Undergraduate Journal of Experimental Microbiology and Immunology (UJEMI) Vol. 29:1-11

Smoking Traditional Cigarettes and Vaping May Not Affect the Diversity Metrics of the Oral Microbiota but Significantly and Distinctively Alters the Taxonomic Composition Relative to Non-Smokers

Jason Bie, Nidhin Biju, Shini Chen, Haohua Li, Sina Safaeian

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Despite decades of anti-smoking initiatives, tobacco remains one of the most commonly used addictive substances in the world. The use of tobacco has strongly been associated with a variety of cardiovascular and respiratory illnesses. Due to their perceived safety over traditional cigarettes, e-cigarettes have surged in popularity in the past five years, especially among the younger populations. However, no consensus has been reached regarding their impact on human health. This study used a bioinformatic approach to study the association of traditional cigarette and e-cigarette usage with the oral microbiome in a cohort of 30 individuals from Houston, Texas, USA. Although no significant differences were observed in core diversity metrics, our findings revealed significant compositional changes in the oral microbiome resulting from smoking when comparing the oral microbiota of cigarette or e-cigarette users with non-smokers. Taxonomic analysis suggests that cigarette smoking increases the abundance of pathogenic genera associated with respiratory and periodontal diseases, while e-cigarette smoking upregulates bacterial species that contribute to oral and respiratory health issues. These results underscore the need for more comprehensive studies to confirm the effects of smoking on oral microbiota in a more diverse population and elucidate the broader effects of the noted dysregulations on overall health.

INTRODUCTION

Despite the anti-smoking policies and campaigns that have run consistently for the last half century, tobacco remains one of the most abused drugs in the world, a cycle that is propagated by the addictive qualities of its active ingredient, nicotine (1). This is problematic as the ingestion of tobacco smoke and its constituents exposes users to several harmful chemicals, including arsenic, carbon monoxide, and formaldehyde (1). Long-term ingestion of these agents can lead to a host of ailments in patients, ranging from cancers to pulmonary disorders such as chronic obstructive pulmonary disease (COPD) which occur due to the mutagenic and pro-inflammatory effects of tobacco constituents and the physical damage caused by inhalation of the smoke (1).

Due in part to the fear of the health complications of tobacco cigarettes (C), e-cigarettes (EC), colloquially referred to as “vapes”, have taken a large segment of the nicotine-product market by being marketed as a healthier alternative to traditional tobacco cigarettes (2). While ECs are believed to be safer for consumption when compared to Cs given that EC compositional compounds are better controlled and typically FDA-approved GRAS (“generally regarded as safe” for consumption) compounds, many studies have correlated EC use with negative health outcomes when compared to a non-smoking population (3-5). For example, EC usage has been associated with higher incidences of asthma, cardiovascular disease, respiratory disease, and myocardial infarction (3).

Despite the mounting evidence of the negative impact of EC usage, how exactly it impacts the diversity and composition of oral microbiota compared to Cs and non-smokers remained unanswered (4–8). Even though small studies on how C and EC use affect the gut and oral microbiomes of humans have been conducted by many researchers, the results have not been conclusive, particularly due to the lack of consensus in the field. While some found no

Published Online: September 2024

Citation: Bie, Biju, Chen, Li, Safaeian. 2024. Smoking traditional cigarettes and vaping may not affect the diversity metrics of the oral microbiota but significantly and distinctively alters the taxonomic composition relative to non-smokers. UJEMI 29:1-11

Editor: Shruti Sandilya and Ronja Kothe, University of British Columbia

Address correspondence to: https://jemi.microbiology.ubc.ca/

significant differences in the alpha and beta diversity of the oral microbiome of EC users compared to non-smoking control, others found that EC usage lowers alpha diversity in saliva samples (4, 9, 10). This inconsistency can be exacerbated by the lack of consensus in the comparison metrics. Some studies compared smokers versus nonsmokers without clarifying whether smokers are EC or C users, while some studies did not compare ECs with Cs (11). Therefore, our study aims to compare both ECs and Cs using non-smokers as a control, hoping it would elucidate the effect of vaping and tobacco on oral microbiome diversity.

Furthermore, smoking can also alter the oral microbiome composition. Previous research has shown that C smokers tend to have an increased abundance of anaerobic bacteria and gram-negative bacilli in the oral microbiota compared to non-smokers (12). However, similar work in EC users has suggested mixed consequences, potentially due to heterogeneity in the chemical compositions used by individual manufacturers such as the presence or absence of acetaldehyde, acrolein, formaldehyde, and other additives in the nicotine-containing e-fluid (13). Nonetheless, given that bacterial fitness varies based on environmental stimuli such that different species will adapt to different environmental niches, it is expected that the distinct chemical composition of C and EC would alter the composition of the oral microbiota.

Stewart *et al.* have investigated the effect of EC and C smoking on the gut and oral microbiota using non-smokers (NS) as a control(9)(9). C users had a significantly different oral and gut bacterial composition compared to EC users, with increased relative abundance of *Prevotella* and decreased *Bacteroides*, while no significant difference was found between the alpha and beta diversity of EC users and non-smoking controls. However, since they collected cross-sectional fecal, buccal swabs, and saliva samples, their investigation has not been focused entirely on the oral microbiome nor did they discuss the specific oral bacterial genera that were altered by EC and C. Therefore, to investigate if true differences exist in oral microbial population and diversity between EC, C, and NS, we decided to continue the study by Stewart *et al.* by using their Houston cohort to perform additional analyses such as core microbiome analysis, indicator taxa analysis, DESeq2, and PICRUSt that were not included in their original study (9). We hypothesized that while both C and EC usage would significantly alter the relative abundance of microbiota in the saliva, the change would be driven by differences in unique bacterial genera in each group; we also expected that the microbial species selected for C and EC usage would be reflective of their abilities to degrade the distinct harmful chemicals present in each cigarette type. To accomplish this, data was processed using QIIME2 and R, leading to insights into changes in microbiome composition at the ASV level. In addition, we conducted PICRUSt analysis to look at alterations of metabolic pathways. We have discovered that C and EC usage did not change the oral taxonomic alpha and beta diversity metrics but significantly altered composition and functional diversity.

METHODS AND MATERIALS

Dataset description. The dataset used in this paper originated from a study by Stewart *et al.* comparing the effects of tobacco smoke and electronic cigarette vapour exposure on the gut and oral microbiota (9). The authors enlisted 10 C smokers, 10 EC smokers, and 10 NS persons from the Houston area. For EC users the participant inclusion criteria involved a minimum of 6 months of continuous daily EC while for cigarette smokers it was set at testing at a level 4 or greater on the Fagerstrom scale for nicotine dependence as well as a minimum intake of 10 cigarettes per day (9). The biometric characteristics of participants in the smoking status groups were also matched with regards to sex, age, diet, height, weight, and ethnicity (9). The authors collected saliva, buccal swabs, and fecal matter from them alongside metadata information pertaining to their smoking habits, demographic information, and biometric characteristics.

Data processing in QIIME2. The V4 regions of the 16S rRNA gene of oral samples were amplified and sequenced on an Illumina MiSeq instrument using the 515F and 806R barcoded Illumina adapters. The metadata and corresponding 16s rRNA sequences from our dataset were imported into the Quantitative Insights Into Microbial Ecology2 (QIIME2) software (version 2023.7.0) (14). Raw sequence reads were demultiplexed for downstream processing and analysis and sequence quality control and denoising were performed using the Divisive

Amplicon Denoising Algorithm 2 (DADA2) (15). Forward reads were truncated to 240 base pairs (bp) and reverse reads were truncated to 190 bp to maintain high sequence quality while removing low-quality regions. An ASV table was generated by clustering similar sequences into ASVs. This table was used for downstream analysis, including alpha and beta diversity analysis, taxonomic classification, and functional analysis.

Data processing using R Studio. All bioinformatic analyses were done in R (version 4.3.2), and basic data wrangling was done using the Tidyverse (version 2.0.0) and Phyloseq packages (version 1.46.0). The ggplot2 package (version 3.5.0) was used for all plots (12). We performed filtration and rarefaction steps by first removing mitochondrial and chloroplast DNA sequences, as well as any row containing "NA" or "not applicable" values. We then removed any ASVs that have less than 5 counts in total, which represent low abundance taxa. We also removed samples with fewer than 100 reads as a quality control. A Rarefaction depth of 95,000 was used based on alpha rarefaction plots. Finally, we created a phyloseq object using unrarefied data only containing the saliva swab samples for downstream analysis.

Alpha and beta diversity analysis. Based on the alpha-rarefaction curve we generated, a sampling depth of 95,000 was selected to maximize sample richness while maintaining a sufficient number of samples for diversity matrix statistical analysis with the Vegan package. Alpha diversity of the saliva swab samples of different smoking habits was assessed through the Observed, Chao1, and Faith's phylogenetic distance metrics in R (16, 17). The Wilcoxon rank sum test was performed to test for significance ($p<0.05$) using the ggpubr package (18). Subsequently, beta diversity between each cohort was assessed by Bray-Curtis Dissimilarity. To calculate statistical significance, the results of beta diversity analysis were evaluated by pairwise permutational multivariate analysis of variance.

Core microbiome analysis. Core microbiome analysis was performed on each smoking category at the species level using the microbiome package (19). First, absolute abundance was converted to relative abundance. Core microbiome was then calculated using a prevalence threshold of 0.05 and detection threshold of 0.001 and visualized as a three-way Venn diagram using the ggVennDiagram package (20, 21).

Indicator Taxa Analysis. Indicator species analysis was conducted in R using the indicspecies package (19, 22–24). Taxonomic data was grouped at the genus level. Multipattern analysis was performed to determine indicator genera that were specific to C smokers, EC smokers and non-smokers. Only the top two indicator genera with the highest indicator values were reported in order to identify the most relevant and high-confidence genera.

DESeq2. Taxonomic differential abundance analysis between C users versus NS as well as EC users versus non-smokers was performed using the DESeq2 package (19, 25). Differential abundance was visualized in volcano and bar plots using non-smokers as the reference group.

PICRUSt Functional Analysis of Pathway Inference and Differential Expression. Functional analysis of predicted dysregulated pathways was performed using PICRUSt 2.0 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (26–30). The QIIME2 package was first used on the rarefied feature table to remove features with a frequency ≤ 5 to enable efficient downstream processing. Subsequently, the PICRUSt2 Qiime plugin was then used to perform functional analysis using the SaTé-Enabled Phylogenetic Placement (SEPP) and Maximum Parsimony (MP) hidden state prediction algorithms. The KEGG Orthology (KO), MetaCyc pathway abundance (pathabun), and Enzyme Commission (EC) outputs were converted to human readable tab-separate values (tsv) files using Qiime export tools. From there, an R code package for PICRUSt analysis and DESeq2 was used on the KO output file to visualize dysregulated pathways in the tobacco smoking and vaping cohorts using the non-smoking group as a baseline. Overall, a heatmap and Log2-fold-change bar plots of pathways with statistically significant dysregulation ($p \leq 0.05$) were produced.

RESULTS

Smoking status did not correlate with changes in alpha and beta diversity measures. To investigate if smoking status and the type of smoking have an impact on the oral microbial diversity of the American population, we first sought to determine whether alpha diversity differed between C smokers, EC smokers and NS. We performed alpha diversity analysis employing three metrics: Observed richness, which takes into account the number of different species observed within the saliva sample of our subject; Chao1 index, which is useful for accounting for low abundance ASUs; and Faith's phylogenetic distance, which factors in the phylogenetic distances between species. Despite our initial hypothesis, we found no significant differences in all alpha diversity metrics examined when comparing C smokers, EC smokers and NS (Wilcoxon rank-sum, $p > 0.05$) (Fig. 1). Our Bray-Curtis dissimilarity analysis also showed three different smoking status groups clustered similarly (Fig. S1).

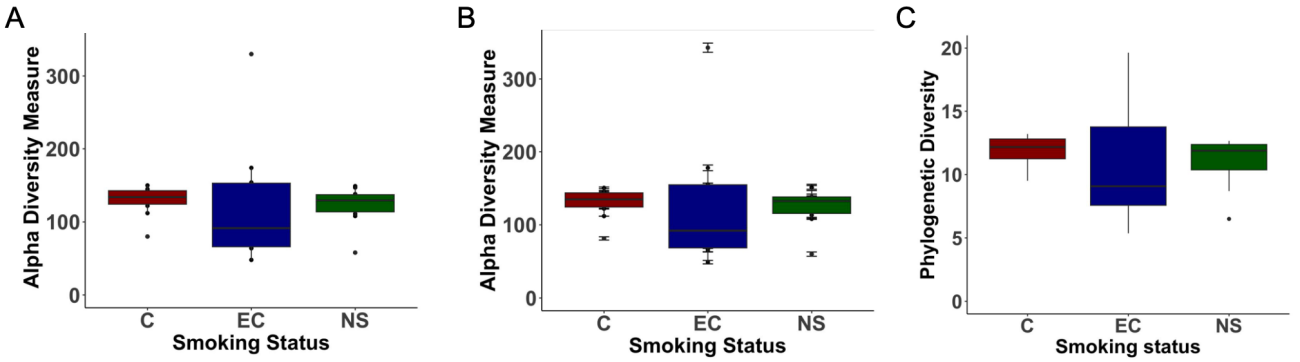

FIG. 1 No significant difference in microbial diversity metrics between smokers, EC smokers, and non-smokers. Alpha diversity measures using (A) Observed, (B) Chao1, and (C) Faith's phylogenetic distance metrics were not statistically different ($p > 0.05$) between C (cigarette users, red, n=10), EC (e-cigarette users, blue, n=10), NS (non-smokers, green, n =10) as per Wilcoxon rank-sum test.

C and EC users had unique oral bacterial species. Subsequently, we explored the three groups at the individual species level. We generated a three-way Venn diagram to compare the core microbiomes of smokers and non-smokers. There were 142 species (28%) common to all three smoking statuses (Fig. 2). Interestingly, there was a greater proportion of core

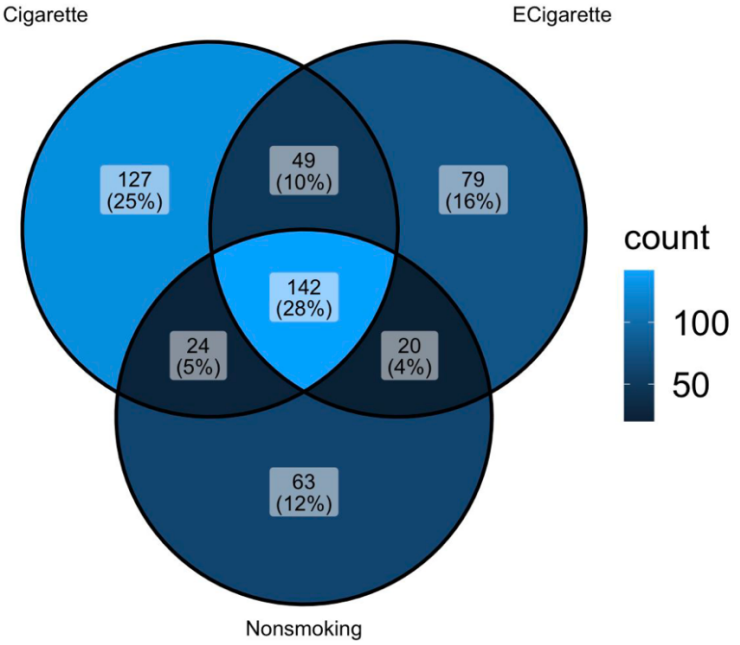

FIG. 2 Relative proportions of compared core microbiomes showed a significant overlap of core taxa between smokers and non-smokers. Core microbiomes were identified using the following parameters: minimum abundance of 0.1% and minimum prevalence of 5%. The core taxa were resolved at the species level. Numbers represent the percentage or frequency of occurrence of particular microbial taxa across multiple samples within a dataset that meet the minimum abundance and minimum prevalence cutoff.

species shared between C and EC users (10%) than between NS and either C or EC users (5% and 4% respectively), supporting the hypothesis that EC usage alters the oral microbiome (Fig. 2). However, further analysis of the taxonomic bar graph analysis revealed no phyla

unique to any smoking status, indicating that the 127 species (25%) unique to C smokers and 79 species (16%) unique to EC users were below the phylum level (Fig. 3).

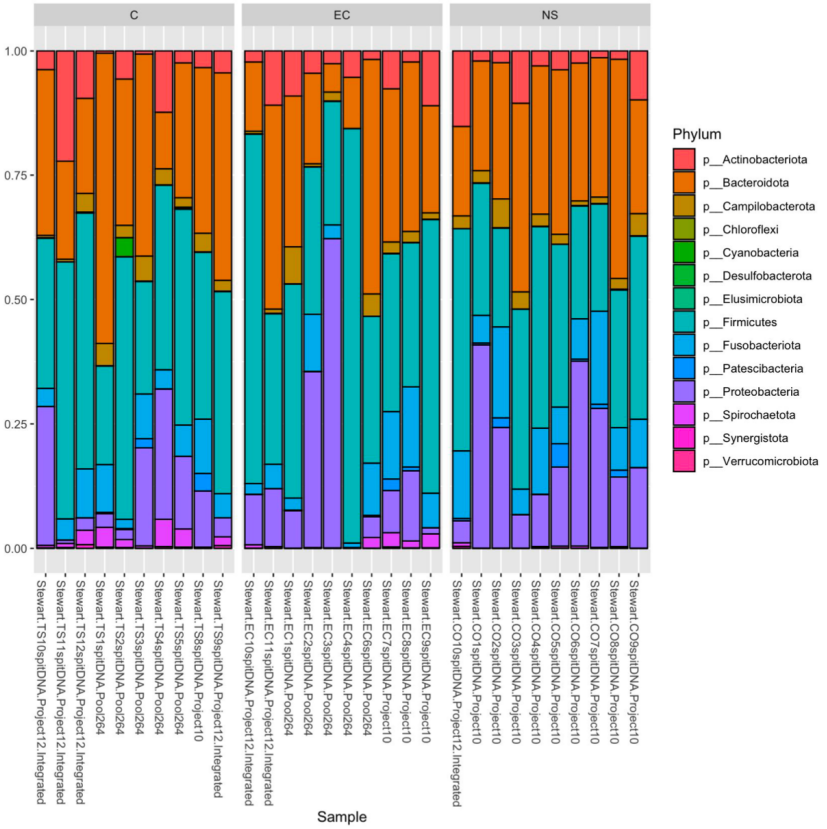

FIG. 3 Cigarette and EC usage demonstrate no obvious differences in the taxonomic composition of the oral microbiome at the phylum level. Bars show the relative frequency of microorganisms at the phylum level for individual samples. Colors indicate different phyla.

Certain species were correlated with respective smoking status. We conducted an indicator species analysis to assess the relative abundance of microbial species and their associations with each smoking status. Taxonomic data were grouped at the genus level, and only genera with indicator values above 0.5 and a p-value below 0.05 were considered to be specific to the smoking group of interest. Eight genera were reported to be specific to cigarette smokers, two genera were specific to EC smokers, and four genera were specific to NS (Table. S1). The two indicative species most specific to each smoking status are summarized in Table 1. There are no overlapping taxa at the genus level, indicating that smoking and different forms of smoking may promote the growth of different taxa in the oral microbiome.

TABLE. 1 Indicator taxa analysis shows unique genus that are most unique to in each population with different smoking status group. Table demonstrates microbial genera that are most correlated with their respective smoking status. Indicator species status is assigned when $P < 0.05$, and only the two genera with highest indicator value are displayed.

Smoking Status	Genus & Species	p-value	Indicator Value
Exclusive to C Smokers	*Clostridiales bacterium*	0.005	0.815
	Tannerella forsythia	0.025	0.761
Exclusive to EC Smokers	*Staphylococcus sp.*	0.035	0.613
	Prevotella sp.	0.045	0.569
Exclusive to NS	*Eubacterium yurii*	0.01	0.682
	Amnipila sp.	0.02	0.665

EC usage enriched more oral microbial species than C usage. We have conducted differential expression sequence (DESeq) analysis, which determines how ASVs from our groups of interests have increased or decreased in abundance relative to a reference, to assess the impact of C and EC smoking on the composition of the oral microbiome. The NS control was used as the reference for this analysis, and differential abundances were visualized as volcano plots. P-adjusted values are considered to be significant when smaller than 0.01. Both

C and EC smoking significantly altered the composition of the oral microbial ASVs and correlated with differences in the abundances of certain microbial species (Fig. 4B-D). Specifically, cigarette smoking is associated with the enrichment of certain ASVs while reducing the abundance of others. It is correlated with a significant increase of the *Streptococcus* genus while a reduction in the prevalence of genera like *Rothia* (Fig. 4B). Compared with cigarettes, EC smoking seemed to select for more microbial taxa. Genera like *Lactobacillus* were enriched with a more than 5-fold increase in abundance, while *Streptococcus* experienced a significant decrease in abundance (Fig. 4D). Again, this confirms that EC and cigarette smokers have a unique profile of oral microbiome composition compared to non-smokers.

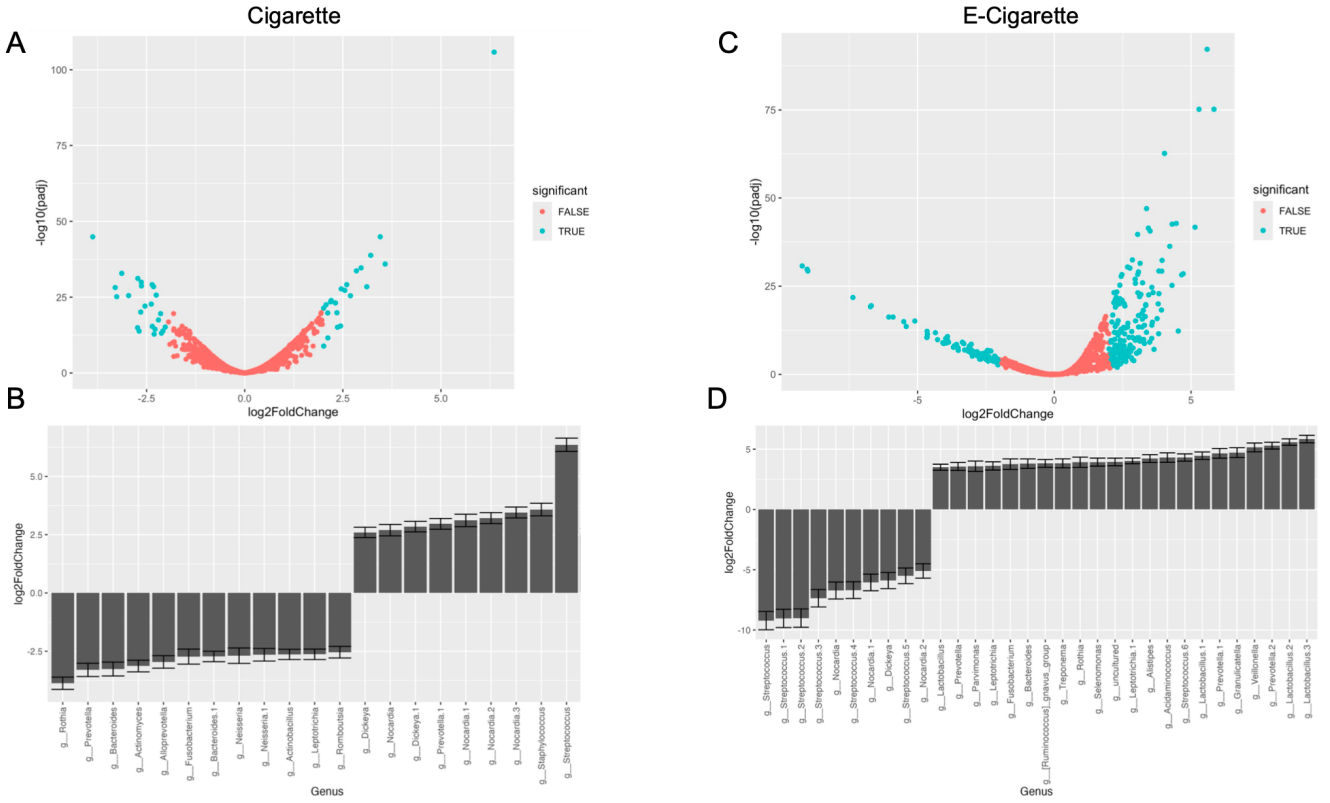

FIG. 4 Cigarette and EC smoking correlates with differences in oral microbial composition compared to non-smokers. Volcano plot demonstrating the log 2 fold change in the abundance of ASVs for the C and EC smoking populations relative to the non-smoking controls. (A, C). Bar plots showing how EC and C smoking correlates with taxonomic abundance at the genus level (B, D). Padj value is considered significant when for interpretability of the plot. (D) ASVs are considered to be significantly different when Padj < 0.01 and a log2fold change > 2.5 (B) or 3 (D), or a Padj < 1*10^-14 and a log2fold change > 2.5 (A) or 3 (C).

Multiple oral metabolic pathways were predicted to be differentially enriched in C and EC smokers compared to NS. PICRUSt2 analysis was done to predict and compare the metabolic pathways active in the saliva microbial community of smokers compared to non-smokers based on the species present (Fig. 5). This predicted that in cigarette smokers, the pathways for chlorosalicylate degradation and glycolysis IV were notably enriched while the pathway for photorespiration was downregulated in the saliva microbial population of smokers compared to non-smokers (Fig. 5A). Furthermore, repeating the analysis in EC smokers compared to non-smokers showed that while chlorosalicylate degradation was predicted to be upregulated as it was in cigarette smokers, EC smokers were expected to have significant downregulation of the pathways for 3-phenylpropanoate, p-cymene, and p-cumate degradation (Fig. 5B).

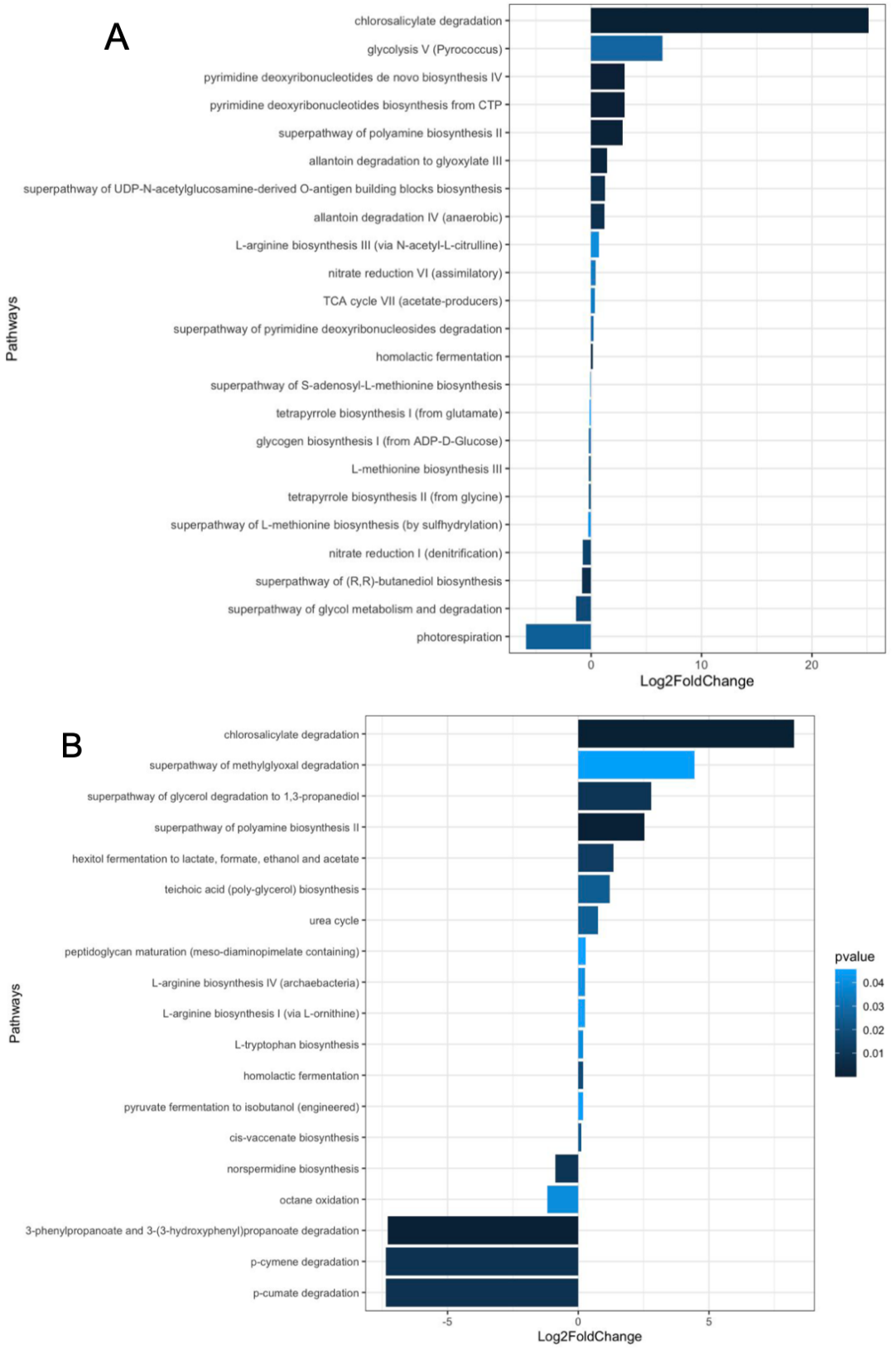

FIG. 5 Tobacco and Electronic Cigarette Smoking May Dysregulate Metabolic Pathways Active in the Salivary Microbial Community. Through PICRUSt2 analysis the metabolic pathways present in the oral microbiota of C smokers (A) and EC smokers (B) were predicted and compared to that of non-smokers.

DISCUSSION

The human oral cavity provides a habitat for more than 600 microbial species, which collectively form what is known as the oral microbiome (31). Oral microbial ecology can be perturbed when exposed to the numerous toxic chemicals of both C and EC smoke (32–34). Prolonged exposure to these toxicants can result in the loss of beneficial species, which may allow the colonization of pathogenic microbes (35). As multiple studies have demonstrated how dysbiosis of the oral microbiome is correlated with many cardiovascular and respiratory diseases (36), it is important to elucidate the impact of cigarette and EC smoking on the diversity, composition, and abundance of organisms of the oral microbiome.

Interestingly, we did not find any significant differences in the alpha diversity of the oral microbiome of C smokers, EC smokers, and NS. Furthermore, our beta diversity analysis suggested that different smoking statuses did not change oral microbiome composition enough to allow for the formation of distinct clusters. This does not align with findings reported by Wu *et al.* and Yu *et al.*, where they have discovered that cigarette smoking is correlated with a significant reduction of oral microbiome alpha diversity (11, 36). Additionally, Pfeiffer *et al.* showed that cigarette smoking seemed to be related to increases in overall oral microbial diversity (37), while Antonello *et al.* discovered no significant changes in smoking on salivary microbiota (38). A possible explanation for the mixed results is the heterogeneity of human microbiomes related to confounding variables like geographical location, lifestyle, age, and sex. The studies listed above are conducted in different regions including Germany, USA, Italy, and Iran, and in different populations with very different lifestyles. This calls attention to the need to control for confounding variables in future studies that can result in drastic changes in oral microbial diversity and composition. If microbiome differences still do not exist after controlling for these confounding variables, then we can be more confident in concluding that cigarette usage does not alter oral microbiome alpha and beta diversity despite the heterogeneity of human microbiomes.

Despite not finding significant differences in alpha or beta diversities between our groups of interest, we have noted differences in the taxonomic composition and relative abundance of the oral microbiome concerning C and EC usage. The differences in bacteria composition are unique to the species level. Specifically, indicator species analysis shows that C usage exclusively correlated with high levels of the *Clostridiales* bacteria and *Tannerella forsythia*, which agrees with our DESeq analysis showing that smoking significantly altered the composition of oral microbial ASVs. *Clostridiales* are not typically found in the normal oral flora and can have severe consequences when inhaled including the development of necrotizing pneumonia, a serious condition characterized by the rapid destruction of lung tissue, often leading to respiratory failure and potentially life-threatening complications (39). It is also known to cause diarrhea and colon inflammation (40). Additionally, *Tannerella forsythia*, a bacterium usually absent in healthy mouths, is frequently observed in patients with periodontal disease (41).

EC usage, on the other hand, exclusively correlated with high levels of the *Staphylococcus* and *Prevotella* genera. *Staphylococcus* is typically not found in normal oral flora and can pose various oral health concerns such as the development of abscesses and gingivitis, which can lead to discomfort and gum disease (42). Additionally, it can potentially cause pneumonia if it gets into the lungs (43). On the other hand, *Prevotella* is commonly found in the mouth. When combined with poor oral hygiene practices, however, it can contribute to the formation of plaque (44). The *Eubacteria yurii* was exclusively correlated with the non-smoking population. As *E. yurii* plays a role in the defence against the colonization of pathogenic microorganisms (45), the loss of *E. yurii* in TC and EC users may increase their likelihood of infection and result in other health consequences.

Interestingly, we found that smoking had distinct effects on different functions of bacteria. For instance, in the case of C smoking, we observed an upregulation of pathways related to chlorosalicylate degradation and glycolysis IV, while photorespiration was downregulated. On the other hand, EC smoking resulted in the upregulation of chlorosalicylate degradation, but downregulation of pathways associated with 3-phenylpropanoate, p-cymene, and p-cumate degradation. 3-phenylpropanoate has been implicated in maintaining the pH required for dental plaque maintenance, suggesting that EC usage may be linked to its degradation (46). On the other hand, p-cymene is an antioxidant

and has anti-inflammatory properties, removing any suggestion that EC usage may be useful against gum disease (46). It is worth noting, however, that many of the other pathways highlighted by our analyses have not been extensively studied, and even those that have been studied lack relevant research on their implications for human health. This highlights the need for further exploration and investigation in this area.

Limitations and Future Directions As noted, the major finding of our paper is that smoking in its various forms does not produce a notable alteration of the overall alpha and beta diversity in the oral microbiota but does change the enrichment of certain species in that space. The key limitation of our findings is rooted in the lack of geographic diversity in the dataset used which makes the overall findings difficult to generalize to all smokers. Similar work should be repeated with more sizable cohorts and more geographically diverse populations to see how broadly these trends hold. Furthermore, to build on this, a future direction would be studying the functions of the differentially abundant taxa and pathways we identified in response to the effects of smoking and vaping. Although dysregulation from natural human homeostasis is typically associated with bad health outcomes, we can only truly grasp the effects of smoking in this context once we have elucidated the effect of the alterations we observe and whether they produce any large-scale effects on the prevalence and occurrence of human morbidities.

Conclusions This study analyzed the effects of traditional smoking (C) and vaping (EC) on the oral microbiome relative to a non-smoking baseline. Our results revealed that although smoking in these forms may not alter the fundamental structure of the oral microbiota with regards to alpha and beta diversity measures, C and EC both may significantly alter the composition of the oral microbiota at the species level and each appears to confer a unique taxonomic fingerprint by promoting the growth of certain key species. Furthermore, through functional analyses, we predict that the metabolic pathways within the oral microbiota of people with differing smoking backgrounds may vary significantly, with certain pathways showing more than 25-fold differences in abundance in cigarette and EC smokers relative to the non-smoking controls. Overall, our work suggests that EC usage induces dysregulation of the oral microbiota compared to non-smokers in a way that is different from C smoking. Furthermore, our results also revealed that C and EC smokers may be at a greater risk of certain oral and respiratory complications based on the identity of the indicator species found to be over-prevalent in their oral microbiota. Given the extensive research conducted on C use over past decades, it underscores the emerging concerns about the long-term effects of EC use as well.

DATA AVAILABILITY

All code used to generate data is available here: https://github.com/safaribread/10-475

ACKNOWLEDGEMENTS

We would like to thank Bessie Wang, Evelyn Sun, and Avril Metcalfe-Roach for their efforts during the course of this project in homing in on our ideas and helping give direction when we were otherwise lost. Without their efforts, this work would not have been achieved in its entirety. We would also like to thank the UBC Department of Microbiology and Immunology for the resources, and Stewart *et al.* for the dataset used in this study.

CONTRIBUTIONS

Analyses:
- Metadata Wrangling: SS, JB
- Qiime Processing: SC, NB, HL
- Alpha/beta diversity and core microbiome analysis in R: SC, JB, NB, HL
- Indicator taxa and Deseq analysis in R: JB
- PICRUSt2 analysis in R: SS

Writing:
- Abstract: SS, JB, HL
- Introduction: SS, JB, SC, NB, HL

- Methods: SS, SC, NB, HL
- Results: SS, JB, SC, NB, HL
- Discussion: SS, JB, SC, NB, HL
- Conclusion: SC
- Limitations and Future Directions: SS, HL
- Figures: SS, SC, HL
- Supplementary: HL

REFERENCES

1. Omare MO, Kibet JK, Cherutoi JK, Kengara FO. 2022. A review of tobacco abuse and its epidemiological consequences. Z Gesundh Wiss 30:1485–1500.
2. Tehrani H, Rajabi A, Ghelichi-Ghojogh M, Nejatian M, Jafari A. 2022. The prevalence of electronic cigarettes vaping globally: a systematic review and meta-analysis. Arch Public Health 80:240.
3. Hamann SL, Kungskulniti N, Charoenca N, Kasemsup V, Ruangkanchanasetr S, Jongkhajornpong P. 2023. Electronic cigarette harms: aggregate evidence shows damage to biological systems. Int J Environ Res Public Health 20.
4. Siegel J, Patel SH, Mankaliye B, Raval AP. 2022. Impact of electronic cigarette vaping on cerebral ischemia: what we know so far. Transl Stroke Res 13:923–938.
5. Espinoza-Derout J, Shao XM, Lao CJ, Hasan KM, Rivera JC, Jordan MC, Echeverria V, Roos KP, Sinha-Hikim AP, Friedman TC. 2022. Electronic cigarette use and the risk of cardiovascular diseases. Front Cardiovasc Med 9:879726.
6. Rao P, Han DD, Tan K, Mohammadi L, Derakhshandeh R, Navabzadeh M, Goyal N, Springer ML. 2022. Comparable impairment of vascular endothelial function by a wide range of electronic nicotine delivery devices. Nicotine Tob Res 24:1055–1062.
7. Neczypor EW, Mears MJ, Ghosh A, Sassano MF, Gumina RJ, Wold LE, Tarran R. 2022. E-Cigarettes and Cardiopulmonary Health: Review for Clinicians. Circulation 145:219–232.
8. Khadka S, Awasthi M, Lamichhane RR, Ojha C, Mamudu HM, Lavie CJ, Daggubati R, Paul TK. 2021. The cardiovascular effects of electronic cigarettes. Curr Cardiol Rep 23:40.
9. Stewart CJ, Auchtung TA, Ajami NJ, Velasquez K, Smith DP, De La Garza R, Salas R, Petrosino JF. 2018. Effects of tobacco smoke and electronic cigarette vapor exposure on the oral and gut microbiota in humans: a pilot study. PeerJ 6:e4693.
10. Wang Y, Wang M, Chen J, Li Y, Kuang Z, Dende C, Raj P, Quinn G, Hu Z, Srinivasan T, Hassell B, Ruhn KA, Behrendt CL, Liang T, Dou X, Song Z, Hooper LV. 2023. The gut microbiota reprograms intestinal lipid metabolism through long noncoding RNA Snhg9. Science 381:851–857.
11. Yu G, Phillips S, Gail MH, Goedert JJ, Humphrys MS, Ravel J, Ren Y, Caporaso NE. 2017. The effect of cigarette smoking on the oral and nasal microbiota. Microbiome 5:3.
12. Huang Q, Wu X, Zhou X, Sun Z, Shen J, Kong M, Chen N, Qiu J-G, Jiang B-H, Yuan C, Zheng Y. 2023. Association of cigarette smoking with oral bacterial microbiota and cardiometabolic health in Chinese adults. BMC Microbiol 23:346.
13. Marques P, Piqueras L, Sanz M-J. 2021. An updated overview of e-cigarette impact on human health. Respir Res 22:151.
14. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336.
15. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 13:581–583.
16. Wickham H. 2016. ggplot2 - Elegant Graphics for Data Analysis, 2nd ed. Springer International Publishing, Cham.
17. Kim B-R, Shin J, Guevarra R, Lee JH, Kim DW, Seol K-H, Lee J-H, Kim HB, Isaacson R. 2017. Deciphering diversity indices for a better understanding of microbial communities. J Microbiol Biotechnol 27:2089–2093.
18. Kassambara A. 2023. *ggpubr: 'ggplot2' Based Publication Ready Plots*. R package version 0.6.0, https://rpkgs.datanovia.com/ggpubr/.
19. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen T, Miller E, Bache S, Müller K, Ooms J, Robinson D, Seidel D, Spinu V, Takahashi K, Vaughan D, Wilke C, Woo K, Yutani H. 2019. Welcome to the tidyverse. JOSS 4:1686.
20. Gao C-H, Yu G, Cai P. 2021. ggVennDiagram: An Intuitive, Easy-to-Use, and Highly Customizable R Package to Generate Venn Diagram. Front Genet 12:706907.
21. Gao C, Chen C, Akyol T, Dusa A, Yu G, Cao B, Cai P. 2024. ggVennDiagram: Intuitive Venn diagram software extended. iMeta 3.
22. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8:e61217.
23. Cáceres M, Legendre P. 2009. Associations between species and groups of sites: indices and statistical inference. Ecology 90:3566–3574.
24. De Cáceres M, Sol D, Lapiedra O, Legendre P. 2011. A framework for estimating niche metrics using the resemblance between qualitative resources. Oikos 120:1341–1350.
25. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550.
26. Mirarab S, Nguyen N, Warnow T. 2012. SEPP: SATé-enabled phylogenetic placement. Pac Symp Biocomput 247–258.
27. Louca S, Doebeli M. 2018. Efficient comparative phylogenetics on large trees. Bioinformatics 34:1053–1055.

28. Ye Y, Doak TG. 2009. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. PLoS Comput Biol 5:e1000465.
29. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, Huttenhower C, Langille MGI. 2020. PICRUSt2 for prediction of metagenome functions. Nat Biotechnol 38:685–688.
30. Douglas GM, Hayes MG, Langille MGI, Borenstein E. 2022. Integrating phylogenetic and functional data in microbiome studies. Bioinformatics 38:5055–5063.
31. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu W-H, Lakshmanan A, Wade WG. 2010. The human oral microbiome. J Bacteriol 192:5002–5017.
32. Aherrera A, Lin JJ, Chen R, Tehrani M, Schultze A, Borole A, Tanda S, Goessler W, Rule AM. 2023. Metal Concentrations in E-Cigarette Aerosol Samples: A Comparison by Device Type and Flavor. Environ Health Perspect 131:127004.
33. Olmedo P, Goessler W, Tanda S, Grau-Perez M, Jarmul S, Aherrera A, Chen R, Hilpert M, Cohen JE, Navas-Acien A, Rule AM. 2018. Metal Concentrations in e-Cigarette Liquid and Aerosol Samples: The Contribution of Metallic Coils. Environ Health Perspect 126:027010.
34. Polosa R, Thomson NC. 2013. Smoking and asthma: dangerous liaisons. Eur Respir J 41:716–726.
35. Nociti FH, Casati MZ, Duarte PM. 2015. Current perspective of the impact of smoking on the progression and treatment of periodontitis. Periodontol 2000 67:187–210.
36. Senaratne NLM, Yung On C, Shetty NY, Gopinath D. 2024. Effect of different forms of tobacco on the oral microbiome in healthy adults: a systematic review. Front Oral Health 5:1310334.
37. Pfeiffer S, Herzmann C, Gaede KI, Kovacevic D, Krauss-Etschmann S, Schloter M. 2022. Different responses of the oral, nasal and lung microbiomes to cigarette smoke. Thorax 77:191–195.
38. Antonello G, Blostein F, Bhaumik D, Davis E, Gögele M, Melotti R, Pramstaller P, Pattaro C, Segata N, Foxman B, Fuchsberger C. 2023. Smoking and salivary microbiota: a cross-sectional analysis of an Italian alpine population. Sci Rep 13:18904.
39. Palmacci C, Antocicco M, Bonomo L, Maggi F, Cocchi A, Onder G. 2009. Necrotizing pneumonia and sepsis due to Clostridium perfringens: a case report. Cases J 2:50.
40. Hernández M, de Frutos M, Rodríguez-Lázaro D, López-Urrutia L, Quijada NM, Eiros JM. 2018. Fecal Microbiota of Toxigenic Clostridioides difficile-Associated Diarrhea. Front Microbiol 9:3331.
41. Chukkapalli SS, Rivera-Kweh MF, Velsko IM, Chen H, Zheng D, Bhattacharyya I, Gangula PR, Lucas AR, Kesavalu L. 2015. Chronic oral infection with major periodontal bacteria Tannerella forsythia modulates systemic atherosclerosis risk factors and inflammatory markers. Pathog Dis 73:1–12.
42. Munoz NR, Ghorbani A, Agwuegbo CC, Vincent Coralde JM. 2023. Dental Infection Causing Methicillin-Resistant Staphylococcus aureus Bacteremia and Spinal Infection: A Case Report. Cureus 15:e51136.
43. Estripeaut D, Sáez-Llorens X. 2009. PERINATAL BACTERIAL DISEASES, p. 979–1020. *In* Feigin and cherry's textbook of pediatric infectious diseases. Elsevier.
44. Tanaka S, Yoshida M, Murakami Y, Ogiwara T, Shoji M, Kobayashi S, Watanabe S, Machino M, Fujisawa S. 2008. The relationship of Prevotella intermedia, Prevotella nigrescens and Prevotella melaninogenica in the supragingival plaque of children, caries and oral malodor. J Clin Pediatr Dent 32:195–200.
45. Whitfield C, Szymanski CM, Lewis AL, Aebi M. 2022. Eubacteria, p. . *In* Varki, A, Cummings, RD, Esko, JD, Stanley, P, Hart, GW, Aebi, M, Mohnen, D, Kinoshita, T, Packer, NH, Prestegard, JH, Schnaar, RL, Seeberger, PH (eds.), Essentials of Glycobiology, 4th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor (NY).
46. Jayasinghe TN, Harrass S, Erdrich S, King S, Eberhard J. 2022. Protein Intake and Oral Health in Older Adults-A Narrative Review. Nutrients 14.