Article

Electronic Cigarette Aerosol Modulates the Oral Microbiome and Increases Risk of Infection

Healthy Gum to Periodontitis: just a "Vapor" away

Oral pathobionts abundance ↑
Haemophilus
Fusobacterium
Rothia
Prevotella
Actinomyces

Host inflammatory response ↑
IL6
IL1β

Cells susceptible for infection

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HIGHLIGHTS

E- cigarette vapors contain high nicotine concentration and other toxic compounds

E-cigarette modulates oral microbiome and increases the abundance of oral pathobionts

E-cigarette aerosol alters host response and promotes gum inflammation

E-cigarette aerosol exposure makes epithelial cells susceptible to infection
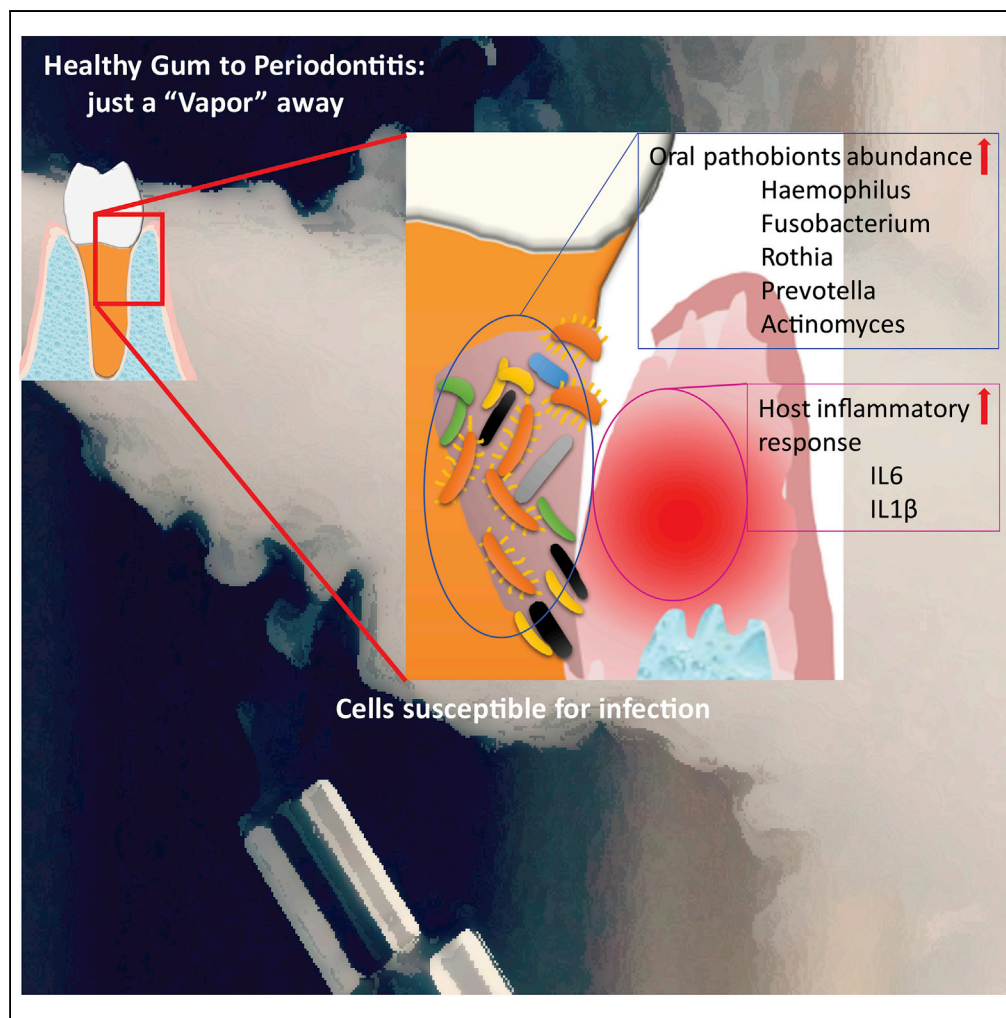
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Article

Electronic Cigarette Aerosol Modulates the Oral Microbiome and Increases Risk of Infection

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SUMMARY

The trend of e-cigarette use among teens is ever increasing. Here we show the dysbiotic oral microbial ecology in e-cigarette users influencing the local host immune environment compared with non-smoker controls and cigarette smokers. Using 16S rRNA high-throughput sequencing, we evaluated 119 human participants, 40 in each of the three cohorts, and found significantly altered beta-diversity in e-cigarette users (p = 0.006) when compared with never smokers or tobacco cigarette smokers. The abundance of *Porphyromonas* and *Veillonella* (p = 0.008) was higher among vapers. Interleukin (IL)-6 and IL-1β were highly elevated in e-cigarette users when compared with non-users. Epithelial cell-exposed e-cigarette aerosols were more susceptible for infection. *In vitro* infection model of premalignant Leuk-1 and malignant cell lines exposed to e-cigarette aerosol and challenged by *Porphyromonas gingivalis* and *Fusobacterium nucleatum* resulted in elevated inflammatory response. Our findings for the first time demonstrate that e-cigarette users are more prone to infection.

INTRODUCTION

Electronic cigarettes (e-cigarettes), non-combustible battery-operated devices, are considered to be a safe and healthier alternative to conventional combustible cigarette smoke (Beaglehole et al., 2019). However, according to the Centers for Disease Control and Prevention (CDC), the US Food and Drug Administration (FDA), and the National Cancer Institute analysis of the 2011–2018 National Youth Tobacco Surveys data, to estimate tobacco product use in US middle and high school students, it was reported that in 2018, the current use of any tobacco product was 27.1% in high school students (4.04 million) and 7.2% in middle school students (840,000); e-cigarettes were the most commonly used product among high school (20.8%; 3.05 million) and middle school (4.9%; 570,000) students (Gentzke et al., 2019). Interestingly, e-cigarette vaping swelled by 77.8% (from 11.7% to 20.8%) among high school students and 48.5% (from 3.3% to 4.9%) among middle school students in the year 2017–2018 (Gentzke et al., 2019). These figures are of great concern due to the mounting popularity of e-cigarette usage among teenagers having never smoked any combustible products.

Unlike traditional cigarettes with tobacco filling, which approximates 24 mg of nicotine per pack (1.2 mg/cigarette), e-cigarette liquid contains nicotine (Cameron et al., 2013) that varies between 6 and 48 mg/mL (Cameron et al., 2013) and is not meant to be smoked completely in one sitting (Etter and Bullen, 2011). Each nicotine cartridge in an e-cigarette can provide on an average 200 puffs equivalent to one to three packs of cigarettes. The nicotine absorption in the body from e-cigarette use depends on the nicotine concentration in the e-liquid, its aerosol mass quantity and deposition, the chemical form of the nicotine, as well as the vaping volume, frequency, and timing (Etter, 2014). As per a 2014 report, the US FDA detected in one of the cartridges the presence of diethylene glycol, a toxic liquid used in explosives and as an antifreeze agent, in addition to, cancer-causing agents, such as tobacco-specific nitrosamines, aldehydes, metals, volatile organic compounds, phenolic compounds, polycyclic aromatic hydrocarbons, flavors, solvent carriers, tobacco alkaloids, and drugs (amino tadalafil and rimonabant) (Cheng, 2014). Moreover,

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recent studies showed that nicotine delivery and emission of some toxicant levels from the latest generation of e-cigarettes were comparable to those from tobacco smoke (Eltorai et al., 2019).

The oral cavity is a gateway and a permanent or makeshift harbor for numerous microbial species to colonize the respiratory and gastrointestinal (GI) tracts (Saxena et al., 2012). The multifactorial pathogenicity of chronic periodontal disease involves a complex interaction of microbial pathogens, host immune response, and genetic and environmental factors, stimulating the host tissue destruction and bone loss (Harvey, 2017). Certain oral bacteria, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, are major perpetrators in periodontal destruction, highly associated with disease progression (Tâlvan et al., 2017). The release of host immune mediators as a primary response to these opportunistic pathogens (pathobionts) and its metabolites favors the disease state. The dysbiosis in microbial communities due to impaired homeostasis as a result of environmental and host metabolic factors can contribute to oral diseases, including dental caries, periodontitis, halitosis, or other medical ailments, such as diabetes, cardiovascular diseases, and cancers (He et al., 2015). Elaborate studies have shown the toxicity of conventional tobacco product consumption to periodontal health (Kumar et al., 2011), albeit with very limited understanding on e-cigarette's effect (Stewart et al., 2018). The e-cigarette aerosol interaction with the host ensues largely in the oral cavity and lungs, where the exposure to nicotine and toxic metabolites of the e-liquid components is the highest and can escalate the host susceptibility to infections (Cheng, 2014). Recent reports associate e-cigarette vaping with vascular endothelial dysfunction causing blood-brain barrier damage with higher risk of cerebrovascular diseases (Sivandzade and Cucullo, 2019). Hence, it is imperative to elucidate the detrimental effect of e-liquids on the host inflammatory responses, which is highly challenging and conflicting.

The lucrative e-cigarette vaping delivery systems, available in a variety of flavors and concentrations, can pose a substantial threat to human health (Gentzke et al., 2019). Wu et al. (2014) have shown that e-liquids induce inflammatory responses and alter innate immune defenses in primary airway epithelial cells. The findings of Sussan et al. (2015) indicated that exposure of C57BL/6 mice to e-cigarette aerosols for 2 weeks can cause impairment of pulmonary bacterial and viral clearance. Accordingly, *in vitro* studies can be extrapolated to recognize responses by electronic nicotine delivery system users, however, without the clinical evidence of smoking history (Kaur et al., 2018). So, to understand the comprehensive health effects of vaping it is essential to conduct clinical research in conjunction with *in vitro* e-cigarette aerosol-exposed model. This will further help us to associate and understand the impact of vaping on human health by gauging the positive and negative controls.

Here, we report the *in vivo* effects of e-cigarette aerosol and its influence on human salivary microbiome and immune health. Furthermore, we evaluate *in vitro* the influence of e-cigarette aerosols on infection efficiency of oral pathogens in pre-cancerous and cancer cell lines using a novel e-cigarette aerosol-generating machine and pro-inflammatory immune mediators.

RESULTS

To assess the *in vivo* influence of smoking on microbial profiles of recruited participants, we stratified 119 subjects according to their smoking status into each of the three cohorts (never smokers [NS, n = 39], e-cigarette users [ES, n = 40], and regular cigarette smokers [CS, n = 40]). The demographic details of all the subjects and smoking history were collected at the baseline for the ES and CS groups (Table S1). The ES and CS cohorts had a nearly equal percentage of male population (~77%–80%). On the other hand, females were higher at 43.6% in the NS cohort. Participants exclusively using e-cigarettes vaped on an average 0.5 e-cigarettes per day, whereas participants exclusively using combustible cigarettes smoked an average of 11 cigarettes per day. There was no significant change in the salivary flow rate across the participants in different cohorts. The severity index of periodontal disease or infection was significantly higher among CS (72.5%), followed by ES (42.5%), and non-smokers (28.2%), as also reflected by the calculated mean pocket depth among cohorts. This was confirmed by the values of bleeding on probing (BoP), one of the markers for inflammation, elevated in CS and receding in e-cigarette vapers and non-smokers, although with no statistical significance. To determine the participants' smoking status, exhaled breath carbon monoxide (CO) levels (ppm) and salivary cotinine levels (ng/mL) were evaluated. Figures 1A and 1B, respectively, illustrate the cohort-based distribution of the smoking biomarkers for CO levels in exhaled breath and salivary cotinine levels in the participants. The CO and the salivary cotinine levels were the lowest in non-smokers and the highest among traditional cigarette users (Table S1).
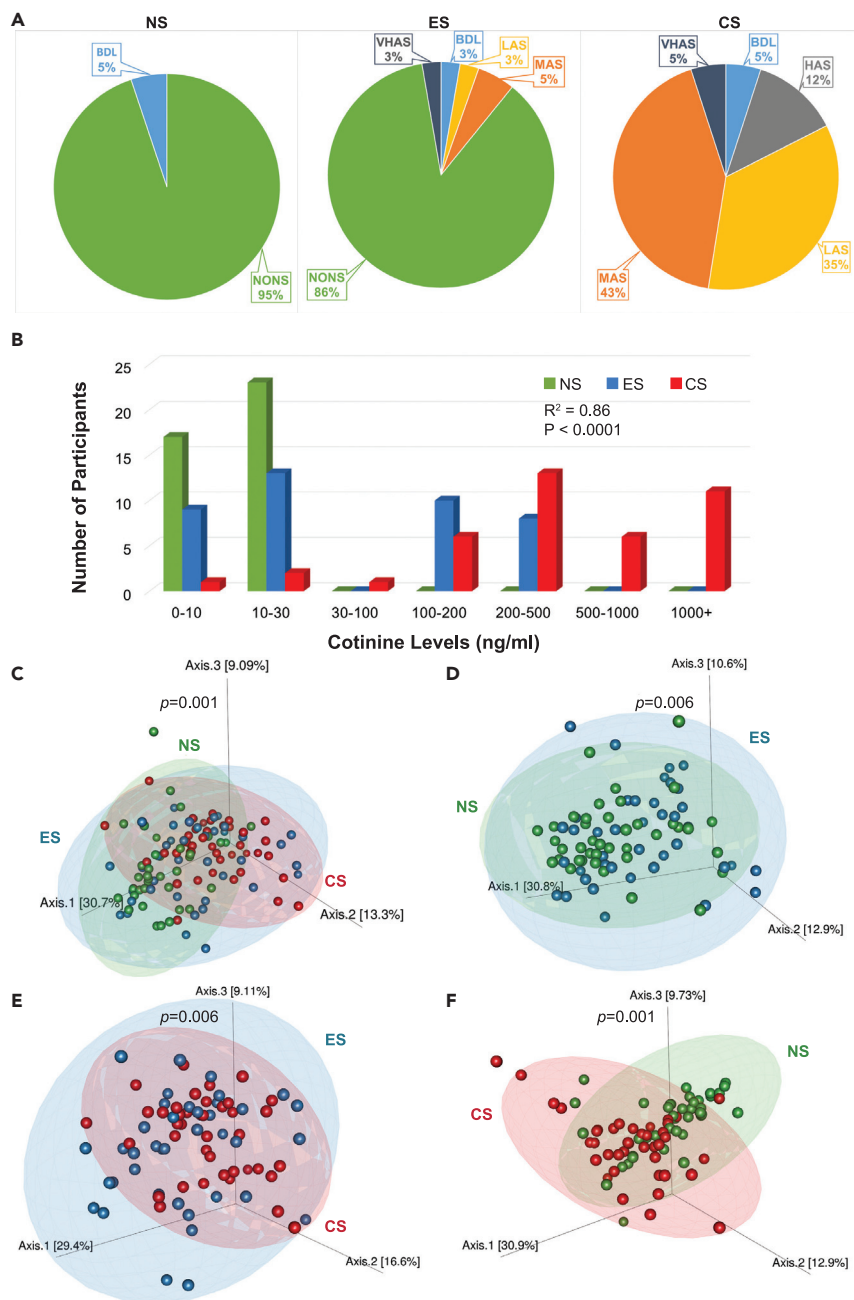
Figure 1. Breath Carbon Monoxide and Salivary Cotinine Levels in NS, ES and CS Cohorts as a Measure of Tobacco Smoke Exposure Resulting in Skewed Bacterial Communities

(A) Levels of breath carbon monoxide (ppm) across the subjects (in percentage) in the non-smokers (NS), e-cigarette users (ES), and cigarette smokers (CS); non-smoker (NONS): 0–6 ppm, borderline (BdL): 7–9 ppm, low addicted smoker (LAS): 10–15 ppm, moderate addicted smoker (MAS): 16–25 ppm, heavily addicted smoker (HAS): 26–35 ppm, and very heavily addicted smoker (VHAS): 36+ ppm.

(B) Distribution of salivary cotinine levels in the participants of the three cohorts.

(C–F) Weighted UniFrac 3D PCoA plots illustrating beta diversity of salivary bacterial taxa across all the samples. (C) Three cohorts, NS, ES, and CS (p < 0.001); (D) between NS and ES cohorts (p = 0.005); (E) between ES and CS cohorts (p = 0.006); and (F) between NS and CS cohorts (p < 0.001). Green, never smokers (NS); blue, e-cigarette vapers (ES); and red, cigarette smokers (CS). Ellipses indicate 95% confidence interval.

Altered Bacterial Richness and Diversity in e-Cigarette Users

The salivary microbiome in 119 participants in the NS, ES, and CS groups was analyzed using 8,254,494 high-quality filtered 16S sequences (mean 68,787 ± 19,758 SD). The salivary microbiome was composed of 11 phyla, 22 classes, 33 orders, 55 families, 99 genera, 162 species, and 911 operational taxonomic units (OTUs).

Principal coordinate analyses (PCoA) were performed to determine the overall microbiome composition in the three cohorts. Based on the weighted UniFrac distance matrix generated from all the samples in each cohort, we observed significant differences ($p < 0.05$, PERMANOVA) in the microbial composition between the three cohorts (Figure 1C) and additionally, between each cohort in an independent combinatorial comparison (Figures 1D–1F). These results suggest that the microbiome structure in each of the three cohorts was distinct. Interestingly, a few of the individual ES cohort samples showed an overlap with some samples in the NS (Figure 1D) and CS (Figure 1E) cohorts, although they were significantly distinct microbiomes. These findings were reiterated by double PCoA that establishes differences in taxonomic signatures (Figure 2C) in context to breath CO levels (Figure 2A) and salivary cotinine levels (Figure 2B) of the participants.

The alpha diversity indices, such as observed OTUs, Shannon index, and phylogenetic diversity were computed to determine the microbial diversity within the three groups (Figure 2D). We observed a significantly higher number of observed OTUs ($p = 0.023$) and phylogenetic diversity ($p = 0.002$) in non-combustible vapers when compared with NS. No significant changes were detected in the diversity indices of the CS cohort when compared with NS and ES users. All the analysis was done using Mann-Whitney test with a confidence level of 99% ($p \leq 0.01$) in each index.

Abundance of Oral Microbial Taxa in e-Cigarette Vapers

The relative abundance of salivary microbiome differed between non-combustible vapers and combustible smokers when compared with healthy non-smoking controls. The five most abundant taxa observed were *Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria,* and *Fusobacteria,* which accounted for 97.5% of the total sequences (Figure 2E). Intriguingly, *Proteobacteria* predominated the ES cohort with significantly lower levels in the CS cohort ($p < 0.0001$). The CS and ES showed significantly elevated salivary *Actinobacteria* compared with NS ($p < 0.0001$ and $p < 0.01$, respectively). However, *Firmicutes* were highly enriched in the saliva of the CS cohort compared with those of the ES ($p < 0.01$) and NS ($p < 0.0001$) cohorts. On the contrary, *Fusobacteria* exhibited significant depletion in the CS cohort when compared with the ES ($p < 0.024$) and NS ($p < 0.001$) cohorts. Another predominant taxon, *Spirochaetes,* proliferated in the CS cohort ($p < 0.005$).

The salivary microbiome in the three cohorts was significantly dominated by eight taxa; *Streptococcus, Veillonella, Prevotella, Neisseria, Haemophilus, Porphyromonas, Rothia,* and *Fusobacterium,* which constituted 79.15% of all the sequences (Figures 3A and 3B). Other differentially abundant taxa identified were *Leptotrichia, Gemella,* and *Capnocytophaga* (Figures 3A and 3B). A hierarchical cluster analysis showed stratification of taxa into four clusters (Figure 3C). Interestingly, the ES salivary microbiota that harbored Cluster Ib and Cluster IIb showed resemblance to those taxa distinct in NS and CS cohorts, respectively (Figure 3C). A similar pattern was also observed in the weighted PCoA plot (Figure 1C). Further analyses revealed these similarities to be closely associated with the nicotine intake (smoking status) by the ES participants. Cumulatively, the taxa observed to be proliferated were *Actinomyces* in the ES cohort, *TM7* in the ES and NS cohorts, and *Granulicatella* in the NS cohort. Moreover, *Neisseria* and *Fusobacterium* were significantly associated with the ES and NS cohorts compared with the CS cohort, $p < 0.0001$, whereas the opportunistic pathogens, *Streptococcus* ($p < 0.01$), *Prevotella* ($p = 0.01$), and *Rothia* ($p = 0.002$) were differentially enriched in the CS cohort. *Veillonella* levels increased significantly by ~4% in ES ($p = 0.008$) and ~4.5% ($p = 0.001$) in CS cohorts than NS cohorts. These findings suggest microbial dysbiosis in non-combustible vapers and combustible smokers.

A heat tree illustrates the relationships of species-specific OTUs in the NS, e-cigarette vapers, and CS (Figure S1). *Streptococcus oralis subsp. tigurinus* clade 071, *Porphyromonas pasteri, Fusobacterium periodonticum,* and *Oribacterium parvum* were depleted significantly in the ES and CS cohorts (Figures S2A and S2B). On the contrary, abundance of *Veillonella rogosae, Granulicatella adiacens,* and *Prevotella* sp. HMT 317 was higher in the NS cohort. *Veillonella dispar, Porphyromonas endodontalis, Fusobacterium nucleatum subsp. vincentii, Prevotella oris,* and *Parvimonas micra* predominated substantially in the ES and
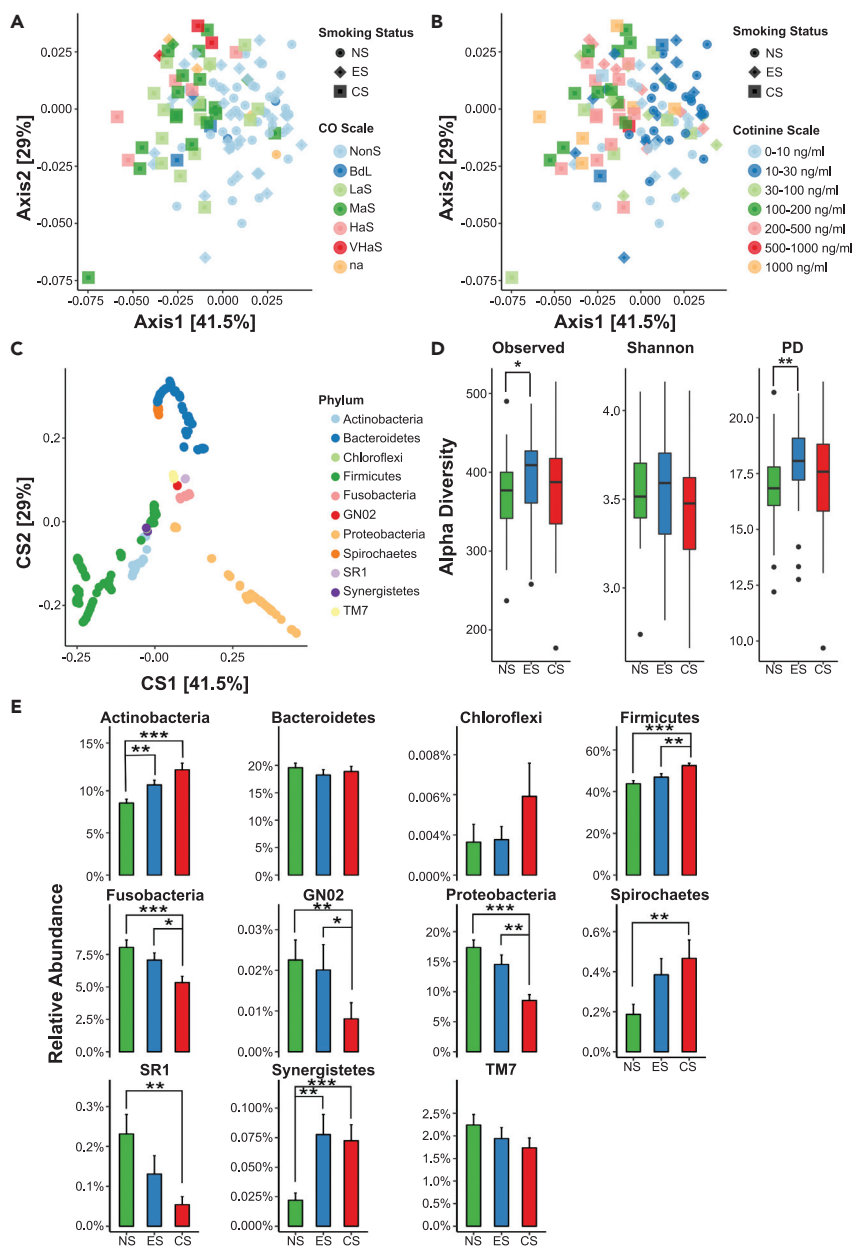
Figure 2. Abundance of Microbiota with Respect to Carbon Monoxide Concentration, Cotinine Levels and Smoking Status

(A–E) Double PCoA plots showing taxa correlating to the smoking status, in terms of (A) breath CO levels (in percentage), (B) cotinine levels, and (C) phyla taxonomy in the non-smokers (NS), e-cigarette users (ES), and cigarette smokers (CS); non-smoker (NonS): 0–6 ppm, borderline (BdL): 7–9 ppm, low addicted smoker (LaS): 10–15 ppm, moderate addicted smoker (MaS): 16–25 ppm, heavily addicted smoker (HaS): 26–35 ppm, and very heavily addicted smoker (VHaS): 36+ ppm, data not available (na). (D) Alpha diversity as measured by observed species, Shannon diversity index, and phylogenetic diversity (PD) of the salivary microbiome in NS (green), ES (blue), and CS (red) cohorts. The line inside the box represents the median, whereas the whiskers represent the lowest and highest values within the interquartile range. Outliers' individual samples are shown as dots. Analysis was done using Mann-Whitney test. (E) Phylum-level relative abundances of the salivary microbiota based on taxonomic inference of 16S rRNA sequences. *Actinobacteria, Firmicutes, Fusobacteria, Proteobacteria,* and *Spirochaetes* were significantly altered. (D and E) Data are represented as mean ± SEM. (*p < 0.05, **p < 0.01, ***p < 0.001).
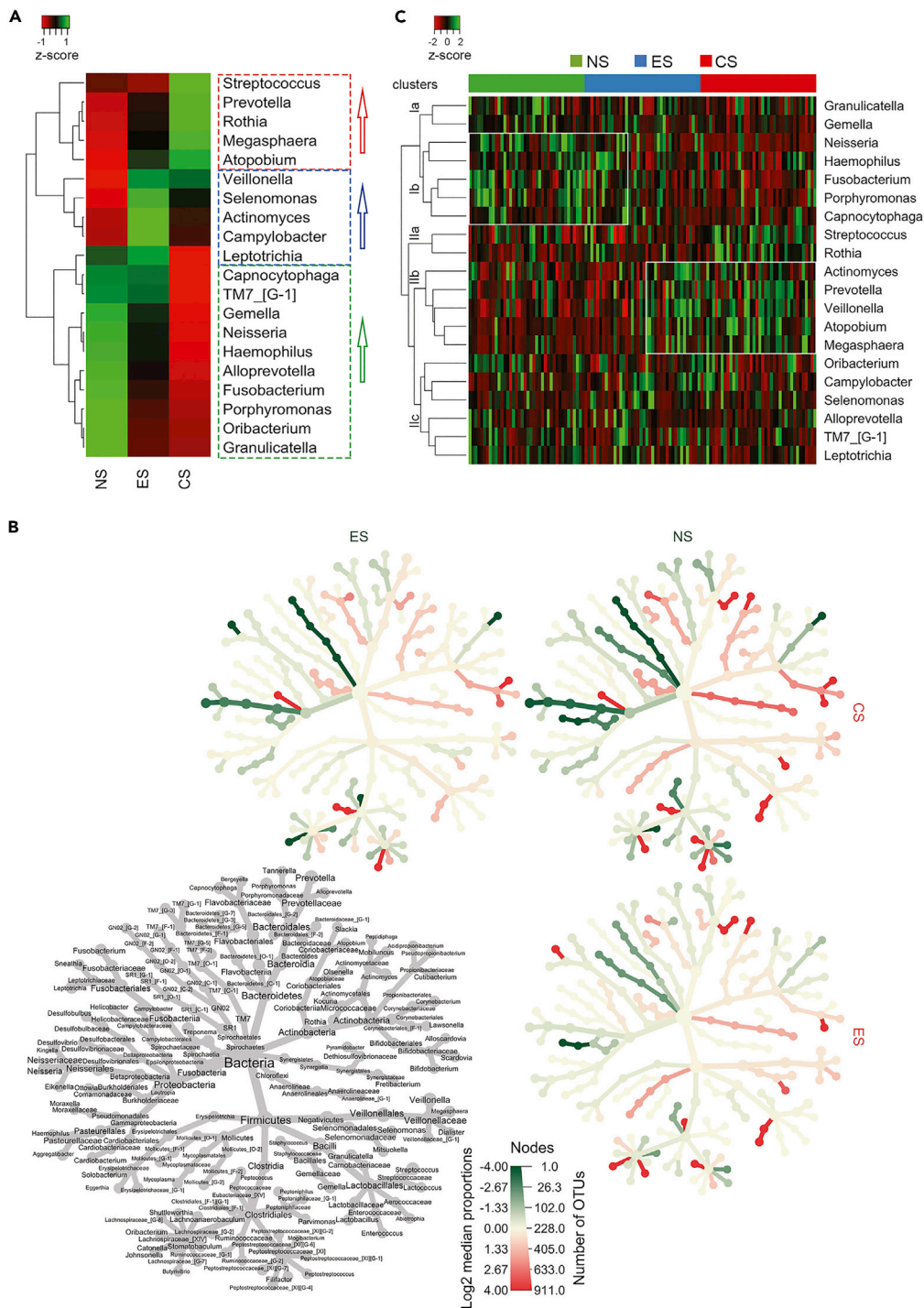
Figure 3. Dysbiotic Salivary Bacterial Genera in NS, ES, and CS Cohorts

(A) Cohort-based microbial abundances observed in NS, ES, and CS cohorts.

(B) Heat tree illustrates the relationship of OTUs up to genus level. Colored branch of the tree denotes significance based on the color of individual cohorts.

(C) Top 20 taxa in the saliva of the subject population across NS, ES, and CS cohorts; rectangular boxes represents two clusters where several taxa in the samples of ES cohort overlap with the samples of NS and CS cohorts.

CS cohorts (Figures S2A and S2B). *Veillonella atypica, Megasphaera micronuciformis, Streptococcus parasanguinis* clade 411, *Prevotella sp.* HMT 311, and *Actinomyces lingnae,* although higher in the ES cohort, significantly expanded in the CS cohort (Figure S2C). *P. gingivalis, Alloprevotella tannerae, Dialister invisus, Corynebacterium durum,* and *Leptotrichia wadei* levels were proliferated in the ES cohort, whereas *Streptococcus salivarius* was higher in the CS cohort.

In vivo Interactions among Oral Taxa and Salivary Cytokines

To explore the salivary inflammatory markers in all participants, 39 in NS cohort and 40 in each of ES and CS cohorts, we analyzed 10 different cytokines using a human multiplex immune assay pro-inflammatory panel. Salivary interleukin (IL)-6 and IL-1β were elevated, although not significant in ES when compared with NS and CS (Figure 4A). Significant reduction in levels of interferon (IFN)-γ and IL-4 were observed in traditional CS compared with NS. Tumor necrosis factor (TNF)-α concentrations were altered significantly in non-smokers (p < 0.05) and considerably in ES (p < 0.1) compared with regular CS. However, other salivary cytokines showed no significant difference among cohorts. We further evaluated the association between inflammatory cytokines and salivary bacteria. Spearman correlation analysis indicated *Porphyromonas, Haemophilus, Catonella,* and *Niesseria* to be positively correlated with cytokines IL-2, IL-13, IL-8, and IL-1β (Figure 4B). In addition, *Lachnoanaerobaculum* and *Stomatobaculum* displayed positive correlation toward inflammatory immune indicators such as IL-2, IL-4, IL-8, IL-13, IL-10, IL-12p70, and IFN-γ (Figure 4B). Positive correlations were observed among *Parvimonas, Peptostreptococcus,* and *Mogibacterium* with IL-8 and IL-1β, and significant correlations were observed in *Fusobacterium, Johnsonella,* and *Catonella* with IL-1β. On the contrary, among a few taxa inverse correlation was observed, i.e., *Desulfovibrio, Dialister, Helicobacter, Peptostreptococcus, Olsenella,* and *Treponema* with TNF-α; *Bacteroides* with IL-6; *Tannerella* with TNF-α, IL-4, and IFN-γ; and *Corynebacterium* and *Olsenella* with IL-8.

To confirm and expand the relevance of the aforementioned *in vivo* findings, we next performed *in vitro* studies using the premalignant (Leuk-1) and malignant (Fadu) cell lines exposed to e-cigarette aerosols. We evaluated the altered pro-inflammatory cytokine response at mRNA and protein levels when co-cultured with the periodontal pathogens, *P. gingivalis* and *F. nucleatum,* the taxa observed to be expanded *in vivo* in the saliva of ES, and with *E. coli* GFP, one of the most established models of infection.

In Vitro mRNA Levels of Pro-inflammatory Cytokines Are Altered in the Presence of e-Cigarette Aerosols

Fadu and Leuk-1 cells were exposed to e-cigarette aerosol or air for 40 min followed by infection with *P. gingivalis* and *F. nucleatum* for 2 h (Figures 5A and 5B). Initially, we exposed malignant Fadu cells to either air or e-cigarette aerosols followed by infection with each of these bacteria. We quantified the mRNA levels of the cytokines, IL-1β, TNF-α, IFN-γ, IL-6, and IL-8 by qPCR. A significant rise in all the five cytokine mRNA levels was detected in e-cigarette aerosol-exposed Fadu cells co-infected with *P. gingivalis* when compared with those exposed to only air co-infected with same bacteria. Intriguingly, TNF-α showed almost a 30-fold increase in expression when compared with the cells exposed only to air. Similarly, higher mRNA levels of cytokines were observed when e-cigarette aerosol-exposed Fadu cells were co-infected with *F. nucleatum.* The expansion in mRNA expression of IFN-γ was highly significant, followed by IL-6, IL-8, and IL-1β, and moderate for TNF-α upon e-cigarette aerosol exposure for Fadu cells. We further assessed the mRNA levels of TNF-α and IL-8 in the Leuk-1 cell line infected with *P. gingivalis* and *F. nucleatum* and observed results similar to that with Fadu cell line (Figures S3A and S3B). Significantly high mRNA expression of IL-8 was observed in e-cigarette aerosol-exposed cells with both bacteria.

Furthermore, we examined whether a similar influence on cytokines is observed when Fadu cells were infected with the classical microbe, *E. coli* GFP (Figure 5C). Upon co-infection with this bacteria, the e-cigarette aerosol-exposed Fadu cells exhibited significantly higher mRNA levels of IL-1β and IFN-γ in contrast to air-exposed bacterial co-infected cells. In parallel, the IL-8 mRNA expression was up-regulated significantly in Fadu cells exposed to e-cigarette aerosol and *E. coli* GFP when compared with cells exposed singly to either air or e-cigarette. TNF-α, IL-8, IFN-γ, IL-1β, and IL-6 mRNA expression was also measured in Leuk-1 cells in the presence of *E. coli* GFP, and e-cigarette aerosols caused a significant rise in cytokine levels (Figure S3C).

The findings suggested augmented cytokine mRNA expression in Fadu as well as Leuk-1 cells upon e-cigarette aerosol exposure, and even more so when co-infected with bacteria, indicating increased susceptibility to infection in cells when exposed to e-cigarette aerosols.
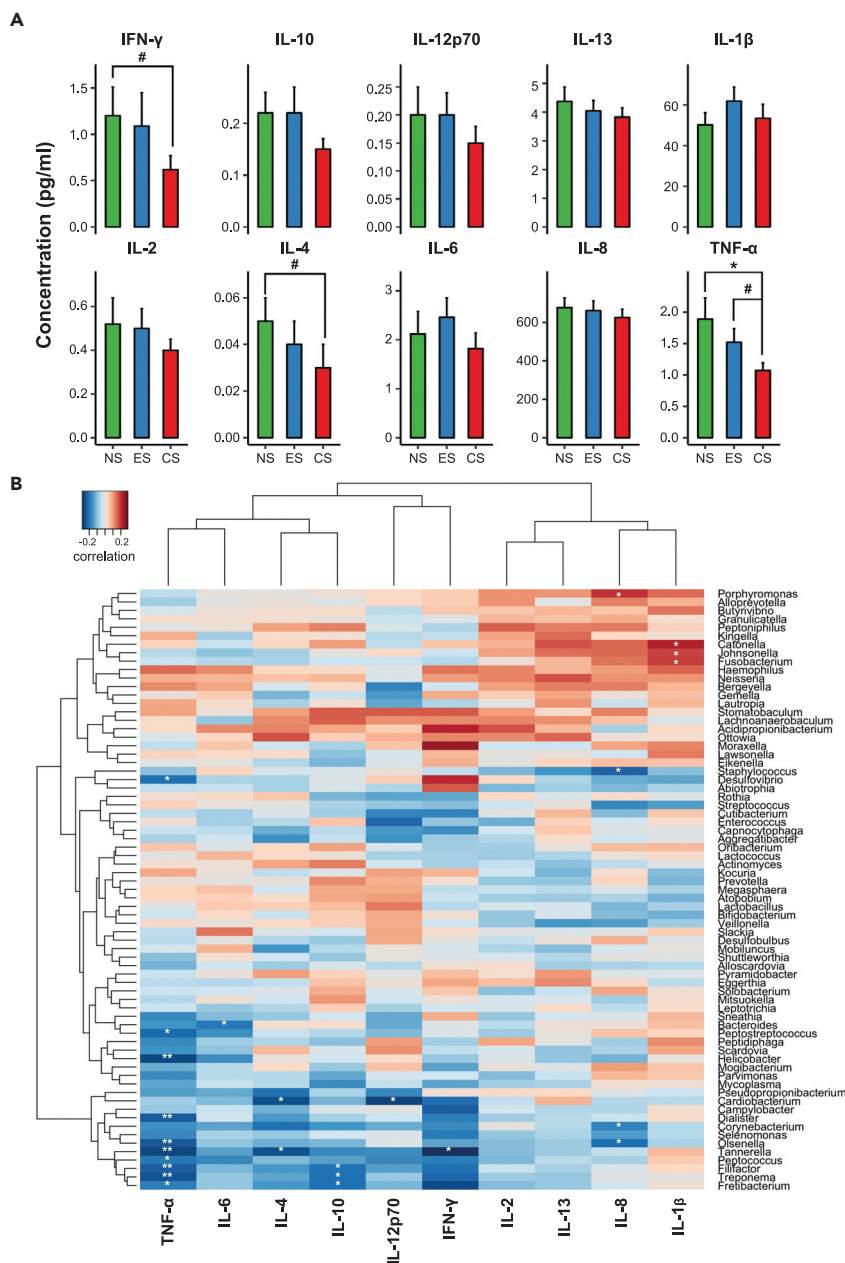
Figure 4. Salivary Inflammatory Cytokine Expression and Its Correlation with Abundant Taxa

(A and B) (A) Levels of 10 different pro-inflammatory salivary cytokines and chemokines in three cohorts, NS, ES, and CS. Data are represented as mean ± SEM. ($^\#p < 0.1$, $^*p < 0.05$). (B) Correlation matrices indicate association of cytokines with the bacterial taxa. Values are represented by colors from blue (negative correlation) to red (positive correlation). Clustering is based on the Spearman rank correlation similarity between cytokines and microbial genera. ($^*p < 0.05$, $^{**}p < 0.01$)

e-Cigarette Aerosol Exposure Increased the Protein Levels of Pro-inflammatory Cytokines *In Vitro*

Fadu cells infected with *P. gingivalis*, showed significant up-regulation of IL-8 protein in the presence of e-cigarette aerosol when compared with air exposure. However, TNF-α protein levels did not significantly change in the cells infected with *P. gingivalis* and *F. nucleatum* (Figures 6A and 6B). On the contrary, under similar conditions with *F. nucleatum*, IL-8 and TNF-α protein levels were differentially up-regulated when compared with air only. However, these protein cytokine levels were not in the detectable range with *E. coli* GFP (data not shown).
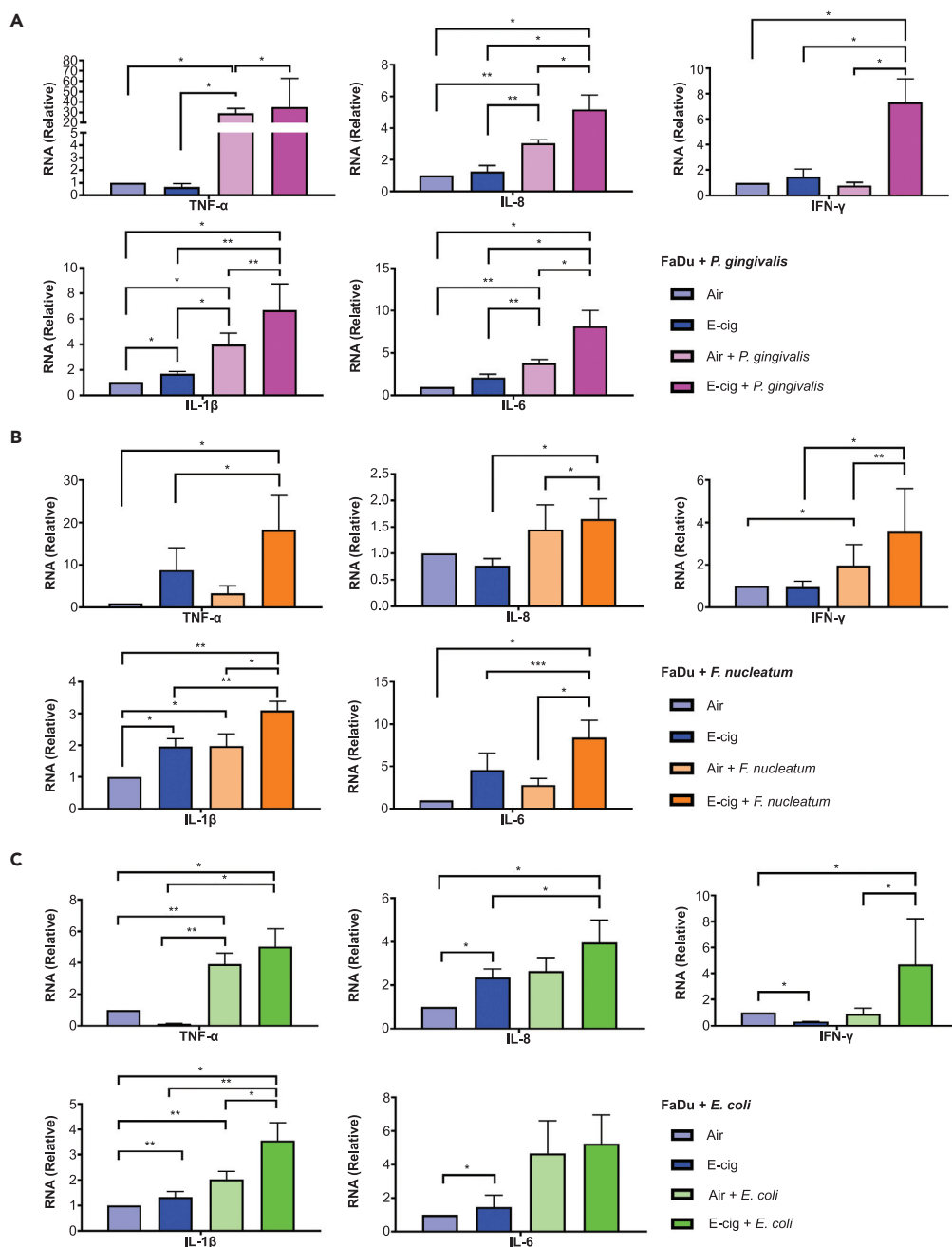
Figure 5. mRNA Expression Levels of Various Cytokines, TNF-α, IL-8, IFN-γ, IL-1β, and IL-6 in Fadu Cells in the Presence of Bacteria and e-Cigarette Aerosols as Determined by qPCR.

(A–C) Significant increase in expression of all cytokine mRNAs was observed with (A) *P. gingivalis* (B) *F. nucleatum,* and (C) *E. coli* GFP. Cells exposed to only air or e-cigarette aerosol were used as controls. Data are represented as mean ± SEM. (*p < 0.05, **p < 0.01, ***p < 0.001).

In addition, when another cell line Leuk-1 was tested, up-regulated IL-8 level protein upon e-cigarette aerosol exposure was observed. Conversely, co-infection of Leuk-1 with *P. gingivalis* did not show a similar effect, where TNF-α levels were not altered (Figure S4A). However, infection with *F. nucleatum* showed higher protein concentration of both TNF-α and IL-8 in e-cigarette aerosol-exposed cells (Figure S4B). Leuk-1 cells co-infected with *E.coli* GFP in the presence of e-cigarette aerosols significantly up-regulated IL-8 protein.
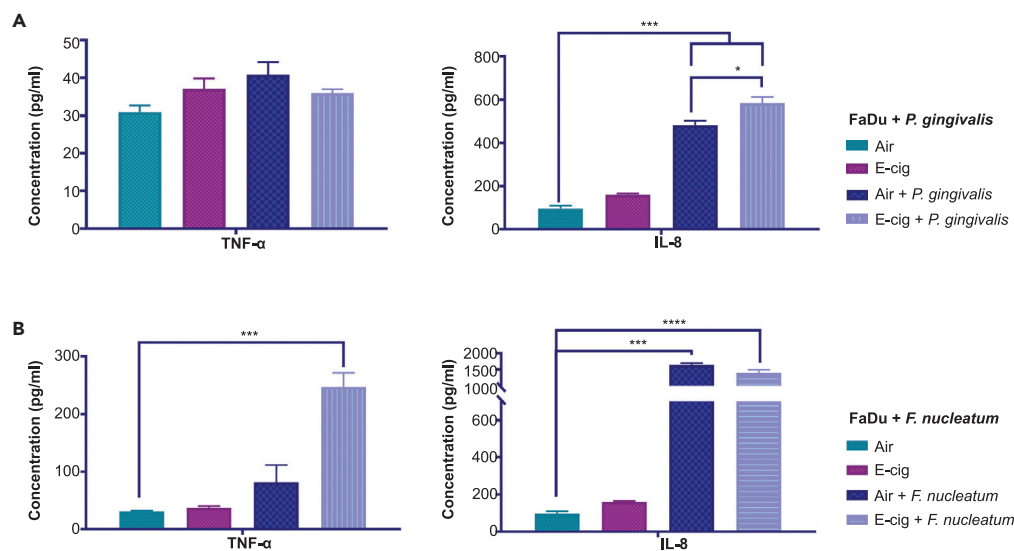
Figure 6. Protein Cytokine Concentration of TNF-α and IL-8 in Bacteria-Treated Fadu Cell-Free Medium upon e-Cigarette Aerosol Exposure as Quantified by ELISA Assay

Cytokine protein levels were up-regulated with e-cigarette aerosol exposure: (A) IL-8 only with *P. gingivalis* and (B) TNF-α only with *F. nucleatum*. Data are represented as mean ± SEM. (*p < 0.05, ***p < 0.001, ****p < 0.0001).

However, the TNF-α protein levels could not be detected with ELISA due to the very low levels of expression (Figure S4C).

Our *in vitro* results clearly indicated that e-cigarette aerosols altered the cytokine concentration at mRNA and protein levels in the presence of periodontal pathogens, as was also confirmed with *E. coli* GFP.

e-Cigarette Aerosols Accelerate Oral Bacterial Infection

To affirm that the e-cigarette aerosol influenced the rate of microbial infection *in vitro*, FaDu cells were co-cultured with either *P. gingivalis* or *F. nucleatum* pre-labeled with fluorescein isothiocyanate and then exposed to air or e-cigarette aerosol, and the infected FaDu cell population was evaluated by flow cytometry. It was observed that *P. gingivalis* and *F. nucleatum* infection in Fadu cells with e-cigarette aerosol contact increased significantly (p < 0.05) by about 65% and 16%, respectively, compared with air only (Figures S5A and S5B). Furthermore, FaDu cells were also infected with *E.coli* GFP in a 1:50 ratio after treating with either air or e-cigarette aerosol. Infection efficiency determined by GFP expression with flow cytometry showed a significant elevation (p < 0.048) of almost 21% in FaDu cells exposed to e-cigarette aerosol than in those exposed only to air (Figure S5C).

DISCUSSION

Although e-cigarettes are conjectured to be an effective alternative to conventional cigarette smoking, statistical data insinuate an exponential rise in e-cigarette usage among college students, and its co-use with alcohol can contribute to negative consequences in the current younger generation (Littlefield et al., 2015). Also, there is limited information on the effects of nicotine content in the e-cigarette on oral microbiota and periodontal infection. This study assessed the influence of different host smoking environments on periodontal health analogous to host immune response and salivary microbial profiles.

We observed that distinct microbial communities harbor the oral cavity of ES, albeit exhibiting some community overlap with either NS or conventional CS participants. Interestingly, the e-cigarette vapers that had overlapping taxa clustering with either the NS or CS cohorts presented moderate to severe periodontitis. In addition, the e-cigarette participants who shared higher abundance of certain microbial communities (Figure 3C) with the non-smoker cohort and with conventional smokers had BoP of 56.09% and 65.49%, respectively, which reflects high inflammatory fingerprint in CS participants. An earlier study has shown an association between periodontal status and the microbiota, with increased counts of several

bacteria including periodonto-pathogens, *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia,* and *Niesseria mucosa* with increased inflammatory biomarkers in the saliva of periodontal disease-positive non-smokers (n = 366) (Lira-Junior et al., 2018). This connotes dependencies for dysbiotic microbial communes on periodontal health status, as signified by pocket depth and inflammatory responses, a measure of bleeding on probing and observed cytokine levels, in addition to salivary cotinine levels. The exposure to nicotine or other components from e-cigarette vaping expanded the abundance of periodontal pathogens. Periodontal microbes are a potential source of oral bacterial consortia that is altered by smoking, as reported earlier in the intervention studies for treatments of periodontal disease and tobacco dependence (Delima et al., 2010).

It is well established that smoking-associated periodontitis is not just a deliberation of poor oral hygiene but extends to provide an appropriate niche for colonization of bacteria such as *P. gingivalis*, *Prevotella intermedia,* and *Actinobacillus actinomycetemcomitans,* facilitating development of periodontal lesions (Eggert et al., 2001). It has been reported that exposure to nicotine modulated the immunological functions via *P. gingivalis*, promoting biofilm formation by interacting with commensal oral bacteria *Streptococcus gordonii* (Hanioka et al., 2019). Moreover, the salivary microbiome exemplifies bacteria from different surfaces in the oral cavity (Gao et al., 2018). Socransky and Haffajee (2005) observed a higher prevalence of eight species, including *P. gingivalis*, in current smokers. Socransky et al. (1998) showed that several bacterial complexes are involved in the etiology of periodontitis. The red complex (*P. gingivalis*, *T. forsythia*, and *T. denticola*) and orange complex (*F. nucleatum* subspecies, *F. periodonticum*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Prevotella nigrescens*, and *Campylobacter rectus*) perio-pathogens indicate high and moderate risks, respectively, of periodontitis (Socransky et al., 1998) as detected in our study.

Moreover, we observed a significant proliferation of specific salivary taxa, to note, *Veillonella* in ES and regular CS when compared with NS. Nonetheless, *Porphyromonas* expanded significantly in ES, whereas *Prevotella* did in conventional CS. The taxon *Granulicatella* was predominant in all the three cohorts. The predominance of these periodontal pathogens in the oral cavity of ES and combustible cigarette users is a reflection of severely compromised periodontal health (p = 0.001) as depicted in Table S1. It has been shown that nicotine and its metabolite cotinine, as well as treatment with a cigarette smoke extract, can alter the function of key periodontal pathogens such as *P. gingivalis* and promote biofilm formation, colonization, and infection (Hanioka et al., 2019). *Granulicatella* is a known commensal of the human oral microbiome, however, which has been implicated in endodontic infection (Siqueira and Rôças, 2006) and is linked to increased risk of systemic diseases, such as pancreatic cancer related to oral inflammation (Farrell et al., 2012).

Cigarette smoking is known to escalate the infection risk of pathogenic or opportunistic bacteria by bringing about physiological and structural changes, increases in bacterial virulence, and immune dysfunction (Bagaitkar et al., 2008). A sophisticated cytokine network regulates the cross talk between periodontal pathogens and immune mediators. The extent of the host inflammatory response to a microbial challenge defines the severity of periodontal disease (Harvey, 2017). IL-1β concentrations were found to be significantly higher along with the progression of periodontal disease (Tâlvan et al., 2017). *Prevotella* was found to be positively associated with the cytokine IL-1β, which suggests a high pro-inflammatory state (Acharya et al., 2017). Previous studies have indicated *Peptostreptococcus* (Riggio et al., 2001) as well as *Mogibacterium* and *Catonella* to be associated with periodontal and endodontic infections (Siqueira and Rôças, 2006). In the present study, *Veillonella* was found to be a highly abundant taxon, specifically with species *V. atypica* and *V. rogosae* being highly elevated in ES and CS cohorts. *Veillonella* are largely commensals, abundantly existing in saliva and on dorsal and lateral surfaces of the tongue (Mager et al., 2003), and have conflicting (i.e., positive, negative, or no) associations with cigarette smoking in patients with periodontitis (Kato et al., 2016). Previous reports suggest that *V. atypica* specifically converts ingested nitrate, largely present in the saliva of tobacco users, to nitrite, which can further be converted to potentially toxic carcinogenic nitrosamines and pro-inflammatory nitric oxide (Stepanov et al., 2008). These compounds can have pathophysiological consequences on oral and systemic health. Furthermore, tobacco exposure suppresses overall immune responsiveness to bacteria and bacterial by-products such as lipopolysaccharides (LPS) (Bagaitkar et al., 2008).

Similar to our *in vivo* results showing increased salivary IL-6 production in ES, our *in vitro* study also asserted up-regulation of IL-6 after exposure to e-cigarette aerosols in Fadu and Leuk-1 cells. Moreover, in the

presence of *P. gingivalis* alone (without e-cigarette aerosols), increased IL-6 expression in Fadu and Leuk-1 cells was detected. *P. gingivalis* is known to affect leukocyte distribution near microbial colonization sites disrupting the innate host surveillance (Hajishengallis et al., 2012; How et al., 2016). The observed enhanced IL-6 expression after infection with bacteria, including *P. gingivalis*, *F. nucleatum,* and *E. coli* GFP, suggests that e-cigarette aerosols enhanced susceptibility to periodontal disease. Both *P. gingivalis* and *F. nucleatum* play pivotal roles in periodontal disease and act on macrophages, neutrophils, and monocytes to induce TNF-α, IL-6, and IL-8 production (Petković et al., 2010). TNF-α secreted by immune cells can block bone formation and inhibit collagen synthesis (Fleetwood et al., 2017). IL-1β synergistically acts with TNF-α in the bone resorption process, and relatively high IL-1β concentrations are reported in the gingival crevicular fluid of patients with severe periodontal disease (Boström et al., 2000). Also, *F. nucleatum*, ubiquitous in oral cavity, is associated with chronic aggressive periodontitis, gingivitis, and endodontic infections, with additional effects such as adverse pregnancy outcomes, GI disorders, and other infections (Han, 2015). Moreover, the LPS of *F. nucleatum* stimulates improved IL-1β and TNF-α production when compared with that of *P. gingivalis* (Lagha and Grenier, 2016). Our *in vivo* results indicated expanded IL-1β levels in the saliva of e-cigarette vapers that parallel the proliferated presence of certain periodontal pathogenic taxa, such as *Porphyromonas* and *Fusobacteria*, in this participating cohort. In addition, *F. nucleatum*, known to stimulate nuclear factor-κB-driven pro-inflammatory responses, induces colon cancer (Lagha and Grenier, 2016). Increasing evidence revealed IL-6, IL-1β, and TNF-α involvement in dental and periodontal disease including inflamed pulp and periapical lesions (Brekalo Pršo et al., 2007) and in stimulation of tissue degradation due to an increase in matrix metalloproteinases (Alevizos et al., 2001). Studies have indicated high IL-6 expression at both transcriptional and translational levels in gingival mononuclear cells of patients with periodontitis (Fujihashi et al., 1993).

The periodontal microbes in tissues likewise stimulate chemokine IL-8 production, which induces a signal for accumulation and activation of neutrophils in local sites (Wilson et al., 1985). Importantly, IL-8 levels were expanded in Fadu cells after co-infection with *E. coli* GFP, *P. gingivalis,* or *F. nucleatum* and increased maximally in cells treated with bacteria and exposed to e-cigarette aerosols. Also, our results have shown an up-regulation of IFN-γ upon co-incubation with e-cigarette aerosols and *E. coli GFP* when compared with air only, e-cigarette only, and combination of air and bacteria. IFN-γ is a key player in immune responses in periodontal disease (Gemmell and Seymour, 2004) and in the activation of immunomodulation in mesenchymal stem cells. *In vivo* studies reported that mice, in the absence of IFN-γ, showed decreased bone loss after *P. gingivalis* infection in oral cavity (Meyle and Chapple, 2015). The limitations of this experimental design are that all *in vitro* experiments were done on cell culture models using oral pathogens, and it will be valuable to include primary cell or 3D oral tissue models and other canonical intracellular pathogen (e.g., *Listeria*) in our future experiments.

The presented data suggest that vaping e-cigarettes causes oral environmental shifts and highly influences the colonization of complex heterogeneous microbial biofilms. More elaborate studies would help in identifying the detrimental effects of e-cigarette aerosols and its toxic components, albeit taking into consideration other confounding factors such as vaping behavior and the dual use of e-cigarettes and conventional cigarettes. With the advent of more advanced versions of e-cigarettes, users have greater control over the quantity of e-liquid usage, power, and airflow settings. In one study, higher plasma nicotine levels were detected in newer generation of e-cigarettes compared with a first-generation e-cigarette device (Farsalinos et al., 2014). Studies are also warranted to evaluate the long-term adverse effects of metal fumes and other contaminants associated with e-cigarette usage. As e-cigarettes have become commercially available only in the past 10–12 years, there is no study that assesses the long-term health hazards associated with their use. However, very recently, the CDC and FDA reported about 215 cases of mysterious lung illnesses, linked to e-cigarette usage and vaping among teens and young adults in 25 states, currently under investigation with state and federal health officials (https://www.cdc.gov/media/releases/2019/s0830-statement-e-cigarette.html, August 30, 2019). Consequently, detailed studies will help to elucidate the mechanism and pathways of host-microbe interactions when in contact with e-cigarette aerosols that could compromise oral, respiratory, and cardiovascular health and its implication in DNA damage with cancer as a potential consequence.

LIMITATIONS OF THE STUDY

The limitations of this experimental design are that all *in vitro* experiments were done on cell culture models using oral pathogens, and it will be valuable to include primary cell or 3D oral tissue models.

We have used three bacteria to study the increase in infection after aerosol exposure; however, it will be helpful if other canonical intracellular pathogens (e.g., *Listeria*) are used in future experiments.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100884.

AUTHOR CONTRIBUTIONS

S.P. carried out sample collection, study design, *in vivo* microbiology and immunology experiments, DNA extraction and 16S sequencing, data analyses and interpretation, and manuscript preparation; B.P. performed *in vitro* cell culture and immunology experiments, study design, qPCR analysis and interpretation, and manuscript preparation; Q.L. performed computational analyses, manuscript preparation, and critical review; J.Y. carried out *in vitro* cell culture and immunology experiments; R.V. carried out subject recruitment; S.M. carried out *in vitro* cell culture and immunology experiments; J.M.G. carried out *in vitro* cell culture experiments and interpretation; S.S. performed DNA extraction; C.X. performed *in vitro* cell culture experiments; M.N.J. performed statistical analyses and critical review; E.Q. carried out subject recruitment; M.B. carried out clinical data analysis; J.L. performed flow cytometry experiments; J.S. performed DNA extraction; F.X. carried out REDCap data entry, technical laboratory work, and statistical analyses; E.A. performed REDCap data entry; Y.G. performed *in vitro* cell culture experiments; D.A. performed oral examination and clinical sample collection; C.G. performed oral examination and clinical sample collection; A.K. performed oral examination, clinical sample collection, and analyses; D.S. performed subject recruitment; Y.A. managed REDCap, clinical data, and electronic messaging system; C.B. provided technical assistance; T.G. assisted in aerosol-generating machine and manuscript preparation; P.C. provided assistance in subject recruitment and clinical sample collection; X.L. and D.S. conceived, designed, supervised, analyzed, and interpreted the study, provided critical review, and prepared the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Electronic Cigarette Aerosol Modulates

the Oral Microbiome and Increases

Risk of Infection

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SUPPLEMENTAL INFORMATION (SI)

TRANSPARENT METHODS

Study design and sample collection

To test our hypotheses, we recruited 119 participants categorized into 3 cohorts for this study, 39 in never smokers (NS), and 40 each in current e-cigarette users (ES), and current traditional cigarette smokers (CS). To verify their smoking status, all subjects were initially screened for their carbon monoxide (CO) levels by an exhaled CO breath test (Smokerlyzer, Covita, Santa Barbara, CA) and salivary cotinine levels using test strips (NicAlert, Craig Medical Inc., Vista, CA) (Marrone et al., 2010). Their periodontal health status was determined by a comprehensive oral examination and only subjects with mild to severe periodontal disease were included in the study. All the subjects signed the informed consent form and filled standardized questionnaires. The Institutional Review Board of New York University Langone Medical Center approved the study. The inclusion criteria were: aged ≥21 years, systemically healthy as evidenced by medical history, and be either a current smoker (smoking at least 10 cigarettes per day for at least 12 months), or nonsmoker (never smoked in their lifetime), or an e-cigarette user (never smoked and using 0.5 to 1 e-cigarette per day for past 6 months). They were diagnosed with mild, moderate or severe periodontal disease, according to the CDC in collaboration with the American Academy of Periodontology (CDC-AAP) (Eke et al., 2013). *Mild periodontitis* was defined as ≥ 2 interproximal sites with ≥ 3 mm attachment loss (AL), and ≥ 2 mm interproximal sites with probing depth (PD) ≥ 4 mm (not on the same tooth) or one interproximal site with PD ≥ 5 mm. *Moderate periodontitis* defined as 2 or more interproximal sites with ≥ 4 mm clinical AL (not on the same tooth), or 2 or more interproximal sites with PD ≥ 5 mm, also not on the same tooth. *Severe periodontitis* defined as having 2 or more interproximal sites with

≥ 6 mm AL (not on the same tooth), and one or more interproximal site(s) with ≥ 5 mm PD (Eke et al., 2013). Also, a minimum of 16 teeth with at least 8 of these teeth must be posterior. Oral and periodontal exams were evaluated by periodontist or dental hygienist, who were calibrated to a single gold standard examiner during 6 calibration sessions, and included: intraoral soft tissue findings, the number of teeth present, PD and gingival recession measured at six sites per tooth (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual and disto-lingual) on all teeth present and the clinical attachment loss (CAL) was determined. Dichotomous measures (0/1) of bleeding on probing (BoP) were assessed after each quadrant of probing. The exclusion criteria were: a medical condition (including uncontrolled diabetes and HIV), subjects who reported taking antibiotics or having a dental professional cleaning within 1 month of the enrollment day, a recent febrile illness that delays or precludes participation, pregnancy/lactation, enrolled in other studies, a history of radiation therapy to head and neck region, presence of oral mucosal lesions suspected of candidiasis, herpes labialis, aphthous stomatitis, premalignancy/malignancy, such as leukoplakia, erythroplakias. In addition, non-smoker participants were excluded in the study when carbon monoxide (CO) level presented >7 parts per million (ppm).

The saliva samples were collected from the qualified subjects for characterizing their microbiome and pro-inflammatory cytokine profiles. The participants were asked to chew paraffin wax pellets (Gleegum, Verve Inc., Providence, RI) to stimulate salivary flow rate and the saliva was collected for 5 min. The saliva was aliquot to desired volumes and stored at -80º C until further analysis.

Microbiome analyses by16S rRNA gene amplicon sequencing

Salivary genomic DNA was extracted using MoBio Power fecal kit as per manufacturer's instructions (MoBio Laboratories Inc., Carlsbad, CA) and stored at −20° C. For 16S library

preparation and sequencing, the V3-V4 region of 16S rRNA gene was amplified from 10 ng per µl of salivary genomic DNA according to modified Illumina 16S metagenomics protocol (Part # 15044223 Rev. B) as mentioned elsewhere (Pushalkar et al., 2018). Amplicon PCR was performed using the primer pair, 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′), each with overhang adapter sequences (IDT, Coralville, Iowa) using 2x Kapa HiFi Hotstart ReadyMix DNA polymerase (KapaBiosystems, Wilmington, MA) in duplicates and amplicons purified by AMPure XP beads. The cycling conditions were 95°C (3 min), with 25 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and final extension of 72°C (5 min). In subsequent PCR, amplicons were barcoded with dual indices from Illumina Nextera XT index kits (Illumina, San Diego, CA) using 2x Kapa HiFi Hotstart ReadyMix DNA polymerase at 95°C (3 min), with 8 cycles of 95°C (30 sec), 55°C (30 sec), 72°C (30 sec), and final extension of 72°C (5 min). The amplicon libraries were purified by AMPure XP beads, quantified by fluorometry PicoGreen assay, and size confirmed on agarose gels. Negative controls were included for all sequencing runs. Equimolar amounts of generated libraries were pooled and quantified fluorometrically. Pooled amplicon library was denatured, diluted, and sequenced on Illumina MiSeq platform using MiSeq Reagent Kit v3 (600 cycles) following 2×300-bp paired-end sequencing protocol.

Bioinformatics for sequence data

A total of 15,299,687 raw sequence reads were generated from 119 saliva samples. Sequences were trimmed using Cutadapt (v1.12)(Martin, 2011) to remove primers and short reads, and then processed in QIIME v1.9.0 (Caporaso et al., 2012, Navas-Molina et al., 2013). The reads were merged by join_paired_ends.py (j=100, p=8), demultiplexed using split_libraries_fastq.py (q = 19 and defaults for other parameters), and clustered into operational

taxonomic units (OTUs) using pick_open_reference_otus.py (m=usearch61) with Human Oral Microbiome Database (HOMD RefSeq, Version 15.1)(Escapa et al., 2018) as reference database. The 8,731,571 quality reads (Mean 72763 ±SD 21204; n=119) with approximate length of 427 bp were clustered into 43158 OTUs. Chimeric sequences were removed using ChimeraSlayer embedded in QIIME. Sequence data were analyzed at various levels of phylogenetic affiliations (phylum, family, genus, species). OTUs present in ≥10% of samples were considered for analyses.

Cytokine measurements by multiplex immunoassay

Salivary cytokine and chemokine levels were quantified using the V-Plex Human Proinflammatory Panel 1 Kit (10-Plex) from Meso Scale Discovery (MSD, Rockville, MD), constituting 10 different markers such as: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, and TNF-α. MSD plates were analyzed on an MS1300 imager (MSD, Meso QuickPlex SQ120) for the electrochemiluminescence. Assays were performed according to the manufacturer's instructions. All standards, control pack and 119 saliva samples were quantified in duplicate.

Cell lines used for *in vitro* study

The squamous cell carcinoma FaDu cell line was obtained from ATCC, whereas the immortalized human oral mucosal epithelial (Leuk-1) cell line was procured from Dr. Peter Sacks at the NYU College of Dentistry, New York, United States. Leuk-1 cells were grown in KBM-2 Keratinocyte Basal Medium (Lonza, Morristown, NJ, USA) supplemented with KGM-2 SingleQuot Supplements & Growth Factors (Lonza) and FaDu cells were grown in Alpha modified Minimum Essential Medium (Corning, USA) with 10% Fetal Bovine Serum (Atlanta

Biologicals, Georgia, USA) and 1% Penicillin-Streptomycin Solution (Hyclone Lab Inc., Logan, UT). The cell lines were maintained in a 5% CO_2 incubator at 37°C.

Bacterial inoculum

Escherichia coli GFP (ATCC 25922*), Fusobacterium nucleatum* (ATCC 10953) and *Porphyromonas gingivalis* (ATCC 33277) were obtained from American Type Culture Collection (Manassas, VA, USA). *F. nucleatum* was maintained on tryptic soy agar with 5% sheep blood (BD biosciences, San Jose, CA, USA) and *P. gingivalis* on CDC anaerobe blood agar plate in Whitley A35 Anaerobic Station (Don Whitley Scientific, West Yorkshire, UK) at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). *E. coli* GFP was cultivated in tryptic soy agar plate or tryptic soy broth supplemented with antibiotic ampicillin (Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 100 μg per ml at 37°C under aerobic conditions. The exponential phase anaerobic cultures of *F. nucleatum* were grown for 16 h in brain heart infusion broth (Fluka Analytical, Munich, Germany) and *P. gingivalis* for 40 h in fastidious anaerobe broth (Lab M Limited, Lancashire, UK) at 37°C. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 min at 4ºC and diluted to a concentration of ~$1×10^8$ CFU per ml with cell culture medium.

In vitro e-cigarette aerosol exposure

FaDu and Leuk-1 at 1×10^6 concentration were seeded in 60 mm tissue culture plates (Celltreat, Pepperell, MA, USA) and incubated for 24 h in a 5% CO_2 incubator prior to e-cigarette aerosol exposure. Fresh media was added prior to each exposure experiment. Cells with or without bacteria were exposed to e-cigarette aerosols at 3.3 V for 40 min at the rate of 2 puffs per min of disposable cigarettes having 2.4% nicotine in a modular incubator chamber (Billups-rothenberg Inc, San Diego, CA, USA). Controls cells (absence of e-cigarette aerosols) were

exposed to filtered air for the same duration. Post-exposure to air or e-cigarette aerosol, fresh culture media was added to cells. *E. coli* GFP, *F. nucleatum* or *P. gingivalis* were inoculated in a ratio of 1:50 (mammalian cell to bacteria) and incubated for 2 h after aerosol or air exposure. The aerosol exposure was performed on rocking shaker (Maxi Rotator, Lab-Line, USA) for uniform contact of e-cigarette aerosol onto liquid media to avoid cells from drying. The incubator was connected to an e-cigarette smoking machine (e~Aerosols, Inc, Central Valley, NY) by a series of tubing and connectors. The cell-free medium was analyzed for ELISA and cells collected immediately after exposure were extracted for total RNA.

Real time PCR (qRT-PCR) for mRNA quantitation

Total cellular RNA was extracted from the cells suspended in RLT buffer with ß-mercaptoethanol using RNeasy Plus Mini-Kit (Qiagen Hilden, Germany). RNA was quantified using Nanodrop 2000 (ThermoFisher, Waltham, MA). cDNA was generated from RNA (400 ng to 1 ug) with Taqman Reverse Transcription Kit (Applied Biosystems, Carlsbad CA, USA). qPCR analysis of IL-1β, IL-8, IL-6, TNF- α and IFN-γ were performed thrice in triplicates using Power SYBR- Green PCR Master Mix (Applied Biosystems Warrington, UK) on an iCycler thermal cycler (Bio-Rad, Hercules, USA). The 2-ΔΔC$_T$ method was used to calculate relative expression levels. FTH1 was used as a reference gene. Measurements were expressed in mean ± SEM.

ELISA assay for *in vitro* cytokine measures

The cell-free culture medium was collected after exposure to air or e-cigarette aerosols either in the presence or absence of bacteria and analyzed for IL-1β, IL-8, IL-6, TNF- α and IFN-γ using an Instant ELISA Kit (Affymetrix eBioscience, Veinna, Austria) as per manufacturer's instructions. In brief, 50 µl of culture medium was diluted according to the protocol and each

dilution was added to a pre-coated well in duplicate. The ELISA plate was incubated at room temperature for 3 h at 400 rpm. After incubation, the wells were washed 6X times with wash buffer following 10-30 min incubation with substrate solution. The enzyme reaction was terminated with 100 µl of stop solution. The cytokines were read at absorbance of 450 nm and quantified based on a standard curve.

Flow cytometry

FaDu cells (1×10^6) were seeded on 60 mm plate for 24 h and replaced with fresh media prior to experiment. *F. nucleatum* or *P. gingivalis* were centrifuged at 6000 rpm for 15 min. The bacterial cells were treated with FITC (0.1mg per ml) in 1X PBS solution and incubated for 30 min in anaerobic chamber. The FITC-labeled bacteria were centrifuge and the pellet washed thrice with 1X PBS buffer. After the final wash, the bacterial cells ($\sim 5 \times 10^7$) were re-suspended in FaDu cell culture medium, incubated for 30 min at 5% CO_2 and exposed to e-cigarette aerosol for 40 min. After exposure, FaDu cells were washed with 1X PBS buffer, trypsinized for 5 min and centrifuged at 1000 rpm for 5 min. The cells (pellet) were washed with 1X PBS, centrifuged and re-suspended in 2 ml of sheath fluid (0.2% Bovine Serum Albumin in EDTA solution).

Using *E. coli* GFP, Fadu cells were exposed to e-cigarette aerosol or air for 40 min followed by 2 h of bacterial infection. The cell culture medium was collected and treated with propidium iodide. In parallel, the adherent cells were scraped off and suspended in 1X PBS and later stained with propidium iodide. Flow Cytometry was performed on BD FACSort Cytometer (BD, Franklin Lakes, NJ, USA). Infected FaDu cell population was analyzed with FlowJo Software (FlowJo Ashland OR, USA).

Fluorescence imaging

Eight-well Millicell EZ chamber slides (Millipore Sigma, USA) were seeded with FaDu cells 24 h prior to aerosol and bacterial exposure. The exponential phase grown *F. nucleatum* and *P. gingivalis* cultures were centrifuged at 6000 rpm for 15 min. The bacterial cells were suspended in FITC solution (0.1mg per ml in 1X PBS) for labeling and incubated anaerobically for 30 min at 37ºC. After incubation, the cells were centrifuged, washed thrice with 1X PBS and bacterial pellet re-suspended in FaDu culture medium. The FITC-labeled bacteria were added to each well of chamber slides (FaDu to bacteria ratio of 1:50) and co-incubated with FaDu cells for 30 min at 37ºC, followed by e-cigarette aerosol exposure for 40 min. Post-exposure, the chamber slides were washed with 1X PBS buffer. The cells were fixed with 3.6% formaldehyde for 10 min and incubated for 2 min with DAPI (Thermo Scientific, Waltham, MA, USA) for nuclei labeling. The chamber slides were mounted with aqueous mounting medium (Vector Labs, Burlingame, CA, USA) and visualized under fluorescence microscope (Nikon, Tokyo, Japan).

Fluorescent microscopy with *E. coli GFP* seeded in Fadu cells were grown on chamber slides for exposure to e-cigarette aerosol for 40 min, followed by infection with bacteria for 2 h. Fresh medium was replaced, and formalin added to cells for 10 min. The residual formalin was removed and washed with 1X PBS. Subsequently, the cells were stained with 200 µl of Hoechst dye for 5 min. Later, the dye was removed; cells were washed twice with PBS and visualized under fluorescent microscope (Nikon Tokyo, Japan).

Statistical analyses

The participants' demographic and periodontal data were entered in NYULMC REDCap and analyzed using IBM SPSS 20.0. ANOVA and Chi-square tests were applied to normally distributed data and a post hoc Tukey test used to determine cohort-based differences. Mean ± SD calculated for quantitative and qualitative variables were expressed as percentages.

For sequence data, Kruskal-Wallis and Mann-Whitney U tests were used to compute the significant distinction in bacterial composition between the three cohorts and within samples. Alpha diversity (Observed OTUs, Shannon Index, Phylogenetic diversity) was computed and plotted using phyloseq (v1.27.2)(McMurdie and Holmes, 2013) and picante (v1.8) (Kembel et al., 2010) in R (v3.5.1). Principal coordinate analyses (PCoA) were performed with weighted Unifrac distance metrics and one-way permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences between cohorts (Adonis, R package Vegan v2.5.4) (Jari Oksanen, 2019). The dissimilarities were further analyzed, if significant, by LEfSe tool to identify differentially altered bacterial taxa between the cohorts by Kruskal-Wallis test (Segata et al., 2011).

For different chemokines and cytokines, a standard t-test with equal variance was used to analyze differences between the cohorts. For association analyses between cytokines and different bacterial taxa found in the study, a Spearman rank correlation was performed.

Statistical analyses for qPCR and ELISA were performed using one-tailed paired Student's t-test. The graphs were generated in Graph-pad prism (San Diego, CA, USA). For all the stated analyses, $p<0.05$ was considered statistically significant.

Data availability

16S rRNA sequence data of this study is publicly available in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA602902.

Quality Control

For adequate quality control, we employed best practices using techniques recently described by us (Berk Aykut, 2019, Pushalkar et al., 2018). All the samples were collected using standard sterile technique. We maintained consistency in DNA extraction techniques and

reagents throughout. All PCR reagents were periodically checked for environmental contaminants using 16S universal primers. All qPCR reactions had appropriate controls (without template) to exclude DNA contaminants.

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Table S1: Socio-demographic and clinical parameters of study subjects in three cohorts.

	Never Smokers	Cigarette Smokers	E-cigarette users	p-value
	(n= 39)	(n= 40)	(n= 40)	
Male (%)	56.4[b]	80.0[a]	77.5[a]	0.04
Age (yr)				
Male, mean (SD)	28.8 (6.1)[a]	46.4 (10.0)[c]	36.0 (9.5)[b]	<0 .001
Female, mean (SD)	38.4 (13.8)	44.6 (12.0)	35.7 (16.6)	0.42
Ethnicity (% Hispanic)	23.1	12.5	17.5	0.48
Race (%)				<0.001
White	25.6	30.0	60.0	
Black	28.2	50.0	32.5	
Asian	43.6	7.5	5.0	
Other	2.6	12.5	2.5	
Electronic cigarette				
E-cigarettes/day, median (IQR)			0.5 (1.0)	
Puffs / day, median (IQR)			140.0 (100.0)	
Tobacco Smoke				
Cigarettes/day, median (IQR)		11.0 (5.0)		
Alcohol Use				
Average drinking (days/week), mean (SD)	1.5 (1.3)	1.9 (1.5)	2.0 (1.1)	0.29
Average # drinks/day, mean (SD)	2.5 (1.2)	3.0 (2.3)	3.4 (4.9)	0.58+
Carbon monoxide (ppm), mean (SD)	1.8 (2.3)[a]	18.8 (9.4)[b]	5.1 (6.9)[a]	< 0.001+
Salivary Cotinine (ng/ml), mean (SD)	11.1 (5.0)[a]	535.9 (358.5)[b]	103.7 (125.3)[a]	<0 .001+
Salivary Flow Rate (g/min), mean (SD)	2.4 (1.2)	2.9 (1.2)	2.2 (1.1)	0.06
Periodontal Status (%) *				0.001
Mild	5.1	0	7.5	
Moderate	66.7	27.5	50.0	
Severe	28.2	72.5	42.5	
Pocket depth (mm, avg. per site) mean (SD)	2.7 (0.4)[a]	3.3 (0.7)[b]	3.0 (0.6)[a,b]	<0.001+
Bleeding on Probing (%), mean (SD)	53.0 (31.8)	64.5 (29.9)	57.2 (31.3)	0.26

*CDC-AAP criteria; +Welch test for heterogeneous variances; Like superscripts indicate homogeneous subsets
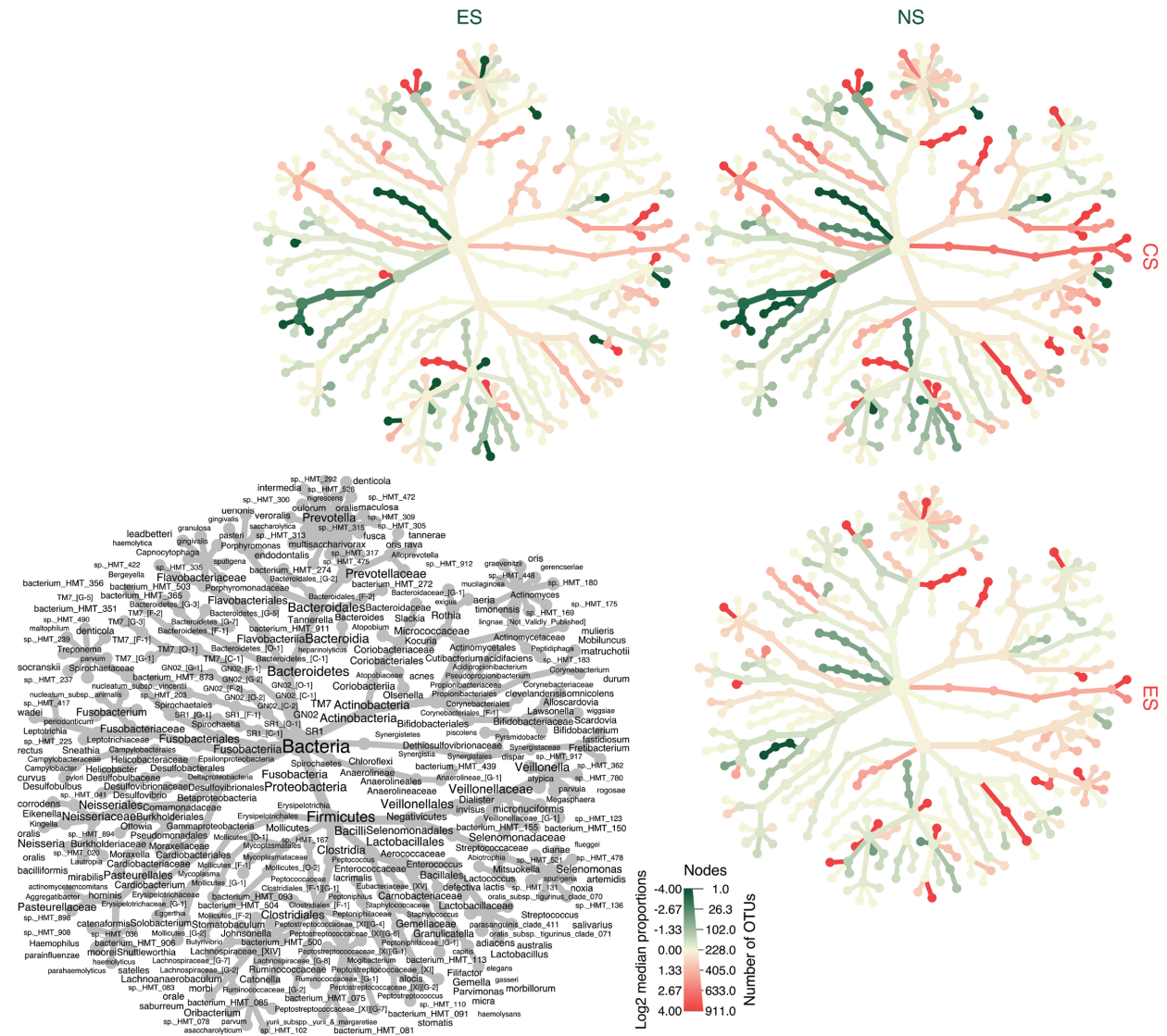
Figure S1

Figure S1. Related to Figure 3: Heat tree illustrates the relationship of species-specific OTUs in NS, ES and CS cohorts. Colored branch of the tree denotes significance based on the color of individual cohorts.
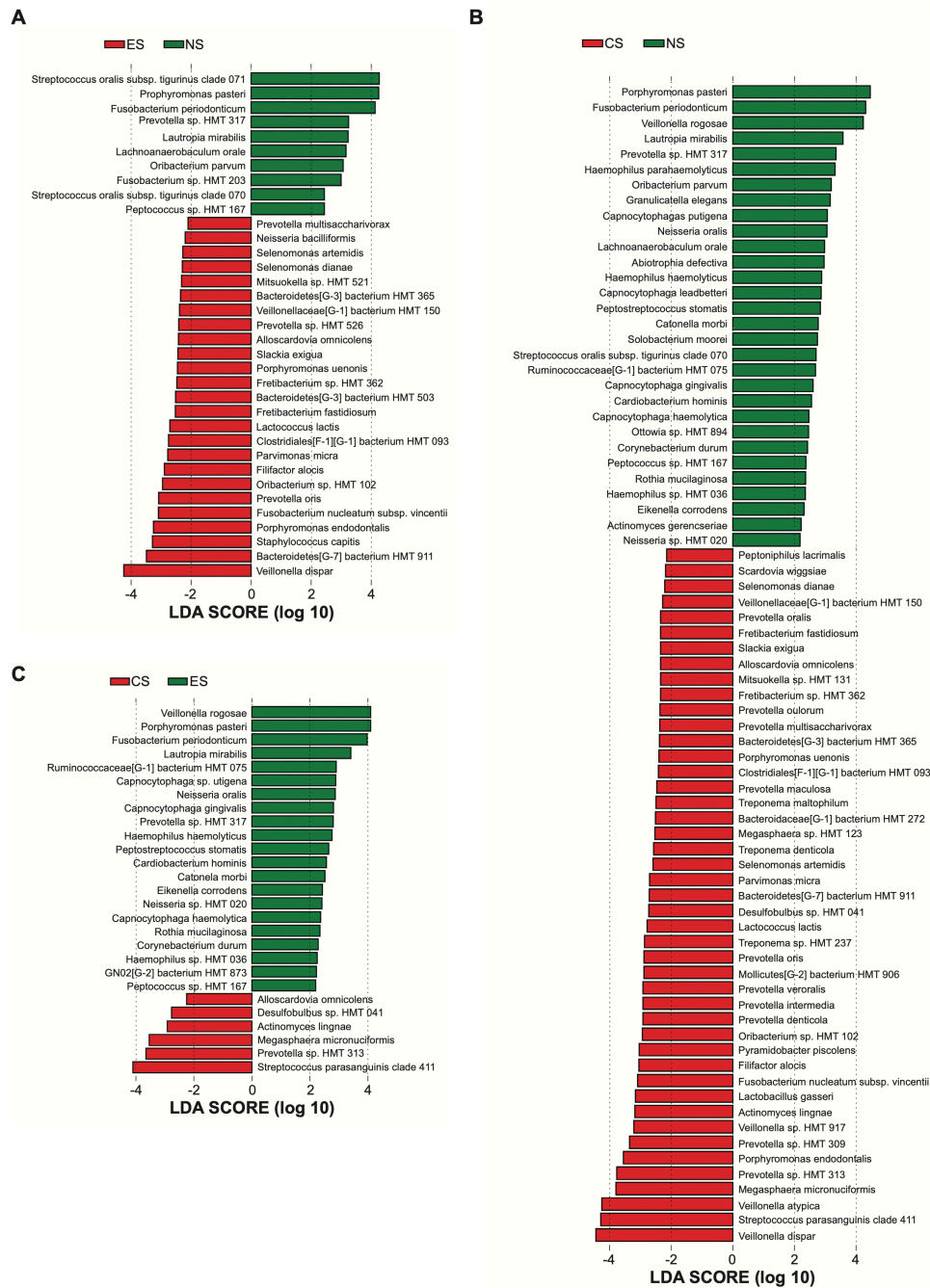
Figure S2

Figure S2. Related to Figure 3: Linear discriminant analysis (LDA) at species level combined with effect size measurements (LEfSe) revealed differentially altered taxa in the saliva samples of **(A)** NS and ES; **(B)** NS and CS; and, **(C)** ES and CS cohorts. *p<0.05* and score ≥ 2.0 were considered significant by Kruskal–Wallis and pairwise Wilcoxon tests.
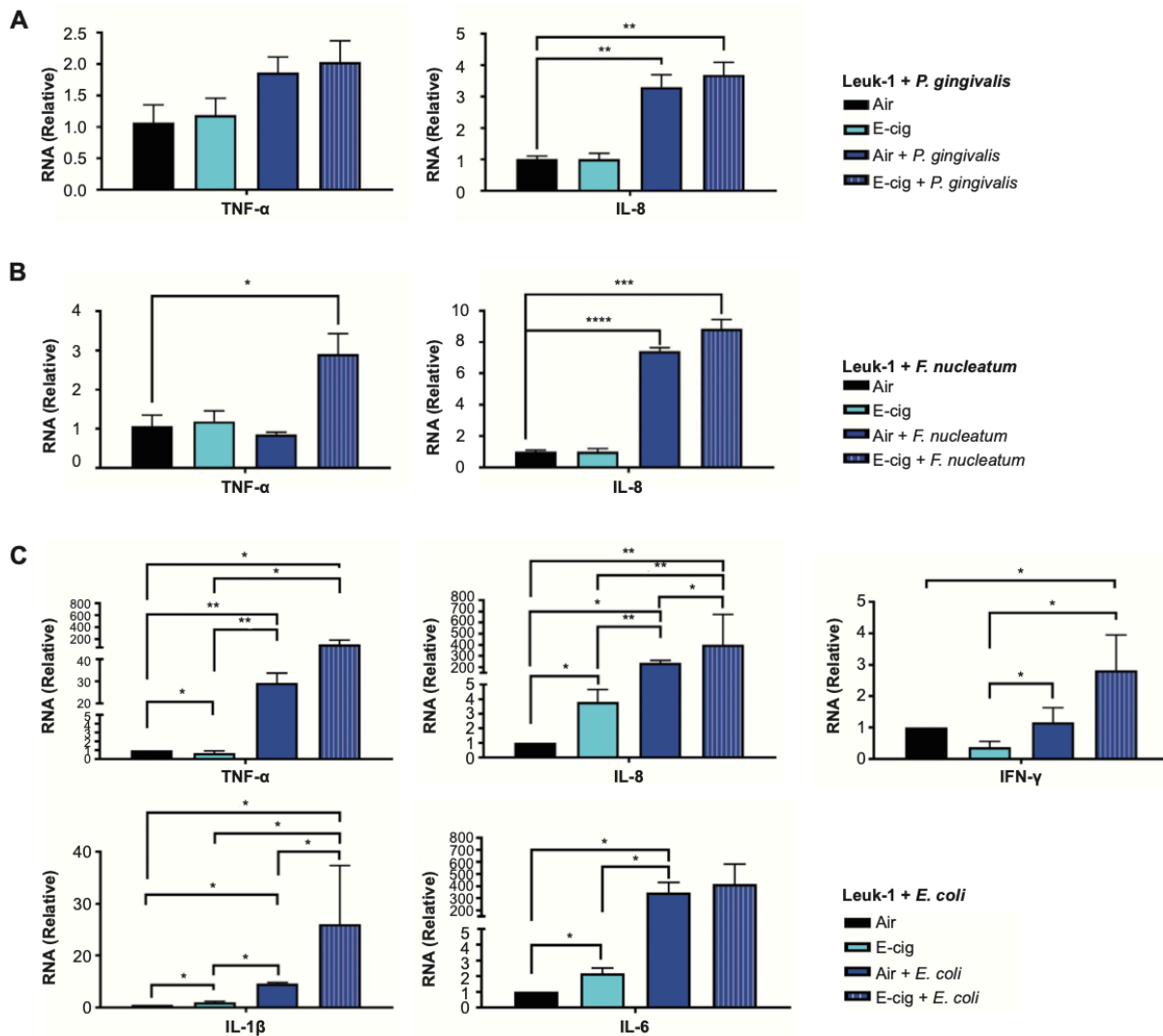
Figure S3

Figure S3. Related to Figure 5: mRNA expression levels of various cytokines, TNF-α, IL-8, IFN-γ, IL-1β and IL-6 in Leuk-1 cells in presence of bacteria and e-cigarette aerosols as determined by qPCR. Significant increase was observed in **(A)** IL-8 with *P. gingivalis,* **(B)** TNF-α and IL-8 with *F. nucleatum,* and **(C)** TNF-α, IL-8, IFN-γ, IL-1β and IL-6 with *E. coli* GFP. Cells exposed to only air or e-cigarette aerosol were used as controls. Data are represented as mean +/- SEM. (*p<0.05, **p<0.01, ***p<0.001).
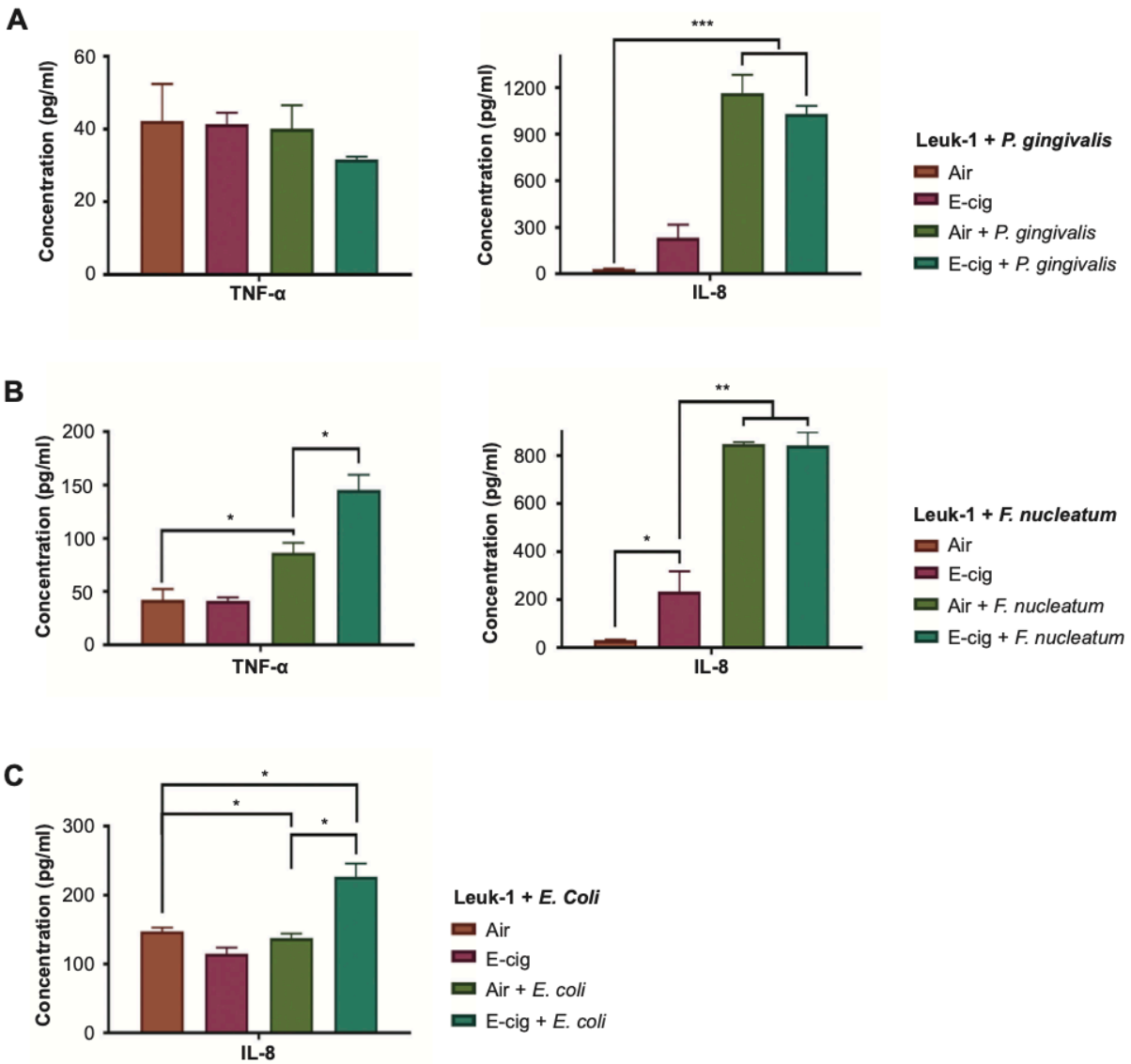
Figure S4

Figure S4. Related to Figure 6: Protein cytokines concentration, TNF-α and IL-8 in bacteria-treated Leuk-1 cell-free medium upon e-cigarette aerosol exposure as quantified by Elisa assay. Cytokine proteins were up-regulated by e-cigarette aerosol exposure: (**A**) IL-8 only with *P. gingivalis* (**B**) TNF-α and IL-8 with *F. nucleatum*, and (**C**) IL-8 only with *E. coli* GFP. Data are represented as mean +/- SEM. (*p<0.05, **p<0.01, ***p<0.001).
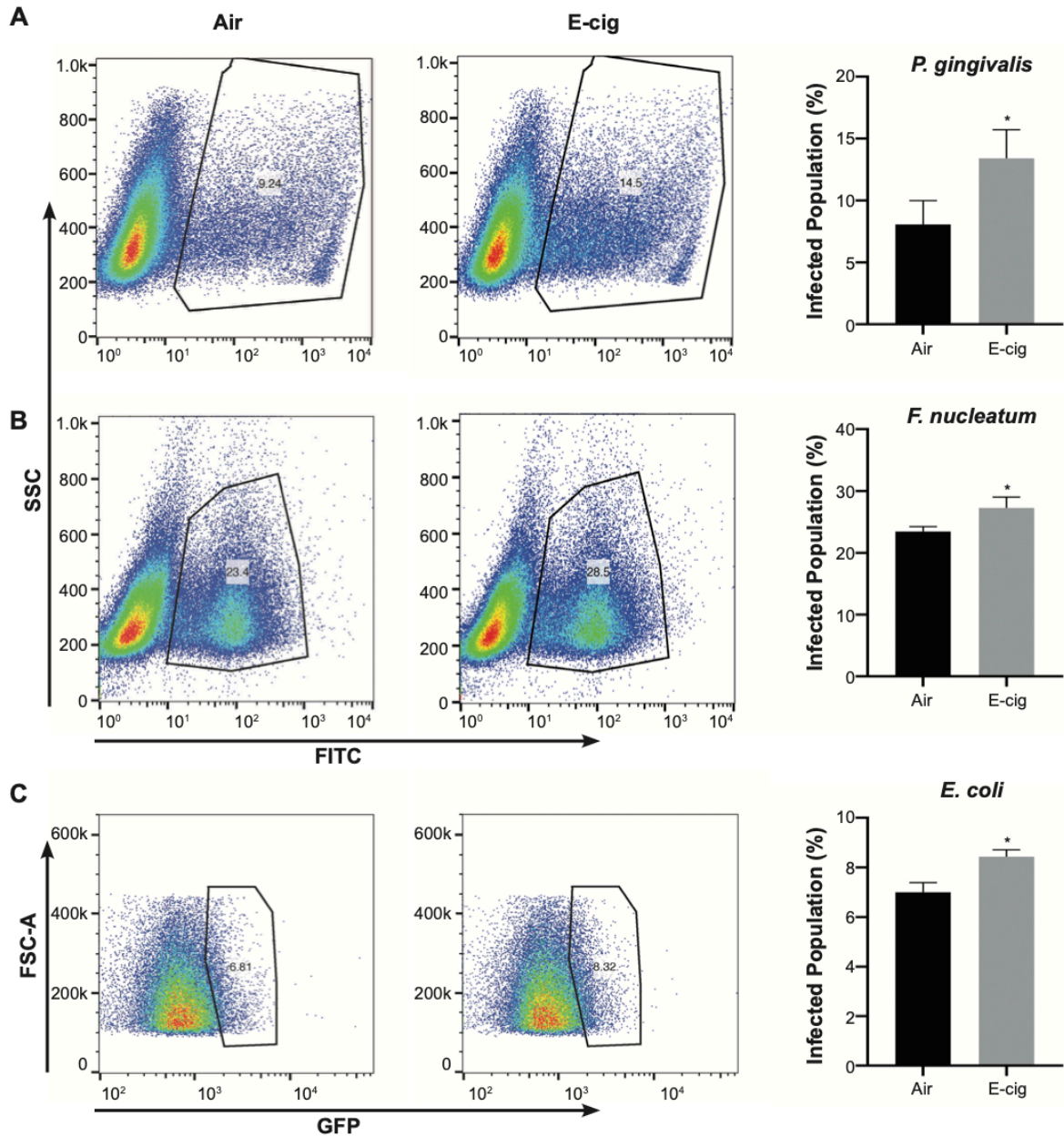
Figure S5

Figure S5. Related to Figure 5 and Figure 6: Flow cytometry plots exhibiting enhanced infection efficiency in Fadu cells after exposure to e-cigarette aerosol in presence of **(A)** *P. gingivalis* **(B)** *F. nucleatum*, and **(C)** *E.coli* GFP, compared to air. Graphical data are represented as mean +/- SEM. (*p<0.05, **p<0.01, ***p<0.001).