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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 x 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 $2\text{-}\Delta\Delta C_T$ 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 (~5×10^7) 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

SUPPLEMENTAL FIGURES

Figure S1

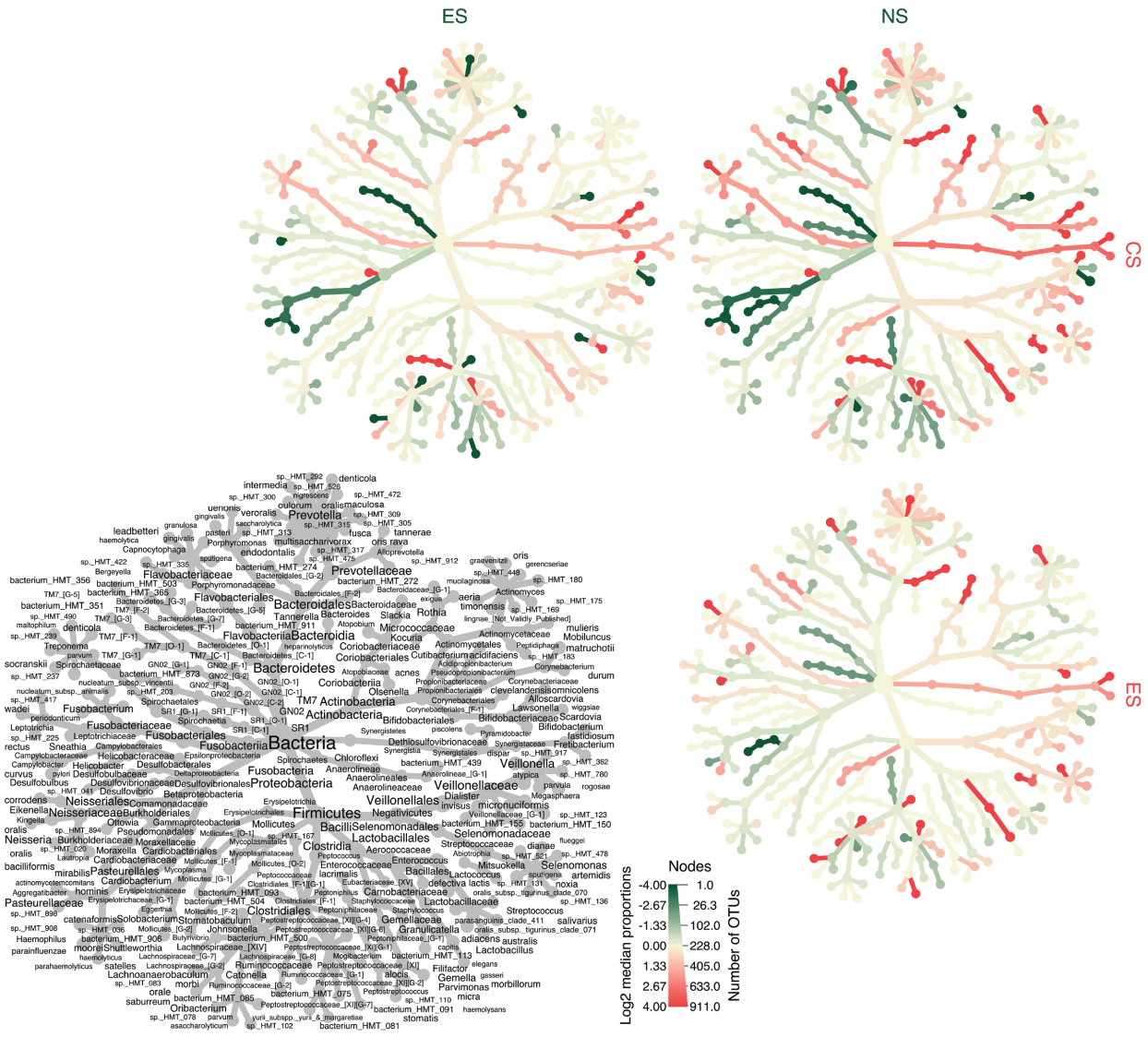

Figure S1. Related to Figure 3: Heat tree illustrates the relationship of species-specific OTUs in NS, ES and CS cohorts. Colored branch of the tree denotes significance based on the color of individual cohorts.

Figure S2

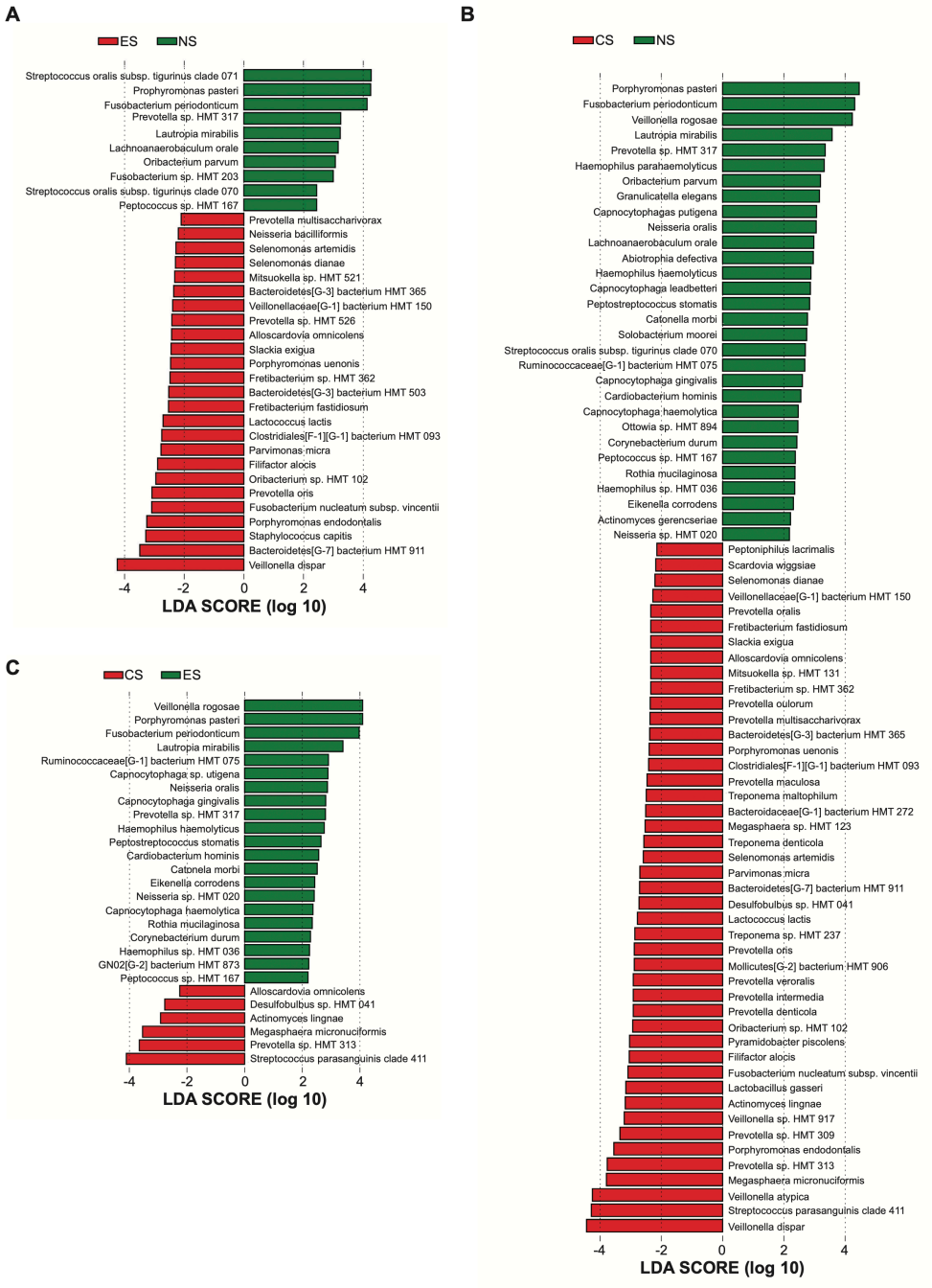

Figure S2. Related to Figure 3: Linear discriminant analysis (LDA) at species level combined with effect size measurements (LEfSe) revealed differentially altered taxa in the saliva samples of **(A)** NS and ES; **(B)** NS and CS; and, **(C)** ES and CS cohorts. $p<0.05$ and score ≥ 2.0 were considered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

Figure S3

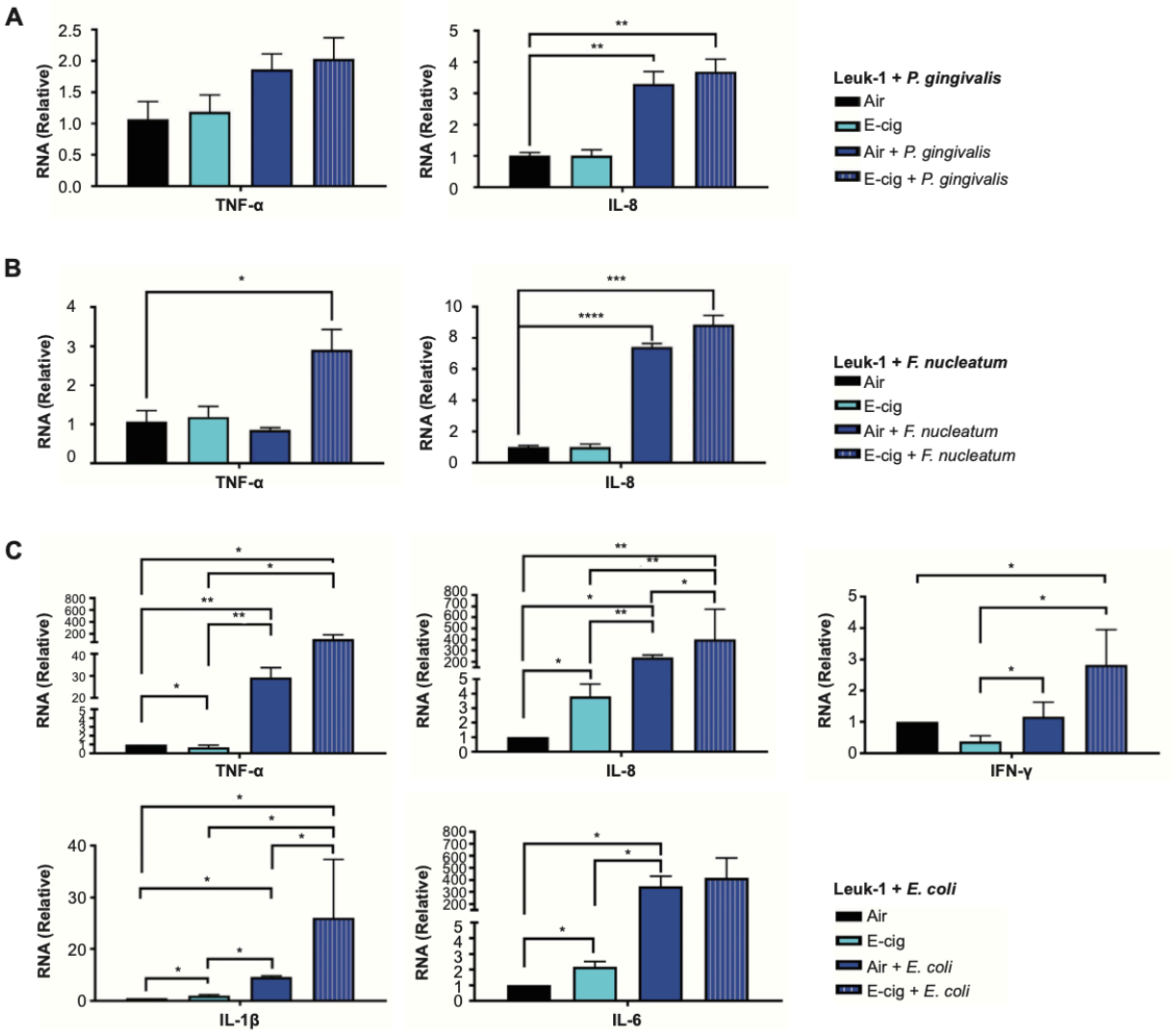

Figure S3. Related to Figure 5: mRNA expression levels of various cytokines, TNF-α, IL-8, IFN-γ, IL-1β and IL-6 in Leuk-1 cells in presence of bacteria and e-cigarette aerosols as determined by qPCR. Significant increase was observed in **(A)** IL-8 with *P. gingivalis,* **(B)** TNF-α and IL-8 with *F. 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

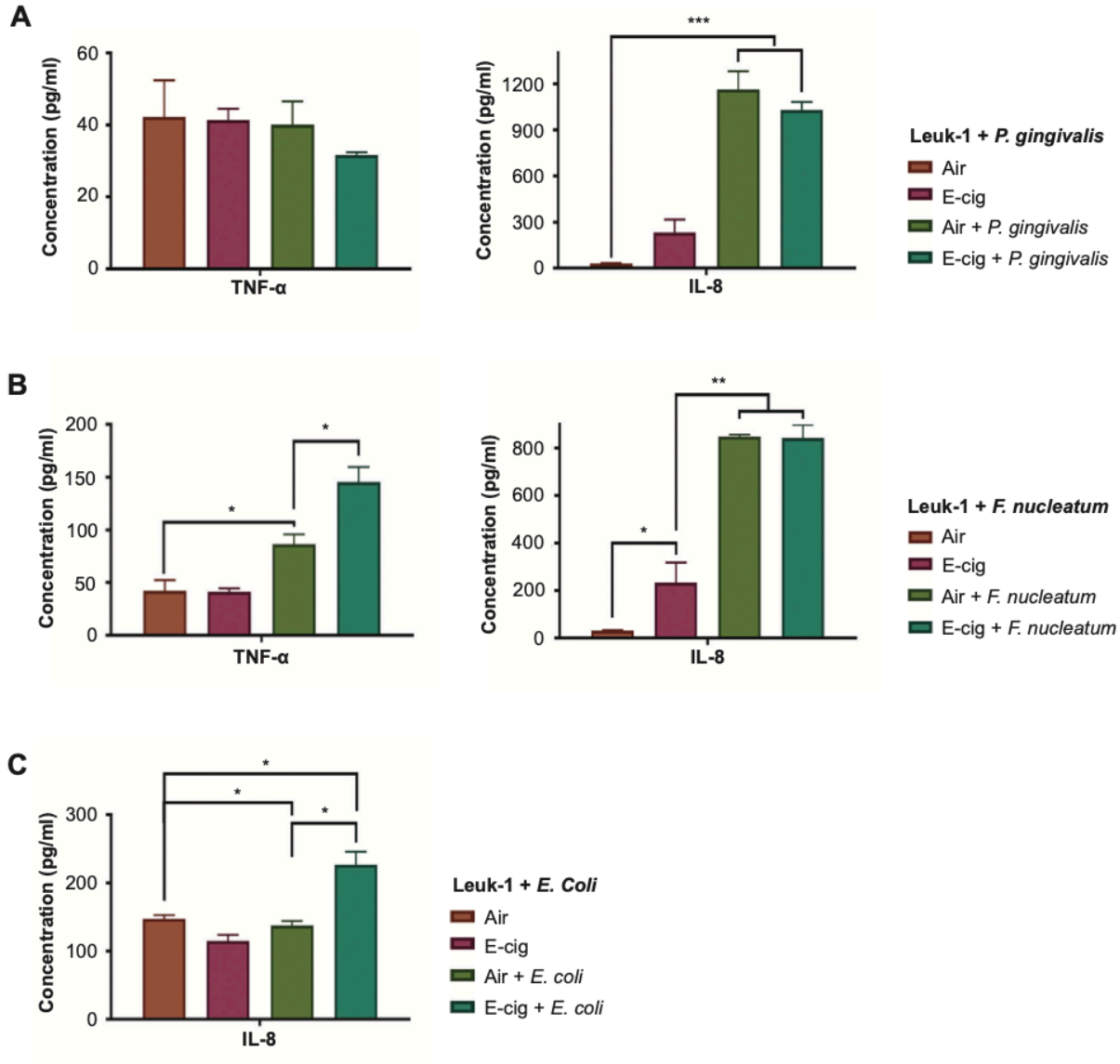

Figure S4. Related to Figure 6: Protein cytokines concentration, TNF-α and IL-8 in bacteria-treated Leuk-1 cell-free medium upon e-cigarette aerosol exposure as quantified by Elisa assay. Cytokine proteins were up-regulated by e-cigarette aerosol exposure: (**A**) IL-8 only with *P. gingivalis* **(B)** 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

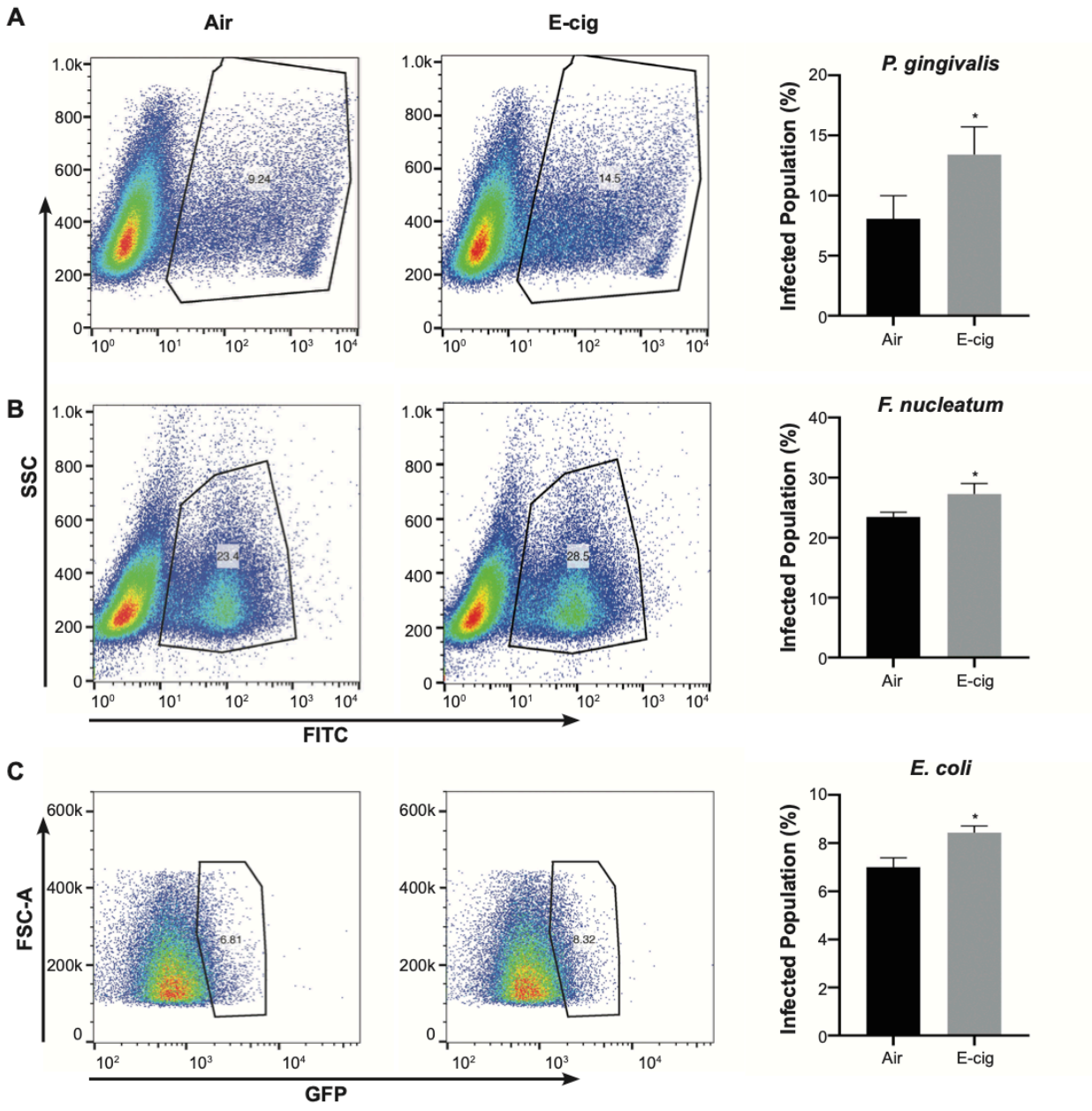

Figure S5. Related to Figure 5 and Figure 6: Flow cytometry plots exhibiting enhanced infection efficiency in Fadu cells after exposure to e-cigarette aerosol in presence of **(A)** *P. gingivalis* **(B)** *F. nucleatum*, and **(C)** *E.coli* GFP, compared to air. Graphical data are represented as mean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