glycyl-L-proline-*p*-nitroanilide (GPPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.642	0.090	0.657	0.090
5	0.620	0.089	0.639	0.090
10	0.611	0.090	0.632	0.090
15	0.613	0.090	0.633	0.090
20	0.614	0.090	0.642	0.091
25	0.620	0.090	0.635	0.090
30	0.637	0.090	0.636	0.090
35	0.639	0.090	0.621	0.090
40	0.631	0.090	0.620	0.091
45	0.630	0.090	0.612	0.091
50	0.639	0.091	0.597	0.092
55	0.636	0.091	0.604	0.092
60	0.650	0.093	0.603	0.094

TABLE XII: Absorbance at 660 nm for *Prevotella intermedia* cells with the chromogenic substrate N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.650	0.102	0.657	0.102
5	0.628	0.102	0.649	0.102
10	0.610	0.102	0.647	0.102
15	0.604	0.102	0.664	0.102
20	0.614	0.102	0.647	0.102
25	0.613	0.102	0.657	0.103
30	0.624	0.102	0.662	0.103
35	0.633	0.102	0.655	0.103
40	0.623	0.103	0.644	0.103
45	0.631	0.103	0.627	0.103
50	0.632	0.104	0.611	0.104
55	0.646	0.105	0.611	0.106
60	0.648	0.106	0.607	0.108

TABLE XIII: Absorbance at 660 nm for *Prevotella intermedia* cells with the chromogenic substrate N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAAPPPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.654	0.103	0.702	0.104
5	0.623	0.103	0.692	0.104
10	0.610	0.110	0.725	0.113
15	0.612	0.117	0.754	0.122
20	0.615	0.120	0.764	0.126
25	0.609	0.118	0.764	0.124
30	0.608	0.116	0.768	0.121
35	0.625	0.118	0.800	0.123
40	0.637	0.119	0.814	0.125
45	0.654	0.120	0.815	0.127
50	0.659	0.121	0.833	0.128
55	0.694	0.124	0.832	0.132
60	0.683	0.121	0.815	0.127

TABLE XIV: Absorbance at 660 nm for *Prevotella intermedia* cells with the chromogenic substrate N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) over 60 minutes with and without nicotine; mean and standard error of the means shown.

	WITHOUT NICOTINE		WITH NICOTINE (1 mg/ml)	
TIME (MIN)	MEAN (n=12)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$	MEAN (n=9)	STANDARD ERROR OF THE MEANS $[\frac{STANDARD\ DEVATION}{\sqrt{n-1}}]$
0	0.640	0.074	0.659	0.075
5	0.632	0.077	0.644	0.079
10	0.603	0.073	0.661	0.074
15	0.609	0.074	0.655	0.075
20	0.600	0.074	0.643	0.075
25	0.605	0.074	0.626	0.075
30	0.620	0.075	0.621	0.076
35	0.619	0.075	0.617	0.076
40	0.619	0.075	0.616	0.077
45	0.613	0.077	0.620	0.078
50	0.598	0.079	0.624	0.081
55	0.589	0.080	0.627	0.083
60	0.581	0.082	0.630	0.085

TABLE XVV: Overall changes in absorbance at 660 nm after 60 minutes of incubation of *Prevotella intermedia* cells with the four synthetic substrates.[c]

	Prevotella intermedia	*Prevotella intermedia with Nicotine*	Mean Change	*P value*
GPPNA	0.650 ± 0.093	0.603 ± 0.094	- 0.047	0.1387
SAAAPNA	0.648 ± 0.106	0.607 ± 0.108	- 0.041	0.1727
SAAPPPNA	0.683 ± 0.121	0.815 ± 0.127	+ 0.132	0.0973
BAPNA	0.581 ± 0.082	0.630 ± 0.085	+ 0.049	0.8351

[c] *P* value <0.05 was considered statistically significant as obtained from a two factor analysis of variance with replication between the two groups. GPPNA: glycyl-L-proline-*p*-nitroanilide; SAAAPNA: N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; SAAPPPNA: N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; BAPNA: N-α-benzoyl-L-arginine-*p*-nitroanilide.

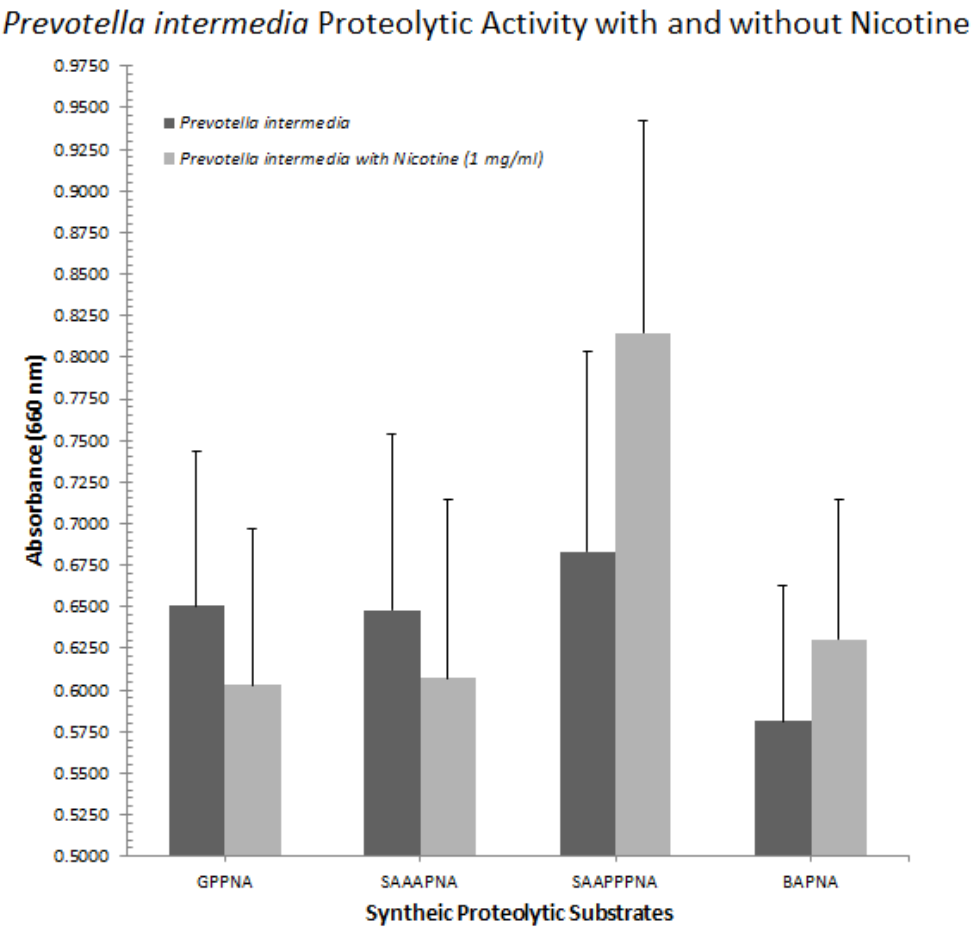

FIGURE 8: Proteolytic activity (mean ± standard errors of the means) of *Prevotella intermedia* cells after 60 minutes of incubation with and without nicotine (1 mg/ml) at an absorbance of 660 nm. Asterisks indicate significant difference ($p<0.05$) between the control and test group. GPPNA: glycyl-L-proline-*p*-nitroanilide; SAAAPNA: N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide; SAAPPPNA: N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide; BAPNA: N-α-benzoyl-L-arginine-*p*-nitroanilide.

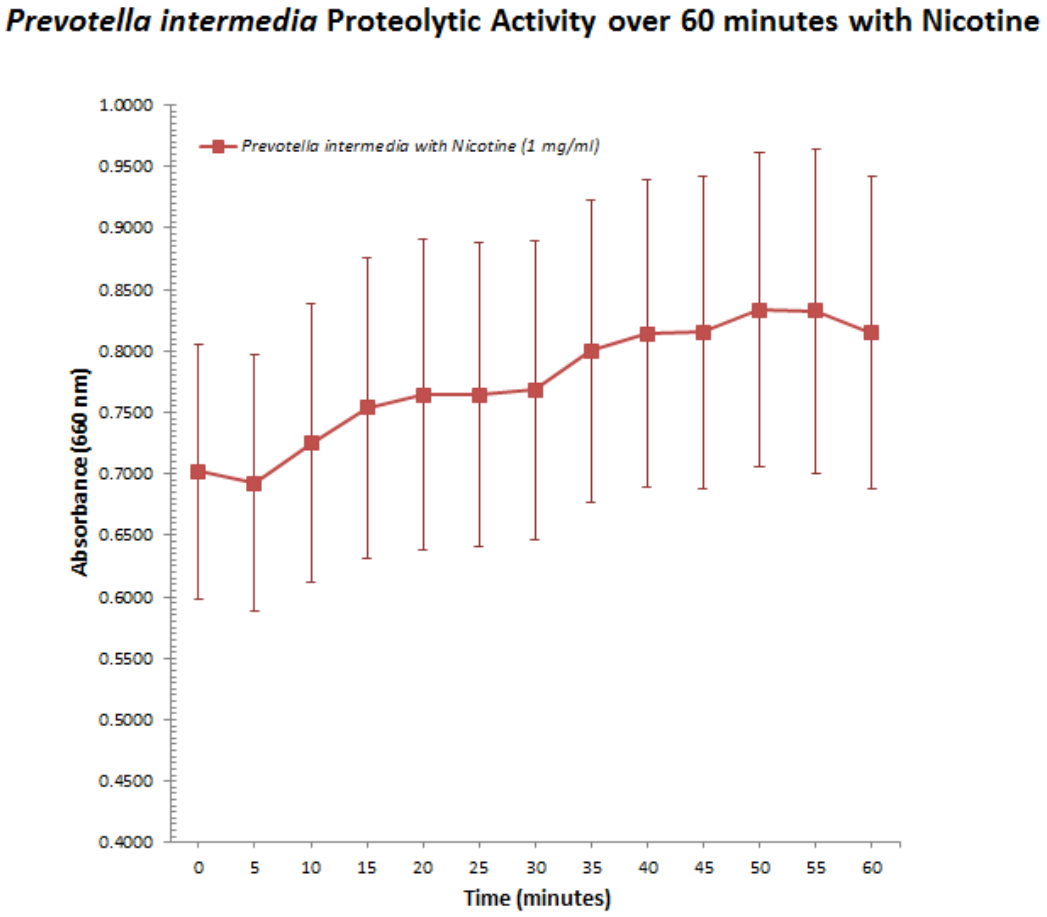

FIGURE 9: Representative proteolytic activity (mean ± standard errors of the means) of *Prevotella intermedia* cells over 60 minutes of incubation with nicotine (1 mg/ml) and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPPPNA) at an absorbance of 660 nm.

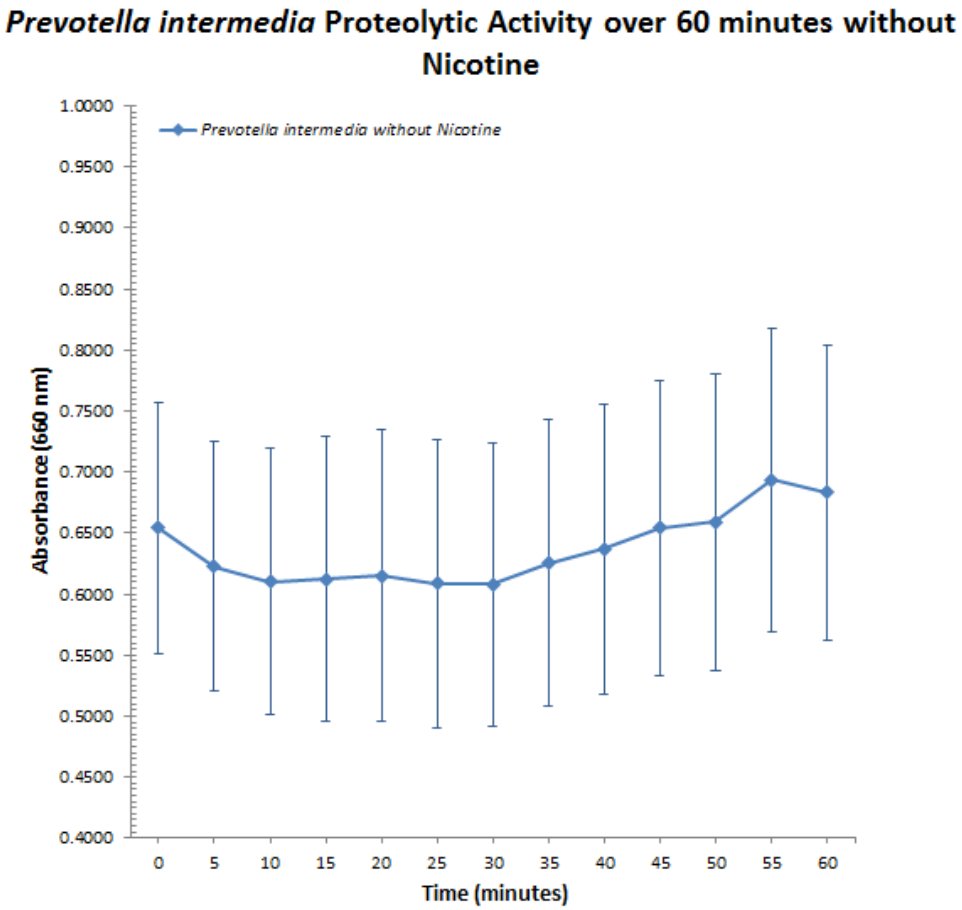

FIGURE 10: Representative proteolytic activity (mean ± standard errors of the means) of *Prevotella intermedia* cells over 60 minutes of incubation without nicotine and N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPPPNA) at an absorbance of 660 nm.

DISCUSSION

Nicotine is one of the main components of cigarette smoke and its effects upon the periodontium and the progression of periodontal disease is well studied. Numerous studies have shown, without a doubt, the effects of nicotine on not only the subgingival microflora but also on the periodontium as well as gingival fibroblasts [10-17, 20-23, 42, 43]. The effects of nicotine and smokeless tobacco on various immune factors had been studied[44-47]. Gregory et al. found that smokeless tobacco users have significantly increased levels of IgA and J chain when compared to non-users [46]. This study ultimately found that smokeless tobacco affects the ability of the secretory component to bind to IgA without a loss in antibody function [46]. In a subsequent study, Gregory et al. examined the effect of smokeless tobacco on mucosal immune factors and found that significantly more salivary IgA, IgA2 and J-chain was seen in users when compared to non-users[45]. This suggests that smokeless tobacco has an effect on secretory epithelial cells which are ultimately responsible for the synthesis of secretory component, lysozyme and lactoferrin along with the packaging of secretory component on IgA[45]. In 1996, Gregory and Gfell reported on the effects of nicotine on the ability of secretory epithelial cells to synthesize secretory component [44]. They found that nicotine significantly decreased the synthesis of secretory component, lactoferrin and lysozyme in the presence of smokeless tobacco or cotinine [44]. From this study they concluded that nicotine, cotinine and smokeless tobacco have an antagonistic effect on the synthesis and secretion of secretory component, lactoferrin and lysozyme [44]. In 2005, Olson et al. examined the effect of nicotine on the release of IL-6 by periodontal ligament fibroblast cells [47]. After exposure of the periodontal ligament fibroblasts to nicotine levels varying from 375 to 1500 µg/ml for a period of 24 hours, the cells exhibited an increase in release of IL-6 [47].

Until now little is known of the effect of nicotine on the virulence factors of periodontopathic bacteria.

Periodontal disease is an inflammatory process induced by supra-gingival and subgingival microbial biofilm. In 1976, Page and Schroeder described the stages of periodontal disease based on histological and ultrastructural features of gingival tissues[48]. The initial lesion most closely correlates with preclinical gingivitis and appears 2 to 4 days after plaque accumulation in previously healthy gingiva and is localized to the gingival sulcus, including the junctional epithelium and the most coronal part of the connective tissue, rarely involving more than 5 to 10% [48, 49]. Characteristics of the initial lesion include: 1) vasculitis subsequent to the junctional epithelium; 2) extravascular fibrin and serum protein; 3) loss of perivascular collagen; 4) increased migration of leukocytes into the junctional epithelium and sulcus; 5) alteration of the coronal part of the junctional epithelium; and 6) increased crevicular fluid [48, 49]. The early lesion correlates with early gingivitis clinically and appears 4 to 7 days after plaque accumulation and is characterized by a dense lymphoid infiltrate[48, 49]. Characteristics of the early lesion include: 1) features of initial lesion are present and progress; i.e., gingival crevicular fluid (GCF) increases with inflammation and crevicular leukocytes peak at 4 to 7 days; 2) collagen loss increases and may reach 60% to 70% in the inflamed tissue; 3) cytoplasmic alterations appear in resident fibroblasts; 4) lymphoid cells accumulate subjacent to the junctional epithelium and make up approximately 75% of the infiltrate resulting in approximately 5 to 15% of the total connective tissue being affected; and 5) basal cells of the junctional epithelium begin to proliferate [48, 49]. Lymphoid cells have a non-specific affinity for inflamed tissue and cellular hypersensitivity may play a role in

the early lesion[48, 49]. The established lesion clinically is related to chronic adult gingivitis and occurs 2 to 3 weeks after the beginning of plaque accumulation[48, 49]. Characteristics include: 1) persistence of the manifestations of acute inflammation; 2) continued loss of connective tissue: fibrosis and scarring may also occur; 3) predominance of plasma cells without appreciable bone loss; 4) extravascular immunoglobulins; and 5) the proliferation, apical migration, and lateral extension of the junctional epithelium [48, 49]. The established lesion may remain stable for extended periods of time[48, 49]. The predominant immunoglobin is IgG with a small but significant amount of IgA and rarely IgM[48, 49].

Different host mechanisms, such as the phagocytic action of neutrophils that migrate continuously through the junctional epithelium into the gingival sulcus typically are able to maintain a normal environment for the host bacterial flora; however, once this balance is disturbed and more pathogenic bacteria accumulate, the host is challenged [50, 51]. In response to this challenge, the epithelial cells within the periodontal pocket initiate the inflammatory response which then leads to cell activation within the connective tissue compartment and the recruitment of neutrophils into the gingival crevice [50, 51]. Pathogenic bacteria induce epithelial cells to secrete IL-1β, TNF-α, IL-6 and IL-8. At the same time, virulence factors that have diffused in the connective tissue, along with as well as IL-1β, TNF-α, IL-6 and IL-8 in turn stimulate macrophages, fibroblasts and mast cells to further produce and release pro-inflammatory cytokines, chemotactic molecules, prostaglandins, histamine and leukotrienes, as well as matrix metalloproteinases (MMPs) that begin the degradation of collagen [50, 51].

Adaptive immunity involves primarily lymphocytes and can be divided into B cells and T cells, both of which have the ability to defend against foreign antigens[50, 52]. B cells primarily produce antibodies, whereas helper T and regulatory T cells help to regulate the immune response by releasing a variety of regulatory cytokines[50, 52]. T helper type 1 (Th1) cells principally secrete IL-2 and interferon-γ (IFN-γ), while T helper type 2 (Th2) cells secrete predominantly IL-4, IL-5, IL-10, and IL-13[50, 52]. Ultimately, whether the Th1 or Th2 response dominates will determine the outcome of the infection and the fate of the tissues at the inflammatory site[52-55]. The most critical factor for the development of Th1 or Th2 phenotype is the cytokines available in the local microenvironment[50, 52].

The other mechanism by which periodontal destruction can progress is through the proteolytic activity of enzymes produced by not only the host but also the periodontopathic bacteria. Examples of proteolytic enzymes include: elastase, dentilisin, dipeptidyl peptidase IV and trypsin-like proteases. Periodontal disease and smoking and the effects upon the periodontium are well studied. Numerous studies have shown, without a doubt, the effects of nicotine, the primary component of cigarette smoke, on not only the subgingival microflora but also on the periodontium as well as gingival fibroblasts [10-17, 20-23, 42, 43].

Previous studies examining the effect of nicotine on the proteolytic activity of cariogenic bacteria have shown a significant difference between nicotine users and non-users and subsequent increases in virulence[56-58]. Based on these previous studies, we examined the effects of nicotine on periodontopathic bacteria. This study of the proteolytic activity of various periodontal pathogens with and without exposure to

nicotine and with various chromogenic substrates showed both statistically significant as well as statistically insignificant increases, as well as decreases in activity (Tables 5, 10, 15). Only *F. nucleatum* showed statistically significant increases in proteolytic activity with all but one of the substrates in the presence of nicotine; however, *P. assacharolyticus* as well as *P. intermedia* showed a statistically insignificant increases along with decreases in proteolytic activity in the presence of nicotine.

The synthetic chromogenic substrates used in these experiments are indicative of specific proteolytic activity. Glycyl-L-proline-*p*-nitroanilide (GPPNA) is typically utilized when dipeptidyl peptidase IV-like activity is to be assessed[40, 59]. Dipeptidyl peptidase IV is a dipeptidyl peptide hydrolase which cleaves peptides with an N-terminal sequence of X-Pro-Y to yield X-Pro and Y[59, 60]. Dipeptidyl peptidase IV has been implicated in the metabolism of connective tissue primarily because of its affinity for the penultimate proline[59, 61]. Specifically, dipeptidyl peptidase IV has been shown to destroy already partially degraded type I collagen thereby contributing to the further destruction of the periodontal ligament and the progression of periodontal disease[62, 63]. The proteolytic activity of *P. assacharolyticus* and *F. nucleatum* demonstrated an increase in activity when incubated with nicotine and GPPNA whereas *P. intermedia* showed a decrease. The only bacterium which exhibited these changes with any statistical significance was *F. nucleatum.*

N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA) is normally used to ascertain elastase-like activity of a microorganism. Elastase has been described as a potent protease with the ability to degrade various connective tissue components such as elastin, proteoglycans, laminin, fibronectin and collagen[64-67]. Studies have shown

that peripheral neutrophils may become hyperactive when exposed to certain periodontopathic bacteria such *F. nucleatum* and may stimulate significant reactive oxygen species generation, cytokine and elastase production which could subsequently result in tissue damage[68, 69]. Katsuragi et al. (2003) noted that *F. nucleatum* may be crucial in the neutrophil-dependent, reactive oxygen species induced damage of tissue within the periodontium as seen in the increased generation of reactive oxygen species upon phagocytosis of the bacterium[69, 70]. When the periodontopathic bacteria were incubated with SAAAPNA, an increase in proteolytic activity was seen with *F. nucleatum* and *P. assacharolyticus* but a decrease in activity was seen with *P. intermedia*, once again with statistical significance seen with the increase in proteolytic activity only with *F. nucleatum*.

N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAAPPPNA) is generally indicative of chymotrypsin-like activity (dentilisin activity) of a bacterium. Dentilisin is a prolyl-phenylalanine specific surface protease and a major pathogen of the red complex bacterium *T. denticola*[71, 72]. Dentilisin hydrolyzes host bioactive proteins[71, 73] and is ultimately cytotoxic to periodontal ligament epithelial cells[71, 74]. Dentilisin, in conjunction with other surface proteins, has been thought to be the direct cause of the cytopathic effects seen on epithelial cells such as loss of desmosomal attachment and pericellular fibronectin[63, 74]. Dentilisin has also been implicated in the migration of *T. denticola* primarily due to its ability to compromise the basement membrane, fibronectin, laminin and type IV collagen allowing for penetration of the host tissues thus contributing, once again, to the progression of periodontal disease [63, 75]. The proteolytic activity of all three of the periodontal pathogens studied demonstrated an increase in

activity when tested with SAAPPPNA; however the increases seen in the proteolytic activity demonstrated no statistical significance with any of the bacteria examined.

Finally, N-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) is utilized when trypsin-like activity of a microorganism is to be assessed. Trypsin cleaves the bond between the arginine and the *p*-nitroaniline to release free *p*-nitroaniline causing a measurable color change. Therefore, it follows, that the trypsin-like activity of the cysteine proteolytic gingipains can be used with BAPNA to determine proteolytic activity of *P. gingivalis* and other proteolytic microorganisms. Trypsin-like activity of the *red complex bacteria* is well studied in the literature and has been reported to have the ability to degrade collagens, proteoglycans, fibronectin and the basement membrane which effectively destroys the periodontal connective tissues thereby enabling invasion of host tissues; inactivate host defense proteins; and impede tissue repair of the periodontal lesion by altering the ability of clot formation or by lysing of the fibrin matrix [76]. The trypsin-like activity of *P. intermedia*, more specifically, has been shown to have the ability to degrade IgG as well as fibronectin which in doing so reduces the effectiveness of the host immune and inflammatory defenses[76-79]. All three of the periodontopathic bacteria studied exhibited an increase in proteolytic activity when incubated with nicotine and subsequently tested with BAPNA; however, once again, only *F. nucleatum* demonstrated this increase with any statistical significance. Since nicotine affects the proteolytic activity of *F. nucleatum, P. intermedia* and *P. assacharolyticus* this may have implications in the progression of periodontal disease.

F. nucleatum is a gram-negative anaerobic bacterium associated with gingivitis and chronic periodontitis but is also implicated in other infections[50, 80, 81]. Not only does

F. nucleatum have the ability to co-aggregate with many plaque bacteria, effectively acting as a sort of *microbial bridge* but it can also initiate physio-chemical changes within the gingival sulcus which allows for pathogenic successors to not only establish themselves but to also allow for subsequent proliferation [50]. Studies have also suggested that *F. nucleatum* facilitates invasion of host cells by *P. gingivalis* [50, 82], has been demonstrated to be a significant marker for destructive periodontal disease in adults [50, 83-85], has been seen more often in active diseased sites and is always present whenever *T. denticola* and *P. gingivalis* are present [50, 86-88]. This confirms the notion that *F. nucleatum* might be required for colonization of subsequent pathogenic successors. Based on the work by Kolenbrander et al., (2002) early and late colonizers of bacterial plaque tend not to co-aggregate with one another but do co-aggregate with *F. nucleatum* which, to some degree, explains why *F. nucleatum* is abundant in diseased as well as healthy periodontal sites [87]. They also described the presence of adhesins on the surfaces of *F. nucleatum*, which appear to be mediated by galactose-binding adhesins, and cognate receptors on the surfaces of those bacteria, such as *P. gingivalis* and *P. intermedia*, which competitively bind with *F. nucleatum* [87, 89].

P. intermedia is a black-pigmented gram-negative obligate anaerobic non-motile rod and is prevalent in patients with chronic periodontitis, aggressive periodontitis and acute necrotizing ulcerative gingivitis[50, 88, 90-92]. Several proteolytic enzymes have been described including trypsin-like serine proteases and a dipeptidyl peptidase IV[50, 93-95]. *P. intermedia* also possess various types of fimbriae which may mediate the adherence of the bacterium to various surfaces [50].

Even though *P. assacharolyticus* is a suitable substitute, based on the similarities of the biochemical and chemical properties, for *P. gingivalis* in most respects based on the evidence provided by Shah and Collins, it is still not identical [6]. Both *P. assacharolyticus* and *P. gingivalis* are gram-negative, obligately anaerobic, non-spore forming, non-motile rods or coccobacilli and both form colonies on blood agar plates which are smooth, shiny (*P. gingivalis* can be rough sometimes), convex and 1 to 3 mm in diameter and darken progressively from the edge of the colony toward the center after 6 to 10 days in the case of *P. assacharolyticus* and 4 to 8 days in the case of *P. gingivalis*[6]. However this is where subtle differences become apparent. *P. gingivalis* colonies are always black pigmented with non-pigmented colonies rarely occurring whereas *P. assacharolyticus* colonies become progressively pigmented due to increased protoheme production [6]. The proteolytic activities of each are different: proteolytic activity for *P. assacharolyticus* is low but gelatin liquefaction is positive and fibrinolytic activity is present and can produce indole; *P. gingivalis* has proteases (i.e., trypsin-like enzyme and collagenase) and can produce indole [6]. Indole formation has been shown to be present in greater numbers in the saliva of patients with periodontitis and is representative of the putrefactive end products normally seen in periodontal breakdown[96-98].

According to Eley et al in 2003, the proteolytic activity of putative periodontal bacteria without the presence of nicotine is varied [76]. They found that *F. nucleatum* did not exhibit any dipeptidyl peptidase IV-like, elastase-like, chymotrypsin-like or trypsin-like activity based on bacterial cell sonicates in the absence of nicotine. In examining the data presented here, there was a statistically significant increase in the proteolytic activity

in the presence of nicotine with the aforementioned proteases (only the chymotrypsin-like activity was statistically insignificant). Eley et al also demonstrated that *P. gingivalis* has an increased protease activity of dipeptidyl peptidase IV and of trypsin-like proteases but no activity of the elastase-like or the chymotrypsin-like protease. Once again, the data presented here suggests an increased, but not statistically significant, trend in proteolytic activity in the presence of nicotine with all four of the proteases examined when incubated with *P. assacharolyticus.* Finally, the authors stated that *P. intermedia* demonstrated an increased protease activity of dipeptidyl peptidase IV and trypsin like proteases and no activity of the elastase-like or the chymotrypsin-like protease in the absence of nicotine. The data presented here suggests that there is an increased trend in the proteolytic activity of the chymotrypsin-like and the trypsin-like proteases and a decreased trend in the proteolytic activity of dipeptidyl peptidase IV and elastase-like activity when incubated with nicotine. These conclusions can only be made as extrapolations of the data that Eley et al presented and based on the known effects of nicotine on the periodontal apparatus and not necessarily from the data presented here directly due to a lack of statistical significance.

SUMMARY AND CONCLUSIONS

Based on the results of this study, it can be concluded that in the presence of 1 mg/ml of nicotine the dipeptidyl peptidase, elastase, chymotrypsin and trypsin-like proteolytic activity of the periodontal pathogens *F. nucleatum* and *P. assacharolyticus* demonstrated a trend of increased activity, albeit without any statistical significance in the case of *P. assacharolyticus*, after a period of 60 minutes. More specifically, however, statistically significant increases in the proteolytic activity of dipeptidyl peptidase, elastase and trypsin-like proteases were seen with *F. nucleatum* in the presence of 1 mg/ml of nicotine. However, the dipeptidyl peptidase, elastase, chymotrypsin and trypsin-like proteolytic activity of *P. intermedia* exhibited a trend in increased activity only with the substrates SAAPPPNA and BAPNA and a trend of decreased activity with GPPNA and SAAAPNA, once again without demonstrating any statistical significance. Therefore, it could be concluded that based on these results nicotine at a concentration of 1 mg/ml may increase the proteolytic activity of periodontal pathogens and thus may increase periodontal disease activity and subsequent periodontal breakdown. Further studies are needed to validate these results utilizing different concentrations of nicotine.

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ABSTRACT

EFFECTS OF NICOTINE ON THE PROTEOLYTIC ACTIVITY OF PERIODONTAL PATHOGENS

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Periodontal disease is the leading cause of tooth loss in adults. Bacterial biofilm on tooth surfaces is the primary initiator of periodontal disease. Various factors contribute to the severity of periodontal disease including the different virulence factors of the bacteria within the biofilm. In the progression of periodontal disease, the

microflora evolves from a predominantly Gram positive microbial population to a mainly Gram negative population. Specific gram negative bacteria with pronounced virulence factors have been implicated in the etiology and pathogenesis of periodontal disease, namely *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* which form the *red complex* of bacteria. The *orange* complex bacteria become more dominant in the maturation process of dental plaque and act to bridge the early colonizers of plaque with the later more dominant *red* complex bacterial and consists of such bacteria as *Campylobacter showae, Campylobacter rectus, Fusobacterium nucleatum* and *Prevotella intermedia*. Perhaps the most investigated contributing factor is the relationship between smoking and periodontal disease. When examining the association between cigarette smoking and interproximal bone loss, greater bone loss is associated with higher cigarette consumption, longer duration (i.e., pack year history) and higher lifetime exposure. The presence of various virulence factors such as the production of a capsular material, as well as the proteolytic activity of the various periopathodontic bacteria has been associated with the pathogenesis of periodontitis. Even though many different enzymes are produced in large quantities by these periodontal bacteria, trypsin-like enzymes, chymotrypsin-like enzymes and elastase-like enzymes, as well as dipeptidyl peptidase-like enzymes, have been thought to increase the destructive potential of the bacterium and mediate destruction of the periodontal apparatus. More specifically, it is hypothesized that the proteolytic activity of other clinically important periodontal pathogens, such as *Fusobacterium nucleatum, Prevotella intermedia and Porphyromonas assacharolyticus*, is increased in the presence of nicotine. The purpose of this study was to determine the effects of nicotine on *F. nucleatum, P. intermedia* and *P. assacharolyticus* proteolytic

activity. Cultures were maintained on anaerobic blood agar plates containing 3% sheep blood. Bacterial cells were harvested from the plates and washed. Washed *F. nucleatum, P. intermedia* and *P. assacharolyticus* cells were incubated with 1 mg/ml of nicotine. Bacterial cells not incubated with nicotine were used as positive controls. Secreted enzymatic activity was measured using the synthetic chromogenic substrates glycyl-L-proline-*p*-nitroanilide (GPPNA), N-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide (SAAAPNA), N-succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (SAAPPPNA) and N-α-benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA) (Sigma-Aldrich Products, St. Louis, MO, USA). Appropriate means and standard deviations were determined for each of the enzymatic activities measured and analysis of variance (ANOVA) was used to compare the groups utilizing a 5% significance level for all comparisons. Results demonstrated that after 60 minutes of incubation of *F. nucleatum, P. intermedia* and *P. assacharolyticus* cells with 1 mg/ml of nicotine and the various synthetic substrates, had the following proteolytic activity for GPPNA: 0.83 ± 0.14, 0.72 ± 0.03 and 0.67 ± 0.10, respectively; SAAAPNA: 0.82 ± 0.06, 0.76 ± 0.05 and 0.68 ± 0.08, respectively; SAAPPPNA: 0.90 ± 0.13, 0.85 ± 0.17 and 0.72 ± 0.03, respectively; and BAPNA: 0.81 ± 0.15, 0.74 ± 0.13 and 0.74 ± 0.16, respectively. In conclusion, the results indicate that in the presence of 1 mg/ml of nicotine, the proteolytic activity of *F. nucleatum* and *P. assacharolyticus* was increased with all of the synthetic substrates (with statistical significance seen only in the increases with *F. nucleatum* and GPPNA, SAAAPNA and BAPNA). The proteolytic activity exhibited an increasing trend in activity for *P. intermedia* with SAAPPPNA and BAPNA but a decreasing trend in activity with GPPNA and SAAAPNA when incubated with 1 mg/ml of nicotine, once

again demonstrating no statistical significance for any of the substrates. Therefore, it could be concluded that based on these results nicotine at a concentration of 1 mg/ml may increase the proteolytic activity of periodontal pathogens and thus may increase periodontal disease activity and subsequent periodontal breakdown. Further studies are needed to validate these results utilizing different concentrations of nicotine.

CURRICULUM VITAE

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