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The effect of *Beta vulgaris* on an *in vitro* oral microbiome of electronic cigarette users

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ABSTRACT

Background: Although touted as a safer alternative to cigarette smoking, electronic cigarette usage has been increasingly linked to a myriad of health issues and appears to impact the oral microbiome. Meanwhile, nitrate supplementation has shown promise as a prebiotic that induces positive effects on the oral microbiome.

Methods: In this pilot study, the impact of nitrate supplementation as a countermeasure to e-cigarette usage was explored using *in vitro* growth and 16S rRNA analysis of microcosms derived from e-cigarette users and nonusers and supplementation with nitrate-rich beetroot juice extract.

Results: The impacts of e-cigarette usage and beetroot supplementation were somewhat limited, with beetroot juice extract supplementation having a significant impact on diversity according to some, but not all, diversity metrics examined. The saliva of the e-cigarette users was depleted in nitrate-reducing Neisseria spp. In terms of differentially abundant individual taxa, the addition of beetroot juice extract to the saliva-derived microcosms had a larger impact on the communities derived from the e-cigarette users compared to that of the nonusers.

Conclusions: Overall, this limited pilot study suggests that beetroot juice extract supplementation may impact the microbiota of e-cigarette users and adds to contemporary research paving the way for more in-depth studies examining the role of nitrate-rich supplements as prebiotics to promote oral health.

KEY FINDINGS

1. In line with previous research, the saliva of e-cigarette users had a lower pH and was depleted of Neisseria spp. generally regarded as health-associated nitrate reducers in the context of oral health.
2. In this pilot study group, the impacts of e-cigarette usage and beetroot supplementation were somewhat limited, with beetroot juice extract supplementation having a significant impact on diversity according to some, but not all, diversity metrics examined.
3. This study suggests that beetroot juice extract supplementation may impact the microbiota of e-cigarette users and adds to contemporary research paving the way for more in-depth studies examining the role of nitrate-rich supplements as prebiotics to promote oral health.

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E-cigarettes; nitrate; oral microbiome; prebiotic; saliva

Introduction

The oral cavity is home to a diverse microbiota of bacteria, archaea, viruses, fungi, and protozoa, which collectively have a profound impact on oral health and an increasingly recognized impact on overall health [1]. These microorganisms live in diverse microenvironments within the mouth, including the teeth, gums, and hard or soft palate [1]. The two most common diseases of the human oral cavity – dental caries and periodontal disease – both have microbial etiologies [2,3]. Furthermore, there is accumulating evidence linking the oral microbiota to systemic conditions such as diabetes, cardiovascular disease, Alzheimer's disease, obesity, and others [4]. Meanwhile, the commensal bacteria of the oral microbiota induce protective responses in the immune system and may prevent the colonization of pathogenic bacteria, in many cases, through the production of antimicrobial compounds [5–7].

Increasing use of electronic cigarettes (e-cigarettes), especially among young people, represents a significant public health concern [8,9]. The use of e-cigarettes impacts oral health and the oral microbiota [10] with e-cigarette usage being associated with increased alpha diversity, increased *Veillonella* and *Haemophilus* spp. in saliva and the buccal mucosa [11], and a higher abundance of periodontal pathogens such as *Porphyromonas gingivalis, Fusobacterium nucleatum, Bacteroidales*, and

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other Gram-negative anaerobes in the subgingival community [12–14]. Aerosols of the main ingredients of e-cigarettes, such as propylene glycol and glycerol, also increased pathogenic biofilms and cariogenic potential and favored the growth of *Streptococcus mutans* over commensal *Streptococcus* spp [15,16]. In addition to impacts on the oral microbiome, *in vitro* and *in vivo* studies of the effects of e-cigarette aerosol showed increases in acid damage to tissues in the oral cavity (including tooth enamel), as well as oxidative stress, DNA strand breakage, and increased concentration of pro-inflammatory cytokines, such as keratinocyte-derived cytokine (KC) and interleukin-6 (IL-6) [17–20]. The altered immune response may also lead to increased oropharyngeal colonization by the pathogen *Staphylococcus aureus* [21]. Given the importance of promoting oral health, identifying substances that can counteract the detrimental health effects of e-cigarettes on the oral microbiome would be valuable.

Recent research illustrated that nitrate and nitrate-containing foods can increase the relative abundance of commensal bacteria and decrease the abundance of pathogenic bacteria, resulting in decreased acid production and increased ammonium production [22–25]. Importantly, these factors may raise the local pH and consequently reduce tooth decay and oral inflammation [22]. The root of the plant *Beta vulgaris*, commonly known as beetroot, is a high-nitrate vegetable which is also rich in polyphenols such as rosmarinic acid, carnosic acid, chlorogenic acid, rosmanol, camphor, and eucalyptol [26,27]. Furthermore, since substances high in nitrate have shown promise in promoting optimal bacterial composition (i.e. within the oral microbiome), testing the effect of exposure to high-nitrate substances, such as *Beta vulgaris*, on the oral microbiome of e-cigarette users could yield important information for the development of potential commercial countermeasures involving the incorporation of dietary beetroot to combat the negative effects of e-cigarettes on the oral cavity.

In this study, oral microcosms were obtained from the saliva of e-cigarette users and nonusers and grown *in vitro* in the presence or absence of *Beta vulgaris* juice extract. 16S rRNA gene sequencing was used to examine differences between the composition of the communities derived from the e-cigarette users and nonusers and to determine the effects of *Beta vulgaris* on the simulated oral microbiome.

Materials & methods

Study group and sample collection

Lakeland Community College (Kirtland, Ohio) Institutional Review Board approved the study and recruitment advertisements for participants (IRB 06231). Nonuser controls ('nonusers') were defined as subjects who self-reported no use of e-cigarettes. Electronic cigarette users ('e-cigarette users') were defined as subjects who reported use of an electronic cigarette device (also known as e-device, vape pen, box mods, pod device, or any other vaping device). A total of 14 subjects were recruited, with 7 participants in the nonusers group and 7 participants in the e-cigarette users group. Participants ranged from 18 years old to 38 years old, with an average age of 24, comprising 8 males and 6 females. Consent was received from all subjects (Supplemental File S1) and the subjects were asked to complete a questionnaire (Supplemental File S2) obtaining metadata regarding gender, age, race/ethnicity, oral health conditions, diet, recent food consumption, e-cigarette and liquid type and frequency of use. For frequencies of use, responses were scored using the following conversions to generate a numeric score for each response: never = 0; a few times a year = 1; 1–3 times per month = 2; 1–2 times per week = 3; 3–4 times per week = 4; 1 time per day = 5; 2 or more times per day = 6. The resulting scores for consumption of chocolate, ice cream, candy and soda were added together for each subject to give an overall 'sugar consumption score'. Due to either lack of differences in response, or relevance to this study, only the metadata categories Group, Gender, Age, Coffee, Alcohol, and Overall Sugar Score were further analyzed here, as described below in the Downstream analyses section. Subjects were asked to not eat or drink anything after midnight the night before collection nor brush their teeth in the morning prior to collection. 10 mL of saliva was self-collected by having participants spit into a provided OMNIgene-oral collection device (DNA Genotek) using the provided instructions (Supplemental File S3). Samples were hand delivered the same day. Upon receipt of the samples, glycerol was added to the saliva samples to 50% and the samples were frozen in liquid nitrogen.

Growth of microcosms

Samples were thawed on ice, the pH of each sample was recorded, and 100 µL of the saliva samples were added to 7 ml of either BHI or BHI +30 mg/mL beetroot juice extract (Alovitox, Inc.) and incubated at 37°C under 95% air/5% CO_2. Aliquots were collected at 0 hours, 3.6 hours, and 5 hours for optical density at 600 nm (OD_{600}) and colony forming unit (CFU) measurements. For CFU measurements, serial dilutions were plated on BHI agar, incubated at 37°C under 95% air/5% CO_2, and colonies were counted after 1 day. Plots of optical density and CFU/mL were generated using Microsoft Excel (v16.92) and GraphPad Prism (v10.4.1). Statistical analyses were performed using GraphPad Prism (v10.4.1). After

the 5 hour collection time point, the remainder of the microcosm samples were centrifuged for 10 min at 1800 × g; cell pellets were decanted and stored at −80°C for sequencing.

16S rRNA amplicon sequencing

Cell pellets and remaining saliva samples were thawed on ice and DNA was extracted using the Zymo Quick-DNA Fungal/Bacterial Miniprep Kit according to the manufacturer's instructions. Sequencing libraries were generated using the 16S Barcoding Kit 24 (Oxford Nanopore Technologies, Inc.) according to the manufacturer's instructions. The 42 samples were sequenced across 4 Flongle Flow Cells (R10.4.1; Oxford Nanopore Technologies, Inc.) on a MinION sequencer (Oxford Nanopore Technologies, Inc.). 24 and 18 samples (i.e. all 42 samples in total), were initially sequenced across two Flongle Flow Cells, respectively. Any samples not reaching the critical point on a rarefaction curve (in this case, about 5000 reads) were further sequenced on the additional two Flongle flow cells such that all samples had sufficient depth to pass the critical point on a rarefaction curve. Therefore, saliva samples C6 and S6, microcosms without beetroot S1 and C7, and microcosms with beetroot S6 were sequenced on two additional Flongle flow cells. Basecalling and demultiplexing were performed in real-time using MinKnow v24.06.16 (Oxford Nanopore Technologies, Inc.). The 16S workflow in EPI2ME (Oxford Nanopore Technologies, Inc.; v5.2.1) was used to perform taxonomic classification of sequencing reads. All parameters were kept at default except Taxonomic Rank, which was set at the S (species) level. Species-level abundance tables (i.e. feature tables) were exported in .csv format and imported into QIIME2 v2024.10.1 [28]. Feature tables from each sequencing run (i.e. the 4 Flongle flow cells) were merged using `qiime feature-table merge`.

Downstream analysis

Downstream analyses were conducted using QIIME2 (v2024.10.1). As the EPI2ME 16S workflow does not provide phylogenetic analysis output files, the RefSeq 16S and 18S database (as of 12/11/2024) was downloaded and 16S sequences matching the species-level features in the dataset from this study, as identified by EPI2ME, were extracted. Using the resulting sequences, a phylogenetic tree was generated using `qiime phylogeny align-to-tree-mafft-fasttree`. Alpha diversity was analyzed using the Shannon, Simpson, Chao1, and Faith's PD metrics. Beta diversity was analyzed using the Bray-Curtis, Jaccard, RPCA [29], Phylogenetic RPCA, Weighted UNIFRAC [30], and Unweighted UNIFRAC methods. Phylogenetic Compositional Tensor Factorization (Phylo-CTF) was performed using Gemelli v0.0.12 [31]. Differential abundance analyses were performed using ANCOM-BC [32] implemented in QIIME2 v2024.10.1 and Songbird v1.0.4 [33] implemented in QIIME2 v2020.6.0. Beta diversity and CTF plots were visualized using Emperor v2024.10.0 [34]. Phylogenetic RPCA and Phylo-CTF ordination plots were visualized using Empress v1.2.0 [35]. All scripts and code from this study are provided in the GitHub repository associated with this project: https://github.com/jonbakerlab/beetroot-microcosms.

Results

Study design and growth of the microcosms

A total of 7 nonusers and 7 e-cigarette users were recruited to this study, self-collected 10 mL of saliva, and filled out the questionnaire. The collected subject metadata is provided in Supplemental Table S1. The initial colony-forming units (CFU/mL) of the saliva was measured on BHI agar and ranged from 1.96×10^5 to 3.36×10^7. The CFU/mL of the collected saliva of the e-cigarette users were not significantly different than those of the nonusers (Figure 1a). The pH of the initial saliva was measured and the saliva of the e-cigarette users had a significantly lower pH than that of the nonusers (Figure 1b). 100 µL of each of the saliva samples was added to 7 ml of BHI with or without 30 mg/mL beetroot juice powder and incubated at 37°C for 5 hours. Growth of the resulting microcosms was monitored using OD_{600} and CFU. The microcosms from the saliva of the e-cigarette users had a lower mean OD_{600} and CFU than those from the saliva of the nonusers and the addition of beetroot to the growth media reduced the mean CFU in the microcosms from both study groups. However, these differences were not substantial enough for a $p < 0.05$ with the following exceptions: the CFU of the nonusers with beetroot was significantly lower than the nonusers without beetroot at 3.6 hours and the CFU of the e-cigarette users with beetroot was significantly lower than that of the nonusers without beetroot at 5 hours (data not shown). Taken together, these results indicate that the addition of beetroot to the growth media did not have a major impact on overall bacterial growth under the conditions tested.

Alpha diversity analyses of saliva and microcosms

To determine the microbial composition of the saliva samples and resulting microcosms grown with or without the addition of beetroot juice powder, 16S rRNA amplicon analysis was performed on the starting saliva samples as well as the 4 microcosms after

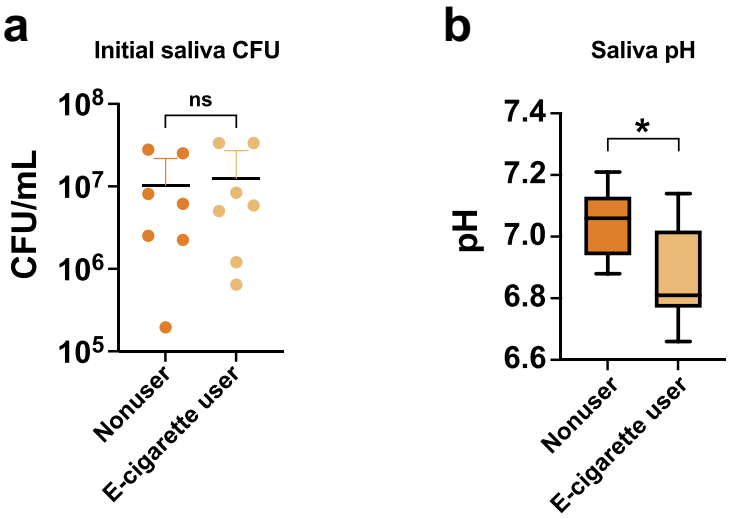

Figure 1. pH of initial saliva and growth of the microcosms. (A) Scatter plot illustrating the initial CFU/mL of the saliva samples. ns, not significant. (B) Box and whisker plot illustrating the pH of the saliva collected from the nonusers compared to the e-cigarette users. Asterisk denotes statistical significance ($p < 0.05$) between the two groups as determined by an unpaired t-test.

5 hours of growth. Supplemental Table S2 is the feature table providing the relative abundances of each bacterial species identified in each sample using the approach employed here. The number of observed features was significantly higher in the starting saliva, with ~ 100 species observed in all saliva samples and ~ 40 species observed in all microcosm samples at a depth of 5000 reads (Figure 2a). This is to be expected, as growth in BHI media ± beetroot in aerobic conditions will select for the growth of certain taxa, with minimal growth of anaerobes and microaerophiles. Correspondingly, alpha diversity of the starting saliva samples was also significantly higher than all microcosm samples using all 4 metrics of alpha diversity employed here (Shannon, Simpson, Chao1, and Faith's PD; Figure 2, panels B and C). Alpha diversity of the saliva of the e-cigarette users was higher than that of the nonusers according to all 4 metrics, however this difference was not statistically significant (Figure 2, panels B and C). Among the microcosms, alpha diversity of the e-cigarette user microcosms was significantly lower than that of the nonusers in the absence of beetroot according to the Shannon or Simpson metrics, but not the Chao1 or Faith's PD metrics; there was no statistical difference between the e-cigarette user and nonuser microcosms under any metrics in the presence of beetroot. Addition of beetroot to the BHI growth media of the microcosms had led to a significant decrease of alpha diversity in both the nonuser and e-cigarette user groups according to the Shannon and Simpson metrics but not the Chao1 or Faith's PD metrics (Figure 2, panels B and C). In addition to study group (e-cigarette user versus nonuser) and presence or absence of beetroot, the impact of subject gender, age, coffee consumption score, alcohol consumption score, and sugar consumption score on alpha diversity was also examined. A higher alcohol consumption score was correlated to a reduction in alpha diversity based on the Shannon and Simpson metrics (Figure S1). Alcohol consumption may represent a confounder in this study, as 5 of the 7 nonusers also reported the highest levels of alcohol consumption across all subjects. Increased coffee consumption correlated to a significantly lower alpha diversity according to the Chao1 metric (Figure S1). No other subject metadata had a significant impact on alpha diversity based on the metrics examined. Taken together, these results indicate that the addition of beetroot reduced the alpha diversity of the microcosms of both the e-cigarette users and the nonusers.

Beta diversity analyses of the saliva and microcosms

Similar to the alpha diversity analyses, beta diversity of the saliva samples was found to be significantly different from the microcosm samples via a pairwise PERMANOVA across all metrics tested (Bray-Curtis, Jaccard, RPCA, phyloRPCA, Weighted UNIFRAC, Unweighted UNIFRAC; Figure 2, panels D-G). However, the beta diversity of the saliva of the smokers versus the nonsmokers was not significantly different, by any of the metrics. In the absence of beetroot, beta diversity between the microcosms of the nonusers and the corresponding e-cigarette users showed a significant difference based on the Bray-Curtis metric. The treatment of beetroot to the microcosms had a significant impact on beta diversity for both the e-cigarette user and nonuser microcosms according to the Weighted UNIFRAC metric and the Bray-Curtis metric for the nonuser microcosms,

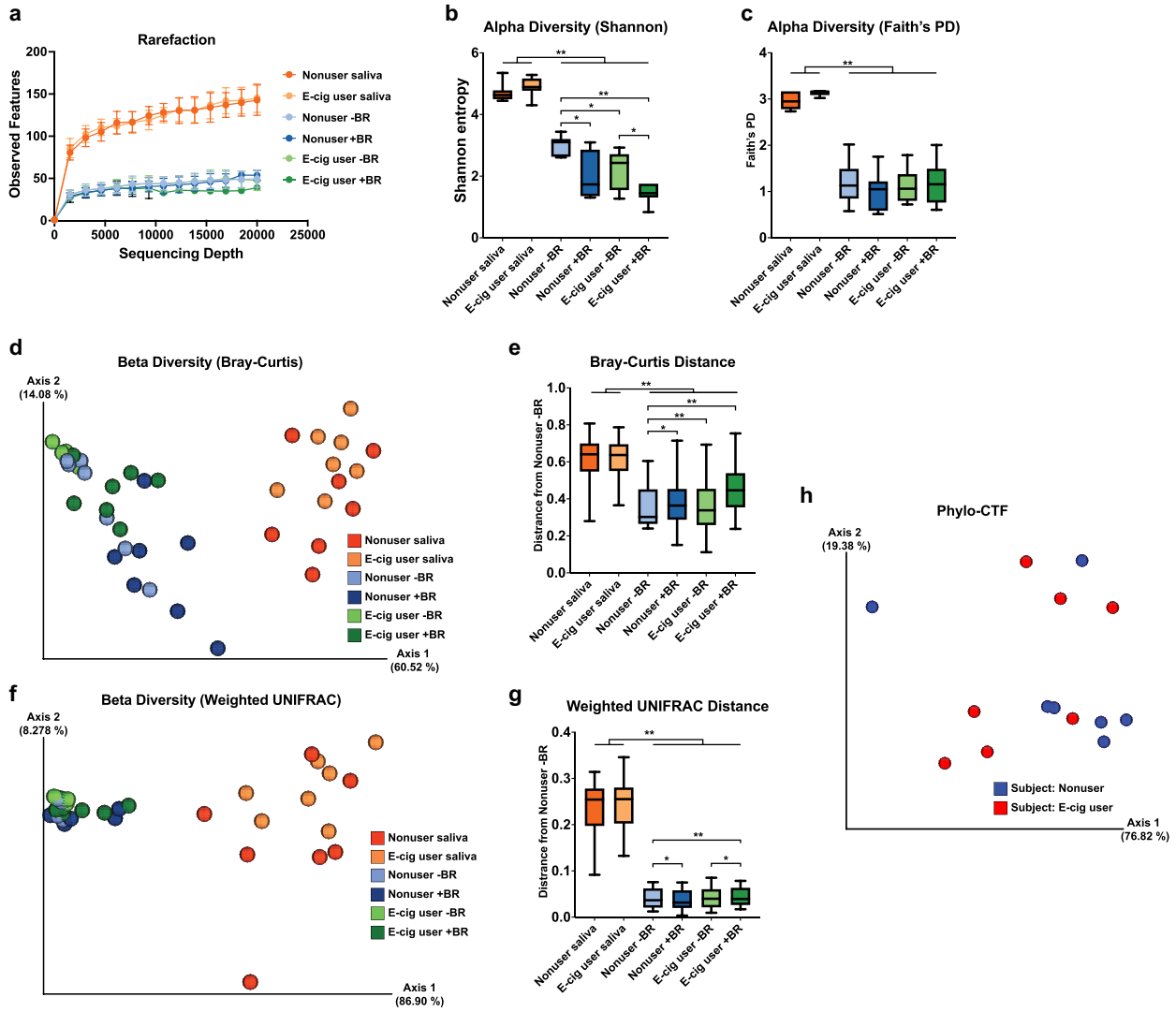

Figure 2. Diversity analyses of the saliva and microcosms. (A) Rarefaction curves illustrating the number of average observed taxonomic features (species) present at increasing sequencing depth for each indicated group of samples; $n = 7$. (B-C) box and whisker plots indicating the alpha diversity of each indicated group of samples based on the Shannon (panel B) and Faith's phylogenetic diversity (panel C) metrics. Asterisks denote statistical significance between indicated groups as determined by a Kruskal-Wallis test (*, q < 0.05; **, q < 0.01; *** q < 0.001) ($n = 7$). (D) PCoA plot of beta diversity according to the Bray-Curtis metric with data points representing individual samples colored by the indicated group of samples. (E) Box and whisker plot indicating the Bray-Curtis distances of the indicated groups of samples from the nonuser microcosms without beetroot juice extract. Asterisks denote statistical significance between indicated groups of samples as determined by a pairwise PERMANOVA (* q < 0.05, ** q < 0.01) ($n = 7$). (F) PCoA plot of beta diversity according to the weighted UNIFRAC metric with data points representing individual samples colored by the indicated group of samples. (G) Box and whisker plot indicating the weighted UNIFRAC distances of the indicated groups of samples from the nonuser growth group. Asterisks denote statistical significance between indicated groups of samples as determined by a pairwise PERMANOVA (*, q < 0.01; ** q < 0.05) ($n = 7$). ±BR = presence or absence of beetroot juice extract (H) PCoA plot of phylo-CTF according to the data points representing individual subjects colored by the indicated group of samples.

exclusively – no other metrics tested proved significant (Figure 2, panels D-G). In addition to study group (e-cigarette user versus nonuser) and presence or absence of beetroot, the impact of gender, age, coffee consumption score, alcohol consumption score, and sugar consumption score on beta diversity was also examined. Alcohol consumption score had an impact on beta diversity based on both the Bray-Curtis and Jaccard metrics (Figure S1). No other subject metadata had a significant impact on beta diversity according to the metrics examined. These data collectively indicate a high degree of subject-to-subject differences (as is common in microbiomes) in the saliva, which translated to individual differences in the microcosms. Compositional tensor factorization (CTF) was employed to tease out differences between study groups where there are repeated measures of the same subject in spite of subject-to-subject variability [31]. Phylo-CTF analysis of the dataset separated the e-cigarette user and nonuser study groups in ordination space better than any of the individual sample beta diversity measures

(Figure 2h), however, the difference was still not significant according to a PERMANOVA. Taken together, beta diversity analyses indicated that the addition of beetroot to the growth media of the microcosms had a modest impact on the beta diversity of these communities.

Differential abundance analysis

Differential abundance analysis of taxonomic features in microbiome datasets is fraught with challenges due to the compositional nature of the data [36]. Different approaches have different pitfalls and yield different results, with no one method being recognized as a best practice [37]. Therefore, two different types of approaches were used to examine differentially abundant species in this dataset: ANCOM-BC [32] and Differential Ranking (DR; implemented using Songbird) [33], with the rationale that differential taxonomic features identified by both independent methods would warrant a higher degree of confidence. Note that Songbird provides highly accurate ranks but does not provide a p-value, q-value, or other means statistical significance determination; therefore, taxa identified as significantly differentially abundant (q-value < 0.01) by ANCOM-BC, and their agreement with Songbird, are mainly what is discussed here. However, the full Songbird rankings are available in Figure S2. According to ANCOM-BC, when compared to the saliva from the nonusers the saliva of the e-cigarette users was enriched in *Prevotella histicola* and depleted in *Neisseria sicca, Streptococcus timonensis, Capnocytophaga sputigena*, and *Neisseria elongata* (Figure 3a). This was corroborated by DR, with *Prevotella histicola* having the highest rank associated with the saliva of the e-cigarette users and *Neisseria elongata, Neisseria sicca*, and *Streptococcus timonensis* having the respective 1st, 7th and 9th ranks associated with the nonusers (Figure 3a). *Capnocytophaga sputigena* did not meet the abundance requirements for Songbird, the DR tool employed here. When comparing the microcosms of the e-cigarette users versus the nonusers, in the absence of beetroot, *Streptococcus thermophilus* was the only species identified by ANCOM-BC as significantly enriched and it also was the 3rd highest ranked taxa identified as associated with the saliva of the e-cigarette users by Songbird (Figure 3b). According to ANCOM-BC there were no differentially abundant features with a q-value <0.01 between the saliva of the nonusers with beetroot and the saliva of the nonusers without beetroot. Interestingly, Songbird indicated that the addition of beetroot increased relative abundance of the nitrate reducing

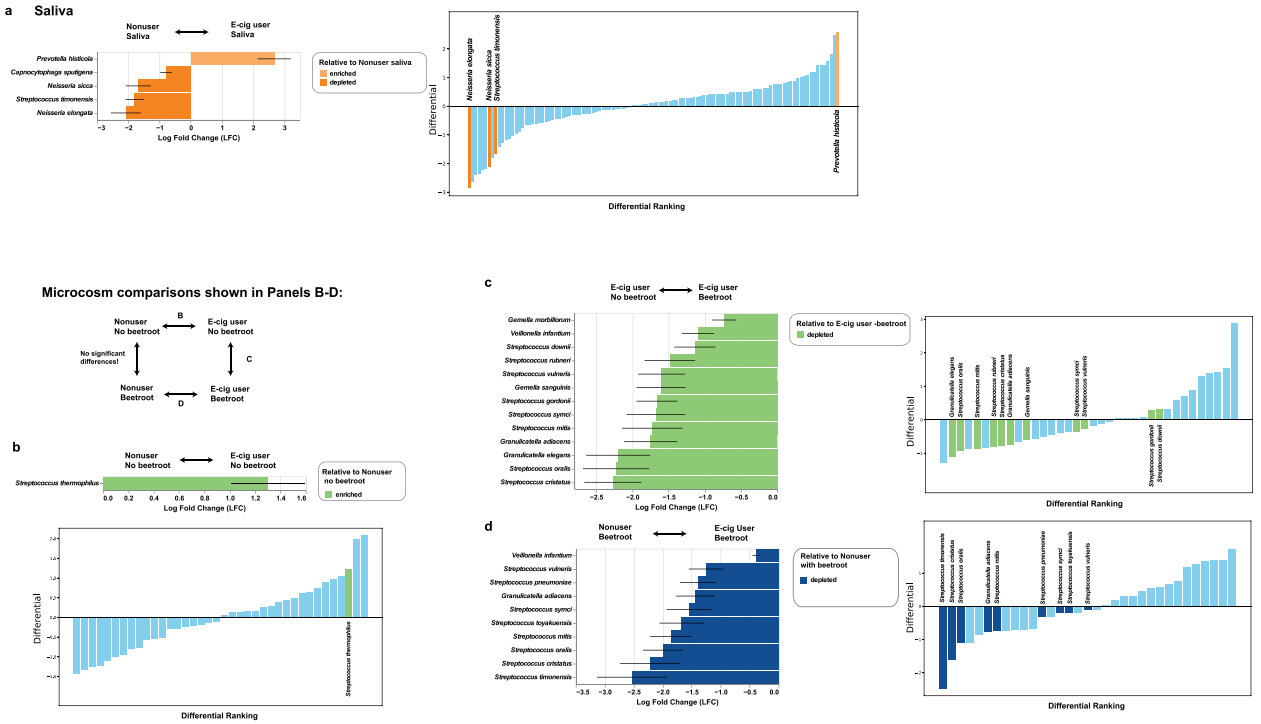

Figure 3. Species-level differential abundance analysis of the saliva and microcosms. For each panel, the bar chart on the left illustrates the results of the ANCOM-BC analysis indicating the log fold change of the indicated taxa between the two indicated conditions. Only taxa with a q-value < 0.01 are shown. The bar plot on the right shows the ranking of the differentials as determined by Songbird, with the taxa identified as significant from the ANCOM-BC analysis highlighted. Note that ANCOM-BC did not identify any taxa that were at a significant differential abundance between the nonusers with beetroot and the nonusers without beetroot microcosms. Bars are colored such that the group of samples where the given taxa have a higher abundance matches that group's color label in Figure 1. Complete Songbird data with all taxa labeled are available in Supplemental Figure S2.

organism, *Haemophilus parainfluenzae* (Figure S2). According to ANCOM-BC, the addition of beetroot to the growth media of the microcosm for the e-cigarette users led to a significant decrease in *Streptococcus cristatus, Streptococcus oralis, Granulicatella elegans, Granulicatella adiacens*, and several other *Streptococcus, Gemella*, and *Veillonella* spp (Figure 3c). These were largely corroborated by Songbird, except for *Streptococcus gordonii* and *downeii* (Figure 3c). When comparing the microcosms grown from the saliva of the e-cigarette users with beetroot versus the nonusers with beetroot, ANCOM-BC identified several *Streptococcus* species including *timonensis, cristatus, oralis*, and *mitis* as most depleted in the microcosms from the e-cigarette users with beetroot, which was in strong agreement with the DRs of this comparison (Figure 3d). Overall, these data indicate a depletion of *Neisseria* spp. in the starting saliva of the e-cigarette users compared to that of the nonusers. Given the number of differentially abundant species, the addition of beetroot seemed to have a larger effect on the microcosms from the e-cigarette users compared to the nonusers. Notably, although they were not identified as significantly different by ANCOM-BC, Songbird identified the nitrate reducers *Haemophilus parainfluenzae* and *Rothia mucilaginosa* as associated with the addition of beetroot, while beetroot appeared to reduce *Veillonella* spp.

Discussion

Although originally touted as a healthier alternative to traditional cigarette smoking, use of e-cigarettes has increasingly been linked to a myriad of negative impacts on health [8,9]. This is particularly concerning given the increase in e-cigarette use, especially among young people [8,9]. Research has shown that, in addition to direct negative effects on user health, use of e-cigarettes also impacts the oral microbiome of the user, which may then indirectly affect the health of the user [10]. Meanwhile, nitrate and nitrate-rich foods have shown promise as an oral prebiotic, while nitrate-reducing bacteria have shown promise as probiotics [23,38]. Several nitrate reducing taxa such as *Rothia, Haemophilus*, and *Neisseria* have also been linked to good dental health [39]. This pilot study aimed to examine the oral microbiome of e-cigarette users versus nonusers and use microcosms derived from these microbiomes to examine the impact of beetroot juice powder – rich in nitrate – on these microcosms; the hypothesis being that the nitrate in the beetroot juice powder may serve to ameliorate some of the ecological changes induced by e-cigarette use.

Although the initial saliva collected from the e-cigarette users did not have a significantly different bacterial load as measured by CFU, the pH of the saliva from the e-cigarette users was significantly lower than that of the nonusers (Figure 1, panels A and B). Several previous studies have also noted more acidic saliva in e-cigarette users compared to nonusers [40,41], which is likely to place these subjects at increased risk for dental caries. Addition of the beetroot juice extract to the microcosms lowered the CFU/mL of the communities in the nonusers but not the e-cigarette users and the optical density of the cultures was not significantly impacted by addition of the beetroot juice extract, indicating that it had minor effects on bacterial growth of the microcosms overall.

Although the initial saliva samples did not have a significantly different alpha or beta diversity between the e-cigarette user and nonuser groups, the resulting microcosms grown from the e-cigarette users had a significantly lower alpha diversity, although this effect was not robust enough to be significant across all tested alpha diversity metrics (Figure 2, panels B and C). This may indicate a higher abundance of bacteria in the saliva from the e-cigarette users that could not grow in the microcosm conditions tested here (e.g. anaerobes). Application of Phylo-CTF to the dataset to reduce the impact of individual subject-to-subject variability did lead to a more pronounced separation between the nonusers and e-cigarette users in ordination space compared to the individual beta diversity metrics (Figure 2h), however, this difference was still not statistically significant. It is possible that a larger sample size would result in more robust statistical differences between the e-cigarette user and nonuser microbiomes. Addition of the beetroot juice extract to the microcosms led to a reduction in alpha diversity and change in beta diversity in both the microcosms derived from the e-cigarette users and nonusers, however, these effects were also not robust enough to be significant across all metrics tested. Again, this may be due to the limited number of samples in this study.

Differential abundance analysis indicated a reduction in *Neisseria* and *Capnocytophaga* spp. in the saliva of the e-cigarette users, which was also seen previously [12,42,43]. *Neisseria* spp., in particular, are nitrate-reducing organisms that are increasingly associated with good oral health [1]. Differences between the microcosms derived from the e-cigarette users without beetroot compared to the nonusers without beetroot were more limited, likely due to the reduction in alpha diversity of these communities compared to the starting saliva. Beetroot juice as a nitrate supplement has been shown to impact the oral microbiota, increasing *Neisseria* spp. at the

expense of *Prevotella* [44,45]. Furthermore, *Rothia* spp [22]. and *Haemophilus* spp [45]. have also been identified as health-associated nitrate-reducing species with increased abundance following nitrate supplementation. Although they were not identified here as significantly increased in the presence of beetroot by ANCOM-BC, they were positively correlated with addition of beetroot according to Songbird (Supplemental Figure S2). The lack of statistical significance of these trends may again be due to the limited sample size of this study. It is notable that the addition of beetroot juice extract appeared to impact the microcosms derived from the e-cigarette users more than those derived from the nonusers (according to ANCOM-BC, 13 taxa with significantly different relative abundances ± beetroot juice extract in the user-derived microcosms versus no taxa with significantly different relative abundances ± beetroot juice extract in the nonuser-derived microcosms). This finding encourages further studies examining whether this indicates that beetroot juice extract supplementation can mitigate some of the negative effects of e-cigarette usage on the oral microbiome.

There are several limitations to this pilot study. First is the small sample size, which limits interpretation of the results. For example, within this study group, the nonusers reported a higher consumption of alcohol. This is not concordant with observations at the population level (i.e. heavier drinkers are more likely to also use e-cigarettes and vice versa). Furthermore, this may confound the results of this study to some degree as alcohol has its own impact on the oral microbiome [46,47]. Notably, however, in this study increased alcohol consumption was associated with reduced alpha diversity, while other studies have observed an increase in alpha diversity [47]. A second limitation of this study was the lack of knowledge about the actual concentration of nitrate and other natural products in the beetroot juice extract. A previous study showed up to 50-fold variability in nitrate concentration across different brands (and even up to 30% lot to lot variability within brands) of beetroot juice extract supplements [48]. Future, more in-depth studies should perform a detailed chemical analysis of the beetroot juice extract supplement being examined. A third limitation was that the growth conditions of the microcosms (planktonic, 95% air/5% CO_2) were not reflective of the diverse environments of the oral cavity and specific niches within the oral microbiome and select for the growth of specific organisms, as illustrated by the large reduction in alpha diversity between the initial saliva samples and the microcosms (Figure 2). For example, the conditions used here did not allow for the growth of anaerobes, which can impact oral health (e.g. the canonical periodontal pathogens *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola*). The freeze/thaw cycle that happened during sample transport may also have impacted the viability of the samples, and some species within, however all samples were treated with the same freeze and thaw steps. The beetroot juice extract itself also contained additional nutrients besides the nitrate. For example, the beetroot juice extract contained 6 g of sugar per 8 g serving, meaning that the BHI media with the beetroot had an additional 2.25% sugar by weight. These metabolites may have impacted the community, however the enrichment of nitrate-reducing bacteria suggests that nitrate may have been a significant driver of the observed changes. Future studies could compare the impact of these metabolites (nitrate, polyphenols, etc.) to the growth of the microcosms on an individual basis. Furthermore, the use of 16S rRNA amplicon sequencing employed here to perform the microbiome analysis, although economical, has several disadvantages. First is that it only detects bacteria, ignoring other members of the microbiota and microcosms such as fungi and viruses, which are also likely to be impacted by e-cigarette usage and by addition of beetroot juice powder. Second is that the PCR amplification can add bias to the taxonomic features detected. Third is the limited taxonomic resolution of 16S rRNA amplicon sequencing compared to metagenomic sequencing (although this is mitigated to some degree here by sequencing of the entire 16S rRNA genes, rather than specific variable region(s)). Amplicon-based microbiome analyses, such as 16S rRNA-based analysis, can also only provide rough estimates of the metabolic pathways encoded by microbial communities as intra-taxa pangenomic differences cannot be resolved. Finally, the self-collection of the saliva and self-reporting of the metadata may also have impacted the results of this study. Using the results of the alpha and beta diversity analysis in this study, a power analysis was conducted to estimate the group sizes needed in a future study to see significant differences between the users and nonusers. Based upon what was observed in the 7 users and 7 nonusers in this study, we would need 47 and subjects in each group (users and nonusers) to observe a significant difference in alpha diversity using the Shannon metric (power 0.8 and $p < 0.05$) and 26 subjects in each group to observe a significant difference (power 0.8 and $p < 0.05$) in beta diversity using the Bray-Curtis metric. Therefore, any follow-up study should include at least 47 subjects per group. Beyond the larger study group size, future, more in-depth research on this topic should also include: 1) the addition of

longitudinal and/or intervention data, if possible; 2) additional microcosm models (e.g. biofilm and/or anaerobic) or an *in vivo* clinical trial of beetroot juice powder as a supplement; and 3) metagenomic analyses to examine the impacts of e-cigarette usage and beetroot juice on the non-bacterial members of the oral microbiome as well as changes in the metabolic repertoire of these microbial communities. Overall, this pilot study suggests that beetroot juice extract may impact the microbiota of e-cigarette users and adds to the growing body of contemporary research paving the way for more in-depth studies examining the role of nitrate-rich supplements as prebiotics to promote oral health.

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Author contributions

CRediT: **Daniela V. Staton:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft; **Jonah Tang:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing; **Matthew Barbisan:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing; **Justin Nussbaum:** Conceptualization, Project administration, Supervision, Writing – review & editing; **Jonathon L. Baker:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The raw reads of the 16S rRNA amplicon sequencing of the saliva and microcosms are available in the NCBI SRA under accession number PRJNA1214875.

All code and scripts used in this study, along with interactive QZV files, are available in the associated GitHub repository: https://github.com/jonbakerlab/beetroot-microcosms.

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