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Nitrate supplementation affects taste by changing the oral metabolome and microbiome

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Nitrate, an inorganic anion found in various foods is also present in saliva and has emerged as a potential prebiotic for the oral microbiome. Salivary glands concentrate nitrate from the bloodstream and release it into the oral cavity via the anion transporter sialin SLC17A5. In previous studies dietary nitrate supplementation altered oral bacteria composition, favouring genera like *Rothia* and *Neisseria* while reducing *Streptococcus, Veillonella, Prevotella*, and *Actinomyces*. The present study hypothesized that taste intensity might adapt to changes in the oral microbiome caused by nitrate supplementation. Participants underwent taste tests before, during, and after supplementation. All subjects showed greater levels of salivary nitrate during supplementation and had higher levels of *Neisseria* compared to before. Subjects were then grouped according to taste tests (before vs. during) as responders (ANOVA $p < 0.05$, $n = 7$), and non-responders (ANOVA $p > 0.05$, $n = 6$) and their salivary metabolome and oral microbiome further analysed. Responders had significantly less 5-amino pentanoate, formate, propionate and butyrate in saliva while non-responders showed no metabolite changes between before and during supplementation. In contrast, non-responders had increased *Capnocytophaga gingivalis* and altered lysosomal degradation pathways. Overall, nitrate supplementation shifted the oral microbiome composition in all subjects and when taste intensity was altered this correlated to bacteria-derived short-chain fatty acid production. This suggests taste perception is affected by the oral microbiome.

Oral microbiome is the second most abundant in human body after the gut[1] with approximately 700 species of microorganisms[2,3]. In contrast to gut microbiome, oral microbiome has demonstrated greater resilience/stability. With the exception of fermentable carbohydrates, the influence of diet on the composition of oral microbiome is minimal[4] and under healthy conditions, probiotics effects only persisted for 2 to 4 weeks following the end of treatment[5,6]. The stability of the oral microbiome is ascribed to the wide variety of environmental niches and the constant supply of nutrients via saliva or from gingival crevicular fluid surrounding teeth[7].

Taste is a complex mechanism, involving tastants acting as agonists on taste receptors located in taste buds on the tongue. There are five basic gustatory molecules: umami, salt, sweet, bitter and sour. These molecules can be classified into two groups based on the activation method: ion-based and G-protein-based taste molecules. Salt and sour compounds fall under the category of ion-based molecules. The defining characteristic of the ion-based group is that the agonist ion enters the cell for activation, either through the channel (epithelial sodium channel for salt) or the receptor (Otopetrin-1 for sour)[8–10]. The second group of taste molecules, including umami, sweet, bitter activate G protein-coupled receptors on the cell surface[11]. Following binding of the G-proteins and activation of intracellular secondary messengers (calcium and cyclic AMP) the epithelial cells signal to nerves by releasing a range of signalling agents such as purinergic or 5-hydroxytryptamine.

Taste receptors are primarily located in the tongue epithelium in taste buds, some superficially on the tongue within fungiform papillae but most taste receptors are located in the posterior part of the tongue, within the foliate and circumvallate papillae[12]. Taste buds consisted of five types of cells: Type I to V cells. Type I cells expressed enzymes and transporters needed to eliminate extracellular neurotransmitters and redistribute ions, thereby closing taste signals from Type II and Type III taste cells and supporting other taste cells[13,14]. Type II cells are responsible for detecting umami, sweet, and bitter tastes[15,16]. Type III cells responded directly to sour and salt taste[8,17]. Type IV cells, which lay at the taste buds base and are located at a distance from taste pores, are typically considered as undifferentiated stem cells of

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their bud cell progeny[18,19]. Type V cells were described in early research papers[20,21] however, type V cells do not represent a widely accepted concept, as they did not exhibit any common features across different species such as fish and frogs.

Within the oral environment the host can influence taste perception in several ways. Firstly the release of tastants (and volatiles) is dependent on the physical properties of the saliva. Secondly the transportation of taste molecules from food particles to taste cells is also regulated by salivary proteins and ions. Thirdly the interaction of flavour molecules with the mouth surfaces will affect their substantivity and intensity[22]. Amylase, an enzyme produced by salivary glands, modulates sweet taste intensity perception of starch-related foods[23]. Similarly, in the context of fatty taste perception, phosphotransferase and simple sugar transport systems displayed increased expression in non-tasters compared to super-tasters[24]. Additionally, the concentration of salivary long-chain free fatty acids was suggested to influence fatty taste threshold[25,26] and lingual lipase, a serous minor gland product, exhibited a weak association with oral fat detection[27,28]. An elevation in salivary sodium concentration was associated with a reduction in salt perception[29,30]. Additionally, bicarbonate ions could reduce salivary proton concentration and consequently affect sour taste threshold[31].

During the transportation of tastants to taste buds to generate taste signals, they must pass through the tongue biofilm (a major part of the oral microbiome). In this context, it is conceivable that oral microbiome will also interact with taste molecules, thereby influencing taste perception. There are at least three potential pathways through which the oral microbiome can affect taste. The first is the accumulation of oral microbiome by-products (such as acetate) forming a viscous diffusion barrier over taste buds, altering the activation of taste receptor. A reduction in tongue biofilm thickness achieved through tongue brushing has been associated with a decrease in the recognition threshold for salt[32,33]. The second mechanism entails the oral microbiome by-products conditioning taste buds, thereby changing the threshold of each taste[34]. The third pathway involves the oral microbiome metabolising tastants (e.g., sucrose) before they arrive at the taste buds[35,36]. This study aims to elucidate which of these mechanisms is pertinent to human taste.

Past research has predominantly focused on elucidating the association between taste thresholds and oral bacteria. For example, studies have shown a high abundance of *Streptococcus mutans* and *lactobacilli* can lead to a decrease in overall taste thresholds[37]. A study[38] revealed significant differences in the abundance of five bacterial genera between supertasters and non-tasters. Supertasters exhibited higher levels of *Actinomyces*, *Oribacterium*, *Solobacterium*, *Catonella*, and *Campylobacter*. Whilst *Streptococcus parasanguinis*, *Streptococcus gordonii* and *SR1 HOT-345* were found to have a weak correlation with sweet, sour, bitter, salt, and umami taste thresholds, and the abundance of these three species associated with taste threshold differences[39]. These findings suggested the influence of the oral microbiome on taste but were not definitive as the underlying mechanisms had not been extensively investigated. Evidence for the third pathway i.e., metabolism of tastants before they arrive at the taste buds have been easier to accrue. Sucrose, a sweet tastant is also a well-recognised substrate in the carbohydrate metabolism of oral microbiome[36]. The metabolism of sucrose via the citric acid cycle was a distinguishing feature of sucrose super-tasters[35]. Intriguingly the use of nitrate supplements for oral cavity rinsing had been observed to alter sucrose metabolism, shifting from lactate to the citric acid cycle although no taste tests were reported[40].

Few studies have shown a cause-effect of microbes on taste because many pre- or probiotics have limited effects on the oral microbiome due to the constant washing effect of salivary flow. Certain lactobacilli strains commonly used in yogurt products as gut probiotics[41] have limited effects under healthy conditions[42]. Instead, this study aims to alter the composition of oral microbiome by altering the availability of prebiotics/substrates in the mouth. One approach is to utilise the entero-salivary cycle. This cycle describes orally consumed substances circulating back to the oral cavity through the uptake in the gastrointestinal system, the transport via the blood system and then delivery through the salivary glands[43,44]. The most widely studied of these was nitrate which is ubiquitous in vegetables and fruits[45,46]. In the oral cavity, nitrates were stable, and bacteria could not directly utilise them. Instead, nitrates were reduced to nitrites, and nitrites were further reduced to ammonia through enzymatic reactions for further use[47]. When bacteria were exposed to excessive amounts of nitrates, it was demonstrated that the composition of the oral microbiome could change. Consequently, microbiome change could alter the metabolome characteristics as bacterial abundance shifts, affecting taste buds perception performance as their surrounding environment changes. Current research has demonstrated the impact of nitrate on oral microbiome at the genus level using 16S rRNA gene sequencing[48–53]. However, the use of shotgun sequencing could provide new insights into nitrate reduction at the species level and the entire functional gene capacity level[54].

In summary, this study aims to use the shift in the oral microbiome caused by the nitrate prebiotic to study possible changes in taste perception.

Results

This study employed sensory, metabolomics, and metagenomics methods to investigate how nitrate supplements affect taste, saliva metabolite profiles, and microbiome composition, while also exploring the relationships between taste, oral metabolites, and microbiome composition.

Dietary nitrate supplementation increased salivary nitrate levels

To determine the best sampling time five participants (case 1–5 in Fig. 1A, B) were recruited to assess the nitrate and nitrite time curve. Each participant provided unstimulated saliva every 30 min for a total duration of 4 h and 30 min. The first two collections were the control points and subjects consumed nitrate supplements after the second collection immediately. Individual variations in nitrate and nitrite time curve (Fig. 1A, B) showed considerable variation, making it difficult to identify an ideal sampling time for maximum nitrate and nitrite levels. Saliva flow rate did not show any significant change ($p = 0.42$ in repeated ANOVA, Supplementary Fig. 2).

For the main study the mean salivary nitrate output increased after 5 days of nitrate supplementation treatment (from 0.41 ± 0.61 to 3.47 ± 2.36 micromolar per minute; $p < 0.001$), whereas salivary nitrite showed no increase ($p = 0.063$) (Fig. 1C, D).

Taste perception changed during nitrate supplementation

In a two-way ANOVA analysis, taste perception data from the third, fourth, and fifth day of each experiment week were compared. Based on the treatment effect, there was no significant change of the whole group but there was when the thirteen subjects were divided into two groups: responders ($n = 7$, $p < 0.05$) whose taste perception changed in the during period compared to either before or after periods and non-responders ($n = 6$, $p > 0.05$) (Fig. 2). Upon evaluating individual tastants (using data solely from the fifth day of each experiment stage), only monosodium glutamate (MSG) revealed a change ($p = 0.049$, Supplementary Fig. 3). However, the data collected from participants at each stage remained insignificant ($p > 0.05$). The two-way ANOVA and Friedman tests yielded results, indicating that nitrate affects taste at varying levels for different participants.

Salivary metabolites changed during nitrate supplementation

For the whole group ($n = 13$), 5-aminopentanoate (0.14 ± 0.083 to 0.082 ± 0.087 millimolar per litre), propionate (0.30 ± 0.19 to 0.184 ± 0.283 millimolar per litre) and butyrate (0.041 ± 0.026 to 0.025 ± 0.015 millimolar per litre) showed significant decreased levels ($p < 0.05$) in both Wilcoxon and Maaslin2 analysis during supplementation period compared to the before period. Splitting these results by subjective taste, except 5-aminopentanoate, propionate, butyrate and formate (0.184 ± 0.227 to 0.040 ± 0.068 millimolar per litre) also showed a significant reduction ($p < 0.05$) in responders group, whereas non-responders did not show any change in the metabolome performance (Fig. 3). After the end of supplementation period, 5-aminopentanoate and propionate returned to pre-

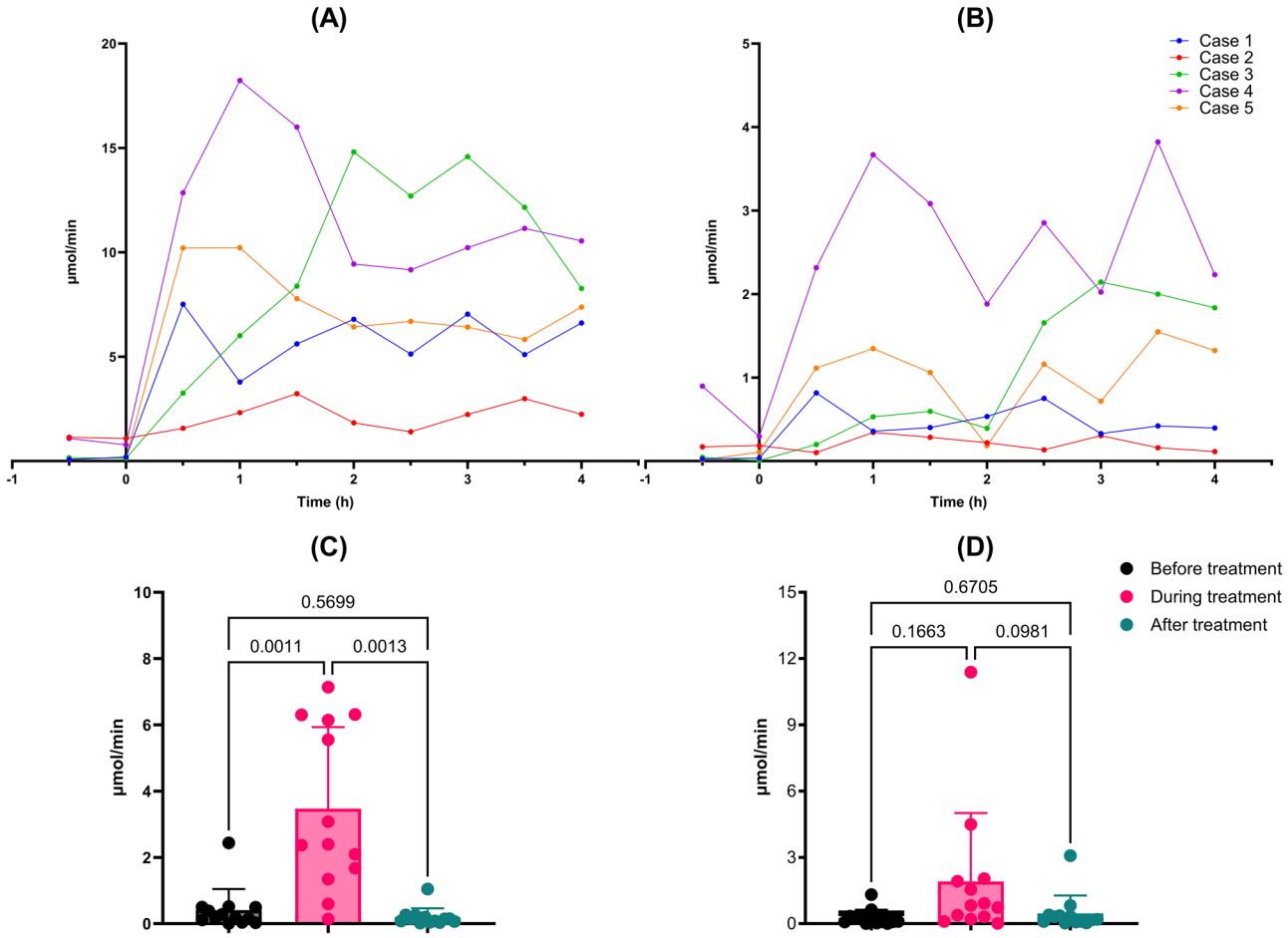

Fig. 1 | Mean salivary nitrate and nitrite outputs (concentration (µMol/L) × flow rate (mL/min),with standard deviation bars). A Salivary nitrate output curves for five subjects immediately after consumption of a single nitrate supplement; **B** salivary nitrite output curves in the same samples. In **A**, **B**, −0.5 and 0 h refer to the timepoints before participants consumed the nitrate supplements. Participants drank the nitrate supplements immediately after providing a saliva sample at the 0 h timepoint. The 0.5 to 4 h timepoints indicate the number of hours after they consumed the nitrate supplements. **C** Salivary nitrate output was significantly increased for the whole group ($n = 13$) whereas **D** salivary nitrite output was unchanged compared to either before or after periods. In **C**, **D**, saliva was collected on the 5th day of treatment. Prior to this, participants had received 5 days of nitrate supplementation. After treatment refers to the day after the last supplement dose was consumed.

supplemental levels while arginine and urea decreased in the responders group (Fig. 3A, C, E, F). In the pre-treatment stage, isopropanol was the only metabolite that differed significantly between responders and non-responders ($p < 0.05$). Responders exhibited a higher isopropanol concentration (0.006 ± 0.002) compared to non-responders (0.004 ± 0.001) (Fig. 3G). No other metabolite differences were observed as significant between the two groups at pretreatment.

Nitrate supplementation affected nitrate-reducing bacteria

The Shannon diversity index ($p > 0.05$) and Bray-Curtis based beta diversity did not have any change during supplementation period (Fig. 4A, B). However, the composition of oral microbiome was affected at the single species level (Fig. 4A, B). In particular, the abundance of nitrate-reducing bacteria which increased following nitrate supplementation (Fig. 4C) in all subjects. For example, *Neisseria flavescens* abundance increased 1.16-fold compared to pre-supplemental levels. Overall, the most abundant species observed after supplementation were *Neisseria flavescens*, *Rothia mucilaginosa 1*, and *Streptococcus mitis*, accounting for 30% of the overall composition (see Supplementary Fig. 4). In non-responders, there were few changes to the oral microbiome composition, with the exception of *Neisseria flavescens*. *Capnocytophaga gingivalis* was the only species to exhibit a significant increase as assessed using Maaslin2 and Wilcoxon tests (Fig. 4D). In contrast, responders showed a more complex response to nitrate supplementation. Aside from *Neisseria flavescens*, five additional species exhibited a significant change ($p < 0.05$) during supplementation. These findings suggested that responders may have a more diverse and dynamic oral microbiome, which could contribute to their altered taste perception (Fig. 4C).

Multiple metabolic pathways were affected by nitrate supplementation

At the single gene level, over a thousand genes were significantly altered (during compared to before periods, $p < 0.05$; 1509 in Wilcoxon and 1210 in Maaslin2) in responders group, whereas non-responder altered fewer genes than responders (450 in Wilcoxon and 439 in Maaslin2). In Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis, 108 pathways were identified as significant and relevant to the project (adjusted $p < 0.05$; nitrogen metabolism adjusted $p = 0.1$, See KEGG enrichment results FINAL). In gene set analysis using the piano package, p-values from different analysis methods (Wilcoxon and Maaslin2) did not affect the results for either responders or non-responders. The heatmap displayed distinct up- and down-regulated pathways for each group (Fig. 5, processed with -log). In the non-responders group, there was a notable increase in the lysosome pathway during supplementation ($p = 0.0002$). Within this pathway, proteases, including cathepsin C, napsin-A, TPP1, and LGMN, were enriched in the positive direction, along with transport vesicles such as

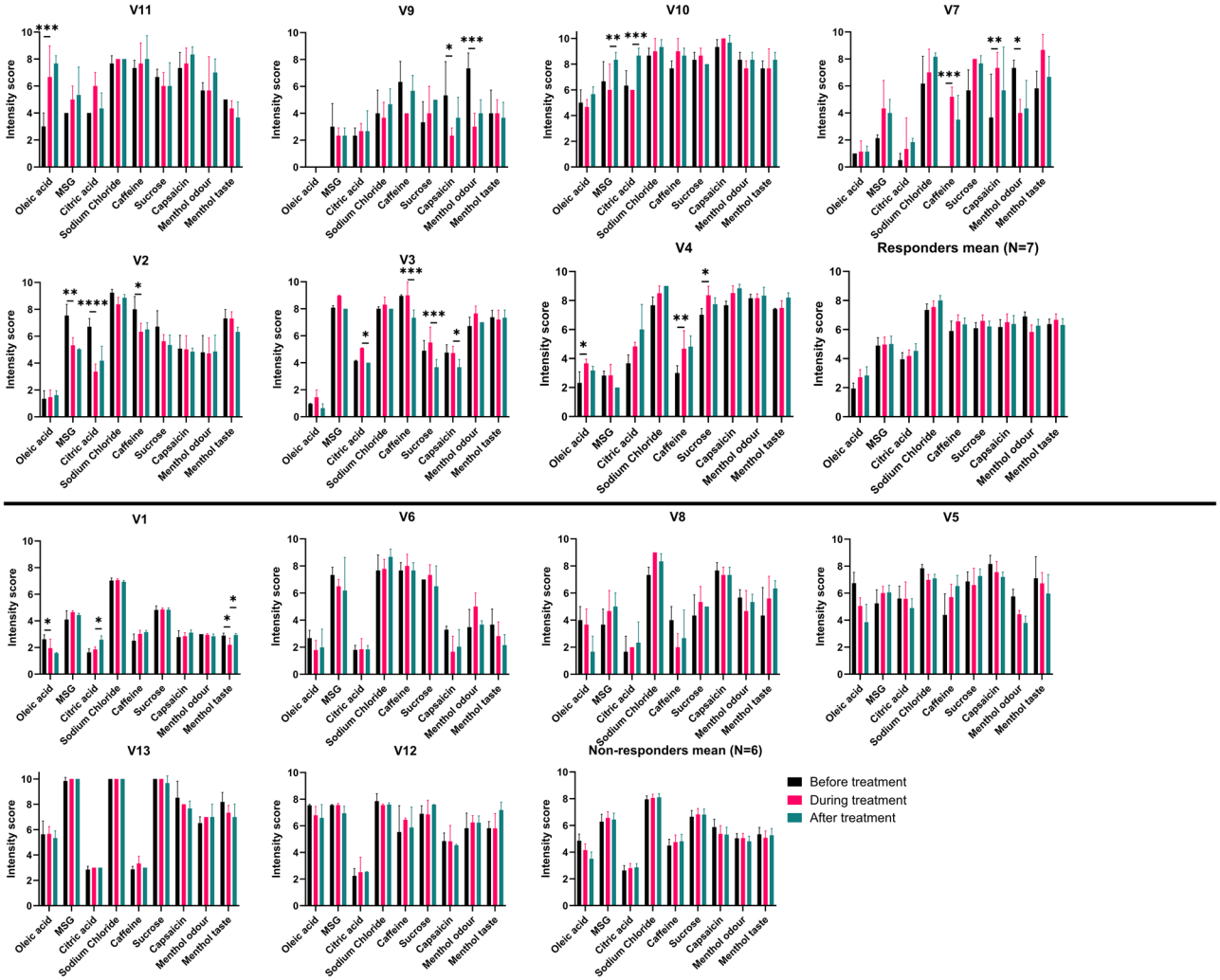

Fig. 2 | Visual analogue results for each subject with standard deviation bar from three consecutive days (3rd, 4th and 5th of each week). Subject's scores were analysed by two-way ANOVA and if $p < 0.05$ were labelled as responders or non-responders if $p > 0.05$. V1, V2 are participant numbers. Top half: responders (p (treatment effect) <0.05). Bottom half: non-responders (p (treatment effect) >0.05). When all responders were compared to all non-responders, there was no difference ($p > 0.05$), although all tastants were different ($p < 0.0001$). Asterisks indicated significant differences between the during-before and during-after conditions. ****$p < 0.0001$, ***$0.0001 < p < 0.001$, **$0.001 < p < 0.01$, *$p < 0.05$.

AP-1, AP-3, and AP-4 (see Supplementary Fig. 5). Interestingly, in the lysosome pathway, AP-1 was the only significant gene in the Wilcoxon analysis ($p < 0.05$), while GNPT and clathrin were significant in the Maaslin2 analysis. The top 10 pathways from the gene set analysis heatmap for both responders and non-responders are available in Supplementary Fig. 6.

Ammonia-urea pathway acted as a possible confounder

To investigate the potential relationship between oral microbiome and oral metabolome in responders and non-responders, significant species and significant metabolisms were identified using Wilcoxon and Maaslin2 tests (Fig. 6A, B). In the responders group, the highest absolute r value was approximately 0.6, indicating a weak moderate relationship between oral microbiome and oral metabolome. Conversely, in the non-responders group, the r value results were superior to those in the responders group. For example, the r value between *Neisseria flavescens* and propionate in non-responders was −0.66, whereas in responders, it was −0.39. However, *Neisseria flavescens* did not cause propionate to have a significant decrease during supplementation. The multi-correlation analysis suggested that the change in metabolites was determined by the alterations in multiple species. There was no contradiction between alpha, beta diversity and multi-correlation results because alpha and beta diversity only consider the absolute change of species (from 1 to 0).

PCA plots revealed a clear separation at the X-axis level (Fig. 6C). In the loading plot (Fig. 6D), it was observed that nitrate output had a positive correlation with urea, while 5-aminopentanoate, formate, propionate, and butyrate displayed a negative correlation with nitrate output. Moreover, in the PCA loading plot and correlation analysis within the non-responder group (Fig. 6D, E), *Neisseria flavescens* and *Capnocytophaga gingivalis* exhibited a strongly positive relationship. Interestingly, although ammonia is a potential precursor of urea, it did not show significance during supplementation ($p = 0.9$ in repeated measure one-way ANOVA, see Supplementary Fig. 7). However, a subtle increase in urea and ammonia was observed during supplementation (Fig. 6F). Finally, the modality score (subjective tastes average score) demonstrated a mild negative relationship with nitrate output, indicating that nitrate supplements may relate to taste perception. To maintain the integrity of the PCA analysis, one subject (V10T) was excluded due to their extreme values, which disproportionately influenced the overall distribution of reads in the score plot (Fig. 6A).

Discussion

This preliminary study provided the first evidence that nitrate supplements could alter taste perception. In a sensory test involving 9 different tastants, 13 subjects were defined into two groups: responders ($n = 7$, whose taste changed) and non-responders ($n = 6$). Specifically, only the responders

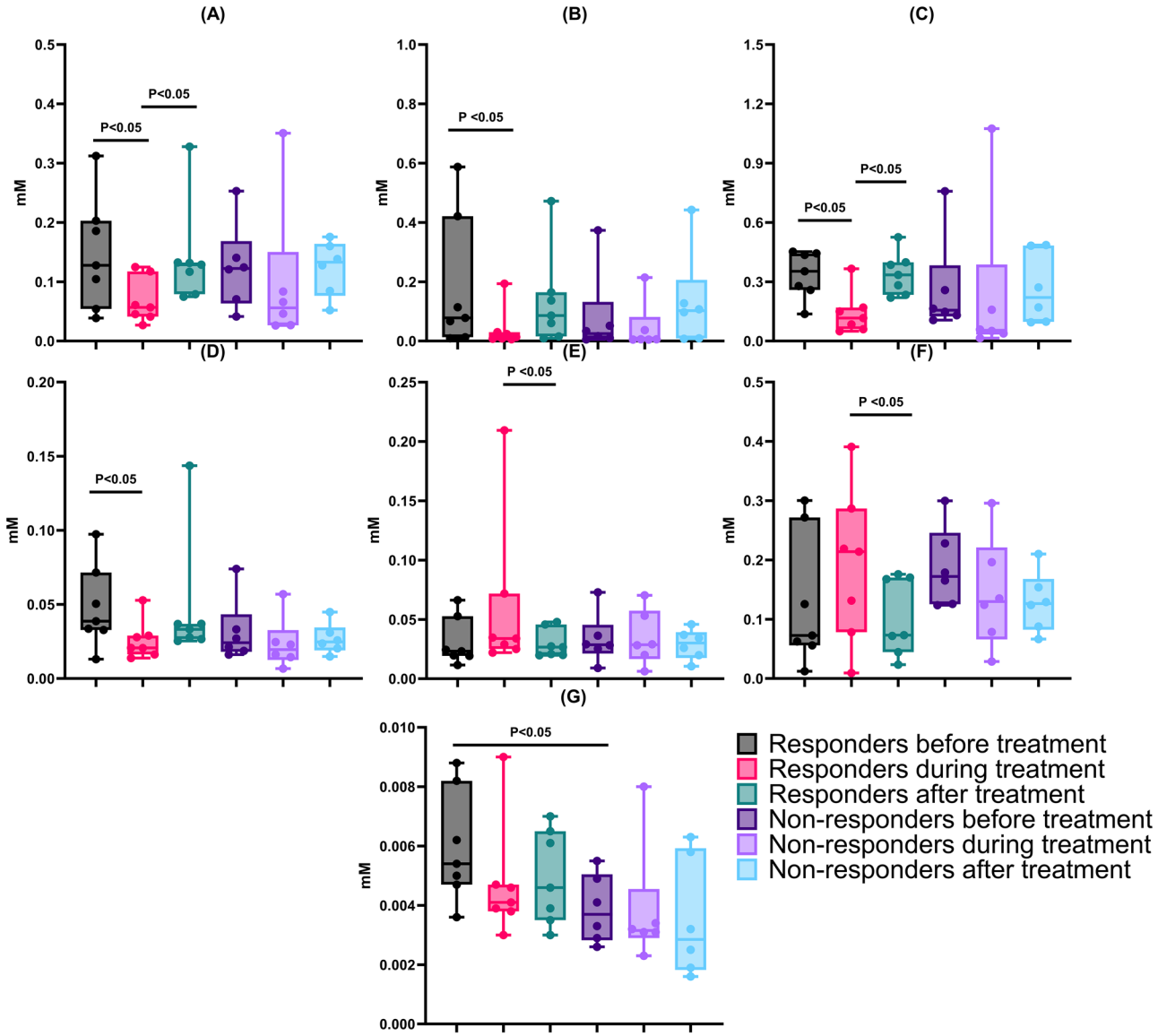

Fig. 3 | Salivary analytes assessed by NMR results which were significant ($p < 0.05$) in both Wilcoxon and Maaslin2. A 5-aminopentanoate; **B** formate; **C** propionate; **D** butyrate; **E** arginine; **F** urea; **G** isopropanol. Unit: millimolar per litre (mM), box plots represent medians and interquartile ranges for responders ($n = 7$) and non-responders ($n = 6$).

exhibited a series of significant changes ($p < 0.05$) in their oral metabolome during supplementation, including a significant decrease in formate, propionate, butyrate, and 5-aminopentanoate. Since taste receptors within taste buds are known to adapt to their environment, we believed this is why the altered metabolome affected taste perception.

This paper was also the first paper which uses shotgun sequencing in a nitrate related study. Despite an observed increase in salivary nitrate levels and a corresponding rise in the abundance of nitrogen metabolism-associated species during supplementation, KEGG enrichment analysis did not identify nitrogen metabolism as a significantly altered pathway (adjusted $p = 0.1$). Notably, several other pathways did exhibit significant changes, suggesting that nitrate's effects may extend beyond the realm of nitrogen metabolism. This finding implied that nitrate's prebiotic potential may be broader than previously thought.

The use of shotgun sequencing extended the functional gene analysis from nitrate reduction gene to the entire oral microbiome, allowing for a comprehensive assessment of the overall effect[47]. By using the gene set analysis via piano package, the number of significantly related pathways ($p < 0.05$) was reduced from 108 to 21 in both responders and non-responders (Supplementary Fig. 6). Among these 21 pathways, lysosome degradation pathway showed a clear increase ($p < 0.05$ in mixed up group and distinct up group in gene set analysis) in non-responders group during supplementation. Within lysosome pathway, many proteases and transport vesicles were enriched (see Supplementary Fig. 5). This finding implicated *Capnocytophaga gingivalis*, a Gram-negative bacterium, as a potential key player in the functional gene landscape. Transport vesicles and proteases were ubiquitous in Gram-negative bacteria[55–57]. Further investigations are required to confirm the presence of genes coding proteases and transport vesicles in *Capnocytophaga gingivalis*, as well as their expression. Another intriguing finding in the PCA was the positive relationship between nitrate output and urea. Early research established that urea serves as a precursor of ammonia[58]. However, recent studies questioned whether urea directly participates in nitrate metabolism[47]. Considering the PCA and correlation results in Fig. 6D, F, it appeared more plausible that urea should be regarded as a product related to nitrate metabolism. Within the KEGG pathway, urea had been identified as a precursor to ammonia in purine metabolism[59]. By integrating these findings with the urea-ammonia and functional gene data,

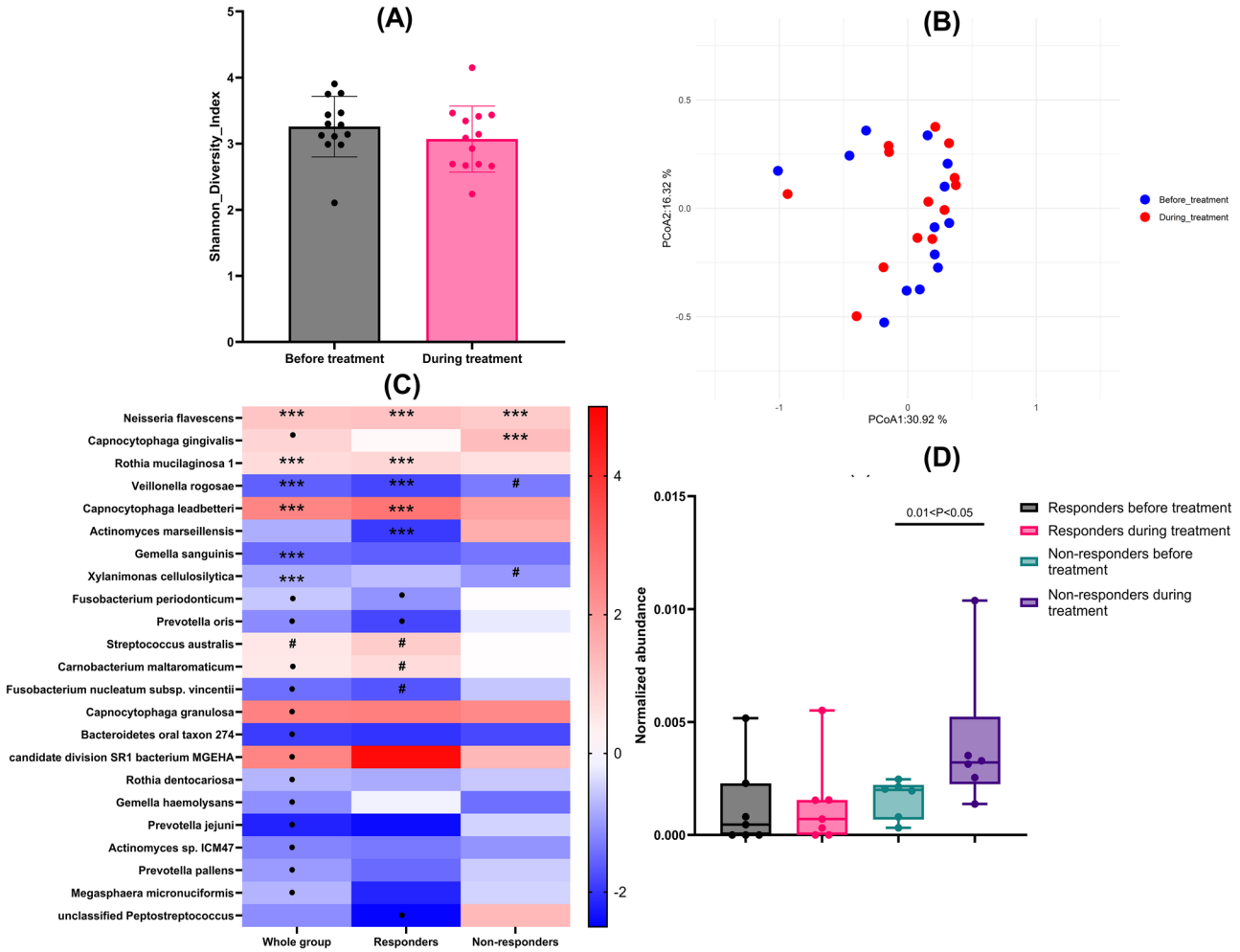

Fig. 4 | Metagenomic analysis. A Shannon diversity index $p = 0.13$ in Wilcoxon test; **B** Bray–Curtis-based beta diversity was visualised using principal coordinate analysis (PCoA). PERMANOVA indicated that there was no separation between the two groups ($p = 0.993$); **C** $\log_2$(A/B) fold change heatmap on selected species. ***Significant in Maaslin2 and Wilcoxon. #Significant in Maaslin2. •Significant in Wilcoxon; **D***Capnocytophaga gingivalis* abundance in each group.

it is likely that *Capnocytophaga gingivalis* could limit the influence on taste intensity.

As expected, the abundance of nitrate-reducing bacteria increased during supplementation which mirrors several previous studies[50,60]. At the species level, *Neisseria flavescens* abundance increased in all subjects, and *Rothia mucilaginosa 1* increased in responders. These changes in species align with previous results, confirming *Neisseria flavescens* and *Rothia mucilaginosa 1* exhibited increased relative abundance during supplementation[49]. However, a novel finding was that only in the non-responder group, was *Capnocytophaga gingivalis* abundance increased ($p < 0.05$) during the nitrate supplement whilst responders did not show any change ($p > 0.05$). This species had been identified before as a major nitrate reduction and nitrite production component of the tongue biofilm[47,61]. Moreover, *Capnocytophaga gingivalis* was associated with oral squamous cell carcinoma[62,63]. The reason why this species increased abundance did not alter the metabolome is unclear, but it was interesting to note there was increased lysosomal degradation in the non-responders which could potentially explain minimal metabolite export.

In this study, we observed that nitrate supplementation altered taste perception intensity, at overall taste level (Figs. 2 and 6D). When examining single tastant, only umami taste perception exhibited a significant change ($p = 0.049$) during supplementation (see Supplementary Fig. 3). The increase in umami taste perception may be attributed to a decreased short-term metabolic effect in the oral cavity, as glutamate can be metabolised directly by the tongue biofilm[36]. Interestingly, the concentration of glutamate substrate and the thickness of the tongue biofilm did not appear to affect the umami threshold[32,64].

In the present study dietary nitrate supplements significantly increased salivary nitrate levels although salivary nitrite was unchanged. The unchanged nitrite could be attributed to the reversible nature of the nitrate reduction reaction in biological metabolism, and nitrite was known to be less stable than nitrate[65]. The salivary nitrate and nitrite measured in this study (3.86 ± 2.85 mM and 1.32 ± 1.32 mM respectively) were similar with those from previous research although the data is more variable than in the earlier work. During supplementation, salivary nitrate concentration could go up to 5–8 mM within 30 min (10 mg/kg; 600 mg for 60 kg person)[45]. In similar papers with the same supplements, nitrate went up to 6.8 ± 3.95 μM and 1.4 ± 1.15 mM for nitrite (one nitrate supplements in morning and one nitrate supplements in evening)[66]. The diversity of salivary nitrate and nitrite was mainly caused by the variability of treatment effect. In the nitrate time curve experiment (Fig. 1C, D), the treatment effect for each individual varied at the same collection time. For example, case 1 spent 30 min to reach the peak whereas case 3 spent 2 h to reach peak level, making it difficult to identify the optimum sampling point for each subject. This variability in the nitrate treatment effect was consistent with observations from previous studies[67–69].

This study had several limitations. The small size of the sample group was necessary for the variety of the analysis (combined genomic, metabolomic and sensory tests) but limited the power of the study. We also used saliva sample for many analyses, but a tongue scrape would have been more

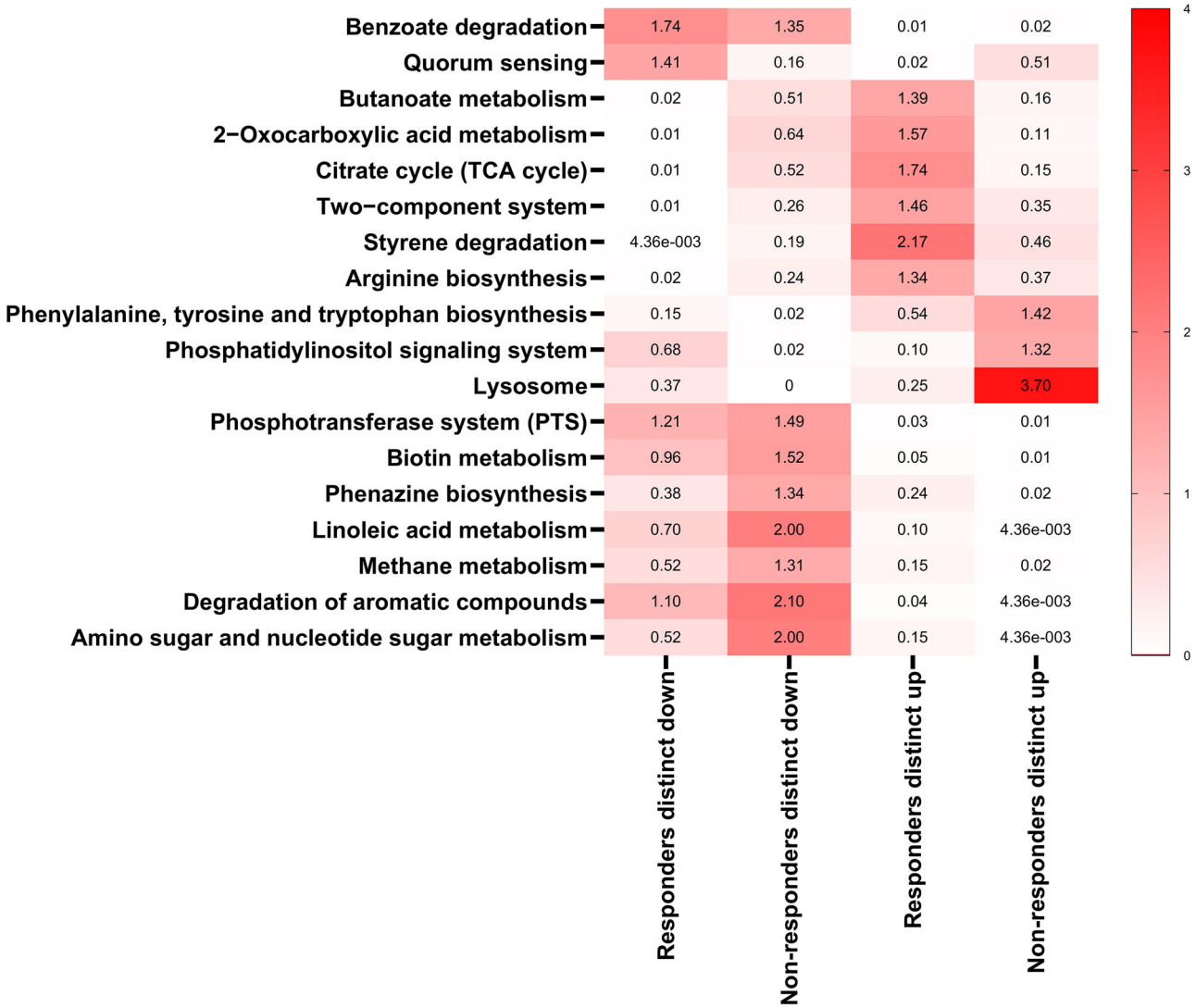

Fig. 5 | Distinct depleted and enriched pathways based on gene set analysis (during treatment vs. before treatment for responders ($n = 7$) and non-responders ($n = 6$)). Cell values represent the p value (−log processed), where −log 0.05 = 1.3.

informative. Additionally, DNA genomics did not give information about altered expression of genes, only RNA transcriptomics can. Finally, the use of a single concentration of each tastant was rather limited, and ideally, a range of concentrations to determine threshold and identification concentrations would have been more sensitive to changes in taste perception.

This paper was the first to show altered taste with dietary nitrate supplementation. Given that this was a relatively novel area of study, it was advisable to begin with prebiotics known to affect oral microbiome through the salivary-entero cycle. The potential for other prebiotics to influence taste perception through similar routes warrants further exploration, considering the possibility that systemic changes in oral microbiome can be induced by prebiotics. This interplay between prebiotics, oral microbiome, and taste can be conceptualised as a sequence of events where prebiotics modulate the metabolism and then the composition of oral microbiome. This shift in oral microbiome, in turn, leads to alterations in the oral microbial gene expression. Ultimately, changes in the oral microbiota's functions might impact taste perception as the taste buds adapted to the nearby oral environment. *Capnocytophaga gingivalis* could prevent the change of taste via lysosome degradation pathway. To conclude, in this study, nitrate supplementation was found to alter overall taste perception and it was possible to design food products containing oral prebiotics to enhance consumers' taste perception.

Methods

Ethics, volunteer recruitment and interventions

Before volunteer recruitment, this preliminary study obtained ethical approval from the King's College London Research Ethics Subcommittee (Reference Number: HR/DP-21/22-26684). Thirteen healthy subjects (8 female and 5 male, aged 18–65) joined the nitrate preliminary study. Participants did not have Covid history within the preceding 3 months or prescribed medicine history within 6 months. They were non-smokers and did not have any taste and smell disorder symptoms. All subjects signed the consent form before they started experiment.

This project consisted of daily taste-tests before, during and after the treatment week (5 days). The initial 2 days were allocated for sensory test training to familiarise participants with the attributes involved in sensory testing. Participants completed sensory tests once a day at the same time. Throughout the treatment week, participants consumed nitrate supplements orally (concentrate from beetroot juice, 400 mg, equivalent to 6.45 mM, sourced from James White Drinks, Ipswich, UK) each morning. Sensory testing was conducted in the afternoon. On the fifth day of each week, volunteer provided a resting whole mouth saliva sample (5 min). Before the sensory tests and saliva collection, all participants did not eat within 1 h or drink within 30 min. Project summary was available in

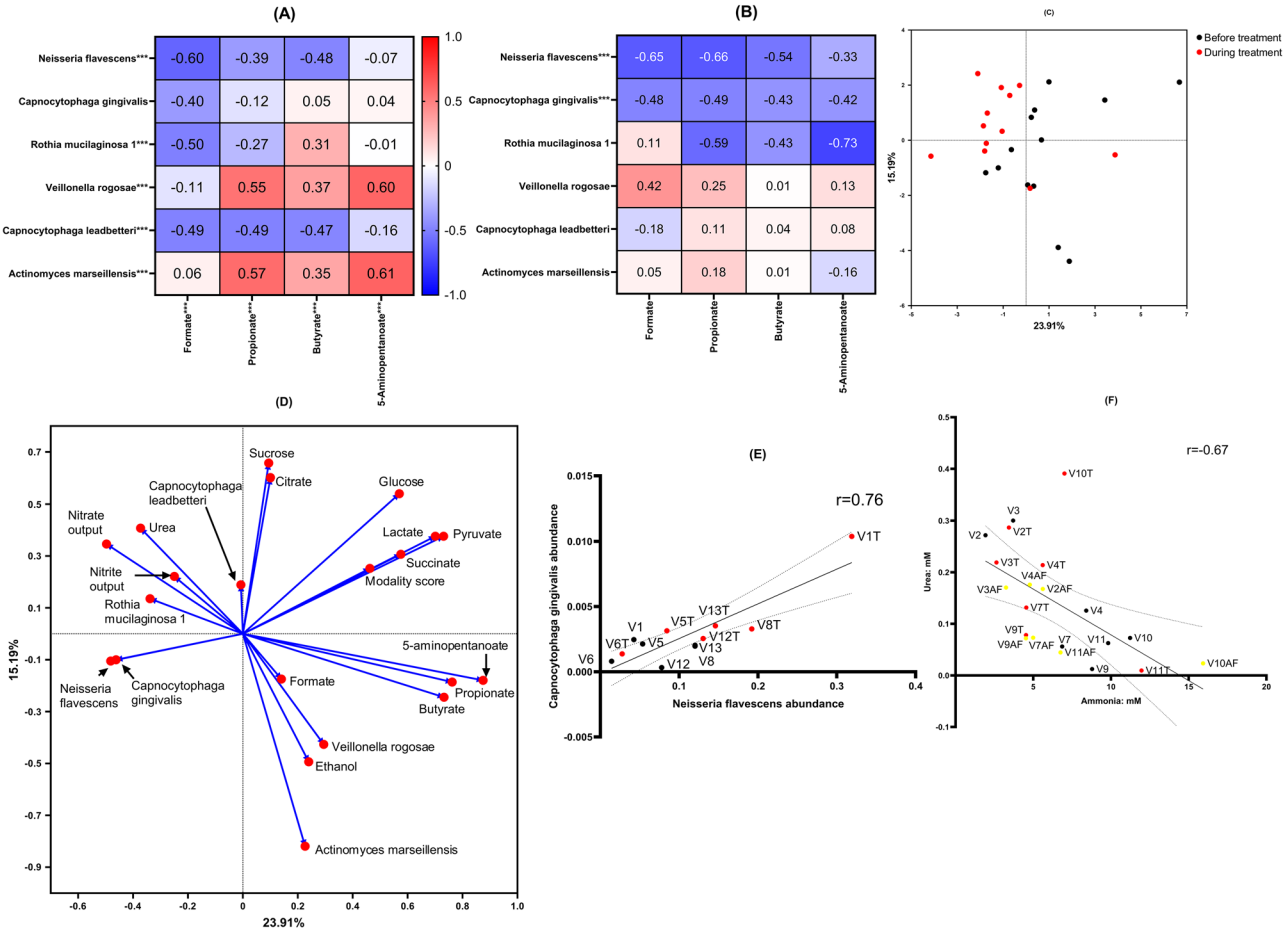

Fig. 6 | Multi-correlation, PCA analysis and ammonia assay results. A Responders multi-correlation Spearman *r* results; **B** non-responders multi-correlation Spearman *r* results; **C** PCA score plot; **D** PCA loading plot. Six species were selected for analysis as they were significant in both Maaslin2 and Wilcoxon tests, in either the responders or non-responders group. Eight metabolites from the NMR analysis were chosen because they were identified as relevant metabolites (see 'Methods' section for further details). The modality score represents the average intensity of the nine tastants based on the fifth-day data for each subject; **E** *Neisseria flavescens and Capnocytophaga gingivalis* correlation analysis in non-responders group; **F** ammonia and urea correlation analysis in responders group. ***Significant in both Wilcoxon and Maaslin2 analysis.

Supplementary Fig. 1. The final assessment day of this project was on the Friday of the after-treatment week (equivalent to the 7th after-treatment day).

Sensory tests

In the sensory test, each volunteer evaluated nine taste solutions with 8 different molecules: 0.05 M oleic acid (fat), 0.08 M MSG (umami), 0.0052 M citric acid (sour), 0.25 M sodium chloride (salt, EMSURE, Darmstadt, Germany), 0.008 M caffeine (bitter), 0.25 M sucrose (sweet), 1 ppm capsaicin (Spice), 100 ppm menthol L pellets (menthol odour and menthol taste, Symrise AG, Holzminden, Germany). Unless specified otherwise, all tastant molecules were bought from Sigma (Gillingham, UK). For hygiene reasons, the menthol odour and menthol taste solutions were prepared separately rather than using the same cup. However, both solutions had the same concentration of menthol pellets.

Eight types of tastant solutions (except oleic acid) were weighed and dissolved in 1.5 L Natural Mineral Water (Buxton, Skelmersdale, UK). Due to the low water solubility of capsaicin and menthol, ethanol (0.095% in capsaicin; 0.475% in menthol odour and taste; SAFC, Darmstadt, Germany) were added to dissolve capsaicin and menthol. Sensory test solutions were stored in the fridge (4 °C) between use. The oleic acid sample was prepared immediately before the sensory test by creating an emulsion through the addition of oleic acid and mixing it with mineral water.

The taste intensity of each tastant solution was evaluated by the generalised labelled visual analogue scale (glVAS). After 30 s rest before each cup to avoid carryover taste, the sensory test solution order started with fatty, umami, sour, salt, bitter, sweetness, spicy, menthol odour and menthol taste. This order was designed to reduce potential taste-taste interactions during the evaluation. Participants were asked to rate the maximum intensity perceived during the holding process on the glVAS scale. To maintain consistency, every day, the sensory test time for each volunteer was kept within 2 h of the previous test time.

Saliva collection

In the fifth day of each week, each volunteer was asked to provide unstimulated saliva sample. Before saliva donation, each volunteer performed a COVID lateral flow test and only if negative were they allowed to provide a saliva sample. Subjects refrained from eating within 1 h and drinking within 30 min before saliva collection. Subjects were given a pre-weighed 50 mL sterilised tube and spitted the saliva sample into the tube. After the saliva donation, the tube was screwed and stored on ice immediately. The saliva sample was weighed to calculate the unstimulated saliva flow rate (ml/min) and then aliquoted and stored (−20 °C).

Nitrate, nitrite and ammonia assay

After thawing the frozen saliva, samples were centrifuged (15,000 × *g* for 3 min) and the supernatant separated, diluted by Phosphate Buffered Saline

(PBS) buffer (Thermo Scientific, Basingstoke, UK) at a dilution ranging from 10 to 1000 times. Then the mixture heated at 100 °C for 10 min to deactivate enzymatic activity.

To determine the nitrate and nitrite concentration, nitrate assay kit (Cell Biolabs, San Diego, USA; Sigma, UK) and nitrite assay kit (Thermo Scientific, UK) were used. Ammonia concentration was detected by ammonia assay kit (Sigma, UK). Different nitrate assay kits yielded comparable results, as demonstrated by the use of a nitrate standard from another kit. For every nitrate and nitrite detection, standard curves, duplicates and fresh standard stock solution were employed to ensure data quality.

NMR

A total of 0.5 ml saliva supernatant from each sample was mixed with 0.125 ml TSP buffer. The TSP buffer consist of 0.03125 ml deionised water, 0.03125 ml 2 mM sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (Sigma, UK), 0.0625 ml deuterium oxide (Sigma, UK), 28.4 mg/ml Na_2HPO_4 (Sigma, UK) and 5.28 mg/ml NaH_2PO_4 (Sigma, UK). Then the mixed solution was moved into a 5 mm NMR tube (Bruker, Coventry, UK). The tubes were sealed and analysed on a 600 MHz spectrometer (Bruker, UK) for ^{1}H 1D-NMR and 2D-NMR (Biomolecular Spectroscopy Centre, King's College London, UK). The concentration of metabolites was measured using Chenomix NMR Suite version 9.0 (Chenomix Ltd., Edmonton, Canada). The 2D-NMR was analysed by using TopSpin version 3.6.5 (Bruker, UK). The final NMR list (37 metabolites in total) was confirmed by 2D-NMR, Human Metabolome Database 5.0, and related paper[70–74]. The setting of 600 MHz spectrometer followed previously published method[57].

DNA extraction, shotgun metagenomic sequencing and metagenomic analysis

After thawing, the before and during saliva sample, GenElute Bacterial Genomic DNA Kit (Sigma, UK) was used to extract the DNA from saliva sample. The average DNA concentration in 26 samples was 85 ng/μL. The extracted DNA samples underwent shotgun sequencing at Novogene (Cambridge, UK), using the NovaSeq 6000 system (Illumina, Cambridge, UK). The DNA sample underwent 150 paired-end sequencing, resulting in an average of 55.8 million reads per sample. Then reads containing adaptors, reads containing undetectable reads over 10% and reads containing low quality (Qscore ≤5) base which is over 50% of the total base were removed from primary reads by Novogene (UK). The barcode for each sample was provided in Supplementary Table 1.

Raw sequencing reads from Novogene (UK) underwent high-quality (HQ) filtering by aligning them to the human reference genome (GRCh38), food-related genomes (Bos Taurus, Nov 2014 version), and Arabidopsis thaliana TAIR10[75]. Subsequently, the HQ reads were mapped and quantified using the METEOR pipeline and the reference gene catalogue of the human oral microbiome HS_8.4_oral gene catalogue[76,77]. Taxonomic annotation of metagenomic species utilised gene homology with previously sequenced organisms[78]. The signals of metagenomic species (i.e., abundances) across samples were calculated as the mean of 50 marker genes[77,78].

Statistics analysis

All statistics analysis and figure creation were conducted using Graphpad (Prism, La Jolla, USA) and RStudio. One-way repeated measures ANOVA with Tukey test was used to access salivary nitrate, nitrite and ammonia level. For the evaluation of personal sensory performances, two-way ANOVA with Tukey test (considering treatment effect and tastants as factors) was utilised. Friedman test was used to evaluate the change of each tastants. Spearman correlation analysis was performed to explore relationship between metabolites and species. Functional gene, species, and metabolite data were analysed using Maaslin2[79] and two-tailed Wilcoxon test was used for comparison in functional gene, species and metabolites analysis with the results from Maaslin2. KEGG enrichment analysis was conducted using clusterProfiler to get gene sets[80]. Gene set analysis was carried out using Piano[81]. PCA was employed to investigate the relationship between taste, metabolites and species. The abundance of functional genes and species in the results was normalised by using total-sum scaling method. The alpha and beta diversity were calculated by vegan package[82]. Beta diversity was visualised using PCoA, and group separation was evaluated by PERMANOVA from the vegan package with 999 permutations.

PCA analysis was conducted to investigate the relationship within oral microbiome, oral metabolome and taste. The selection of oral metabolites was based on previous papers. Urea was chosen as an indicator of oral alkalisation and acid neutralisation[36]. Glucose and ethanol were considered an important precursor in nitrate reducing bacteria metabolism and the key product in oral carbohydrate metabolism[36,83]. During the nitrate supplementation, nitrate could alter the sucrose metabolism from lactate to citric acid cycle[35,40]. Selected metabolites sucrose, succinate, citrate, lactate, and pyruvate were based on citric acid cycle, along with four other metabolites identified in NMR analysis: 5-aminopentanoate, formate, propionate, and butyrate for further analysis. To reduce the taste diversity into a suitable level, the average intensity of the nine tastants from the fifth day data for each subject was calculated and utilised in the PCA analysis. This approach was chosen as flavour can be considered a single modality in neuro-related flavour studies[84].

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data generated or analyzed during this study were included in this published article and its Supplementary Information files. Raw sequencing data had already been submitted to the European Nucleotide Archive (ENA) under accession code PRJEB74139.

Code availability

The KEGG enrichment analysis, Maaslin2, and Piano script were available in the Supplementary Material.

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Author contributions

S.Z. contributed to the design of the study, undertook experiments, analysed the data, and wrote the manuscript. F.C. mapped the species abundance and functional gene abundance. H.C. improved the code in functional gene analysis. T.D. contributed to analysis of the data, supervision of the project, and the final draft of the manuscript. S.S. analysis of the data, supervision of the project, and final draft of the manuscript. G.C. contributed to the design of the research, analysis of the data, supervision of the project, and the final draft of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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