Thesis

Effects of tobacco smoke and electronic cigarette aerosol exposure on the oral microbiota

submitted by

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in partial fulfillment of the requirements for the degree of

Doktorin der gesamten Heilkunde

(Dr.^{in} med. univ.)

at the

Medical University of Graz

executed at the

D&R Institute of Hygiene, Microbiology and Environmental Medicine

under the supervision of

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Graz, 7th June 2024

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University‘s regulations and procedures concerning plagiarism.

Graz, 7th June 2024 Olga Nyezhentseva m.p.

Acknowledgments

I would like to extend my sincere thanks to those who have supported me and made the thesis possible.

First of all, I would like to express my deepest gratitude to my supervisors, Univ.-Prof. Ivo Steinmetz and Ass.-Prof. Karl Dichtl, for their generously provided knowledge, expertise, thoughtful commennts and recommendations. I feel incredibly lucky to have been able to work under their guidance.

Besides, I am also thankful to my family and friends for being a source of strength and inspiration. Their relentless support and profound belief in my abilities have helped alleviate challenges during completing my research.

Zusammenfassung

Einleitung

Elektronische Zigaretten gelten weithin als sicherere und gesündere Alternative im Vergleich zu traditionellen verbrennbaren Tabakzigaretten. Sie werden häufig als Hilfsmittel zur Raucherentwöhnung eingesetzt und haben insbesondere bei jüngeren Menschen an Popularität gewonnen.

Um den potenziellen Einfluss der Verwendung von elektronischen Zigaretten auf die Mundgesundheit zu untersuchen, führten wir eine Überprüfung der vorhandenen Literatur durch. Unser Ziel war es, die Mundmikrobiome von Tabakrauchern und elektronischen Zigarettenbenutzer*innen mit denen von Nichtraucher*innen zu vergleichen.

Die Mundhöhle beherbergt eine vielfältige Mikroorganismenpopulation, die jeweils spezifische Nischen besetzt. Dieses symbiotische Mikrobiom spielt eine entscheidende Rolle bei der Abwehr externer Krankheitserreger, wodurch die Stabilität des Ökosystems erhalten und die normale Entwicklung von Mundgeweben und des Immunsystems unterstützt wird. Die anfänglichen Besiedler der Mundoberflächen bestehen hauptsächlich aus fakultativ anaeroben Bakterien, darunter verschiedene Arten von *Streptokokken* und *Actinomyces*. In der subgingivalen Region, wo der Sauerstoffgehalt niedriger ist, kommt es zu einer Verschiebung der Bevölkerungszusammensetzung, wobei eine Zunahme der Prävalenz strikter Anaerobier, insbesondere solcher, die zur Familie *Bacteroidaceae* gehören, zu beobachten ist.

Methodik

Entsprechend der Zielsetzung wurde ein Scoping Review erstellt. Die Literaturrecherche wurde in der MEDLINE-Datenbank der National Library of Medicine unter Verwendung von PubMed nach relevanten Artikeln mit Hilfe von Schlüsselwörtern wie "electronic cigarette", "electronic nicotine delivery system", "vaper", "tobacco smoke", "oral microbiota" und ähnlichen Varianten durchgeführt. Anhand definierter Einschlusskriterien wurden 43 Studien kritisch bewertet und einbezogen.

Ergebnisse

Die Exposition gegenüber Tabakrauch und elektronischem Zigarettenaerosol führt zu Veränderungen im oralen Mikrobiom. Der Einfluss der Exposition gegenüber elektronischem Zigarettenaerosol auf die Entwicklung einer oralen Dysbiose wurde umfassend nachgewiesen. Darüber hinaus ist die mikrobielle Reichhaltigkeit und Vielfalt

des oralen Mikrobioms sowohl bei Benutzern elektronischer Zigaretten als auch bei Tabakraucher*innen im Vergleich zu Nichtraucher*innen deutlich höher.

Bei Tabakraucher*innen dominieren die Hauptphyla *Proteobacteria, Bacillota (Firmicutes), Bacteroidetes* und *Fusobacteria*, wobei die wichtigsten Gattungen wie *Neisseria*, *Streptococcus, Prevotella, Fusobacterium* und *Porphyromonas* vorherrschen. Im Gegensatz dazu werden bei Nichtraucher*innen die Hauptphyla *Actinobacteria, Proteobacteria, Bacillota (Firmicutes), Bacteroidetes* und *Fusobacteria* dominant, wobei vorherrschende Gattungen wie *Corynebacterium, Neisseria, Streptococcus, Actinomyces* auftreten. Die Hauptgattungen *Neisseria, Prevotellaceae, Campylobacter* in der Gruppe der elektronischen Zigarettenraucher*innen wiesen höhere relative Häufigkeiten im Vergleich zu denen der Tabakraucher*innengruppe auf, während Taxa wie *Porphyromonas, Fusobacterium, Veillonella* und *Haemophilus* in den Mundhöhlen von Tabakraucher*innen erhöht waren.

Schlussfolgerung

Zusammengenommen können wir aus den Daten der gesichteten Literatur die Schlussfolgerung ziehen, dass das Rauchen sowohl von brennbaren Zigaretten als auch von elektronischen Zigaretten die orale Dysbiose beschleunigt, was die Entwicklung von Erkrankungen der Mundhöhle begünstigt.

Abstract

Background

Electronic cigarettes are widely perceived as a safer and healthier option compared to traditional combustible tobacco cigarettes. They are commonly used as aids for smoking cessation and have gained popularity, particularly among younger individuals.

To explore the potential impact of electronic cigarette use on oral health, we conducted a review of the existing literature. Our aim was to compare the oral microbiomes of tobacco smokers and electronic cigarette users with those of non-smokers.

The oral cavity harbors a diverse array of microorganisms, each occupying specific niches. This symbiotic microbiota plays a crucial role in defending against external pathogens, thus maintaining the stability of the ecosystem and supporting the normal development of oral tissues and the immune system. Initial colonizers of oral surfaces primarily consist of facultative anaerobic bacteria, including various species of *Streptococci* and *Actinomyces*. In the subgingival region, where oxygen levels are lower, there is a shift in population composition, with an increase in the prevalence of strict anaerobes, particularly those belonging to the *Bacteroidaceae spp*.

Methods

A scoping review was conducted to fulfill the objectives of this study. A systematic literature search was performed in the MEDLINE database of the National Library of Medicine using PubMed. Relevant articles were identified using keywords such as “electronic cigarette,” “electronic nicotine delivery system,” “vaper,” "tobacco smoke," and "oral microbiota." Inclusion criteria were predefined, resulting in the inclusion of 43 studies in this review.

Results

Exposure to both tobacco smoke and electronic cigarette aerosol leads to alterations in the oral microbiome. The impact of electronic cigarette aerosol exposure on the progression of oral dysbiosis has been extensively demonstrated. Furthermore, the microbial richness and diversity of the oral microbiome are notably higher in both electronic cigarette users and tobacco cigarette smokers compared to non-smokers.

In tobacco cigarette smokers, the predominant phyla include *Proteobacteria, Bacillota (Firmicutes), Bacteroidetes*, and *Fusobacteria,* with major genera such as *Neisseria, Streptococcus, Prevotella, Fusobacterium*, and *Porphyromonas* prevailing. Conversely, in non-smokers, *Actinobacteria*, *Proteobacteria, Bacillota (Firmicutes),*

Bacteroidetes, and *Fusobacteria* become the dominant phyla, with prevalent genera including *Corynebacterium, Neisseria, Streptococcus, Actinomyces*. The main genera *Neisseria*, *Prevotellaceae*, and *Campylobacter* in the group of electronic cigarette smokers showed higher relative abundances compared to those in the group of tobacco cigarette smokers. However, taxa such as *Porphyromonas, Fusobacterium, Veillonella*, and *Haemophilus* were increased in the oral cavities of tobacco cigarette users.

Conclusion

Taken together the data of the reviewed literature, it can be concluded that smoking combustible cigarettes as well as electronic cigarettes leads to oral dysbiosis, which might facilitate progression of oral cavity diseases.

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List of Abbreviations

ABC transporters	ATP-binding cassette transporters
ANOVA	analysis of variance
ANOSIM	analysis of similarities
CS	Cigarette smokers
CFU	Colony Forming Unit
db-RDA	Distance-based redundancy analysis
DESeq2 analysis	A differential expression analysis of RNA-Seq data
EC	Electronic cigarette
EPS	extracellular polymeric substance
GFP	Green fluorescent protein
HOMD	Human Oral Microbiome Database
HGs	Hunter-gatherers
LDA	Linear discriminant analysis
Methanogens	Methane-producing organisms
NS	Never smokers
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PubMed	Public Medical Literature Online
rRNA	ribonucleic acid
SVG	Single-virus genomics
SCFA	Short chain fatty acids
TFs	Traditional farmers
WCs	Western controls
WEIRD	Western educated industrialized rich and democratic
TM7	*Candidatus Saccharibacteria oral taxon TM7x*

Glossary
Cigarette smokers
Class
Colonization
Current smokers
Electronic cigarette aerosol
Electronic nicotine delivery system
Family
Genus
Human oral stomatotype
Microbial abundances
Microbial niches
Never smokers
Oral cavity
Oral microbiome
Order
Pathogenic species
Phylum
Species
Tobacco cigarette smoke
Vaping
Vaper

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1. Introduction

1.1 Electronic cigarettes as a new challenge for human health

The urgent need for comprehensive comparisons between the effects of electronic cigarette aerosol and tobacco cigarette smoke on the human oral microbiota arises from the escalating use of electronic cigarettes among youth globally and the ever-evolving landscape of vaping products and promotional strategies employed by the tobacco industry [1, 2]. The design of visually appealing cigarette packaging tailored to specific demographic groups reflects a calculated approach to shaping consumer perceptions and behaviors. Furthermore, electronic nicotine delivery systems have been marketed with a perceived healthy “halo”, a tactic crucial in promoting these products. The strategic use of visually striking colors and names, known to resonate with younger consumers, remains prevalent in marketing campaigns [3]. Thus, it is crucial to address and deter the consumption of nicotine delivery devices in future to safeguard public health.

1.2 Electronic cigarettes as smoking cessation tools

The term "smokeless tobacco" encompasses a spectrum of products, including electronic nicotine delivery systems, or electronic cigarettes. Initially positioned as aids for smoking cessation and promoted as safer alternatives to traditional cigarettes, electronic cigarettes are non-combustible devices engineered for nicotine delivery.

The concept of the electronic cigarette traces back to a patent secured by Herbert A. Gilbert in 1963. However, the modern electronic cigarette as we know it today was invented by the Chinese pharmacist Hon Lik in 2003, and it was subsequently introduced to the market the following year. Available in various formats mimicking traditional cigarettes, pens or USB flash drives, electronic cigarettes function by vaporizing an electronic liquid (e-liquid) mixture containing nicotine, flavorings, propylene glycol, vegetable glycerin, and other additives. The resulting aerosol is then inhaled by the user. International export of electronic cigarettes commenced in 2005-2006, with the first global patent granted in 2007 [4].

Since their introduction to the American market in 2007, electronic cigarettes have experienced exponential growth, becoming a multi-billion-dollar industry [5].

Systematic reviews and meta-analyses indicate lifetime prevalence rates of electronic cigarette vaping at 24% in America, 26% in Europe, 16% in Asia, and 25% in Oceania, with current prevalence rates standing at 10% in America, 14% in Europe, 11% in Asia, and 6% in Oceania [6].

The surge in electronic cigarette use among young individuals can be attributed to the availability of enticing flavors and the proliferation of discreet electronic cigarette models designed to resemble USB flash drives. Marketing strategies continue to capitalize on visually appealing colors and names, which subtly convey sensory or health-related attributes, appealing particularly to younger demographics [7]. Moreover, studies highlight the influence of product packaging alterations on consumers' perceptions of healthfulness and harm, irrespective of any changes to the physical product itself.

1.3 The human oral microbiome in health

1.3.1 Role of the oral microflora in human population

The human body and its microbiome coexist as a cohesive superorganism, developed through millions of years of coevolution, featuring mutual adaptation and functional synergy, resulting in substantial advantages for both entities. The evolutionary process has engendered a highly diverse oral microbiome, comprising over 700 species of microorganisms [8, 9]. Within a health-promoting community, niche saturation plays a pivotal role, wherein select species dominate, impeding the invasion of pathogenic organisms and preserving mucosal well-being [10, 11]. Commensal microflora in the oral cavity forms bacterial biofilms, acting as barriers against the attachment of harmful microorganisms to mucosal surfaces. Numerous studies have explored the microbiomes of diverse oral sites, including saliva, tongue, buccal mucosa, dental surfaces, gums, palate, subgingival and supragingival plaque, as well as the pharynx and tonsils[12, 13, 14]. While general similarities are observed across these sites, minor distinctions exist; for instance, both types of plaque exhibit elevated levels of the genus *Corynebacterium* [12, 13, 14], whereas saliva and buccal mucosa show increased levels of the phylum *Bacillota (Firmicutes)* compared to plaque [8, 15]. Despite extensive research, the extended Human Oral Microbiome Database (HOMD) reveals that only 57% of oral bacterial species have official names, 13% have been cultured but remain unnamed, and 30% remain uncultivated [16]. The oral cavity harbors several distinct niches, each shaped by variations in oxygen

levels, nutrient availability, and saliva's pH-modulating properties, fostering the proliferation of diverse organisms and influencing both local and systemic functions [17, 18].

Commensal bacteria employ protective mechanisms, including the production of bacteriocins, peptides with antimicrobial properties, to selectively target competing bacteria, thereby restraining their growth [19].

1.3.2 Factors determining the composition of the oral microbiome

Saliva production rate is a crucial determinant of oral microbiome composition, regulating pH levels and nutrient accessibility. Additionally, surface type and oxygen levels significantly influence microbial makeup [20]. For instance, non-shedding tooth surfaces develop two types of plaque, each generating distinct biofilms due to differences in oxygen availability: obligate anaerobic organisms predominate in subgingival plaque, while facultative anaerobic organisms prevail in supragingival plaque [21].

Recent studies have unveiled variations in the oral microbiome across global regions, with a focus on "WEIRD" (Western, educated, industrialized, rich, and democratic) nations and Chinese populations [22, 23]. Moreover, within specific populations, diverse microbiome composition patterns are evident among generally healthy individuals [10, 24]. Oral rinse samples offer insights into how external factors shape the oral microbiome; variations in drinking water composition and dietary patterns directly impact oral health. A study involving 1,319 samples from healthy adolescents in Spain revealed correlations between water ionic makeup and oral microbiome structure [10]. Samples from regions with elevated alkalinity and sulfate (SO4) and sodium (Na) levels exhibited higher quantities of genera such as *Porphyromonas* and *Flavobacterium,* while those from areas with lower ion levels showed elevated levels of *Veillonella, Pseudomonas*, and *Ralstonia*. Additionally, dietary patterns, such as those observed in hunter-gatherer (HGs) and traditional farmer (TFs) populations in the Philippines, influence microbiome composition, with HG samples exhibiting greater alpha-diversity compared to Western controls (WCs) [10, 25]. Similarly, there was a notable gradient in the prevalence of the core oral genera *Neisseria* and *Haemophilus*: HG samples showed elevated levels of *Neisseria* and diminished levels of *Haemophilus*, whereas the opposite trend was observed in WC samples [25, 26]. TF samples again displayed intermediary levels between the two extremes [10, 26]. Despite demonstrating good oral health, samples from HG exhibited elevated levels of several

species commonly identified as oral pathogens associated with gingivitis and periodontitis according to Western standards [25, 26]. Functional assessments revealed a heightened presence of vitamin B5 biosynthesis pathways in HG samples, with a lesser extent observed in TF samples [26]. Given the higher consumption of vitamin B5-rich foods among Americans, the authors posit that its absence in hunter-gatherer diets may result in the selection of organisms capable of independently synthesizing it [25, 26]. Conversely, the study found that WC samples, and to a lesser extent, TF samples, showed higher levels of urease activity, primarily attributed to *Haemophilus spp*. Urease aids in mitigating pH drops caused by bacterial breakdown of sugars into acidic compounds, thus making its prevalence in WC samples, characterized by sugar- and starch-rich diets, plausible [25, 26]. The authors suggest that organisms typically considered oral pathogens in Western societies may actually constitute part of the healthy microbiomes of diverse populations such as hunter-gatherer communities [26]. Furthermore, they propose that pathogenic strains of these organisms could be selected based on dietary nutrient availability [26]. While food and water play significant roles, numerous other factors may influence the microbiome. It is conceivable that a limited number of symbiotic equilibrium states exist between a human host and its microbiome, arising from diverse diets and lifestyles [10, 25, 26].

1.3.3 Human oral stomatotype

Certain research findings suggest the potential existence of balanced symbiotic states between a human host and their oral microbiome, referred to as "stomatotypes" in one study, reminiscent of the term "enterotype" but specific to the oral cavity [27, 28].

The health-associated stomatotype encompasses diverse taxa combinations [27]. Recent studies identified at least two compositional patterns: one characterized by elevated levels of *Proteobacteria* genera *Neisseria* and *Haemophilus*, and the other exhibiting higher abundances of *Bacteroidetes* genus *Prevotella* and *Firmicutes* genus *Veillonella* [10].
Table 1 outlines bacterial genera associated with different stomatotypes.

Table 1 *Consensus stomatotype-driving genera. Stomatotype numbers have been assigned randomly. The second column lists the genera, along with relevant notes on associations between the organisms. The third column provides literature references that support these stomatotype associations.*

Stomatotype	**Genus**	**Bibliography**

Stomatotype 1	*Neisseria* *Haemophilus*	[Willis JR [15]; Zaura E [29]; De Filippis F, Vannini L [30]; Takeshita T, Kageyama S [31]
Stomatotype 2	*Prevotella* *Veillonella*	[Zaura E [29]; De Filippis F, Vannini L [30]; Takeshita T, Kageyama S [31]
Variable Stomatotypes	*Streptococcus* —varies depending on study and species	[Willis JR [15]; Zaura E [29]; De Filippis F, Vannini L [30]; Takeshita T, Kageyama S [31]
	Gemella — co-occurs with *Streptococcus* and *Porphyromonas*	[De Filippis F, Vannini L [30]; Takeshita T, Kageyama S [31]]
	Porphyromonas— may co-occur with *Streptococcus*, *Gemella*, or *Neisseria*	[De Filippis F, Vannini L [30]; Takeshita T, Kageyama S [31]]
	Rothia — co-occurs with varying species of *Streptococcus*, depending on study	[De Filippis F, Vannini L [30]; Takeshita T, Kageyama S [31]]

Since the microbiomes of healthy individuals from various unique populations worldwide remain unexplored, our understanding of the potential equilibria of microbial abundances leading to specific stomatotypes remains incomplete.

A random subset of 500 samples from an oral microbiome dataset [10] and clustered them into two stomatotypes using the weighted Unifrac distance measure [15] were selected (Figure 1). Type 1 samples, depicted by circles, demonstrate a strong association with *Neisseria* and *Haemophilus*, while Type 2 samples, depicted by squares, show a strong association with *Prevotella* and a weaker association with *Veillonella* [11, 15].

In this case, the factors influencing the "variable stomatotype" exhibit diverse associations. *Streptococcus* exhibits a distinct gradient but doesn't align with either stomatotype, along with *Gemella* and *Rothia*, which typically co-occur with *Streptococcus* but show variable associations here. *Rothia* exhibits a stronger association with stomatotype

1. However, *Porphyromonas*, previously identified to co-occur with *Streptococcus, Gemella* or *Neisseria*, does not demonstrate such associations here but is strongly linked with stomatotype 2 [15].

Interestingly, less dominant taxa exhibit high personalization in the oral cavity; while *Streptococcus* predominates most habitats, *Haemophilus* is abundant in the buccal mucosa, *Actinomyces* prevails in supragingival plaque, and *Prevotella* thrives in immediately adjacent (low oxygen) subgingival plaque [11].

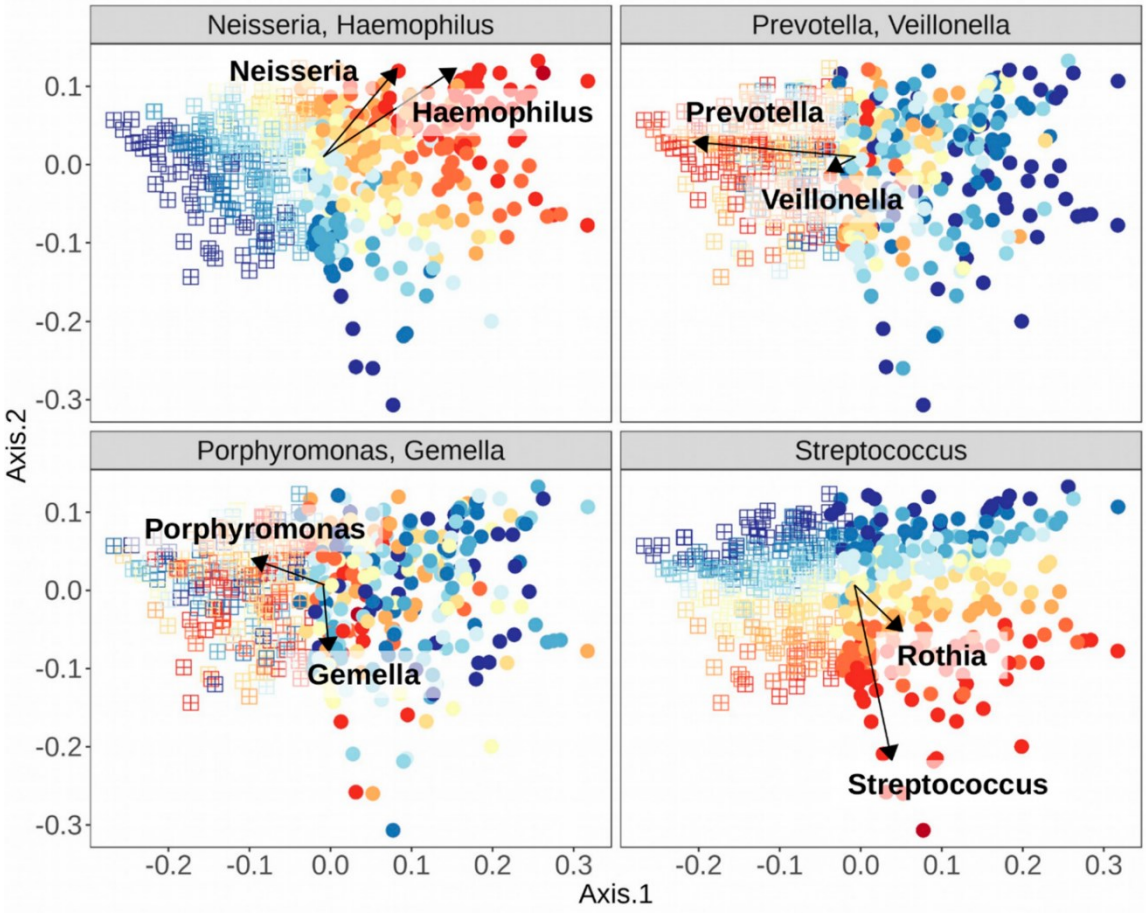

Figure 1 *Variations in the abundance gradients of key genera shaping the consensus stomatotype [10]*

While numerous studies and reviews have focused on the results of respiratory and cardiovascular systems, additional data is required to comprehend the impacts of electronic cigarettes on the oral microbiome and elucidate the differences in oral community profiles between vapers and conventional cigarette smokers. The primary focus of this thesis is to identify and review existing literature to address a specific question: what are the differences between the effects of tobacco smoke and electronic cigarette vapor on the oral microbiome?

2. Material and Methods

2.1 Literature research

For conducting the literature review, we developed a search strategy tailored to identify relevant studies addressing the research question. Subsequently, we systematically searched and analyzed the literature sources.

2.2 Search strategy

An exhaustive literature search was undertaken within the MEDLINE database of the National Library of Medicine using PubMed as the search engine. The search terms employed included “electronic cigarette”, “electronic nicotine delivery system”, “vaper”, "tobacco smoke", "oral microbiota", and similar variations.

Initially, the search yielded over 76 papers meeting the specified criteria. However, articles solely focusing on policies, regulations, or other unrelated topics were excluded. This refinement resulted in a selection of 43 scientific research articles specifically addressing the effects of smoking on the oral microbiota. These articles were further categorized into three thematic sections. The first set of references encompassed articles elucidating the role of the human oral microbiome in health and disease. The second section (18 articles) pertained to the health implications of electronic cigarette usage, while the third section (25 articles) focused on the impact of tobacco cigarette consumption on human health.

Each article underwent screening to determine its relevance to our review, with a particular emphasis on oral microbiota to gain insights into its implications for human health and the effects of tobacco and electronic cigarette smoking on oral microbiome. Considering the recent surge in research interest regarding the health effects of electronic cigarettes and advancements in methodological approaches, the majority of cited articles were published between 2018 and 2023.

3. Results

3.1 Effects of tobacco smoking on oral microbiota

Apart from inducing anaerobic, acidic, or selectively toxic conditions, smoking significantly affects human immunity, thereby influencing the host's ability to resist colonization by pathogens [32]. The chemotactic mobility and phagocytic function of oral polymorphonuclear leukocytes are diminished in smokers, which are crucial for host defense against pathogens, thus fostering a more conducive environment for oral pathogens and elevating the risk of diseases like periodontitis [33, 34, 35].

The reduction in specific xenobiotic biodegradation pathways in smokers suggests significant functional deficiencies that may have health implications. Oral bacteria, being the initial contact point with cigarette smoke upon entry into the body, might play a key role in breaking down associated toxic compounds. Studies have revealed a depletion of degradation pathways for substances like toluene, nitrotoluene, styrene, chlorocyclohexane, and chlorobenzene in smokers, potentially leading to adverse health effects due to alterations in the oral bacterial community [36].

Long-term oral health relies on maintaining a stable, health-compatible microbiome, with any imbalance leading to dysbiosis, triggering exaggerated inflammatory responses and the development of various diseases [37]. Cigarette smoking represents a well-established anthropogenic risk factor for periodontal disease, impacting the function and proliferation of various periodontal cells (including gingival fibroblasts, periodontal membrane cells, and periodontal ligament cells), hindering autoimmune defenses, and exacerbating inflammatory reactions, ultimately causing damage to the alveolar bone and increasing the risk of oral cancer [38, 39, 40].

Smoking alters the relative proportions of microbiome members, disrupting the microbial ecology of the mouth through various mechanisms such as antibiotic effects and oxygen deprivation [41]. It significantly impacts colonization within different microbial niches of the oral microbiome and affects bacterial composition at all stages of plaque development [42, 43, 44]. Smokers exhibit earlier and more extensive colonization by pathogenic species, weaker correlations between marginal and subgingival ecosystems, and heightened pro-inflammatory responses in cases of periodontitis, contributing to the development and persistence of the disease [45]. Additionally, smokers experience diminished capacity for the subgingival microbiome to recover following episodes of

disease, reducing the resilience of the ecosystem and its ability to resist future disease [43, 44].

An extensive evaluation of oral microbiome community composition and individual taxon abundance in smokers revealed significant differences in microbiome composition based on smoking status, with distinct alterations observed in various taxa across different smoking statuses [36].

The overall microbiome composition varied depending on smoking status (never, former, and current), as demonstrated by principal coordinate analysis based on UniFrac phylogenetic distances [36]. Significant differences were observed in the abundance of *Proteobacteria* across smoking statuses, with a notable depletion in current smokers compared to never smokers. Apart from *Proteobacteria*, the relative abundance of the phylum *Actinobacteria* was higher in current smokers compared to never smokers[36]. *Betaproteobacteria* and *Gammaproteobacteria* were notably lower in current smokers, as were several genera including *Neisseria, Haemophilus*, and *Aggregatibacte*r[36]. Additionally, several taxa not belonging to *Proteobacteria*, such as the class *Flavobacteriia* and genera *Capnocytophaga*, exhibited significantly lower abundance in smokers [36]. At the same time, such genera, in class-level analysis, as *Corynebacterium* (*Actinobacteria*), *Porphyromonas* and *Prevotella* (*Bacteroidetes*), *Leptotrichia* (*Fusobacteria*), and *Peptostreptococcus*, *Abiotrophia* and *Selenomonas* (*Bacillota)*) were not altered [36].

In current smokers compared to never smokers, there was a rise in the abundance of genera like *Atopobium (Coriobacteriia), Bifidobacterium (Actinobacteria), Lactobacillus*, and *Streptococcus (Bacilli).* The phylum *Bacillota (Firmicutes)* was notably more prevalent in current smokers, encompassing 42 operational taxonomic units (OTUs) within *Actinobacteria* (typically enriched in current smokers), 95 OTUs from Bacilli (mostly from the Streptococcus genus, generally enriched in current smokers), 32 OTUs from Clostridia (typically depleted in current smokers), 25 OTUs from Proteobacteria (generally depleted in current smokers), and 27 unclassified OTUs. In contrast, only 17 OTUs were distinguished as differentially abundant between former and never smokers [46]. Microbiota functionality, inferred through metagenomes using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm, follows a two-step process [46]. Firstly, in the "gene content inference" step, gene content is computed for each organism in a reference phylogenetic tree, constructing a table of predicted gene family abundances for each organism in the 16S-based phylogeny. Secondly, in the “metagenome inference” step,

the resulting gene content predictions for all microbial taxa are combined with the relative abundance of 16S rRNA genes in one or more microbial community samples, adjusted for expected 16S rRNA gene copy number, thereby generating the anticipated abundances of gene families in the entire community [46].

Remarkably, paths belonged to aerobic metabolism, like the citric acid cycle and electron transport-linked phosphorylation, were found to be diminished in current smokers, while pathways independent of oxygen, such as glycolysis, fructose metabolism, galactose metabolism, sucrose metabolism, and photosynthesis, were enhanced in current smokers [36]. Furthermore, the copiousness of xenobiotic biodegradation paths were notably changed in current smokers, with certain reactions showing enrichment (such as polycyclic aromatic hydrocarbon degradation, xylene degradation, and drug metabolism) and others showing depletion (such as styrene degradation, toluene degradation, nitrotoluene degradation, chlorocyclohexane degradation, and chlorobenzene degradation) compared to never smokers[36]. Bacterial genera that displayed alterations in current smokers were linked to many of these paths (Figure 3).

At the phylum level, there was a notable depletion of Proteobacteria and an enrichment of *Bacillota (Firmicutes)* and *Actinobacteria* in current smokers compared to never smokers[36]. These substantial differences at the phylum level were attributed to variations in Operational Taxonomic Unit (OTU) abundance across entire phyla between current and never smokers[36]. Further analysis of inferred metagenomes suggested that smoking could potentially affect oral microbial ecology by influencing oral oxygen availability, while simultaneously impacting microbial degradation of xenobiotics.

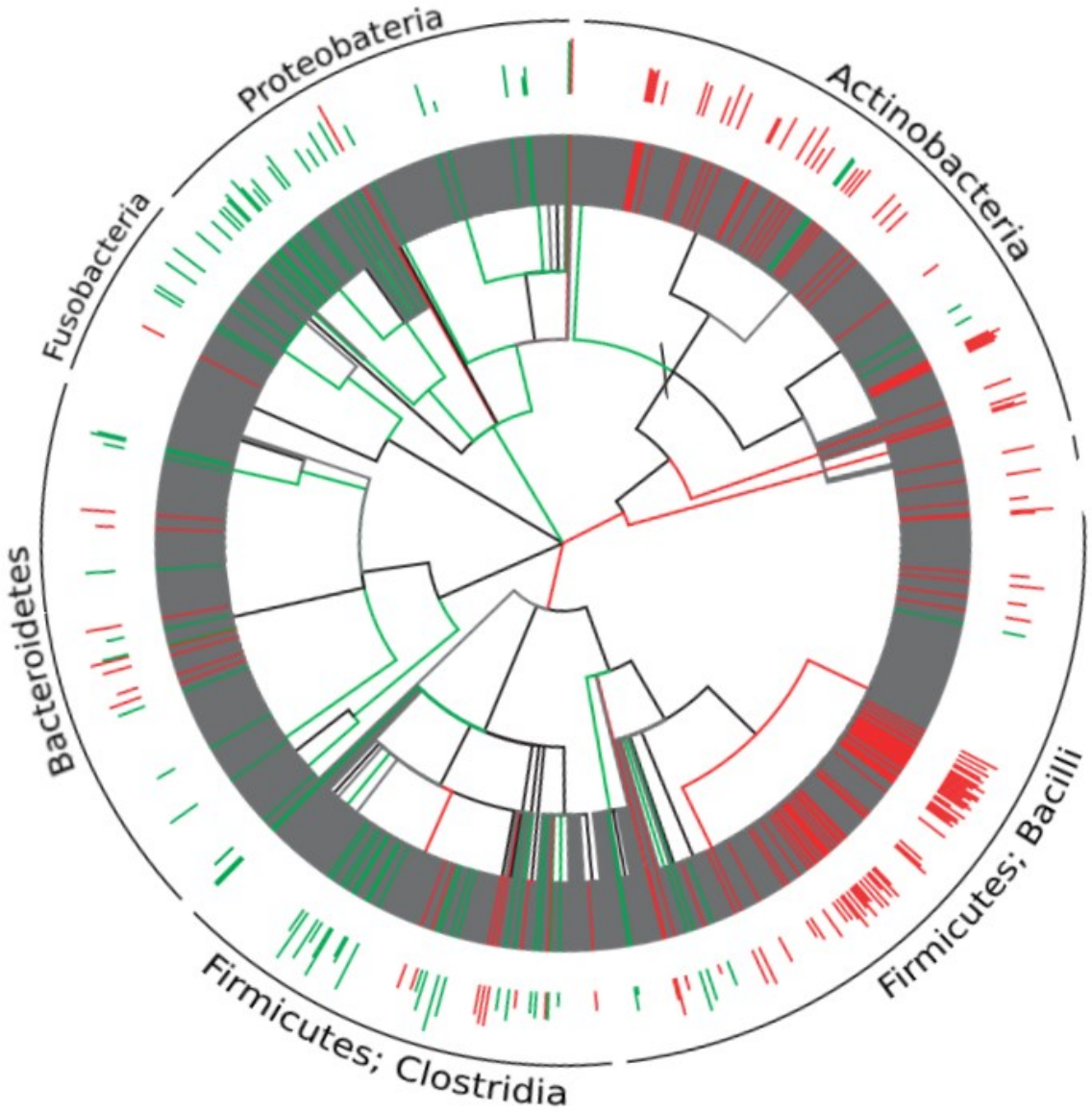

Figure 2 *A visual representation using a cladogram displays oral microbiome Operational Taxonomic Units (OTUs) linked with smoking status. In this representation, red branches signify taxa or OTUs more abundant in current smokers, while green branches indicate those less abundant. The bars on the graph depict log2 fold changes in counts between current and never smokers, with red bars indicating a positive change and green bars indicating a negative change, as identified through DESeq2 analysis [36]*

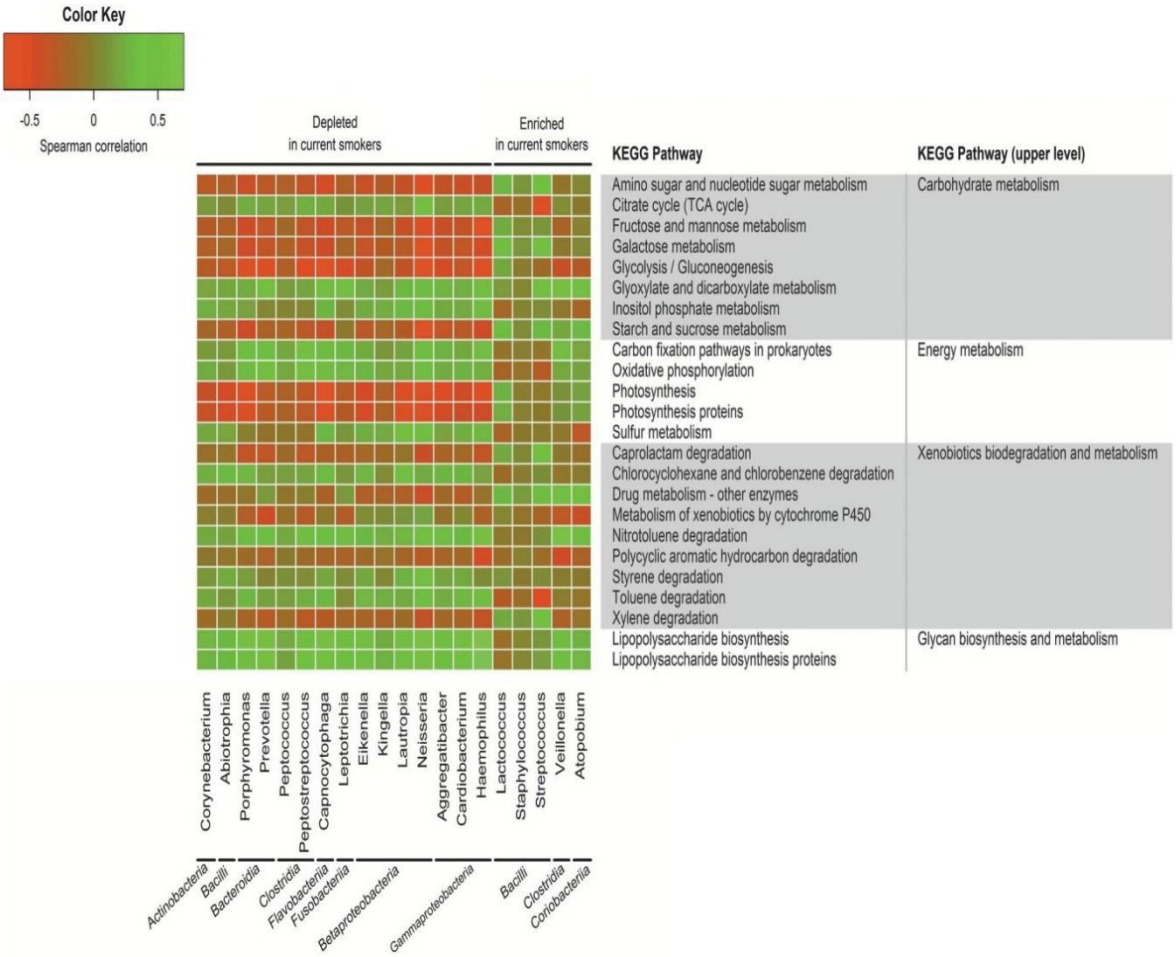

Figure 3 *Bacterial taxa linked with smoking status are associated with multiple gene functional pathways [36]*

In smokers, there is a noticeable increase in anaerobic bacteria, such as those found within the *Veillonella* genus and *Actinobacteria* OTUs originating from anaerobic *Actinomyces spp., Rothia mucilaginosa, Bifidobacterium longum*, and *Atopobium spp. Conversely*, there is a reduction in the abundance of aerobes like *Neisseria subflava* and *Corynebacterium* [44]. This finding corresponds with the oxygen deprivation hypothesis proposed by Mason et al., who observed higher levels of anaerobic bacteria and lower levels of aerobic bacteria in subgingival plaque samples from smokers compared to non-smokers [44]. Interestingly, certain anaerobic OTUs, including *Leptotrichia spp., Veillonella parvula,* and *Peptostreptococcus spp.,* exhibit depletion in smokers, possibly due to specific antibiotic toxicants in cigarette smoke or indirect effects such as

competition for colonization with smoking-enriched bacteria or co-aggregation with smoking-depleted bacteria [44]. However, since these studies were predominantly observational, further investigation is necessary to elucidate the precise mechanisms behind these intriguing findings.

Several reported observations align with the progression toward a diseased state: *Neisseria* and *Eikenella* are found in lower abundance in oral mucosa from periodontitis patients, while *Streptococcus* species are more prevalent in oral sites transitioning toward periodontal disease compared to healthy oral sites [33-35, 47].

3.2 Comparative characterization of some oral species exposed to electronic cigarette aerosol and tobacco smoke (*in vitro* studies)

Moving on to *in vitro* studies, a laboratory-based electronic cigarette aerosol-exposure model was utilized to investigate the impact of vaping on the oral microbiome. This study's data contribute to understanding how vaping affects the oral microbiome by comparing groups influenced by tobacco smoke with control groups. Six species - *Streptococcus oralis* (ATCC 35037), *Streptococcus sanguinis* (10556), *Streptococcus mitis* (49456), *Actinomyces naeslundii* (12104), *Neisseria mucosa* (25997), and *Veillonella parvula* (17745) - were seeded onto sintered hydroxyapatite disks and incubated under aerobic conditions in a 1:1 (v/v) mixture of SHI medium and artificial saliva [37]. The SHI medium, originally developed to support supragingival bacterial communities, can maintain in vitro biofilms representative of a diverse inoculum with high reproducibility, capturing 60-80% of the taxonomic abundance from the original community [48]. Pathogen-rich biofilms consisted of *Fusobacterium nucleatum* (10953), *Porphyromonas gingivalis* (33277), *Filifactor alocis* (35896), *Selenomonas sputigena* (35185), *Selenomonas noxia* (43541), *Campylobacter gracilis* (33236), *Prevotella intermedia* (25611), *Parvimonas micra* (33270), and *Tannerella forsythia* (43037) and were incubated under anaerobic conditions [37].

Cigarette smoke extract was prepared by passing the smoke from two cigarettes through 5 ml of artificial saliva, while electronic cigarette vapor was created by vaping two cartridges of nicotine-free or 6 mg nicotine electronic cigarettes into 5 ml of artificial saliva until depletion [37]. Following this, 1% solutions of cigarette smoke extract and electronic

cigarette vapor were applied to the biofilms for 24 hours, after which total RNA was isolated. Enriched mRNA served as a template for polyadenylation and complementary DNA synthesis [37]. Microbial libraries were then sequenced on the Illumina HiSeq 4000 platform using 150-bp paired-end sequencing [37]. Raw fluorescence images were processed using the Illumina base-calling pipeline. A bacterial gene catalog from the subgingival microbiome of twenty healthy vapers consisted of 9730 functionally annotated microbial genes [37]. In the microbiome influenced by electronic cigarettes, carbohydrate metabolism emerged as the predominant functionality, these genes account for 12 to 36% of gene abundance in each individual. Within this pathway, genes encoding kinases (glycerol, glycerate, and fructose), lysine and butyrate fermentation, and ATP-binding cassette (ABC) transporters were found to be highly abundant [37]. Protein and amino acid metabolism were also significant pathways, collectively representing 3 to 12% of each individual's metagenome [37]. Genes involved in biofilm formation, including cell wall and capsule synthesis, peptidoglycan, membrane transport, and flagella synthesis, exhibited a relative abundance of 4 to 7% across all 20 individuals [37].

Network analysis unveiled a densely interconnected topology characterized by multiple tightly clustered hubs [37]. The most significant hub, comprising 203 species from genera like Actinomyces, Capnocytophaga, Filifactor, Fusobacterium, Treponema, Tannerella, Prevotella, Selenomonas, and Streptococcus, constituted a core microbial community[37]. This hub ranked prominently in both betweenness centrality and node degree, suggesting their potential role in resource and information flow within the ecosystem (Figure 4). Overall, both phylogenetically and functionally, the oral microbiome of vapers demonstrated remarkable homogeneity, indicating that electronic cigarettes might impose a selective pressure on the subgingival ecosystem [37].

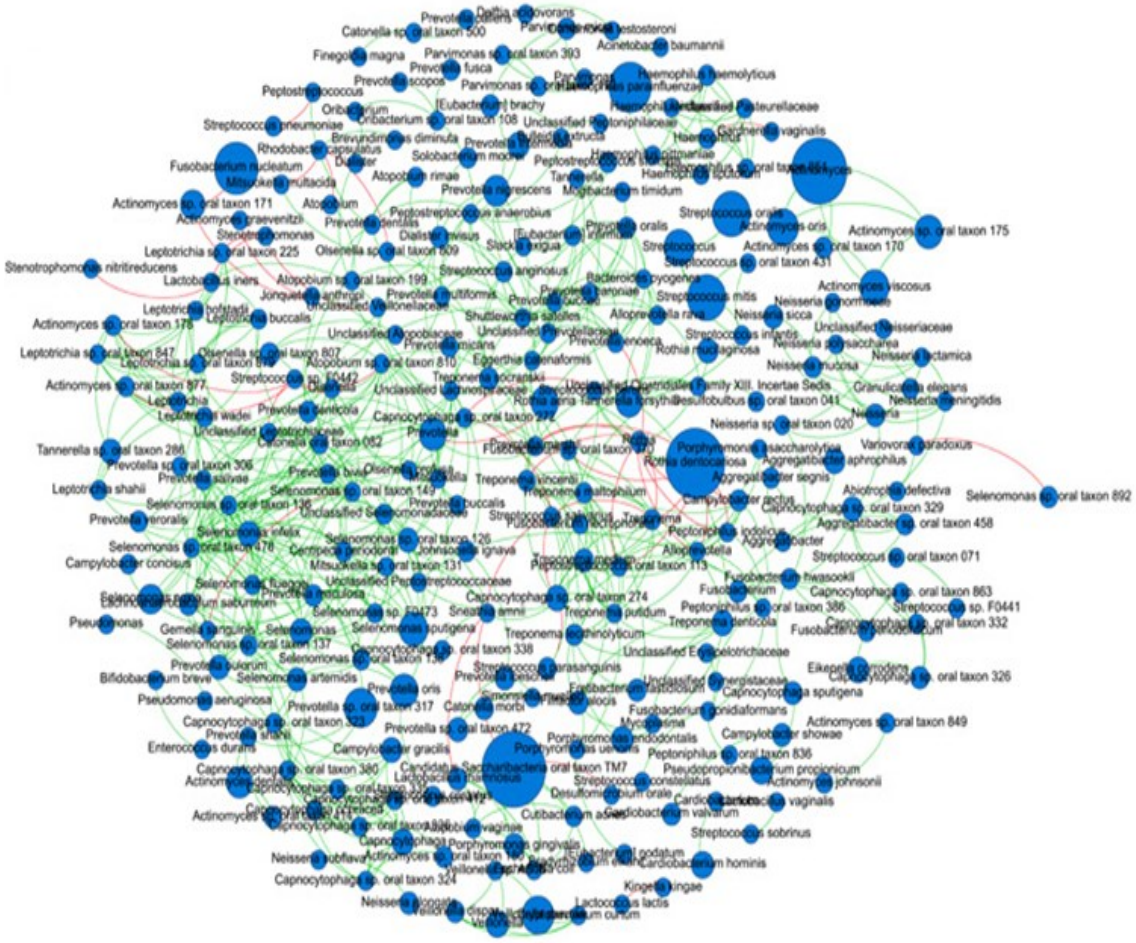

Figure 4 *The functional traits of the subgingival microbiome among electronic cigarette users are depicted through network graphs. Each graph includes nodes, represented by circles sized according to proportional abundance for each group, with edges depicted as lines. Nodes signify microbial genes within species-level Operational Taxonomic Units (OTUs), while edges represent Spearman's rho correlations. Green edges denote positive correlations, whereas red edges signify negative correlations. Only edges showing a correlation coefficient (Spearman's rho) of ≥0.80 and a significance level of $p<0.05$ are depicted [37]*

Oral commensal bacteria, including streptococci such as *Streptococcus gordonii*, *Streptococcus intermedius, Streptococcus mitis*, and *Streptococcus oralis*, are vital in forming oral biofilms on both soft and hard surfaces within the mouth [49]. A study examining the effects of flavorless electronic cigarette aerosol and conventional cigarette smoke on the survival and growth of these streptococci revealed a significant toxic effect from cigarette smoke, whereas the flavorless electronic cigarette aerosol showed a much lower toxic effect on these bacteria (Figures 5A, 5B) [49]. Without exposure to either substance (0 puffs), the number of colony-forming units (CFUs) per agar plate ranged from 37 to 63 for *Streptococcus gordonii*, 25 to 42 for Streptococcus intermedius, 35 to 70 for

Streptococcus mitis, and 65 to 84 for *Streptococcus oralis* [49]. When exposed to flavorless electronic cigarette aerosol, with or without nicotine, the bacterial colonies were comparable to those exposed to air, although significant differences ($p < 0.01$) were observed between aerosols with and without nicotine for *Streptococcus gordonii* and *Streptococcus mitis* at 75 and 50 puffs, respectively (Figures 5A, 5B)[49]. In stark contrast, bacteria exposed to 50 or 75 puffs of cigarette smoke resulted in no colony formation [49]. All four species were capable of forming biofilms after exposure to air (control) and flavorless electronic cigarette aerosol with or without nicotine, but not after exposure to cigarette smoke[49]. Specifically, compared to air exposures (0 and 75 puffs), 75 puffs of flavorless electronic cigarette aerosol with or without nicotine allowed for biofilm formation and growth, regardless of the overall architecture of bacterial communities for all four species [49].

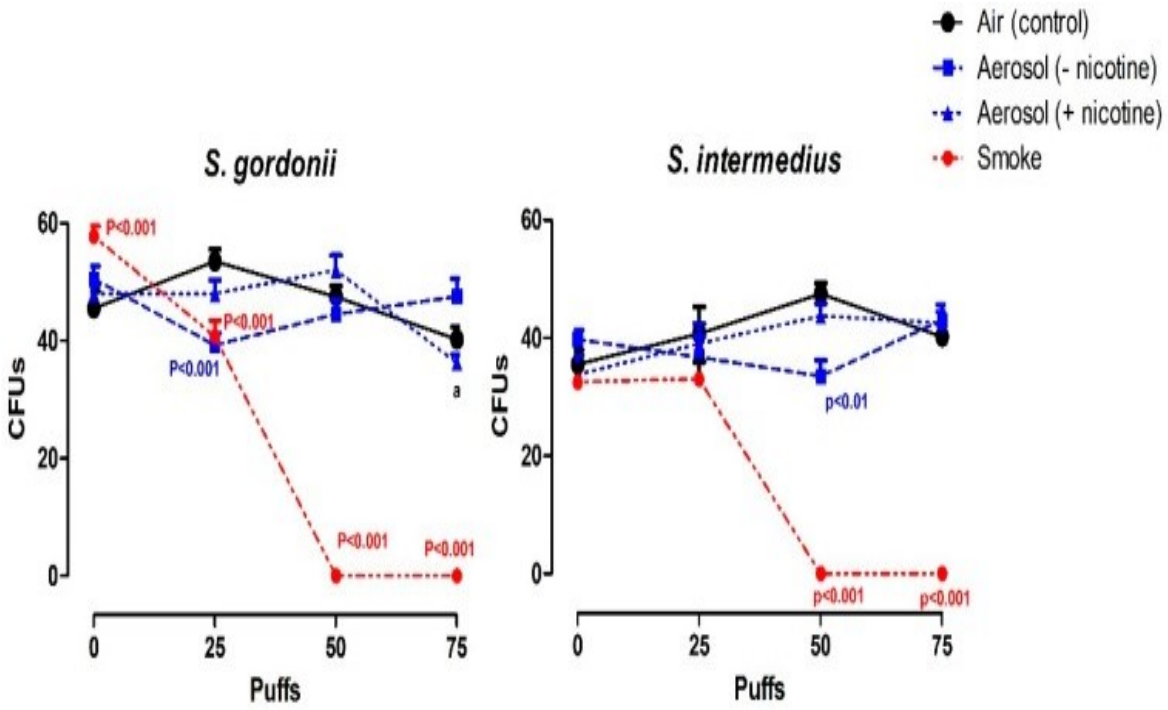

Figure 5 (A) *Effect of air, flavorless electronic cigarettes aerosol (±20 mg/mL nicotine), or smoke (Marlboro® Red cigarette) on CFU counts. Each data point represents the Mean ± standard error (SE), with a sample size (n) of 4, which is the average of triplicates obtained from each quadrant of an agar plate. The p values indicate the significance compared to the control group [49]*

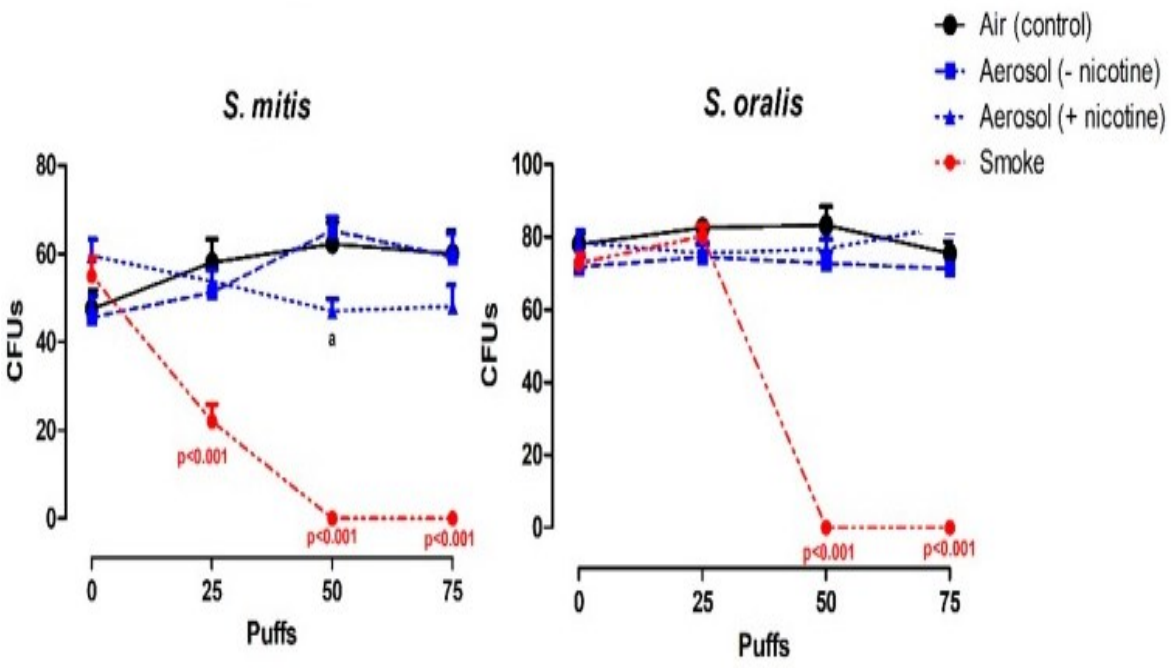

Figure 5 (B) *Effect of air, flavorless electronic cigarettes aerosol (±20 mg/mL nicotine), or smoke (Marlboro® Red cigarette) on CFU counts. Each data point represents the Mean ± standard error (SE), with a sample size (n) of 4, which is the average of triplicates obtained from each quadrant of an agar plate. The p values indicate the significance compared to the control group [49]*

3.3 Comparative analysis of oral microbiota in vapers, tobacco cigarette smokers and non-smokers: insights from clinical research

The study aimed to investigate the difference among the oral microbiomes of twenty vapers, twenty-five tobacco cigarette smokers, and twenty-five non-smokers (controls) [37]. From these 70 participants, one billion sequences were obtained. Multivariate analysis revealed statistically significant group separation between tobacco cigarette smokers, vapers, and non-smokers based on both functional and taxonomic profiles [37]. In comparison to both tobacco cigarette smokers and non-smokers, vapers showed enrichment of 284 genes ($p < 0.05$), associated with processes such as alanine and arginine biosynthesis, polyamine metabolism, central carbohydrate metabolism, one-carbon metabolism, mono-, di-, and oligosaccharide metabolism, fermentation, cell division, and cell cycle [37]. This suggests that while central metabolic processes are shared among all three groups, specific pathways contributing to these processes differ among them [37]. Additionally, the bacterial

metagenome of electronic cigarette users exhibited higher abundances of genes related to ATP-binding cassette transporters, RNA processing and modification systems, and various virulence factors compared to tobacco cigarette smokers and non-smokers [37]. These factors included those involved in cell wall and capsular polysaccharides, peptidoglycan and lipopolysaccharide biosynthesis, stress response, quorum sensing and biofilm formation, resistance to antibiotics and toxic compounds, flagellar motility, and siderophores (Figure 6) [37]. Notably, several studies have shown that biofilm formation increased in response to both cigarette smoke extract and electronic cigarette vapor extract across various species, including *H. influenzae, S. pneumoniae, P. aeruginosa, S. aureus, Streptococcus gordonii, Porphyromonas gingivalis*, and *Candida albicans*. This suggests a potential increase in bacterial adhesion to epithelial cells and subsequent biofilm formation. Furthermore, methicillin-resistant *Staphylococcus aureus* exposed to cigarette smoke extract exhibited increased hydrophobicity and altered surface charge, leading to enhanced adherence to epithelial cells and decreased susceptibility to antimicrobial peptides [49, 50].

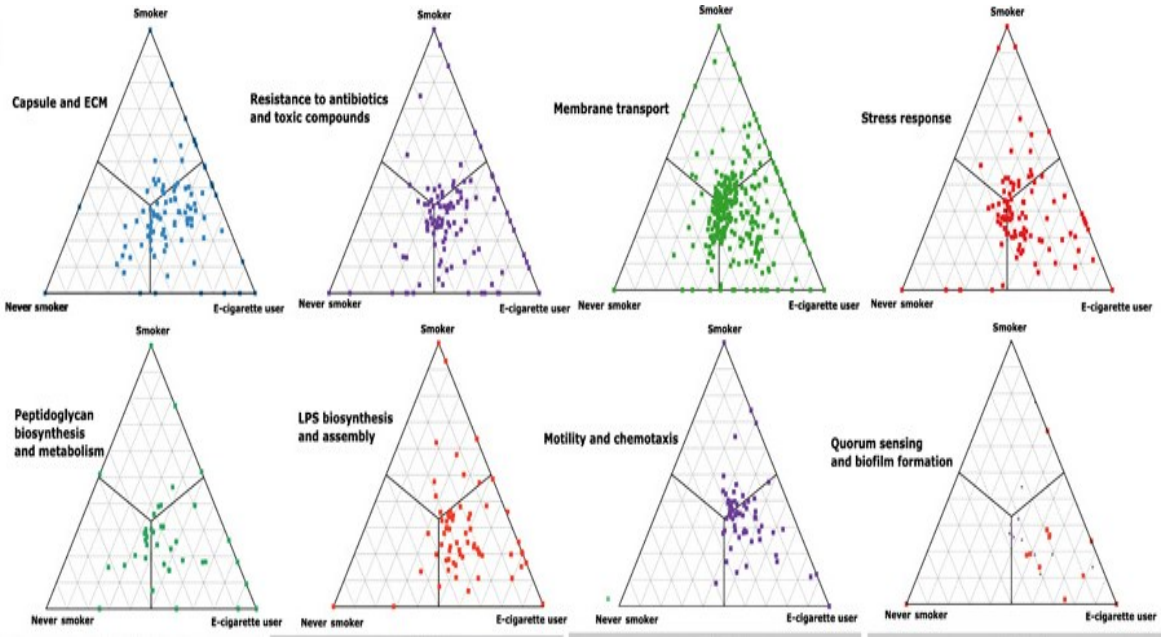

Figure 6 *Variations in both composition and roles of the microbial community are observed among electronic cigarette users (blue), tobacco smokers (red), and nonsmokers (green). Subjects' microbial profiles are grouped based on their exposure type, resulting in three statistically significant clusters [37]*

Electronic cigarette use led to higher levels of Gram-negative facultative anaerobes, while tobacco smoking favored Gram-negative anaerobes[37]. Notably, *Candidatus Saccharibacteria oral taxon TM7x* and species from various genera, including *Abiotrophia, Aggregatibacter, Eikenella, Granulicatella, Cardiobacterium, Hemophilus, Johnsenella, Kingella, Lachoanaerobaculum, Lautropia, Leptotrichia, Mogibacterium, Ottowia, Parvimonas, Peptostreptococcus, Rothia, Rhodobacter, Selenomonas,* and *Veillonella*, were significantly more abundant in vapers compared to both non-smokers and tobacco cigarette smokers [37]. A striking finding was that 70% of the metagenome in vapers was shared by over 80% of subjects, contrasting with only 40% and 50% sharing among tobacco cigarette smokers and non-smokers, respectively. Furthermore, the core microbiome of vapers encompassed 96% of species with significantly higher abundances compared to the other groups, suggesting a distinct impact of electronic cigarette aerosol on the oral microbiome compared to tobacco cigarette smoke [37]. The Random Forest Classifier achieved high accuracy in predicting vapers (90% sensitivity, 97% specificity), followed by controls (88% sensitivity, 92% specificity), and tobacco cigarette smokers (84% sensitivity, 95% specificity) [37]. Additionally, 49 genes associated with cell wall synthesis, peptidoglycan biosynthesis, and enzymes within the protein and amino acid metabolism pathway were identified as discriminators of the microbiome influenced by electronic cigarette aerosol [37].

Since their introduction, electronic cigarettes have been marketed as a strategy for reducing tobacco harm [37]. The microbial shift due to the partial or complete replacement of conventional cigarettes with electronic cigarettes was quantitatively assessed in a study involving 120 participants divided into five groups: smokers, non-smokers, electronic cigarette users, former smokers currently using electronic cigarettes, and dual users of both cigarettes and electronic cigarettes [37]. Dual users had been using electronic cigarettes for 6 to 12 months, while former smokers had quit smoking between 3 months to 1 year prior to the study. Functional and phylogenetic analyses showed no significant difference between former smokers and dual users (p values of 0.27 and 0.35, respectively, ANOSIM) [37]. Further pairwise comparisons revealed that, both phylogenetically and functionally, the microbiomes of dual users and former smokers were more similar to those of electronic cigarette users than to never smokers and smokers [37]. Additionally, the microbiomes of dual users and former smokers were significantly more similar to electronic cigarette users

than to never smokers and smokers, as indicated by both phylogenetic and functional analyses ($p < 0.00001$; Figure 7, A and B) [37].

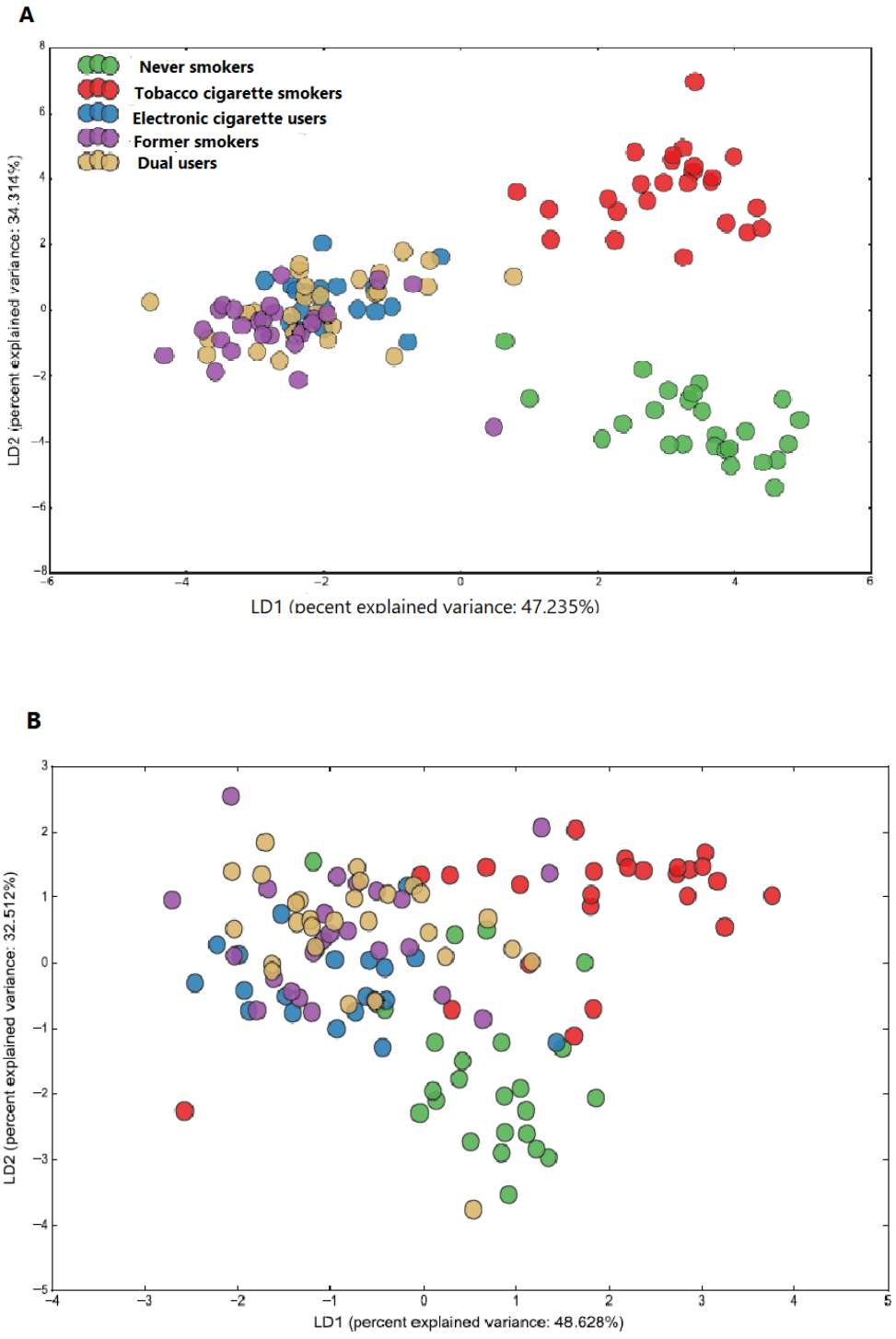

Figure 7 *The combined impact of smoking and electronic cigarettes on the microbiome is investigated. Linear discriminant analysis (LDA) of Bray-Curtis dissimilarity indices based on the relative abundances of species-level OTUs (A) and functional genes (B) is conducted among periodontally and systemically healthy individuals. Participants are categorized into*

groups based on their smoking habits: exclusive electronic cigarette users (blue), exclusive tobacco cigarette smokers (red), dual users (who use both tobacco cigarettes and electronic cigarettes simultaneously, shown in tan), former smokers who currently use electronic cigarettes (purple), and nonsmokers (green). The analysis reveals three statistically significant clusters, with dual smokers and former smokers aligning with exclusive electronic cigarette users (p=0.003, MANOVA/Wilks) [37]

To delve deeper investigate this phenomenon, a Random Forest machine learning classifier was utilized, trained on the microbiomes of electronic cigarette users and tobacco smokers, with dual users and former smokers serving as the validation se[37]t. The classifier categorized dual users as electronic cigarette users with 68% sensitivity and 72% specificity, and former smokers with 76% sensitivity and 83% specificity[37]. This indicates that any potential harm reduction from electronic cigarettes might not be influenced by changes in the microbiome.

The examination of interconnected relationships unveiled a dense hub comprising multiple species among electronic cigarette users, prompting an exploration into the metagenome for potential biological mechanisms. Particularly, the impact of electronic cigarette aerosol on biofilm formation was investigated by examining the metatranscriptome for relevant genes categorized in the KEGG classification [37]. Electronic cigarettes stimulated or upregulated two-component response systems, particularly histidine kinase sensors. Additionally, quorum sensing (via the LuxR system) was induced, along with the expression of genes encoding pellicle proteins [37].

In a different study, 119 participants were divided into three groups based on their smoking status: never smokers (NS) (n=39), electronic cigarette users (EC) (n=40), and regular cigarette smokers (CS) (n=40) [51]. The severity index of periodontal disease or infection was notably higher among CS (72.5%), followed by EC (42.5%) and NS (28.2%), as indicated by the average pocket depth in each group [51]. This was supported by bleeding on probing values, a marker of inflammation, which were elevated in CS and lower in both vapers and non-smokers [51]. The salivary microbiome of the participants was analyzed using high-quality filtered 16S sequences, revealing an oral microbiome consisting of 11 phyla, 22 classes, 33 orders, 55 families, 99 genera, 162 species, and 911 OTUs. The microbiome structure varied distinctly between cohorts, with differences observed between EC and CS compared to healthy NS. The five most abundant taxa were *Bacillota (Firmicutes), Proteobacteria, Bacteroidetes, Actinobacteria*, and *Fusobacteria,* accounting for 97.5% of the total sequences (Figure 8)[51]. *Proteobacteria* were most prevalent in the

EC group but significantly lower in the CS group ($p<0.0001$). *Actinobacteria* levels were significantly higher in both CS and EC compared to NS ($p<0.0001$ and $p<0.01$, respectively). On the other hand, *Bacillota* (*Firmicutes*) were highly enriched in the saliva of the CS group compared to EC ($p<0.01$) and NS ($p<0.0001$). *Fusobacteria* showed significant depletion in the CS group compared to EC ($p<0.024$) and NS ($p<0.001$). Additionally, *Spirochaetes* were found to proliferate in the CS group ($p<0.005$) [51].

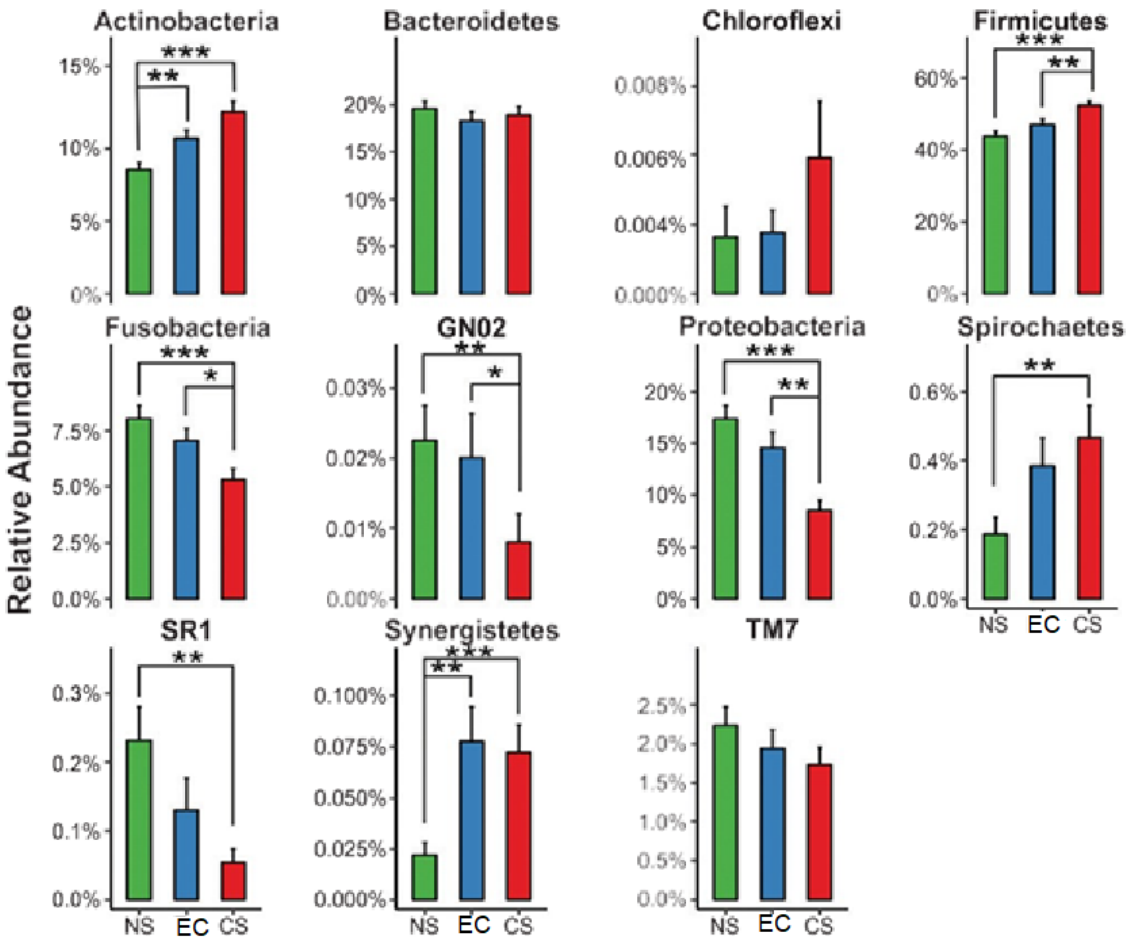

Figure 8 *The proportional distribution of salivary microbiota at the phylum level, inferred from taxonomic analysis of 16S rRNA sequences, are compared across different smoking statuses: nonsmokers (NS, green), electronic cigarette users (EC, blue), and conventional cigarette smokers (CS, red) cohorts. Significant alterations were observed in Actinobacteria, Bacillota (Firmicutes), Fusobacteria, Proteobacteria, and Spirochaetes. Data are presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001) [51]*

The microbial communities present in the saliva of the three groups was predominantly composed of eight taxa: *Streptococcus, Veillonella, Prevotella, Neisseria, Haemophilus, Porphyromonas, Rothia,* and *Fusobacterium*, collectively constituting 79.15% of all the

sequences (Figures 9 and 10)[51]. Additionally, other taxa such as *Leptotrichia, Gemella*, and *Capnocytophaga* were identified as differentially abundant.

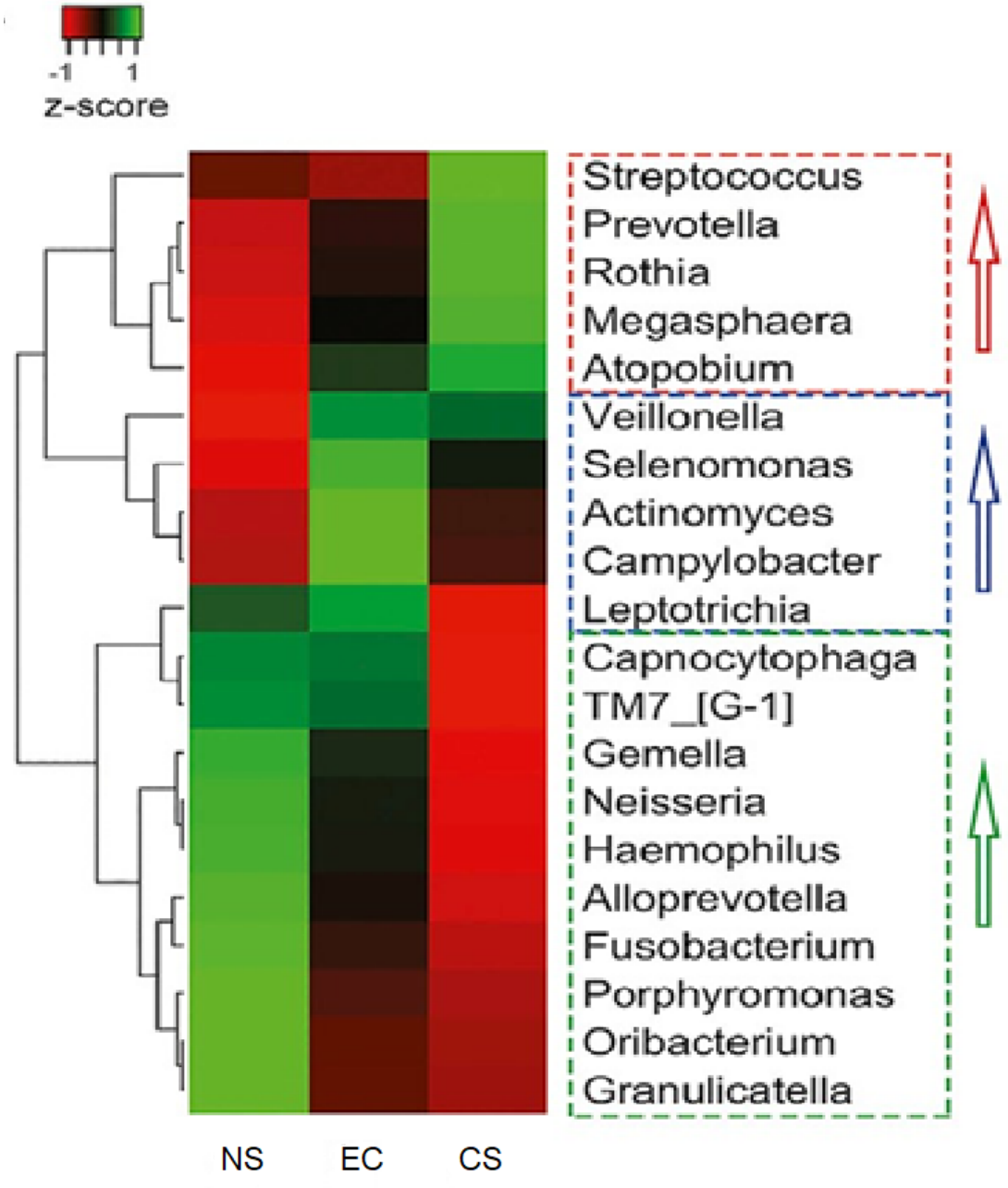

Figure 9 *Microbial abundances observed across cohorts categorized as nonsmokers (NS), electronic cigarette users (EC), and conventional cigarette smokers (CS). Green, never smokers (NS); blue, electronic cigarette vapers (ES); and red, cigarette smokers (CS) [51]*

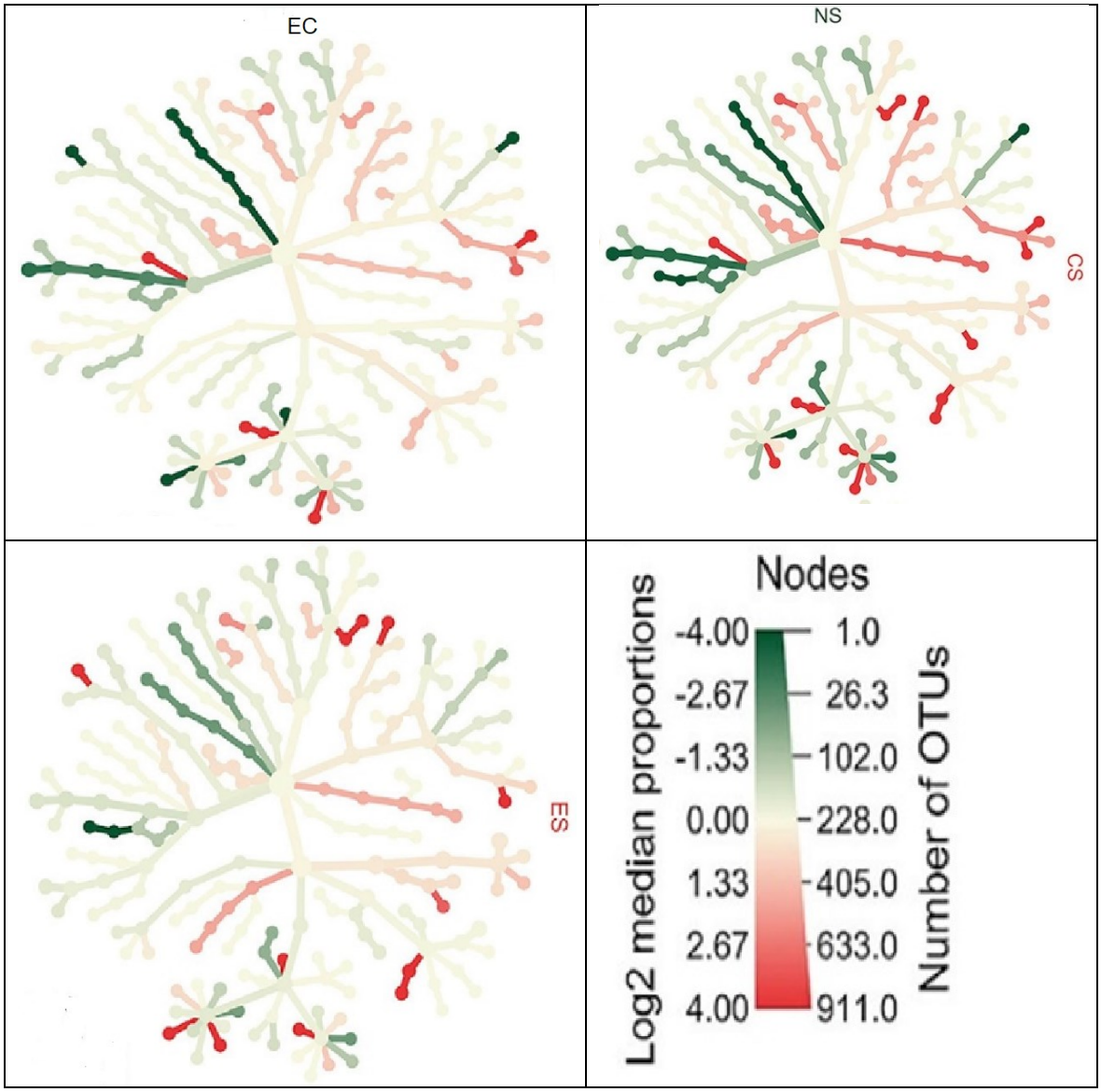

Figure 10 (part 1) *Dysbiotic salivary bacterial genera in NS, EC, and CS cohorts Heat tree illustrates the relationship of OTUs up to genus level. The heat tree visually represents the relationship of Operational Taxonomic Units (OTUs) up to the genus level. Colored branches on the tree indicate significance based on the respective cohorts [51]*

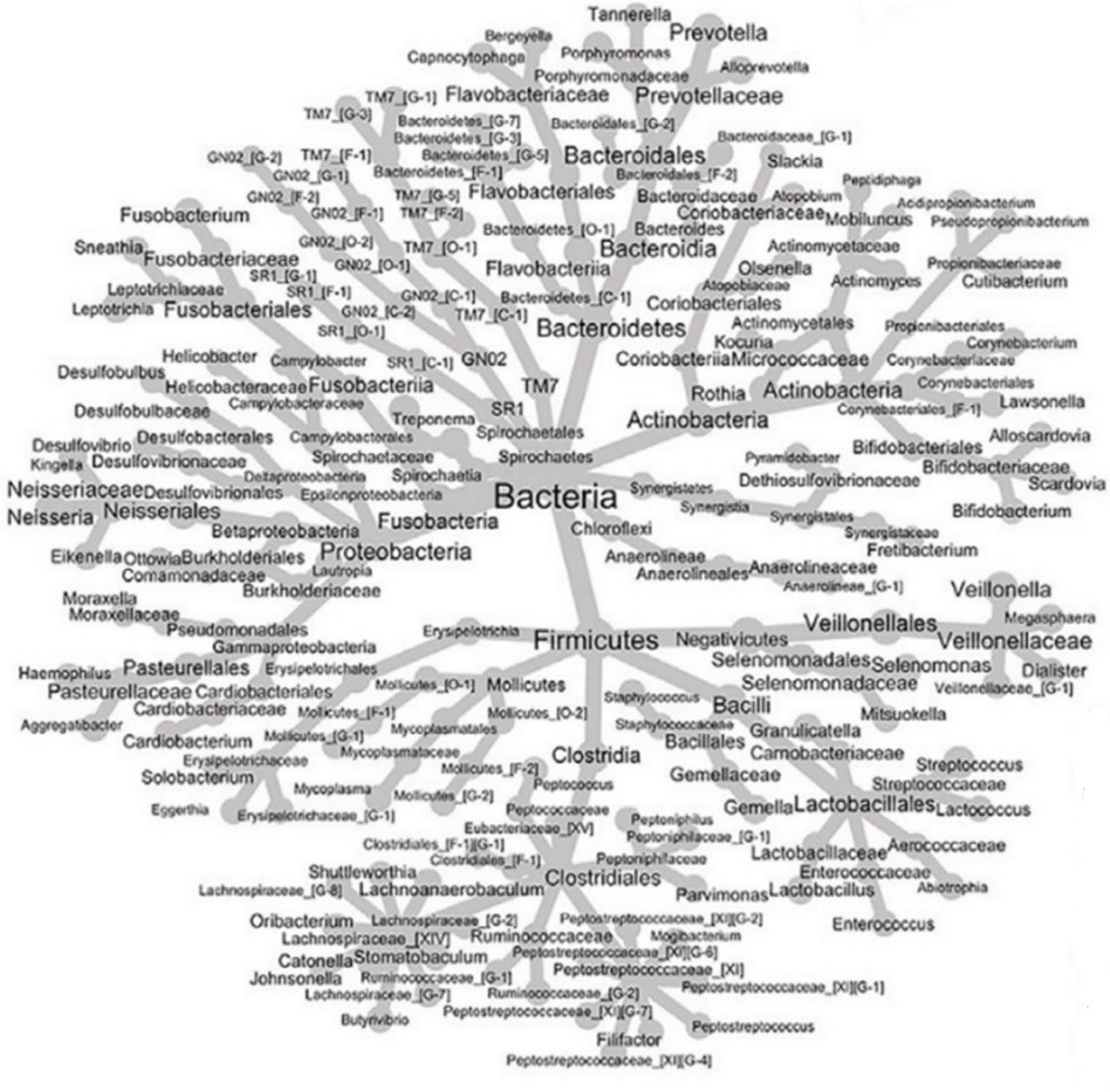

Figure 10 (part 2) *Dysbiotic salivary bacterial genera in nonsmokers, electronic cigarette smokers, and tobacco cigarette smokers cohorts [51]*

A hierarchical cluster analysis revealed the stratification of taxa into four clusters (Figure 11)[51]. Interestingly, the salivary microbiota of electronic cigarette (EC) users, which harbored Cluster Ib and Cluster IIb, exhibited similarities to those taxa distinct in non-smokers (NS) and cigarette smokers (CS) cohorts, respectively[51]. Further analyses unveiled that these similarities were closely associated with nicotine intake (smoking status) among the EC participants[51]. Collectively, the taxa observed to proliferate included Actinomyces in the EC cohort, *TM7* in both the EC and NS cohorts, and *Granulicatella* in the NS cohort. *TM7*, bacteria from the *Saccharibacteria* phylum, is a ubiquitous member of the human oral microbiome. Typically found at low abundance (approximately 1% of the whole oral microbial population based on culture-independent molecular analyses) *TM7*

members were detected to increase, sometimes reaching up to 21% of the whole oral bacterial population, in patients with severe gingivitis and periodontal disease. Strong evidence suggests that *TM7* interacts with *Actinomyces* and plays a critical, although poorly understood, role in the development of the human oral microbiome community composition in health and disease [52].

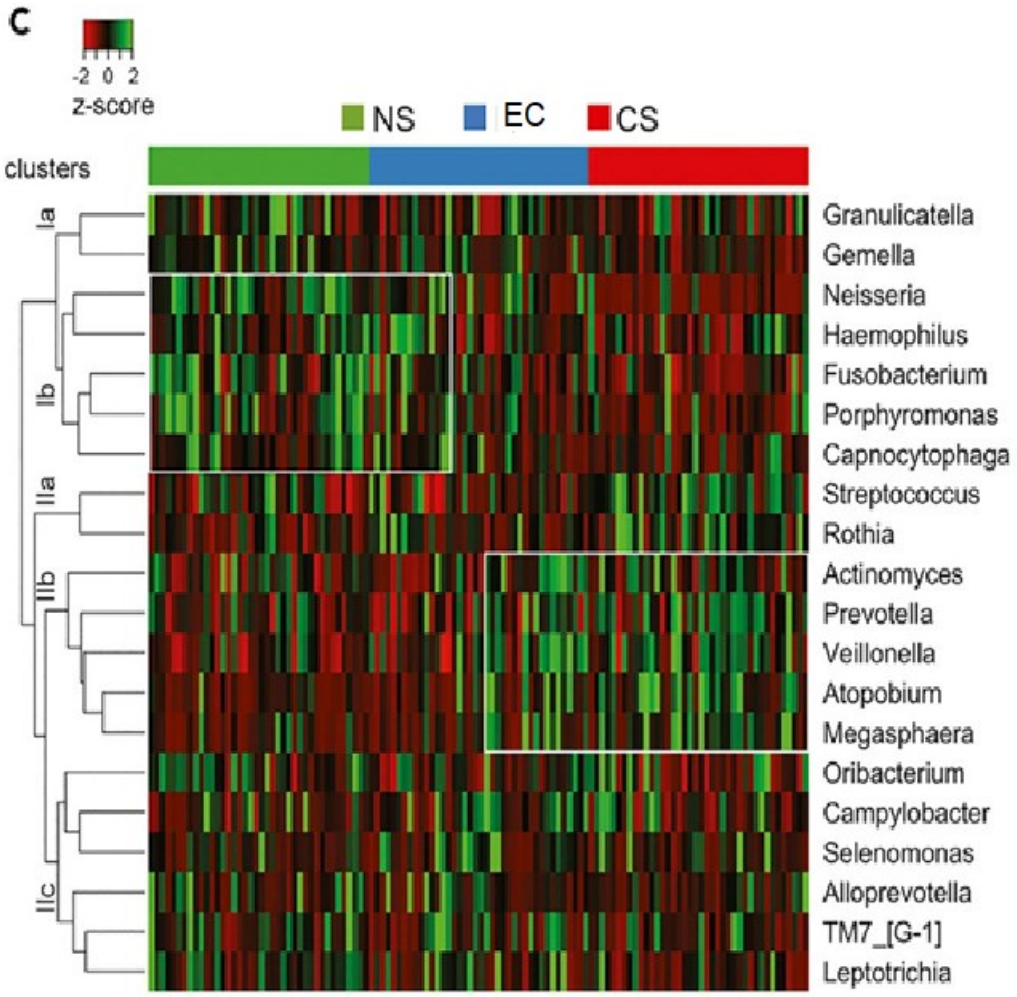

Figure 11 *The twenty most prominent taxa found in the saliva of the subject population across nonsmokers (NS), electronic cigarette users (EC), and conventional cigarette smokers (CS) cohorts are displayed. Rectangular shapes depict two groups where multiple taxa in the samples of the EC group intersect with those of the NS and CS groups [51]*

Furthermore, *Neisseria* and *Fusobacterium* were strongly linked to the EC and NS cohorts compared to the CS cohort ($p<0.0001$)[55]. In contrast, opportunistic pathogens like *Streptococcus*, *Prevotella*, and *Rothia* were significantly enriched in the CS cohort [55]. *Veillonella* levels rose significantly, by about 4% in the EC group and by approximately 4.5% in the CS group, compared to the NS group. These results suggest microbial dysbiosis in both non-combustible vapers and combustible smokers[55].

In both the EC and CS groups, there was a signi ficant depletion of *Streptococcus oralis subsp. tigurinus* clade 071, *Porphyromonas pasteri, Fusobacterium periodonticum,* and *Oribacterium parvum* [55]. Conversely, *Veillonella rogosae, Granulicatella adiacens*, and *Prevotella spp. HMT 317* were more prevalent in the NS group. *Veillonella dispar, Porphyromonas endodontalis, Prevotella oris, Fusobacterium nucleatum subsp. vincentii,* and *Parvimonas micra* were more abundant in the EC and CS groups[55]. Furthermore, *Veillonella atypica*, *Megasphaera micronuciformis*, *Prevotella spp. HMT 311*, *Streptococcus parasanguinis* clade 411, and *Actinomyces lingnae* significantly increased in the CS group, although they were also higher in the EC group. The EC cohort showed elevated levels of *Porphyromonas gingivalis, Dialister invisus*, *Alloprevotella tannerae, Corynebacterium durum*, and *Leptotrichia wadei*, whereas *Streptococcus salivarius* was more prevalent in the CS cohort [55].

Smoking appears to have a dominant effect over genetic factors like ethnicity in shaping the microbiome [44]. Smokers exhibited a remarkable uniformity in their microbial profiles, as demonstrated by notable clustering based on lineage in principal coordinate analysis (PCoA) ($p<0.001$, analysis of similarity [ANOSIM]) [44].

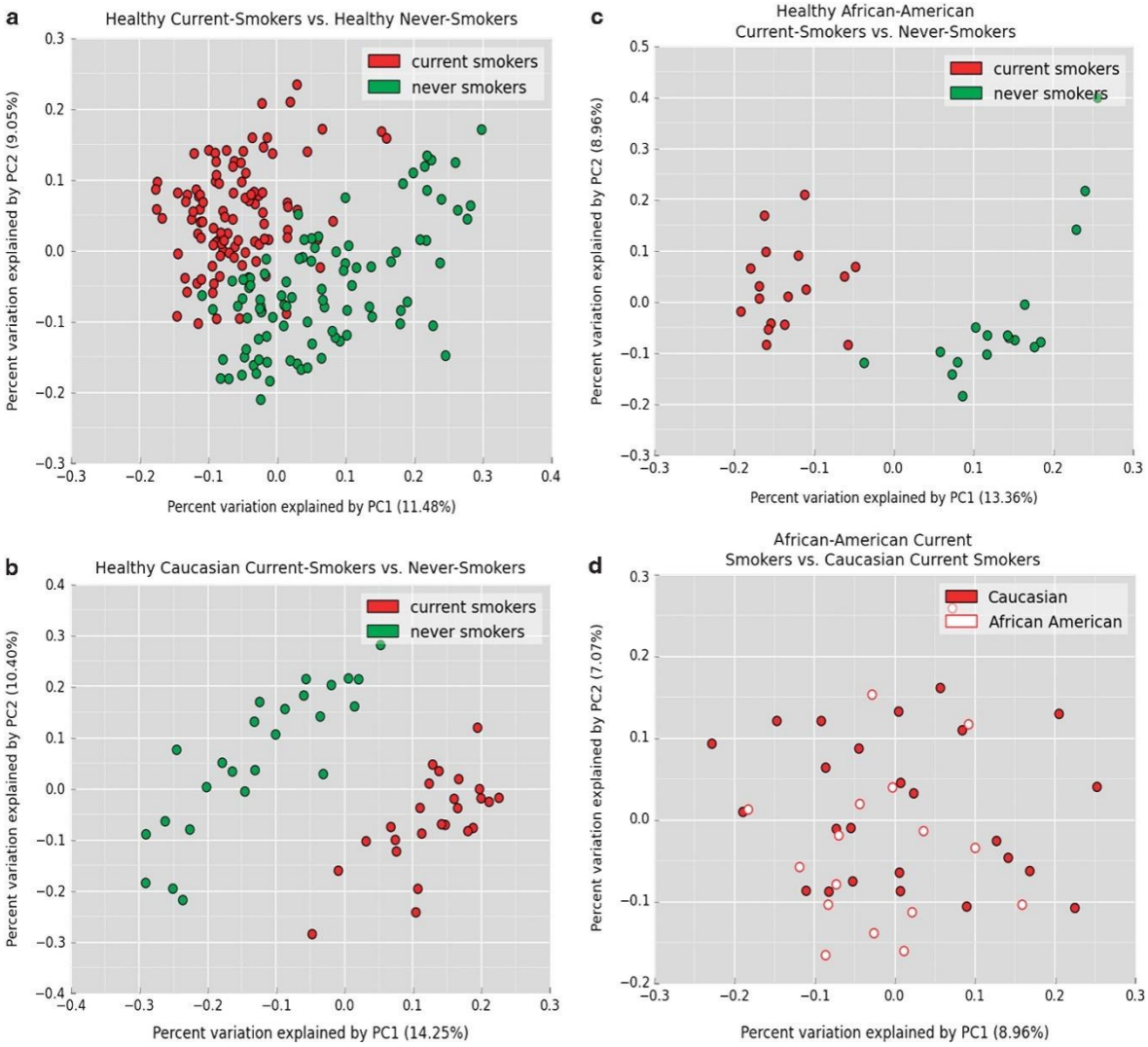

Figure 12 *PCoA plots of the UniFrac distance are presented for all samples categorized by smoking status. Panel (a) displays all 200 samples, while a subset of 50 Caucasians and 34 African Americans is shown in panel (b). Panel (c) focuses on the 34 African American samples. In all panels, significant clustering based on tobacco exposure is observed, regardless of ethnic background. Panel (d) depicts a subset of 17 African American and 25 Caucasian smokers, which does not exhibit significant clustering based on tobacco exposure[44]*

A biofilm consists of microbial cells and an extracellular polymeric substance (EPS) matrix, with varying organisms exhibiting different EPS production levels that increase with biofilm age [53].

Comparative analysis of bacterial acquisition and colonization between 15 current smokers and 15 nonsmokers was conducted during marginal and subgingival plaque formation over a 7-day period. The bacterial biofilm was sampled four times, collecting undisturbed plaque samples at 1, 2, 4, and 7 days, yielding 12,000 near-full-length, high-quality sequences for

analysis[42]. UniFrac distances were utilized to assess the similarities between samples, constructing a common phylogenetic tree from 16S rRNA gene sequences to compute branch lengths for each community [42]. Principal-coordinate analysis of UniFrac distances explored smoking's impact on marginal and subgingival plaque community compositions. The sequences represented various phyla, with *Bacillota (Firmicutes)* accounting for 83.4% of all sequences and different operational taxonomic units (s-OTUs) observed in marginal and biofilms beneath the gumline in individuals who do not smoke and those who currently smoke [42]. Individuals who smoke exhibited higher microbial diversity during initial colonization in both types of plaque ($p<0.05$), followed by a decline over 7 days ($p<0.05$). Among nonsmokers, 71% of the community remained unchanged over 7 days, while in smokers, only 46% exhibited stability. Principal-component analysis showed notable clustering of bacterial sequences based on tobacco exposure in both plaque communities, suggesting distinct patterns of bacterial acquisition and colonization between smokers and non-smokers[42]. The composition varied between smokers and non-smokers, with smokers exhibiting a broader range of species/phylotypes. In non-smokers, the subgingival community was predominantly composed of *Streptococcus*, *Veillonella, Neisseria, Abiotrophia*, and *Selenomonas*, making up 70% of the genera present [42]. Conversely, smokers showed a different profile, with *Streptococcus, Veillonella, Selenomonas, Campylobacter, Pseudomonas, Dialister, Abiotrophia, Neisseria,* and *Prevotella* collectively constituting this percentage [42]. Moreover, smokers obtained multiple phylotypes from genera such as *Lactobacillus, Pseudomonas, Fusobacterium, Leptotrichia, Centipeda, Synergistes, Propionibacterium*, and *Cardiobacterium*, which were absent in non-smokers [42]. In marginal plaque, smokers consistently exhibited higher levels of *Streptococcus, Haemophilus, Selenomonas, Kingella, Lachnospira*, and *Pseudomonas* along with lower levels of *Neisseria, Rothia, Actinomyces*, and *Lautropia* compared to non-smokers ($p<0.05$) [42]. During this observation period, only smokers acquired the genera *Lactobacillus, Treponema,* and *Pseudomonas* [42]. To sum up, smokers demonstrated consistently higher average levels of certain genera in marginal plaque and acquired specific genera during the observation period, highlighting the influence of tobacco exposure on bacterial composition throughout plaque development [42].

To ascertain the impact of electronic cigarette aerosol on microbial infection rates *in vitro*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* were suspended in FaDu cell (malignant cell line) culture medium, incubated for 30 minutes at 5% CO2, treated with

fluorescein isothiocyanate labeling, and subsequently subjected to either air or electronic cigarette aerosol for 40 minutes. Subsequently, the infected FaDu cell population was analyzed using flow cytometry [51]. The findings revealed a significant increase in *Porphyromonas gingivalis* and *Fusobacterium nucleatum* infection in FaDu cells exposed to electronic cigarette aerosol, with respective increments of approximately 65% and 16% compared to exposure to air alone (Figures 13, A and B) [51].

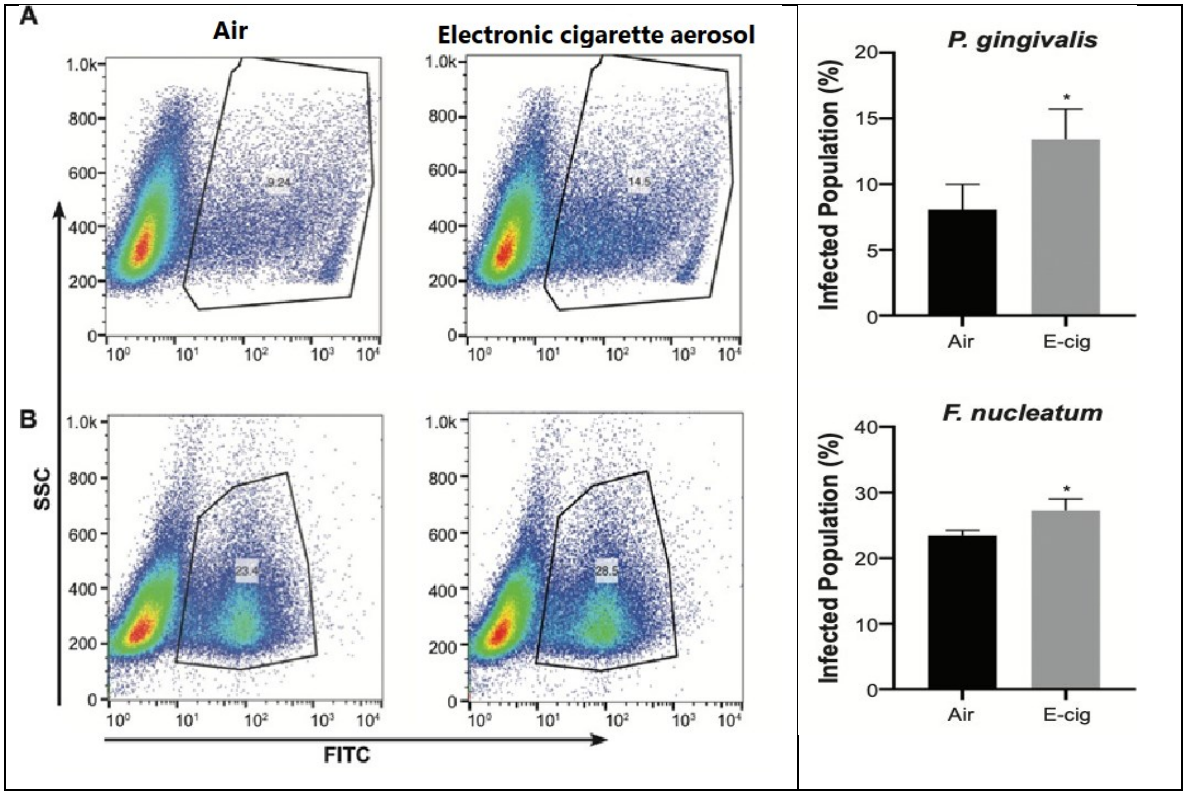

Figure 13 *Flow cytometry graphs demonstrate increased infection rates in FaDu cells following exposure to electronic cigarette aerosol in the presence of (A) Porphyromonas gingivalis and (B) Fusobacterium nucleatum. Graphical representation shows mean values with standard error of the mean +/- SEM indicated. (*p<0.05, **p<0.01, ***p<0.001) SSC (side scatter) Side scatter measurement provides information about the granularity of a cell. Cellular components that increase side scatter include granules and the nucleus [51]*

Furthermore, FaDu cells were infected with E. coli that expressed green fluorescent protein (GFP) at a ratio of 1:50 following treatment with either air or electronic cigarette aerosol (Figure 14) [51].

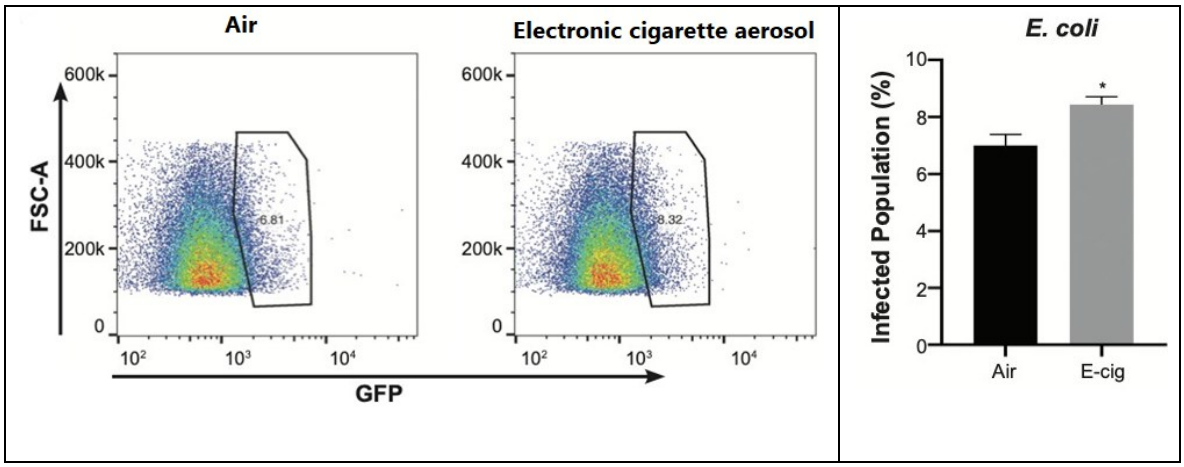

Figure 14 *Flow cytometry graphs display increased infection efficiency in FaDu cells when exposed to e-cigarette aerosol alongside Escherichia coli GFP, as opposed to air exposure. Graphical data are presented as mean values with standard error of the mean (SEM) indicated, with significance levels annotated (*p<0.05, **p<0.01, ***p<0.001) [51].*

Analysis of infection efficiency, assessed by GFP fluorescence with flow cytometry, revealed a notable increase ($p < 0.048$) in FaDu cells exposed to electronic cigarette aerosol compared to those exposed solely to air [51].

A previous study has identified a link between periodontal health and the microbiota, noting increased levels of various bacteria, including periodontal pathogens such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*, and *Neisseria mucosa*, in non-smokers with periodontal disease (n=366) [54]. The use of electronic cigarettes, and the resulting exposure to nicotine or other components, was found to elevate the prevalence of periodontal pathogens[55]. Periodontal microbes may serve as a reservoir for oral bacterial communities influenced by smoking, as previously observed in intervention studies addressing periodontal disease and tobacco dependence [55].

4. Discussion

Given the surging popularity of electronic cigarette (EC) smoking as a perceived safer alternative to traditional tobacco cigarette smoking, recent statistical data suggest a notable increase in EC use among young individuals, potentially carrying adverse implications for the current generation [56]. This review examines the oral microbiota composition of EC users, CS, and NS, shedding light on the impact of both electronic and conventional cigarette smoke on bacterial diversity and abundance.

Studies in the literature reveal distinct microbial communities inhabiting the oral cavity of EC smokers. Notably, EC vapers exhibiting taxa clustering similarities with either non-smokers or tobacco cigarette users often present with moderate to severe periodontitis. Additionally, EC users sharing higher microbial community abundance (Figure 10 [part 2]) with non-smokers or tobacco cigarette smokers displayed bleeding on probing rates of 56.09% and 65.49%, respectively.

Periodontal disease linked to smoking is widely acknowledged not only as a consequence of poor oral hygiene but also as an environment favorable for the colonization of bacteria like *Porphyromonas gingivalis, Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*, thus facilitating the development of periodontal lesions [57]. Nicotine exposure has been shown to affect immunological functions through *Porphyromonas gingivalis*, promoting biofilm formation by interacting with commensal oral bacteria such as *Streptococcus gordonii* [58]. A higher prevalence of eight species, including *Porphyromonas gingivalis*, was observed in current smokers, indicating an increased risk of periodontitis. The presence of the red complex (*Porphyromonas gingivalis, Tannerella forsythia, Treponema dencicola*) and orange complex (*Fusobacterium nucleatum subspecies, Fusobacterium periodonticum, Peptostreptococcus micros, Prevotella intermedia, Prevotella nigrescens*, and *Campylobacter rectus*) periopathogens indicates high and moderate risks, respectively, of periodontitis [51].

Moreover, *Porphyromonas* significantly expanded in EC users, while *Prevotella* was predominant in CS users. The presence of these periodontal pathogens in the oral cavity of EC and combustible cigarette users reflects severely compromised periodontal health. Studies suggest that nicotine and its metabolite cotinine, along with treatment with cigarette smoke extract, can modify the function of key periodontal pathogens such as *Porphyromonas gingivalis*, thereby promoting biofilm formation and infection [59].

Granulicatella, typically a commensal of the human oral microbiome, has been implicated in endodontic infection and associated with increased risks of systemic diseases such as pancreatic cancer related to oral inflammation [60, 61].

Cigarette smoking elevates susceptibility to infections caused by pathogenic or opportunistic bacteria through the disruption of physiological and structural protective mechanisms. Various pathways have been investigated to understand smoking's disruptive impact on the immune system, including disturbances to innate and adaptive immune responses, modifications in respiratory tract microbiota, and effects related to pathogens. Exposure to cigarette smoke or EC aerosol fosters anaerobic conditions, promoting anaerobic microorganisms such as *Veillonella* and *Fusobacterium* while suppressing aerobes such as *Neisseria* [46].

Proposed pathogen-related effects contributing to increased infection rates and altered outcomes include heightened bacterial virulence (harmfulness) with smoke exposure, enhanced pathogen adherence to respiratory tract membranes, and increased antibiotic resistance [62]. *Peptostreptococcus, Mogibacterium,* and *Catonella* are linked to periodontal and endodontic infections [61]. *Veillonella*, particularly *Veillonella atypica* and *Veillonella rogosae*, were notably abundant in both EC and groups of individuals who smoke tobacco cigarettes. *Veillonella* bacteria are predominantly commensal organisms found in high numbers in saliva and on the surfaces of the tongue. Associations have been noted between *Veillonella* and cigarette smoking in patients with periodontitis [63, 64]. Prior research indicates that *Veillonella atypica* is known to convert ingested nitrate, commonly found in the saliva of tobacco users, into nitrite[65, 66]. Nitrite can then be further metabolized into potentially harmful nitrosamines and the pro-inflammatory nitric oxide, which could have implications for both oral and cardiovascular systemic health [65, 66].

This review suggests that vaping electronic cigarettes induces shifts in the oral microenvironment and significantly influences the development of complex and heterogeneous microbial biofilms. With the advent of more advanced electronic cigarette models, users now have increased control over factors such as electronic-liquid consumption, power settings, and airflow. As an illustration, a study revealed elevated plasma nicotine levels in more recent electronic cigarette models in contrast to earlier models of electronic cigarettes [67].

However, an exception to this narrative is provided by an *in vitro* study, which indicates that flavorless electronic cigarette aerosol with or without nicotine has minimal to

no toxic effect on the growth of four oral commensal streptococci[68]. Colonies (CFUs) of all four species exposed to flavorless electronic cigarette aerosol with or without nicotine were able to reach comparable numbers and dimensions as their untreated counterparts. Similarly, streptococci exposed to this aerosol could form biofilms comparable to untreated controls. In contrast, exposure to cigarette smoke severely inhibited or entirely eradicated colony and biofilm growth. Interestingly, nicotine does not seem to be responsible for this inhibition, as electronic cigarette aerosol had a greater nicotine concentration per puff compared to cigarette smoke. While data on CFUs and colony size suggest that aerosol (with or without nicotine) has minimal impact regarding bacterial viability and proliferation, it is worth considering biofilm formation appears to be somewhat impeded when bacteria, especially *S. mitis*, are exposed to aerosol lacking nicotine. However, this effect seems to be reversed upon the introduction of nicotine into the aerosol, suggesting that the base humectants (vaporized propylene glycol and glycerol) might inhibit bacterial survival and growth, while nicotine (at the tested dosage) may promote them[68].While flavorless electronic cigarette aerosol appears to have no effect on the ability of four commensal streptococci to develop biofilms, it is essential to examine the influence of both conventional cigarette smoke and electronic cigarette aerosol on mixed-species biofilms[68]. This helps comprehend the effects of these environmental factors in an open system [68].

Furthermore, the cytotoxicity and genotoxicity of electronic cigarette vapor, and the role of electronic liquid nicotine content in mediating its harmful effects, were assessed using a panel of normal epithelial and head and neck squamous cell carcinoma (HNSCC) cell lines [69]. It was demonstrated that the cytotoxic effects of electronic cigarette aerosol are mediated by both nicotine and non-nicotine components. Numerous studies have identified hazardous substances in electronic cigarette aerosol and heated electronic liquid emissions, including toxic flavoring compounds, carcinogenic carbonyl compounds like formaldehyde and acetaldehyde, oxidants, reactive oxygen species, heavy metals, and volatile organic compounds such as toluene [70-76].

Despite being promoted as a secure substitute for conventional cigarettes, doubts persist regarding the prolonged safety of electronic cigarettes. Additional research is needed to assess the possible long-term adverse effects of metal fumes and other contaminants linked to electronic cigarette use.

4.1 Conclusion

The perception of electronic cigarettes has largely been shaped by their purported benefits for pulmonary and cardiovascular health, such as reduced structural damage and a lower risk of malignancies. However, the impact on the microbiome has received little attention in much of the literature reviewed. This review underscores the need to question the lack of health risks associated with and the harm reduction narrative advanced by marketing campaigns. Emerging evidence suggests that electronic cigarette use significantly alters the composition of the oral microbiome, potentially leading to changes associated with disease. Further research is imperative to fully understand and quantify the microbiome-related risks posed by the growing trend of electronic smoking.

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