A.Y. Shchipkova[1], H.N. Nagaraja[2], and P.S. Kumar[1]*

[1]Division of Periodontology, College of Dentistry, The Ohio State University, 305 W. 12th Ave., 4111 Postle Hall, Columbus, OH 43210, USA; and [2]Department of Statistics, The Ohio State University; *corresponding author, kumar.83@osu.edu

Subgingival Microbial Profiles of Smokers with Periodontitis

ABSTRACT

The subgingival microbiome is largely uncultivated, and therefore, cultivation-based and targeted molecular approaches have limited value in examining the effect of smoking on this community. We tested the hypothesis that the subgingival biofilm is compositionally different in current and never-smokers by using an open-ended molecular approach for bacterial identification. Subgingival plaque from deep sites of current and never-smokers matched for disease was analyzed by 16S sequencing. Smokers demonstrated greater abundance of *Parvimonas, Fusobacterium, Campylobacter, Bacteroides,* and *Treponema* and lower levels of *Veillonella, Neisseria,* and *Streptococcus.* Several uncultivated *Peptostreptococci, Parvimonas micra, Campylobacter gracilis, Treponema socranskii, Dialister pneumosintes,* and *Tannerella forsythia* were elevated in this group, while *Veillonella* sp. oral clone B2*, Neisseria* sp. oral clone 2.24, *Streptococcus sanguinis,* and *Capnocytophaga* sp. clone AH015 were at lower levels. The microbial profile of smoking-associated periodontitis is distinct from that of non-smokers, with significant differences in the prevalence and abundance of disease-associated and health-compatible organisms.

KEY WORDS: bacteria, subgingival, molecular, DNA, smoking, periodontitis.

INTRODUCTION

Evidence indicates that chronic periodontitis results from the presence of complex microbial communities in the subgingival sulcus (Socransky and Haffajee, 2005), and that smoking significantly increases the risk for the development of extensive and severe disease (Tomar and Asma, 2000; Johnson and Hill, 2004). Although both bacterial plaque and smoking play important roles in the pathogenesis of periodontitis, associations between smoking and subgingival bacterial species or consortia have not been well-elucidated.

Earlier investigations have used traditional methods for bacterial identification—for example, culturing or targeted DNA-based assays (PCR, real-time PCR, DNA-DNA hybridization) (Stoltenberg *et al.,* 1993; Darby *et al.,* 2000; Bostrom *et al.,* 2001; Haffajee and Socransky, 2001; van Winkelhoff *et al.,* 2001; Van der Velden *et al.,* 2003; Apatzidou *et al.,* 2005; Natto *et al.,* 2005). However, recent investigations have revealed that the subgingival microbiome is largely uncultivated, and that several uncultivated phylotypes may be important in disease etiology (Kumar *et al.,* 2006). Because culturing selects for organisms that grow under certain conditions and targeted molecular approaches are limited to the examination of only previously known species, these tools are inadequate for a comprehensive examination of this complex ecosystem. An exploration of this microbiome by a comprehensive, quantitative, open-ended molecular approach will advance our understanding of the effect of smoking on the composition of the subgingival biofilm.

Researchers have extensively used 16S cloning and sequencing to examine several host-associated ecosystems (Bittar *et al.,* 2008). The presence of large tracts of variable sequences within the 16S rRNA gene provides unique bacterial 'signatures' that allow for accurate bacterial identification, while sequencing a finite number of clones from each sample provides quantitative information on the relative abundance of each organism within a community. We have previously used this assay to provide representational information on previously unknown and unsuspected bacteria associated with periodontal health and disease in non-smokers (Kumar *et al.,* 2005, 2006).

The purpose of the present investigation was to explore the subgingival microbiome of current and never-smokers with periodontitis using 16S cloning and sequencing for bacterial identification.

METHODS

Participant Selection

Approval for this study was obtained from the Office of Responsible Research Practices at The Ohio State University. Fifteen current smokers (tobacco

exposure of 10 pack-years or more) and 15 age- and sex-matched never-smokers with generalized moderate to severe chronic periodontitis (attachment loss ≥ 6 mm and probe depths ≥ 5 mm in 30% or more of sites) were identified following clinical and radiographic examination and after informed consent had been obtained. Exclusion criteria for both groups included diabetes, HIV infection, use of immunosuppressant medications, bisphosphonates, or steroids, antibiotic therapy, or oral prophylactic procedures within the preceding 3 mos, and fewer than 20 teeth in the dentition.

Data Collection

Smoking status and tobacco exposure were assessed by questionnaire. All participants were examined by a single periodontist. Probe depths and attachment levels were recorded throughout the mouth on 6 sites *per* tooth by means of a PCP-UNC 15 probe. Bleeding on probing and plaque levels were recorded on a binary scale (presence/absence) for each surface.

Sample Collection and DNA Isolation

Subgingival plaque samples were collected and pooled from 4 non-adjacent proximal sites demonstrating at least 6 mm of attachment loss and 5 mm of probe depths. We collected samples by inserting 15 sterile endodontic paper-points (Caulk-Dentsply, Milford, DE, USA) into the pockets for 10 sec, following isolation and supragingival plaque removal. Samples were placed in 1.5-mL microcentrifuge tubes and frozen until further analysis. Bacteria were separated from the paper-points by the addition of 200 μL of phosphate-buffered saline (PBS) to the tubes, followed by vortexing. The points were then removed, and DNA was isolated with a Qiagen DNA MiniAmp kit (Qiagen, Valencia, CA, USA) using the tissue protocol according to the manufacturer's instructions.

Amplification of 16S rDNA

Bacterial 16S rRNA genes were amplified from the community DNA with universal bacterial primers A17 (5′-GTT TGA TCC TGG CTC AG- 3′) and 317 (5′AAG GAG GTG ATC CAG GC 3′) (Biosynthesis, Lewisville, TX, USA). PCR was performed as previously described (Kumar *et al.,* 2005). The PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA).

Cloning and Sequencing

The 16S amplicons generated by PCR were cloned into *E. coli* with the use of a commercially available kit (TOPO TA cloning kit, Invitrogen, San Diego, CA, USA). Inserts of the correct molecular size (≅ 1500 bp) were confirmed by PCR amplification. The products were purified with a Millipore kit (Millipore, Billerica, MA, USA) and sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit) with an ABI 3730 instrument.

Sequence Analysis

Partial sequences of 1100 to 1300 bp were obtained from each amplicon. The sequences generated were compared with the GenBank database for genus-level identification. Sequences belonging to the same genus were then aligned to each other, and a similarity matrix was constructed from the alignments by the method of Jukes and Cantor (1969). Phylogenetic trees were constructed by the 'neighbor joining' method. Sequence similarity of 95% was used to delineate genera, and sequences with 98% or greater similarity within a genus were considered the same species. MacVector software (MacVector, Cary, NC, USA) was used to generate alignments and similarity matrices, and in phylogenetic tree construction.

Statistical Analysis

A minimum of 100 clones were identified from each sample. Statistical analysis was carried out with JMP (SAS Institute Inc., Cary, NC, USA). A variance-stabilizing transformation was used to create normal distribution of the data. The proportion (p) of each species in the community of each subject was expressed as:

$$X = \sin^{-1}(\sqrt{p}).$$

We used a two-sample *t* test to compare the means of this transformed variable X between current and never-smokers. Fisher's exact test was used to test for the presence or absence of species and genera in smokers and non-smokers. Regression analysis with an interaction term was used to examine the effect of smoking on inter-bacterial relationships. Reported p-values correspond to two-tailed tests.

RESULTS

Fifteen current smokers and 15 never-smokers with generalized moderate to severe chronic periodontitis were recruited. There were no differences in clinical and demographic characteristics between the two groups except for tobacco exposure and bleeding on probing (Table 1).

In total, 3157 clones were identified, representing 197 species/phylotypes in current smokers and 176 in never-smokers. The mean number of species in individuals who smoked was 37.1 ± 10.5, while never-smokers demonstrated 36.8 ± 9.1 species (p = 0.67, *t* test). There was no difference in the number of as-yet-uncultivated phylotypes between current and never-smokers (38.8% and 44.5%, respectively, p = 0.09, two-sample *t* test on transformed clone numbers). In both current and never-smokers, the taxa *Synergistes, Lachnospira,* and *Desulfobulbus* were composed entirely of as-yet-uncultivated phylotypes, and *Selenomonas, Neisseria, Veillonella, Eubacterium,* and *Catonella* were predominantly uncultivated (Fig. 1).

Current smokers demonstrated significantly greater levels of the genera *Parvimonas, Campylobacter, Treponema, Bacteroides,* and *Fusobacterium,* while never-smokers demonstrated higher levels of *Streptococcus, Veillonella,* and *Neisseria*

Table 1. Clinical and Demographic Characteristics of Participants (mean ± standard deviation)

	Current Smokers (N = 15)	Never-smokers (N = 15)	p-value
Mean age (yrs)	52.3 ± 2.1	50.2 ± 3.3	0.53
Gender (male)	11	11	
Tobacco exposure (mean pack-yrs ± SD)	18.3 ± 2.7	0	0.007
Clinical parameters at sampled sites			
Mean plaque index (± SD)	2.3 ± 0.6	2.6 ± 0.4	0.32
Mean bleeding on probing (% ± SD)	37.0 ± 2.8	52.8 ± 1.2	0.043
Mean clinical attachment levels (mm ± SD)	7.0 ± 0.6	6.8 ± 0.8	0.56
Mean probing depths (mm ± SD)	6.7 ± 0.3	7.1 ± 0.5	0.72

Significant differences in tobacco exposure and bleeding on probing were seen between the two groups (two-sample *t* test) (AYS, HNN, PSK).

($p < 0.05$, two-sample *t* test) (Fig. 2A). The genus *Neisseria* was detected more frequently in never-smokers ($p = 0.01$, Fisher exact test), while *Bacteroides* were detected more frequently in current smokers ($p < 0.05$) (Fig. 2B).

Significant differences were observed in both the prevalence and abundance of certain species between current and never-smokers (Table 2). *Parvimonas micra* and *Campylobacter gracilis* formed a large fraction of the subgingival microbial community in smokers, but were not as abundant in never-smokers ($p < 0.05$ and $p < 0.01$, respectively; two-sample *t* test). Current smokers also demonstrated higher levels of *Treponema socranskii* ($p < 0.05$), *Dialister pneumosintes* ($p < 0.01$), and *Peptostreptococcus* sp. oral clones BS044, FG014, AP24, and 2002–69396 97 ($p < 0.05$). Never-smokers demonstrated greater levels of *Veillonella* sp. oral clone B2 ($p < 0.05$), *Neisseria* sp. oral clone 2.24 ($p < 0.05$), and *Streptococcus sanguinis* ($p < 0.01$). *Tannerella forsythia* and *Parvimonas* sp. oral clone FG014 were detected only in current smokers, while *Capnocytophaga* sp. oral clone AH015 was found only in never-smokers ($p < 0.05$, Fisher's exact test).

Significantly opposing patterns of co-colonization were observed between *Parvimonas* and 3 other genera in current and never-smokers (Fig. 3). In both groups, these interactions were significant ($p < 0.05$). In smokers, a positive co-association was observed between *P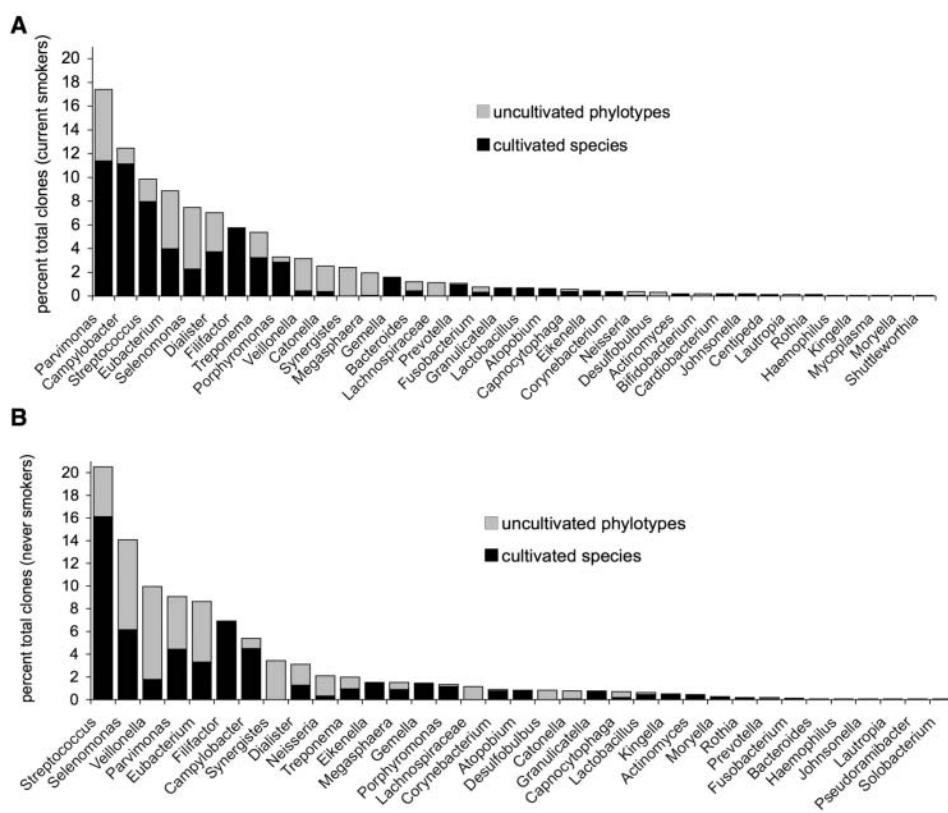arvimonas* and *Streptococcus* in each individual, while a negative co-association was observed among *Parvimonas, Synergistes,* and *Porphyromonas.*

Figure 1. Distribution of cultivated species and uncultivated phylotypes by genus in 15 current smokers (**A**) and 15 never-smokers (**B**). There was no difference in the number of cultivated or as-yet-uncultivated organisms between the two groups ($p > 0.05$, two-sample *t* test on transformed data) (AYS, HNN, PSK).

DISCUSSION

The subgingival microbiome of smokers has previously been examined by either culturing or molecular methods targeting bacterial species identified by cultivation (Zambon *et al.,* 1996;
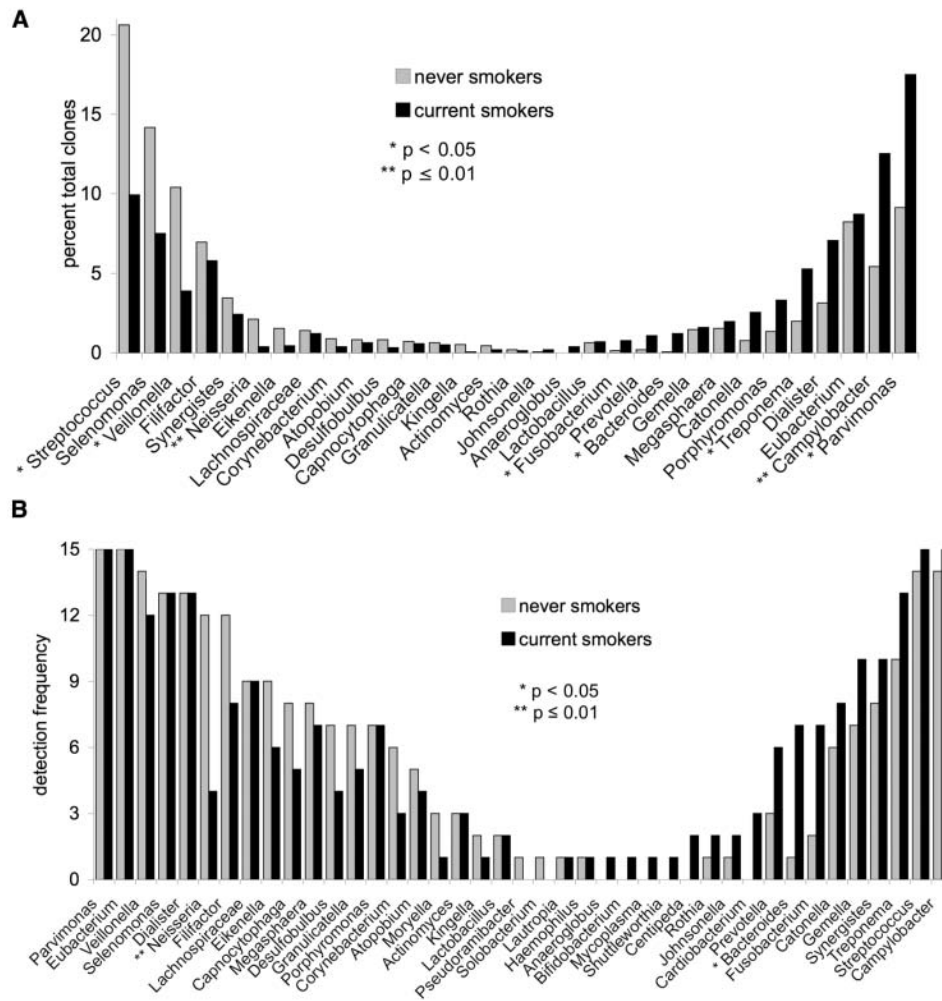
Figure 2. Distribution of 3157 clones by genus in 15 current and 15 never-smokers. Levels of genera are shown in **(A)** and detection frequency in **(B)**. Genera predominant in never-smokers are arranged on the left, and those predominant in current smokers are arranged on the right. Significant differences in abundance ($p < 0.05$, two-sample t test on transformed variable) and prevalence ($p < 0.05$, Fisher's exact test) of certain genera were observed between the two groups (AYS, HNN, PSK).

these disease-matched groups allowed us to explore the association between smoking and the disease-associated biofilm.

A large fraction of the subgingival microbiome in both smokers and non-smokers was uncultivated; however, the numbers of uncultivated organisms were significantly lower in the present study as compared with those reported in earlier investigations (Kumar *et al.,* 2006). Improvements in high-throughput sequencing have made it consistently possible to obtain high-quality sequences that encompass nearly the entire 16S rRNA gene. Because the gene is a mosaic of conserved and variable sequence tracts, longer sequences allow for highly stringent classifications of species and phylotypes. Thus, analysis of the data supports the importance of using longer sequences for phylogenetic analysis.

Uncultivated phylotypes of *Peptostreptococci* clustered with *P. micra* within the same genus; similarly, *Bacteroides* and *Tannerella* formed a genus-level cluster. The deep sites of current smokers exhibited significantly lower levels of *Veillonella, Strepto-coccus,* and *Neisseria* than non-smokers. These genera are reported to be abundant in health-associated biofilms, and their levels decrease in disease (Paster *et al.,* 2001; Kumar *et al.,* 2005, 2006), suggesting that in smokers periodontitis is associated with a greater depletion of beneficial bacteria than in non-smokers. Smokers also demonstrated a greater abundance of *Parvimonas, Campylobacter, Treponema, Bacteroides,* and *Fusobacterium*—genera that are consistently associated with disease (Socransky *et al.,* 1998; Kumar *et al.,* 2005). Analysis of the data, taken together, suggests that periodontitis in smokers is associated with a microbial community that is preferentially enriched for disease-associated pathogens. We have also reported that these disease-associated organisms decrease in levels following smoking cessation (Delima *et al.,* 2010). Thus, pathogen enrichment may be a mechanism by which smoking increases the risk for severe and extensive periodontitis, and this requires further study with a longitudinal study design to examine disease onset and progression in current and never-smokers.

Shiloah *et al.,* 2000; Haffajee and Socransky, 2001; van Winkelhoff *et al.,* 2001; Van der Velden *et al.,* 2003; Darby *et al.,* 2005). These closed-ended or selective approaches showed no clear association between smoking and changes in the subgingival microbiome. In the present study, we used cloning and sequencing of bacterial 16S rRNA genes, allowing for the identification and enumeration of as-yet-uncultivated organisms as well as novel and previously unsuspected species.

Epidemiological evidence indicates that current smokers have greater extent and severity of disease as compared with never-smokers (Tomar and Asma, 2000). It has been suggested that the greater severity of periodontitis in smokers as compared with non-smokers may account for differences in subgingival bacterial profiles (Haffajee and Socransky, 2001). Therefore, in the present investigation, current and never-smokers with similar severity and extent of attachment loss and probing depths were selected. Comparing bacterial profiles of the deep sites from

Table 2. Species and Phylotypes Demonstrating Significant Differences in Detection Frequency and Levels between 15 Current and 15 Never-smokers, Arranged in Order of Decreasing Overall Prevalence

Species/Phylotype(s)	Percent Total Clones N = 3157	Detection Frequency		Mean, Median (range)	
		Current Smokers N = 15	Never-smokers N = 15	Current Smokers	Never-smokers
*Parvimonas micra**	7.3	13	12	10.4, 5.6 (0, 35.9)	3.8, 1.7 (0, 15.7)
*Campylobacter gracilis***	3.4	12	7	5.5, 5.5 (0, 13.7)	1.5, 0 (0, 5.1)
Veillonella sp. oral clone VeillB2*	2.3	9	12	1.3, 1 (0, 4.7)	3.3, 2.6 (0, 9.6)
*Treponema socranskii**	1.7	10	7	2.7, 2 (0, 7.0)	0.6, 0 (0, 1.9)
*Dialister pneumosintes***	1.5	10	4	2.5, 2 (0, 7.8)	0.5, 0 (0, 4.3)
Peptostreptococcus sp. oral clone BS044*	1.3	7	3	2.4, 0 (0, 11.6)	0.3, 0 (0, 2.9)
Peptostreptococcus sp. oral clone AP24*	1.2	1	6	0.1, 0 (0, 1.9)	2.2, 0 (0, 13.2)
Streptococcus sanguinis[##]**	1.1	2	9	0.1, 0 (0, 1.0)	2.2, 0 (0, 9.5)
Peptostreptococcus sp. 2002–69396 97[#]*	0.9	9	2	0.9, 0 (0, 5.3)	0.8, 0 (0, 9.7)
Neisseria sp. clone 2.24*	0.8	2	7	0.1, 0 (0, 1.0)	1.3, 0 (0, 8.6)
Peptostreptococcus sp. oral clone FG014[#]*	0.5	5	0	1.1, 0 (0, 7.3)	0, 0 (0, 0)
Tannerella forsythia[#]*	0.2	4	0	0.5, 0 (0, 2.9)	0, 0 (0, 0)
Capnocytophaga sp. oral clone AH015[#]*	0.2	0	4	0, 0 (0, 0)	0.3, 0 (0, 2.3)

Mean, median, and range are shown; p-values for prevalence are indicated by pound signs ([#] $p < 0.05$, and [##] $p < 0.01$), and significant differences in levels are indicated by asterisks (* $p < 0.01$, ** $p \leq 0.01$) (*t* test and Fisher's exact test) (AYS, HNN, PSK).

Several opposing patterns of bacterial co-colonization were observed in smokers and non-smokers. The most robust of these associations was between *Streptococci* and *Parvimonas,* two abundant genera in both current and never-smokers. Smokers with high levels of *Streptococci* also exhibited high levels of *Parvimonas,* in contrast to non-smokers. *Streptococci* normally form a large fraction of a health-associated microbiome (Aas *et al.,* 2005), and recent evidence suggests that they play a critical role in preventing colonization of this niche by pathogens (Stingu *et al.,* 2008; Van Hoogmoed *et al.,* 2008). It is possible that this protective function of *Streptococci* is impaired in smokers, leading to an altered pattern of co-colonization. In the present study, higher levels of *Parvimonas* also correlated with lower levels of *Synergistes* and *Porphyromonas* in current smokers, but not in never-smokers. *Parvimonas* normally co-aggregates with *Porphyromonas* (Kremer and van Steenbergen, 2000), and *in vitro* investigations indicate that it is essential for growth of *Synergistes* (Downes *et al.*, 2009). Analysis of the data, taken together, suggests that smoking may alter normal inter-bacterial relationships, thus contributing to preferential colonization by certain species. Longitudinal studies that examine the acquisition and colonization of bacteria in smokers are warranted for better elucidation of the effect of smoking on subgingival bacterial inter-relationships.

P. micra (formerly *Peptostreptococcus micros)* was the most abundant species overall, and was detected in both smokers and non-smokers with equal frequency. However, the levels of this species were significantly greater in the deep sites of smokers. This Gram-positive, micro-aerophilic organism is found in polymicrobial infections such as intracranial abscesses, sinus infections, and periodontitis

(Murdoch *et al.,* 1988; Brook, 2006). *P. micra* possesses several virulence factors that contribute to its pathogenic potential. The cell wall of *P. micra* has been shown to induce a potent inflammatory response in macrophages (Tanabe *et al.,* 2007), and it produces proteases that enable it to penetrate the basement membrane (Grenier and Bouclin, 2006). Further, *P. micra* demonstrates a carbohydrate-mediated co-aggregation with *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Kremer and van Steenbergen, 2000).

C. gracilis is a non-motile, micro-aerophilic, Gram-negative organism that has been consistently found in polymicrobial anaerobic infections such as liver, foot, and lung abscesses (Johnson *et al.,* 1985), as well as in periodontal and endodontic infections (Sassone *et al.,* 2008). Evidence regarding the pathogenic mechanisms of these organisms is limited; however, they demonstrate a high level of resistance to several antibiotics, including penicillins and cephalosporins (Johnson *et al.,* 1985), which may contribute to their persistence in chronic infections. Further studies are needed to explore the role played by these organisms in smoking-related periodontitis.

In summary, the microbial profile of smoking-related periodontitis is distinct from that of non-smokers. More differences were observed in abundance than in prevalence of species, suggesting that smoking preferentially enriches an indigenous microbial community for pathogens, while depleting it of health-compatible commensals. Opposing patterns of bacterial co-colonization were observed in smokers as compared with non-smokers, providing evidence for altered inter-bacterial interactions within this community.
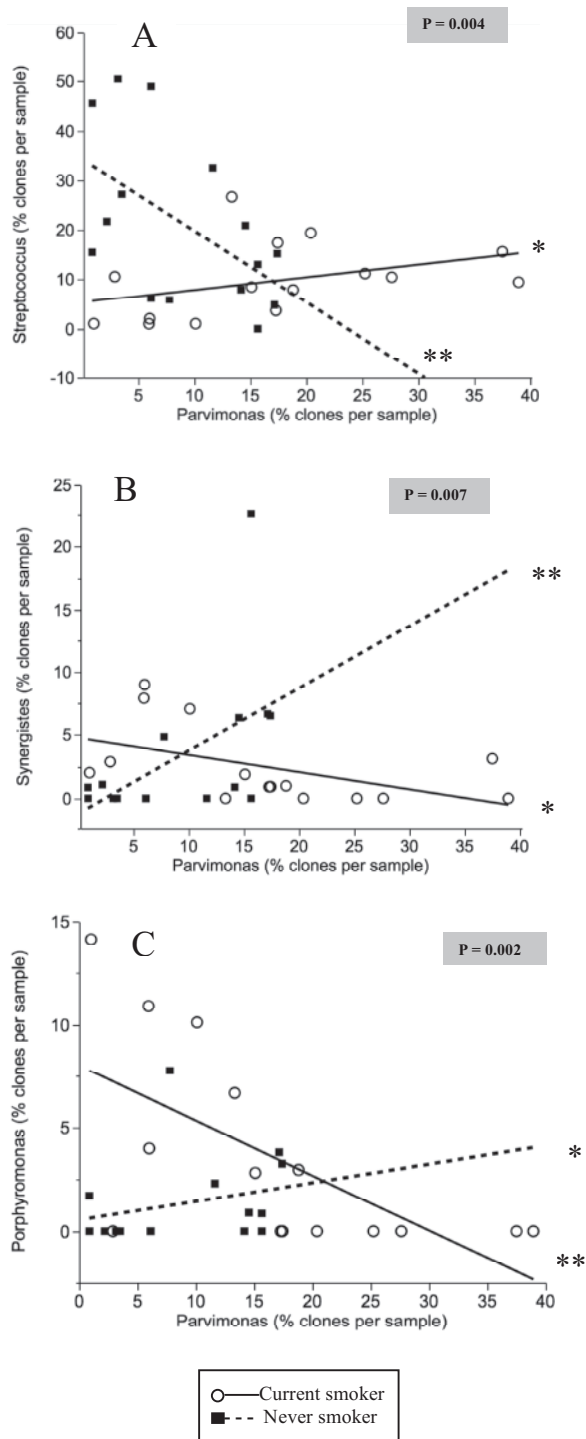
Figure 3. Best linear fits for co-association between genera in current and never-smokers. P-values highlighted in grey correspond to the null hypothesis that the two slopes are the same. P-values corresponding to the null hypothesis that each slope is zero are also shown (*p < 0.05, **p < 0.01) (AYS, HNN, PSK).

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