Smoking decreases structural and functional resilience in the subgingival ecosystem


Abstract
Aims: Dysbiotic microbial communities underlie the aetiology of several oral diseases, especially in smokers. The ability of an ecosystem to rebound from the dysbiotic state and re-establish a health-compatible community, a characteristic known as resilience, plays an important role in susceptibility to future disease. The present investigation was undertaken to examine the effects of smoking on colonization dynamics and resilience in marginal and subgingival biofilms.

Materials and methods: Marginal and subgingival plaque and gingival crevicular fluid samples were collected from 25 current and 25 never smokers with pre-existing gingivitis at baseline, following resolution, after 1, 2, 4, 7, 14 and 21 days of undisturbed plaque formation and following resolution. 16S cloning and sequencing was used for bacterial identification and multiplexed bead-based flow cytometry was used to quantify the levels of 27 immune mediators.

Results: Smokers demonstrated an early pathogenic colonization that led to sustained pathogen enrichment with periodontal and respiratory pathogens, eliciting a florid immune response. Smokers also demonstrated greater abundance of pathogenic species, poor compositional correlation between marginal and subgingival ecosystems, and significantly greater pro-inflammatory responses following resolution of the second episode of disease.

Conclusions: The ability of the subgingival microbiome to “reset” itself following episodes of disease is decreased in smokers, thereby lowering the resilience of the ecosystem and decreasing its resistance to future disease.

Bacteria colonize the tooth soon after eruption and establish communities in two distinct, yet geographically connected habitats—the supragingival area and the subgingival sulcus (Listgarten 1976). It is known that the subgingival and the supragingival (marginal) biofilms are compositionally different—a feature known as community structure; and that these compositional differences lead to different functionalities (Page 1986). One function is maintaining equilibrium with the host immune response; dysbiosis within this community leads to disruption of this equilibrium and results in disease (Hajishengallis & Lamont 2012). The ability of an ecosystem to rebound from the dysbiotic state and re-establish a health-compatible community, a characteristic known as resilience, plays an important role in susceptibility to future disease (Wardwell et al. 2011).

Conflict of interest and source of funding statement
The study was supported by a research grant from Philips Oral HealthCare. Marcelo Aspiras, Marilyn Ward and Marko de Jager are employees of the funding agency.

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
suggesting an aetiological role for loss of resilience in disease causation. In addition, gingivitis is a necessary precursor to periodontitis (Page & Schroeder 1976, Page 1986). It is established that smokers are at high-risk for periodontal disease and that one in two smokers are likely to develop extensive and severe disease (Bergstrom 1989, Bergstrom et al. 2000, Tomar & Asma 2010, Bizzarro et al. 2013). It is possible that smoking increases the risk for disease by promoting dysbiosis and decreasing community resilience, thereby shifting the equilibrium of host-bacterial interactions. However, the effects on smoking on these parameters of ecological health are largely unknown.

We and others have previously demonstrated that smoking promotes acquisition and stable colonization of pathogens in a nascent ecosystem (Kumar et al. 2011) and that disease-associated microbiomes are pathogen-enriched communities in these high-risk individuals (Shchipkova et al. 2010, Bizzarro et al. 2013). Therefore, the purpose of this investigation was to extend the timeline of the previous investigation to examine the effects of smoking on structural resilience (as measured by community diversity, succession and response to disturbance) and functional resilience (as measured by the host response) following episodes of perturbation and stress.

Clinical study design and procedures

Baseline clinical data, marginal plaque, subgingival plaque and gingival crevicular fluid (GCF) samples were collected from those qualifying for the study, following which professional dental cleaning (oral prophylaxis) was administered and oral hygiene instructions were provided (Fig. 1). Subjects returned 7–10 days after this initial visit, when gingival health was confirmed (mean Gingival Index <0.25) and clinical data, plaque and gingival crevicular fluid samples were collected; following which professional oral prophylaxis was administered once again. Acrylic stents were fabricated to cover three teeth and 2 mm of marginal gingiva in two contra-lateral quadrants (a total of six teeth) and subjects were instructed to wear this while brushing for the duration of the study. Subjects returned 24 h later and clinical data, marginal plaque, subgingival plaque and GCF samples were collected; following which oral prophylaxis was administered to zero plaque. Subjects returned after 48 h; when 2-day old data and samples were collected, followed by oral prophylaxis. A similar protocol was followed to collect 4-day, 7-day, 14-day and 21-day samples. Professional oral prophylaxis was provided at this visit and subjects returned 7–10 days later for clinical data and sample collection.

Examiner calibration

All subjects were examined by one of two periodontists (CRM, PSK) calibrated to a Gold Standard examiner. The two examiners were found to have an agreement coefficient (κ-statistic) of 0.94 for PD and 0.96 for CAL, PI and GI with the Gold Standard examiner.

Data collection

Smoking status and tobacco exposure was assessed by questionnaire and salivary cotinine measurements. PD, BOP and CAL were recorded throughout the mouth on six sites.

Methods

Subject selection

Approval for this study was obtained from the Office of Responsible Research Practices at The Ohio State University (Protocol number: 2007H0259). Twenty-five current smokers (defined as those who smoked more than 100 cigarettes in their lifetime and are currently smoking) and 25 never smokers (defined as those who had never smoked a single cigarette in their lifetime) with pre-existing gingivitis (mean baseline gingival index greater than 2.0 [Loe and Silness 1963] attachment loss ≤1 mm and probe depths ≤4 mm at all sites) were recruited and written informed consent obtained. Smoking status was assessed by questionnaire and salivary cotinine levels. Exclusion criteria included diabetes, medical conditions that required use of prophylactic antibiotics, current or planned pregnancy, HIV infection, long-term (greater than 3 months) use of medications known to cause gingival changes, (e.g. immunosuppressants, phenytoin, calcium channel blockers, aspirin, NSAIDS, bisphosphonates or steroids), antibiotic therapy or oral prophylactic procedures within the last 3 months and less than 20 teeth in the dentition. Subjects were discontinued from the study if dictated by a contributory change in medical or dental status, use of antibiotics and antibacterial mouthrinses, or non-compliance with wearing the stent while brushing.

Clinical study design and procedures

Baseline clinical data, marginal plaque, subgingival plaque and gingival crevicular fluid (GCF) samples were collected; following which professional dental cleaning (oral prophylaxis) was administered and oral hygiene instructions were provided (Fig. 1). Subjects returned 7–10 days after this initial visit, when gingival health was confirmed (mean Gingival Index <0.25) and clinical data, plaque and gingival crevicular fluid samples were collected; following which professional oral prophylaxis was administered once again. Acrylic stents were fabricated to cover three teeth and 2 mm of marginal gingiva in two contra-lateral quadrants (a total of six teeth) and subjects were instructed to wear this while brushing for the duration of the study. Subjects returned 24 h later and clinical data, marginal plaque, subgingival plaque and GCF samples were collected; following which oral prophylaxis was administered to zero plaque. Subjects returned after 48 h; when 2-day old data and samples were collected, followed by oral prophylaxis. A similar protocol was followed to collect 4-day, 7-day, 14-day and 21-day samples. Professional oral prophylaxis was provided at this visit and subjects returned 7–10 days later for clinical data and sample collection.

Examiner calibration

All subjects were examined by one of two periodontists (CRM, PSK) calibrated to a Gold Standard examiner. The two examiners were found to have an agreement coefficient (κ-statistic) of 0.94 for PD and 0.96 for CAL, PI and GI with the Gold Standard examiner.

Data collection

Smoking status and tobacco exposure was assessed by questionnaire and salivary cotinine measurements. PD, BOP and CAL were recorded throughout the mouth on six sites.
per tooth using a PCP-UNC 15 probe. Turesky modification of the Quigley Hein plaque index (Turesky et al. 1970) and Loe and Silness gingival index (Silness & Loe 1964) were used to record plaque levels and gingival health status at six sites/tooth respectively.

**Sample collection**

Marginal plaque samples were collected and pooled from all test teeth by scraping the cervical 2 mm of each mesial inter-proximal surface (buccal and lingual) with a 204S scaler and wiping onto paper points. Gingival crevicular fluid (GCF) samples were collected from 12 sites by inserting Periopaper (Oraflow Inc., Smithtown, NY, USA) into the mesial sulcus for 30 s. GCF volume was measured with a Periotron 8000 (Harco Electronics Limited, Winnipeg, Canada) and samples immediately frozen at −20°C. Subgingival plaque samples were collected from the same sites by inserting one sterile endodontic paper point (DENTSPLY-Caulk, Milford, DE, USA) into each mesial sulcus for 10 s, followed by scraping with a curette. Samples were placed in 1.5 ml microcentrifuge tubes and frozen at −20°C until further analysis.

**DNA isolation**

Subgingival bacteria were separated from the paper points by adding 200 µl of phosphate-buffered saline (PBS) to the tubes and vortexing. Marginal bacteria were separated from the points by bead beating for 60 s. DNA was isolated with a Qia-gen DNA MiniAmp kit (Qiagen, Valencia, CA, USA).

**Amplification of 16S rDNA**

Bacterial 16S rRNA genes were amplified previously described (Kumar et al. 2005). The PCR products were purified with the Qiaquick PCR purification kit (Qiagen). The 16S amplicons generated by PCR were cloned into E.coli using a commercially available kit (TOPO TA cloning kit, Invitrogen, San Diego, CA, USA). Inserts were purified with a Millipore kit (Millipore, Billerica, MA, USA) and sequenced with an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit) using an ABI 3730 instrument.

**Sequence analysis**

Partial sequences of 1200–1400 bp were obtained from each amplicon. Sequences were clustered into species-level operational taxonomic units (s-OTUs) at 97% sequence similarity and assigned a taxonomic identity by alignment to locally hosted version of the Greengenes database (DeSantis et al. 2006) using the RDP Classifier. Unifrac and community diversity metrics were computed as previously described (Lozupone et al. 2007). All analyses were conducted using the QIIME pipeline (Caporaso et al. 2010).

**Cytokine assay**

Periopaper strips were thawed on ice and GCF was eluted as previously described (Kumar et al. 2011). Cytokine analysis was done using a commercially available multiplexed bead-based assay designed to quantify multiple cytokines. A panel of 27 cytokines was selected. Briefly, 27 distinct sets of fluorescently dyed beads (Bio-rad laboratories, Inc., Hercules, CA, USA) were conjugated with monoclonal antibodies and incubated with 50 µl of GCF. 25 µl of biotinylated detection antibody and 50 µl of reporter were sequentially added. The level of each cytokine was analysed by measuring the fluorescence of each bead type as well as the fluorescent signal from the reporter on a Bio-Plex 200 detection system.

**Data normalization and statistical analysis**

A minimum of 100 sequences was identified from each sample. A variance stabilizing transformation was used to create normal distribution of the species and genus level data (Shchipkova et al. 2010). Diversity and equitability estimates were made using s-OUT data. Repeated Measures ANOVA was used to compare the levels of each species and immune mediator over the five visits. A “cytokine score” was computed to reduce inter-subject variability in immune mediator levels (Bauer et al. 2006). To achieve this, the absolute concentration of each analyte per microlitre of GCF was normalized across all visits for each subject, such that the maximum value was 1. Thus, irrespective of inter-subject variations in immune mediator levels, the values ranged from zero to one for each subject. A “bacterial score” was similarly computed by normalizing the proportion of each genus across all visits for each subject. Multivariate analysis was used to examine the correlation between bacterial and cytokine scores during induction and resolution of gingivitis. Reported p-values correspond to Spearman’s correlation coefficients. All statistical analyses were carried out with JMP (SAS Institute Inc., Cary, NC, USA).

**Results**

**Subject demographics and clinical characteristics of natural and experimental disease**

The clinical and demographic characteristics are shown in Table 1. Clinically, there were no significant differences between current and never smokers in mean gingival inflammation or plaque accumulation (Fig. 2a,b). However, cluster analysis of the individual subjects revealed that more smokers developed gingival inflammation earlier than never smokers (Fig. 2c). Based on these clinical findings, the samples were re-distributed into the following groups for further analysis: Natural gingivitis (GI>2), Resolution 1 (GI<1), Healthy phase (GI<1), Transition phase (2 > GI>1), Experimental gingivitis (GI>2) and Resolution 2 (GI<1). There were no differences in gingival or plaque indices between natural and experimental gingivitis or between resolutions of each disease state.

**Bacterial shifts in marginal and subgingival biofilms**

A total of 66,068 near full-length, chimera-depleted sequences were used in all analyses. These sequences represented 164 and 293 species level operational taxonomic units (s-OTUs) in the marginal biofilm;
and 235 and 311 s-OTUs in the subgingival biofilm of never and current smokers respectively. The transition from disease to health was accompanied by a decrease in diversity in both current and never smokers; the diversity increased during transition to disease and decreased following resolution of disease (Fig. 3a,b). Smokers demonstrated greater subgingival bacterial diversity than non-smokers during naturally occurring gingivitis and during resolution from both disease states (Fig. 3b). Smokers also demonstrated greater bacterial diversity than non-smokers in marginal plaque during resolution following experimental gingivitis (Fig. 3a). These differences were statistically significant ($p < 0.05$, repeated measures ANOVA).

Microbial-mucosal characteristics of health and disease in never smokers

In never smokers, health (Resolution 1. Healthy phase, Resolution 2) was characterized by low diversity biofilm communities (Fig. 3a,b) that were dominated by species belonging to Veillonella, non-mutans Streptococcus, Neisseria, Actinomyces, Haemophilus and Abiotrophia in marginal plaque and Actinomyces, Abiotrophia, Selenomonas, Neisseria, non-mutans Streptococcus and Veillonella in subgingival plaque (Fig. 3C). Species belonging to these genera constituted 75% of the communities in these habitats. Transition to disease was accompanied by a slight but insignificant increase in diversity, with decrease in levels of Veillonella, Streptococcus, Neisseria and Abiotrophia and increase in the levels of Selenomonas, Dialister, Campylobacter, Eubacterium, Lachnospira and Parvimonas in both subgingival and marginal biofilms. The establishment of gingivitis was characterized by further decreases in levels of Veillonella, Streptococcus, Neisseria and Abiotrophia and increases in the species belonging to the genera Corynebacterium, Actinomyces, Haemophilus, Capnocytophaga, Dialister, Campylobacter, Eubacterium and Selenomonas in both habitats. In the subgingival biofilm, gingivitis was accompanied by an increase in Treponema in addition to the other species. The microbial communities in naturally occurring and experimental gingivitis were remarkably similar as were the communities following resolution from these two disease states ($p > 0.05$, Repeated Measures ANOVA). Significant correlations were observed in the composition and abundance of species in the marginal and subgingival communities during each phase of colonization (Fig. 4a). Host immune mediators exhibited a distinct clustering in response to this colonization ($p < 0.0001$, factor analysis of Principal Components Analysis, Fig. 5). Two distinct clusters were observed—cluster 1 was composed of IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IL-17, IL-1ra, FGF, PDGF, MIP-1a and Eotaxin; whereas cluster 2 was formed by RANTES, GCSF, GM-CSF, VEGF, TNF-α, IFN-γ, IL-1b, IL-2, IL-4, IL-9 and IL-12. Health was associated with low levels of Cluster 1 cytokines and high levels of Cluster 2 cytokines. Transition to disease was accompanied by high levels of both Cluster 1 and Cluster 2 cytokines as well as low levels of MIP-1b, MCP-1 and IP-10. Established gingivitis was associated with high levels of Cluster 2 cytokines. Strong positive correlations were observed between Abiotrophia, Veillonella, Streptococcus, Gemella and Neisseria and the cytokines IP-10, IL-10, IL-6, as well as between Selenomonas, Parvimonas, Megaplasma, Lachnospira, Eubacterium, Dialister and Campylobacter and RANTES, IL-4, VEGF, G-CSF, IFN-γ, IL-1b, IL-2, GM-CSF, TNF-α, IL-9 (Fig. 6a). Strong negative correlations were observed between these cytokines and Abiotrophia, Veillonella, Streptococcus, Gemella and Neisseria.

Microbial-mucosal characteristics of health and disease in current smokers

The three health-compatible marginal communities were similar in composition and diversity; however, in contrast to the never smokers, Resolution 1 and Resolution 2 in smokers exhibited greater subgingival diversity than the Healthy phase (Fig. 3b). In addition, these two states were characterized by significantly greater subgingival diversity than in non-smokers. The health-compatible subgingival communities of smokers were dominated by species belonging to Parvimonas, Gemella, Treponema, Eubacterium, Pseudomonas, Dialister, Selenomonas, Veillonella and Streptococcus, whereas the marginal biofilm was dominated by Streptococcus, Veillonella, Selenomonas, Pseudomonas and Haemophilus (Fig. 3c). Transition to disease was accompanied by a slight but insignificant increase in diversity, with decreases in levels of Veillonella and Streptococcus, and increase in the levels of Selenomonas and Dialister in marginal biofilms and Treponema, Prevotella, Campylobacter, Eubacterium, Dialister and Selenomonas in subgingival biofilms. The establishment of gingivitis was characterized by fur-

Table 1. Demographic features of study population

<table>
<thead>
<tr>
<th></th>
<th>Never smokers</th>
<th>Current smokers</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>26.5 ± 5.5</td>
<td>25.5 ± 3.5</td>
<td>0.20</td>
</tr>
<tr>
<td>Gender – female (%)</td>
<td>77.6</td>
<td>56.4</td>
<td>0.18</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>80.0</td>
<td>85.0</td>
<td>0.03</td>
</tr>
<tr>
<td>African-American</td>
<td>6.0</td>
<td>8.0</td>
<td>0.06</td>
</tr>
<tr>
<td>Asian</td>
<td>6.0</td>
<td>3.0</td>
<td>0.035</td>
</tr>
<tr>
<td>Other</td>
<td>8.0</td>
<td>4.0</td>
<td>0.013</td>
</tr>
<tr>
<td>Education (average years/individual)</td>
<td>13 ± 3</td>
<td>14 ± 2</td>
<td>0.25</td>
</tr>
<tr>
<td>Dental prophylaxis (number of annual visits/individual)</td>
<td>0.96</td>
<td>0.97</td>
<td>0.23</td>
</tr>
<tr>
<td>Brushing frequency (times per day/individual)</td>
<td>1.57</td>
<td>1.28</td>
<td>0.23</td>
</tr>
<tr>
<td>Frequency of flossing (times per day)</td>
<td>1.0</td>
<td>1.0</td>
<td>0.23</td>
</tr>
<tr>
<td>Mean number of teeth</td>
<td>28.0</td>
<td>28.0</td>
<td>1</td>
</tr>
<tr>
<td>Mean probing pocket depth (mm)</td>
<td>3.7 ± 0.8</td>
<td>4.3 ± 1.2</td>
<td>0.25</td>
</tr>
<tr>
<td>Mean clinical attachment loss (mm)</td>
<td>1.1 ± 0.3</td>
<td>0.8 ± 0.7</td>
<td>0.18</td>
</tr>
<tr>
<td>Tobacco exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pack years</td>
<td>0</td>
<td>5.6 ± 2.4</td>
<td>0.0015</td>
</tr>
<tr>
<td>Salivary cotinine (ng/ml)</td>
<td>0.0</td>
<td>23.3 ± 3.7</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
Fig. 2. Gingival and Plaque indices of 25 current and 25 never smokers. (a) Shows the Gingival Index and (b) the Plaque Index. The inset demonstrates the same data for the two episodes of disease and resolution only. Both groups demonstrated an increase in plaque and gingival indices over the observation period. (c) Shows the clustering of the subjects by the extent of gingival inflammation (GI<1 – Red, 1 < GI<2 – Green and GI>2 – Blue). Smokers demonstrated an earlier onset of clinically visible inflammation (1 < GI<2 and GI>2) than non-smokers.
ther decreases in levels of *Veillonella*, *Pseudomonas* and *Streptococcus*, and increases in the species belonging to the genera *Parvimonas*, *Megasphaera*, *Treponema*, *Capnocytophaga*, *Dialis-ter*, *Campylobacter* and *Selenomonas* in both habitats. The microbial communities in naturally occurring and experimental gingivitis were significantly different, with greater abundances of *Eubacterium*, *Treponema*, *Parvimonas* and *Campylobacter* and lower levels of *Veillonella* and *Strep-tococcus*. The composition of the communities also differed significantly during resolutions from these two disease states ($p < 0.05$, $**p < 0.01$, repeated measures ANOVA). Significant differences were observed in the levels of several genera during the different periods of observation (repeated measures ANOVA on transformed variable, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Fig. 3. Shannon Diversity Index and genera in current and never smokers. Diversity of marginal biofilm is shown in (a) and subgingival biofilm in (b). Smokers demonstrated an increased diversity between the two episodes of gingivitis as well as the two resolution states ($p < 0.05$, $**p < 0.01$ repeated measures ANOVA), and showed a greater diversity than never smokers ($**p < 0.01$). Distribution of sequences by genus in marginal and subgingival biofilms of current (red) and never smokers (green) is shown in Figure 3c. Circles are sized by relative abundance of each genus. Genera accounting for >0.5% of total sequences are shown. Significant differences were observed in the levels of several genera during the different periods of observation (repeated measures ANOVA on transformed variable, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Discussion

Experimental gingivitis as a model system of microbial-mucosal interactions

The human body plays host to biofilms in several mucosal niches; and these communities have an integral role in maintaining health and causing disease in these environments (Rakoff-Nahoum et al. 2004). It is often difficult to examine the shifts in microbial communities in diseases such as gingivitis. Strong positive correlations were observed between *Streptococcus*, *Selenomonas*, *Rothia*, *Pseudomonas* and *Propionibacterium* and the cytokines IL-12, IL-17, RANTES, MIP-1A, IL-13, Eotaxin, IL-1b, IL-2, IL-4, IL-6, IL-7, IL-9; as well as between *Veillonella*, *Gemella*, *Lautropia* and the cytokines IL-12, IL-17, RANTES (Fig. 6b).

© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd
that occur in these ecosystems, either because they are not easily accessible or because transitions from health to disease cannot be easily monitored. The subgingival microenvironment provides an easily accessible niche with a defined microbial population and therefore serves as an ideal model to examine mucosal-microbial interactions. Furthermore, gingivitis is a reversible, bacterially induced inflammation that occurs both naturally and can be induced and resolved in a controlled manner (Loe et al. 1965, Theilade et al. 1966). In addition, numerous studies indicate that there are no differences in either the clinical characteristics or bacterial profiles between natural and experimental gingivitis (Loesche & Syed 1978, Syed & Loesche 1978, Moore et al. 1987). Hence, a longitudinal examination of the clinical, microbiological and immunological changes that occur during onset and resolution of naturally occurring and experimentally induced gingivitis allowed us to examine community resilience following two subsequent episodes of bacterially induced inflammation. To the best of our knowledge, this is the first evidence on the effect of smoking on altering community resistance and resilience in any ecological niche in the human body.

### Smoking and parameters of ecosystem health

Three important parameters that affect the health of an ecosystem are biodiversity, disturbance and succession. In non-smokers, marginal and subgingival biofilms of Resolution 1 and Resolution 2 closely resembled that of Healthy phase; with bacterial profiles similar to health-compatible communities described in previous studies (Tanner et al. 1998, Kumar et al. 2006), indicating that these communities reach steady-state within 48 h and continue to exist stably in this health-compatible state. Several examples of syntrophy and mutualism were observed in these communities (Fig. 4a,b). It has previously been shown that Veillonella and Streptococcus share a nutritional syntrophy, in that Veillonella are reliant on the lactate that is produced by the Streptococci as a food source (Kuramitsu et al. 2007).

In this study, Streptococci and Veillonella dominated the marginal and subgingival ecosystems in health. An increase in plaque biomass (Fig. 2) was accompanied by decline in levels of Streptococci by 50% on day 4 (transition phase) and on day 7, the levels of Veillonella declined to comparable levels (Fig. 3c). Another interesting instance of mutualism was apparent between Streptococcus, Actinomyces and Selenomonas. Oral Streptococci appear to be important for Actinomyces colonization, and Selenomonas are poor colonizers unless Actinomyces naeslundii is present (Kolenbrander et al. 1989, Palmer et al. 2001). In the present investigation, surprisingly high levels of Selenomonas were found within 24 h in certain subjects, and correlated strongly with the levels of Streptococci and Actinomyces. These subjects also developed gingivitis earlier (Fig. 2c). Selenomonas are acknowledged as periodontal pathogens and are important in gingivitis (Tanner et al. 1989). Taken together, the data suggest that high levels of Streptococci
and Actinomyces may predispose to early colonization with pathogens and may increase susceptibility to disease. The immune response to this community was predominantly anti-inflammatory, with high levels of IP-10, IL-1ra, IL-10 and IL-15. IP-10 is a powerful inhibitor of VEGF and is an anti-angiogenic factor (Bodnar et al. 2006), and IL-1ra, IL-10 and IL-15 are anti-inflammatory factors previously found in high levels in healthy gingiva (Johnson & Serio 2007). High levels of Streptococcus, Neisseria, Veillonella, Gemella and

Fig. 5. Principal component analysis (PCA) of cytokine scores during each observation period. Cytokines are coloured by lowest score (green) to highest score (red), based on their concentrations in each subject during each observation period. Host immune mediators exhibited a distinct clustering in response to colonization in both current and never smokers ($p < 0.0001$, factor analysis of Principal Components Analysis).
Abiotrophia correlated strongly with these anti-inflammatory mediators. Thus, the central characteristics of a health-compatible community in non-smokers appear to be cooperativity, low diversity and low immunogenicity. The switch from health to disease is triggered by a change from aerobic to anaerobic environment (due to an increase in plaque mass and biofilm thickness), which then triggers a nutritionally driven cascade of events within the biofilm and then extended to the host.

In contrast, the development of the marginal and subgingival ecosystems in smokers was characterized by rapid microbial succession with high levels of periodontal and systemic pathogens, and was accompanied by high levels of pro-inflammatory cytokines (Figs 3 and 5). We have previously reported that smokers acquire large numbers of periodontal pathogens belonging to the genera *Fusobacterium*, *Selenomonas*, *Diaisler*, *Treponema* and *Parvimonas* and systemic pathogens including *Pseudomonas* and *Haemophilus* during early biofilm formation and that there is a large amount of fluctuation in these communities (Kumar et al. 2011). The present investigation demonstrates that this early pathogen acquisition leads to earlier pathogen enrichment in the community. It is well documented that smoking decreases oxygen tension (Hanioka et al. 2000), increases subgingival temperature (Dinsdale et al. 1997), reduces the Eh (Kenney et al. 1975) and increases the amount of free iron (Weinberg 1999). It is also well known that these factors promote parasitic symbiosis (Wilson et al. 1992), preferential colonization with periodontal pathogens (Dinsdale et al. 1997) as well as colonization by species with poor iron acquisition abilities (Weinberg 2000). Taken together with previous research, this study demonstrates that smoking creates an environment that does not support niche saturation by early colonizers, leading to a highly diverse, pathogen-enriched, unstable community that is susceptible to disruption and therefore highly pro-inflammatory. This is also supported by the clinical data (Fig. 2C) that more smokers developed gingival inflammation earlier than non-smokers.

**Smoking and community resilience**

A fundamental requirement for maintaining a healthy oral ecosystem is an understanding of the community’s response to stress and perturbation. This study investigated the effects of smoking on the structural and functional resilience of marginal and subgingival biofilms following two episodes of disease, using diversity, compositional succession and pro-inflammatory potential as markers of resilience. In non-smokers, a remarkable similarity was observed between Resolution 1 and Resolution 2 clinically, microbiologically and immunologically (Figs 3, 4 and 5). Both communities were 90% similar in composition, exhibited low diversity and elicited a low pro-inflammatory response, suggesting...
that these communities are highly resilient following perturbation. However, in smokers, the community in Resolution 2 demonstrated higher levels of the pathogens Treponema, Selenomonas, Parvimonas, Eubacterium, Pseudomonas and Lactobacillus when compared with Resolution 1. The community was also more diverse compared to Resolution 1. In addition, the lowest correlations were observed between the marginal and subgingival OTUs during Resolution 2. It has previously been demonstrated that subgingival colonization is significantly affected by the composition of marginal plaque (Gomes et al. 2008, Meulman et al. 2012). Thus, the present investigation indicates that with each episode of disease, the interactions between these two geographically connected habitats decreases. The pro-inflammatory response to this community was also significantly higher as compared to Resolution 1. The clinical implications of this finding are enormous. Our data suggests that smoking increases susceptibility to disease by lowering the ability of the ecosystem to “reset” itself, thereby lowering its resilience. Given that sustained and repeated episodes of gingivitis predispose to periodontitis (Lang et al. 2009), this reduction in resilience could be a mechanism by which smoking increases the risk for periodontitis and warrants further investigation.

In summary, smokers demonstrated an early pathogenic colonization that leads to sustained pathogen enrichment, eliciting a florid immune response. Oral microbial communities in smokers also demonstrate a decreased resilience to repeated episodes of disease; with greater abundance of pathogenic species, poor compositional correlation between the marginal and the subgingival ecosystems and significantly greater pro-inflammatory responses. Our data suggest that repeated episodes of such insults might increase susceptibility to future disease in smokers. Further studies examining the patterns of gene expressions within these communities are necessary to understand the effect of smoking on aetiopathogenesis of periodontal diseases.

References
© 2014 John Wiley & Sons A/S. Published by John Wiley & Sons Ltd


Address:
Purnima Kumar
4111 Postle Hall
305, W 12th Avenue
Columbus, OH 43210
USA
E-mail: kumar.83@osu.edu

**Clinical Relevance**

**Scientific rationale for the study:** It is known that smoking increases the risk for periodontitis; however, the mechanisms are not fully elucidated.

**Principal findings:** Our data indicate that smokers have a lower resilience in their subgingival microbial community, therefore, this community does not fully return to a health-compatible state following resolution form gingivitis. This loss of microbial resilience leads to a greater pro-inflammatory host response, which increases with each episode of gingival inflammation. This appears to be a mechanism by which repeated episodes of gingivitis may predispose a smoker to destructive periodontal disease.

**Practical implications:** Prevention of gingivitis is extremely critical in a smoker.