Dietary nitrate supplementation alters the oral microbiome but does not improve the vascular responses to an acute nitrate dose

Running Title: Beetroot juice improves markers of oral health

Mia Burleigh[1], Luke Liddle[1], David J. Muggeridge[2], Christopher Monaghan[1], Nicholas Sculthorpe[1], John Butcher[3], Fiona Henriquez[1], Chris Easton[1]

[1]Institute of Clinical Health and Exercise Sciences, University of the West of Scotland, Blantyre, G72 0LH, Scotland.
[2]Institute of Health Research & Innovation, Division of Biomedical Science, University of the Highlands and Islands, Inverness, IV2 3JH, Scotland.
[3]Department of Life Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, G4 0BA, Scotland.

Corresponding author: Dr Chris Easton
University of the West of Scotland
Blantyre
G72 0LH
Scotland
T: 01698 283 100
E: chris.easton@uws.ac.uk

Abstract

Nitrate (NO_3^-) contained in food and beverages can transiently increase nitric oxide (NO) availability following a stepwise reduction to nitrite (NO_2^-) by commensal bacteria in the oral cavity. We tested the hypothesis that regular ingestion of dietary NO_3^- would influence the oral microbiome, the capacity to reduce NO_3^- to NO_2^- in saliva, and the vascular responses to an acute dose of NO_3^-. The abundance of bacterial species on the tongue, the availability of NO markers, and vascular function were assessed in 11 healthy males before and after 7 days of supplementation with NO_3^--rich beetroot juice and a NO_3^--depleted placebo. As expected, saliva and plasma NO_2^- and NO_3^- were significantly elevated after NO_3^- supplementation (all $P < 0.05$) but not placebo. We found that NO_3^- supplementation increased salivary pH (7.13 ± 0.54 to 7.39 ± 0.68, $P = 0.043$) and altered the abundance of some bacteria previously implicated in NO_3^- reduction: *Neisseria* (from 2% ± 3% to 9% ± 5%, $P < 0.001$), *Prevotella* (from 34% ± 17% to 23% ± 11%, $P = 0.001$) and *Actinomyces* (from 1% ± 1% to 0.5% ± 0.4%). Despite these alterations to the oral microbiota, an acute dose of NO_3^- increased salivary and plasma NO_2^-, reduced systolic blood pressure and increased the response to flow mediated dilation to a similar extent before and after 7 days of supplementation ($P > 0.05$). Our study establishes that supplementing the diet with NO_3^- for a sustained period can alter the oral environment in favour of health but does not impact the response to an acute NO_3^- dose. Acute ingestion of NO_3^- results in transient improvements in vascular function but the dietary induced adaptations to the oral bacteria did not enhance these effects.

1. Introduction

The metabolic and immunological activity of the hundreds of species of bacteria that live in and on the human body can directly influence biological function and health. The presence of dysbiotic microbiomes has been linked to various pathologies which include allergies, asthma, inflammatory diseases, obesity, cardiovascular disease and the metabolic syndrome [1]. Conversely, certain commensal microbes from the genera *Granulicatella, Actinomyces*, *Veillonella*, *Prevotella*, *Neisseria*, *Haemophilus, and Rothia* are thought to contribute to the generation of nitric oxide (NO) [2,3]. Myriad biological processes are critically dependent on NO, including host defence via antimicrobial actions [4], regulation of mucosal blood flow and mucus generation [5], regulation of smooth muscle contraction [6,7], cerebral blood flow [8], glucose homeostasis [9], and mitochondrial function [10].

Inorganic nitrate (NO_3^-) is regularly consumed in the diet through foods such as beetroot and green leafy vegetables [11]. NO_3^- enters the gastrointestinal tract where it is rapidly absorbed, enters the circulation, and is secreted in the saliva [12]. Here, it can interact with bacteria concentrated on the dorsal surface of the tongue [2]. Some species of bacteria use the NO_3^- as an alternative electron acceptor which reduces the ion to nitrite (NO_2^-). The NO_2^- in saliva is then swallowed and enters the stomach. In the acidic environment of the stomach, NO_2^- forms nitrous acid which is further converted to nitrosating species and subsequently to bioactive NO in the presence of ascorbic acid [13]. This pathway is known as the enterosalivary NO_3^--NO_2^--NO pathway [14]. Alternatively, NO_3^- and NO_2^- can be stored in the blood and tissues for conversion to NO when endogenous production of NO via the NO synthases (NOS) is limited [15]. The ingestion of NO_3^--rich beetroot juice has been shown to increase the availability of NO and improve exercise performance in simulated altitude [16], reduce blood pressure (BP) [17], enhance endothelial function [6], and is protective against models of ischemia/reperfusion injury [18]. On the other hand, a recent meta-analysis reported that NO_3^- supplementation has only small and trivial effects on exercise performance [19].

We have shown previously that individuals with a higher abundance of NO_3^- reducing bacteria were able to generate more salivary NO_2^- and at a faster rate following the ingestion of NO_3^--rich beetroot juice [20]. In contrast, when the enzymatic activity of bacteria in the mouth is disrupted by antibiotic use or rinsing the mouth with anti-bacterial mouthwash, the BP lowering effects of NO_3^- are abolished [14,21–23]. Oral microbiota live in regulated communities [24] in which they can use quorum sensing and potassium ion channel mediated electrical signalling to communicate and rapidly respond to environmental stimuli [25]. This allows them to maintain the functional and structural integrity of their ecosystems via replication and alterations to their gene expression [26,27]. The composition of an individual's diet can rapidly alter the conditions of the oral cavity by varying substrate availability for commensal bacteria and environmental factors such as pH.

Given the malleability of the oral environment, previous research has sought to determine the effects of dietary NO_3^- supplementation on the oral microbiome with a view to optimising the enterosalivary pathway to improve health. In an animal model, Hyde and colleagues [28] found that the abundance of the NO_3^- reducer *Haemophilus parainfluenzae* increased following $NaNO_3^-$ supplementation. In hypercholesteremia patients, Velmurugan et al. (2016) reported that 6 weeks of beetroot juice increased the abundance of *Neisseria* and *Rothia*. Recently, Vanhatalo and colleagues [30] expanded these findings in healthy young and older adults showing that 10 days of beetroot juice supplementation increased the abundance of *Neisseria* and *Rothia* with concomitant reductions in *Prevotella* and *Veillonella.*

Whilst it is has been shown that NO_3^- supplementation can alter the microbiome, it is presently unclear how this impacts the capacity to reduce NO_3^- following a dietary load. Based on our previous findings [19], one may hypothesise that an increased abundance of NO_3^--reducing bacteria will increase salivary and plasma NO_2^- production and enhance the acute vascular responses to dietary NO_3^-. Therefore, our primary objective was to assess the effects of 7 days of beetroot juice supplementation on the abundance of NO_3^--reducing bacteria in the oral cavity and assess the impact of these changes on NO

metabolites and markers of vascular function in healthy adults immediately following a NO_3^- dose.

2. Methods

2.1. Ethical approval

The study was approved by the School of Science and Sport Ethics Committee at The University of the West of Scotland. All procedures described were conducted in accordance with the Declaration of Helsinki 1974 and its later amendments.

2.2. Participants

Eleven healthy males (age 30 ± 7 years, stature 179 ± 7 cm, and body mass 86.9 ± 14.1 kg) volunteered and provided written informed consent prior to participating in the study. All participants were in good cardiovascular and oral health and did not report any use of antibacterial mouthwash or antibiotics for at least 6 months prior to study commencement. They were free from non-prescription medication including those known to interfere with stomach acid production and were not taking any prescribed medication. Health status was confirmed by completion of a medical questionnaire and The World Health Organisation's oral health questionnaire was used to ascertain oral health status.

2.3. Experimental Design

Participants were required to attend the laboratory on four separate occasions for this placebo-controlled, single blind randomised crossover study. The study comprised two separate 7 day dietary supplementation phases, each preceded by a baseline trial (day 0) and completed with a post-supplementation trial (day 8). In one arm of the study, participants ingested 70 ml of NO_3^--rich beetroot juice (~6.2 mmol NO_3^-) (Pro-Elite Shots, James White Drinks Ltd., Suffolk, England) in the morning and 70 ml in the evening. In the other arm, participants ingested the same volume of NO_3^--depleted beetroot juice (Placebo shots, James White Drinks Ltd., Suffolk, England). Both versions

of the beetroot juice were identical in taste and appearance. The supplementation phases were separated by a prolonged washout period (4 weeks) as it is currently unclear how long it takes the oral microbiome to return to baseline following modification via dietary NO_3^-. All experimental trials were identical with the exception that an acute NO_3^- response test was carried out on days 0 and 8 of the NO_3^--rich beetroot juice phase but not the placebo phase. The decision to exclude this protocol from the placebo phase was based on the premise that the oral microbiome is highly responsive to dietary stimuli [31,32] and a large amount of NO_3^- on day 0 might have altered the post-supplementation markers in the placebo phase. Participants were informed that the acute NO_3^- response test would be implemented in one of the two testing arms and they were not aware that this was only in the NO_3^--rich phase.

2.4. Procedures

Prior to the first trial, participants were briefed on procedures and were provided with a food diary in which they recorded all foods consumed 7 days prior to the trial and during the supplementation period. This diary was used to replicate diet in the week preceding the second supplementation phase. Participants arrived at the laboratory on the morning of each trial in a fasted and euhydrated state after consuming 500 ml of water 1 h before each trial. Participants were instructed to avoid strenuous exercise for 24 h and caffeine for 12 h before each trial. On the morning of each trial, participants were requested not to brush their teeth and tongue or chew gum. They were also requested not to use mouthwash throughout the study and report any changes in health status. Participants provided assurance of their compliance with these instructions via completion of a checklist on each visit.

Anthropometric characteristics were recorded at the beginning of each visit using conventional methods. Following this, participants lay supine for the remainder of the experiment. The posterior dorsal surface of the tongue was swabbed for 1 min with a sterile Hydraflock swab (Puritan HydraFlock Swabs, Puritan Diagnostics LLC, Guilford, Maine, USA.). This area of the tongue is known to harbour NO_3^- reducing bacteria and is

the area of the oral cavity in which the majority of NO_3^- reduction activity occurs [2]. The swabs were transferred to transport tubes containing 0.85 ml of buffered sterile saline and 0.15 ml of glycerol and subsequently frozen and stored at -80 ºC

No further measurements were collected for 30 min to ensure plasma [NO_2^-] had stabilised following the change in body posture [33]. Subsequently, heart rate (HR) was measured via telemetry (Polar Electro, Oy, Finland) and systolic BP (SBP) and diastolic BP (DBP) were recorded in triplicate using an automated device (Orman M6, Intelli-Sense. Hoofdorp, Netherlands). Mean arterial pressure (MAP) was calculated using the following equation:

$$MAP = (2 \times DBP + SBP) / 3$$

Endothelial function of the brachial artery was then assessed by flow mediated dilation (FMD), described in detail below. Venous blood was collected via venepuncture from the forearm in 4 ml aliquots in vacutainer tubes containing ethylenediaminetetraacetic acid (BD vacutainer K2E 7.2mg, Plymouth, U.K.). Samples of whole blood were immediately centrifuged for 10 min at 4000 rpm at 4°C (Harrier 18/80, Henderson Biomedical. UK) following collection. Samples of unstimulated saliva were concurrently collected via an oral swab (Saliva Bio Oral Swab (SOS) Salimetrics, Pennsylvania, USA) placed under the tongue for 3 min. Swabs were transferred to a collection tube (Sartedt, Aktiengesellschaft & Co, Numbrecht, Germany) and centrifuged at 4000 rpm for 10 min at 4°C (Harrier 18/80, Henderson Biomedical. UK). Following centrifugation, the samples of plasma and saliva were immediately stored at -80ºC for later analysis of NO_3^- and NO_2^- content via ozone-based chemiluminescence. The swabs were analysed and found to contain negligible levels NO_3^- and NO_2^-.

149 *2.4.1. Acute Nitrate Response Test*

150 On days 0 and 8 of the NO_3^--rich supplementation phase, participants completed an acute
151 NO_3^- response test following completion of the procedures described above. In this
152 component, participants ingested 2 x 70 ml of NO_3^--rich beetroot juice (~12.4 mmol NO_3^-
153 , James White Drinks Ltd., Suffolk, England). A sample of saliva was collected 90 min
154 after ingestion followed by a blood sample, and measurements of BP and FMD at 150
155 min. This protocol facilitated the comparison of NO_3^- metabolism before and after the
156 expected alteration of the oral microbiome.

157

158 *2.4.2. Flow Mediated Dilation*

159 On the contralateral arm to that used for blood collection, the endothelium-dependent
160 vascular responses of the brachial artery were assessed by high-resolution ultrasound
161 imaging and automated vessel diameter measurements. Ultrasound images were recorded
162 using a Vivid 7 ultrasound machine (GE Vingmed, Horten, Norway) with a L10 11MHz
163 linear array transducer. A straight, non-branching segment of the brachial artery above
164 the antecubital fossa was identified and imaged in the longitudinal plane with
165 simultaneous capture of blood flow gated pulse wave using Doppler imaging. The
166 Doppler gate was set to encompass the majority of the width of the artery and was angle
167 corrected at 60°. The brachial artery diameter was initially recorded for 1 min (baseline).
168 A cuff on the upper forearm (distal to the imaging site) was then inflated to supra-systolic
169 pressure (220 mmHg) for 5 min using a rapid cuff inflator (Hockansen, Bellevue, WA,
170 USA). The cuff was then rapidly deflated and the same segment of the brachial artery was
171 imaged for 5 min with concurrent measurement of blood flow.

172

173 Automatic edge detection software (Brachial Analyzer, Medical Imaging Applications
174 LLC, Coralville) was used to measure the diameter of the brachial artery and blood flow
175 using the envelope of the Doppler spectral traces and to calculate hyperaemic shear. The
176 area under the curve for the hyperaemic shear data was then measured up to the point of
177 maximal arterial dilation using the Reimann sum technique. The change in brachial artery
178 diameter was calculated using a 3 s average and expressed as percentage change from

baseline. As FMD changes are partly dependent upon vessel diameter, the absolute diameter changes were also calculated. The coefficient of variation (CV) for the FMD measurement in our laboratory is 5.6%.

2.4.3. Analysis of saliva and plasma samples

The pH of saliva samples was measured in duplicate with a circular electrode pH-meter 1140 Mettler Toledo (Greisensee, Switzerland) which has a precision of 0.01 pH unit. The measured pH value was not accepted until an unchanged pH value was observed for a period of at least 7 s. Calibration of the pH meter was performed before analysis and after every 10 samples using buffers with known pH (4.01 and 7.00). The electrode was rinsed with deionised water between samples.

For the analysis of plasma and saliva [NO_2^-], tri-iodide reagent (2.5 ml glacial acetic acid, 0.5 ml of 18 Ω deionised water, and 25 mg sodium iodide) was placed in a glass purge vessel heated to 50°C and connected to a NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was created by injecting 100 µL of NO_2^- solutions at various concentrations up to 1000 nM (plasma) and 3000 nM (saliva). Samples were thawed in a water bath at 37°C and 100µL of the sample was injected immediately into the purge vessel in duplicate. Saliva samples were initially diluted with deionised water at a ratio of 1:100 before injection. The NO_2^- content was calculated via the area under the curve using Origin software (version 7.1).

For the analysis of [NO_3^-], vanadium reagent (24 mg of vanadium tri-chloride and 3 ml of 1 M hydrochloric acid) was placed into the purge vessel and heated to 90°C. A standard curve was created by injecting 10-25 µL NO_3^- solutions at concentrations up to 100 µM for both plasma and saliva. Plasma samples were initially de-proteinised using 1 M zinc sulfate ($ZnSO_4$) at 1:10 w/v and 1 M sodium hydroxide (NaOH) at a 1:1 ratio. 200 µL of plasma was added to 400 µL of $ZnSO_4$ and 400 µL of NaOH. Each sample was vortexed for 30 s prior to being centrifuged for 5 min at 4000 rpm and the supernatant was injected into the purge vessel. The NO_3^-concentration was calculated as previously described for NO_2^-.

2.4.4. 16S Metagenomic Sequencing

DNA from the tongue swab samples was isolated (Illumina MasterPure kit, Epicentre, Madison, WI, USA) before shipping to a commercial analysis centre (Omega Bioservices, Norcross, GA, USA). The libraries were prepared using an Illumina 16S Metagenomic Sequencing kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer`s protocol. The V3-V4 region of the bacterial 16S rRNA gene sequences were amplified using the primer pair containing the gene-specific sequences and Illumina adapter overhang nucleotide sequences. Samples were prepared by combining 12.5 ng of the DNA sample with 12.5 µL of 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA) and 5 µL of 1 µM of each primer. The full-length primer sequences were: 16S Amplicon PCR Forward Primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 16S Amplicon PCR Reverse Primer (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC).

Samples were initially subjected to denaturation at 95°C for 3 min followed by 25 x 30 s cycles of denaturation (95°C), annealing (55°C) and extension (72°C), and a final elongation of 5 min at 72°C. The PCR product was cleaned up from the reaction mix with Mag-Bind RxnPure Plus magnetic beads (Omega Bio-tek, Norcross, GA). A second index PCR amplification, used to incorporate barcodes and sequencing adapters into the final PCR product, was performed in 25 µL reactions, using the same master mix conditions as described above. Samples were further subjected to 8 x 30 s cycles of denaturation (95°C), annealing (55°C), and extension (72ºC) followed by a 5 min elongation step at 72°C. The library of approximately 600 bases in size was checked using an Agilent 2200 TapeStation and quantified using QuantiFluor dsDNA System (Promega). Following this, libraries were normalised, pooled and sequenced on the MiSeq (Illumina, San Diego, CA) using the 2 x 300 bp paired-end read setting.

240 *2.4.5. 16s rRNA Gene Data Analysis*

241 Quality filtered data received from the sequencing centre were interrogated using the
242 Qiime 1.8 database [34]. Sequences were clustered *de novo* and binned into operational
243 taxonomic units (OTU) based on 99% identity. Taxonomy was assigned using the RDP
244 classifier trained to the GreenGenes database (October 2013 release). After removal of
245 singleton reads from the dataset, 964,418 sequences remained with an average of 21918
246 sequences per sample. Alpha diversity metrics were calculated by subsampling the OTU
247 table ten times at a depth of 1420 reads per sample. The mean values across the ten
248 subsampled OTU tables were used in diversity calculations. Only species of NO_3^--
249 reducing bacteria that comprised at least 0.01% of the total oral microbiome were
250 included in the subsequent statistical analyses.

251

252 **2.5. Statistics**

253 The Statistical Package for the Social Sciences (SPSS Version 24.0. Armonk, NY: IBM
254 Corp) was used for statistical analysis. GraphPad Prism version 5 (GraphPad Software
255 Inc., San Diego, USA) was used to create the figures. The distributions of data were
256 assessed using the Shapiro Wilk test and non-parametric tests were used where data were
257 not normally distributed. A two-way repeated measures analysis of variance (ANOVA)
258 was used to assess the main effects of time (pre- (day 0) and post-supplementation (day
259 8)) and study arm (placebo vs NO_3^-) and interaction effects on plasma and salivary NO_3^-
260 and NO_2^-, pH, BP measurements, and the abundance of NO_3^--reducing bacteria. For the
261 acute NO_3^--response tests, a two factor ANOVA was used to determine the main effects
262 of time (pre- (day 0) and post-supplementation (day 8)) and measurement (before and
263 after the acute ingestion of beetroot juice) and their interaction on plasma and salivary
264 NO_3^- and NO_2^-, pH, and BP measurements. *Post-hoc* analysis was conducted following a
265 significant main effect or interaction using paired samples t-tests with Bonferroni
266 correction for multiple pairwise comparisons. The alpha level for declaring statistical
267 significance was set at $P \leq 0.05$. Data are presented as mean ± standard deviation (SD)
268 unless otherwise stated. Probability values are expressed with 95% confidence intervals
269 (95% CI) where appropriate.

3. Results

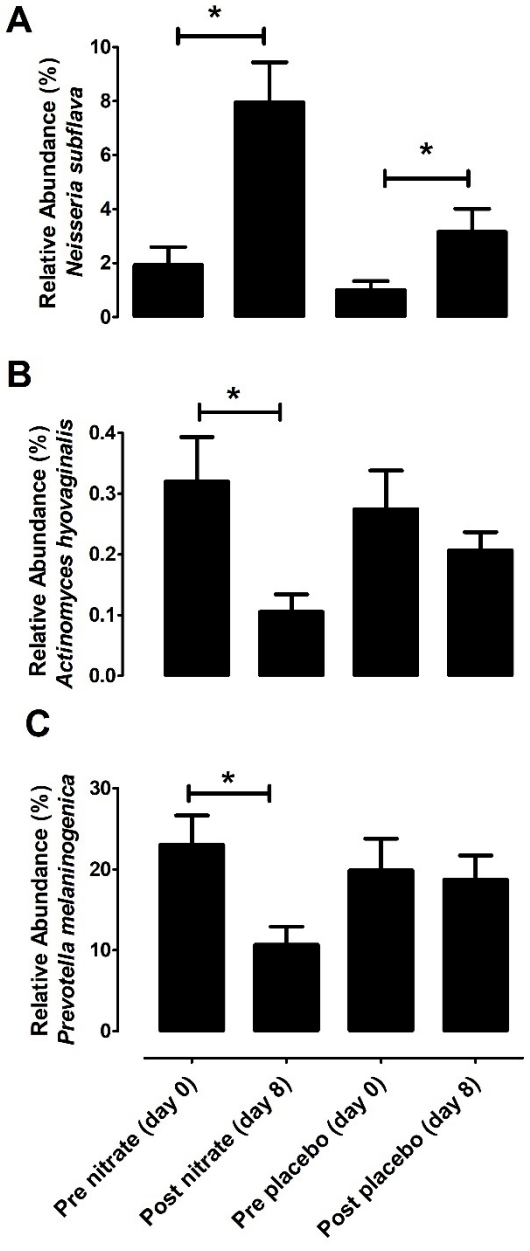
3.1. Impact of 7 days of NO_3^- supplementation on bacterial abundance

Alpha diversity data are presented in Table 1. The Shannon diversity index and the number of observed OTU's were similar between study arms and did not change following supplementation (all $P > 0.05$). The abundance of the most prevalent (>1% relative abundance) at each measurement point are included in the supplementary data.

The most abundant five phyla on the tongue across all four visits were Bacteroidetes (38.1 ± 3.5%), Firmicutes (30.7 ± 2.3%), Fusobacteria (12.1 ± 1.4%), Proteobacteria (11.3 ± 4.6%), and Actinobacteria (3.7 ± 0.6%). The relative abundances of Bacteroidetes, Firmicutes, Fusobacteria, and Actinobacteria did not change after NO_3^- or placebo supplementation and did not differ at baseline between study arms (all $P > 0.05$). There was a main effect of 'time' ($P = 0.009$), and 'study arm' ($P = 0.04$) on Proteobacteria. The abundance of Proteobacteria significantly increased following NO_3^- supplementation (P = 0.011, 95% CI 2.5% - 15.5%) but not placebo ($P > 0.05$). Proteobacteria did not differ at baseline between study arms ($P > 0.05$). The relative abundance of the phyla at each measurement point are included as supplementary data.

Dietary NO_3^- supplementation altered the abundance of four genera of bacteria on the tongue (Table 1). Dietary NO_3^- supplementation reduced the relative abundance of *Prevotella* ($P = 0.021$, 95% CI 2.1% – 20.3%), *Streptococcus* ($P = 0.029$, 95% CI 0.4% – 6.1%) and *Actinomyces* (P = 0.028, 95% CI 0.1% - 1.1%) with no change following placebo and no differences at baseline between study arms (all $P > 0.05$). The abundance of *Neisseria* increased from baseline in both the NO_3^- supplementation arm ($P < 0.001$, 95% CI 4.4 – 9.5%) and the placebo ($P = 0.006$, 95% CI 0.9% – 4.2%). There were no differences at baseline between study arms ($P > 0.05$). The magnitude of the increase in *Neisseria* was greater in the NO_3^- supplementation arm compared to the placebo ($P = 0.001$, 95% CI 2.9% - 8%).

At species level, there were significant effects of time and an arm*time interaction effect on the relative abundance of *Prevotella melaninogenica (*P = 0.03, P = 0.01*)* and *Neisseria subflava* (Fig. 1). There was also a significant main effect of 'time' on *Actinomyces hyovaginalis* (P = 0.01). The relative abundance of *Prevotella melaninogenica* and *Actinomyces hyovaginalis* were lower after 7 days of NO_3^- supplementation compared to pre-supplementation (P = 0.001, 95% CI 6.7% – 20% and P = 0.002, 95% CI 0.1% - 0.3% respectively) and at both time points in the placebo arm (both $P < 0.005$). The relative abundance of *Prevotella melaninogenica* and *Actinomyces hyovaginalis* did not differ at baseline between study arms and were unaltered by 7 days of placebo supplementation (all $P > 0.05$). The relative abundance of *Neisseria subflava* increased from baseline after 7 days of NO_3^- supplementation ($P < 0.001$, 95% CI 3.5% – 8.6%) and also after 7 days of placebo (P = 0.008, 95% CI 0.7% – 3.6%). The magnitude of the increase in *Neisseria subflava* was greater in the NO_3^- supplementation arm compared to the placebo (P = 0.001 95% CI 2.3% - 7.3%). There was no difference in the relative abundance of *Neisseria subflava* at baseline between the NO_3^- and placebo supplementation arms ($P > 0.05$). There were no other differences in any other species or genera of bacteria that are thought to contribute to NO_3^- reduction (all $P > 0.05$).



316

317 Fig. 1. The % relative abundance of bacterial species that were significantly altered between pre-
318 and post-supplementation. (A) *Neisseria subflava*, (B) *Actinomyces hyovaginalis* and (C)
319 *Prevotella melaninogenica*. * denotes significant change from baseline ($P < 0.05$). Only within
320 condition differences are shown for clarity.

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322

323

3.2. Impact of 7 days of NO_3^- supplementation on salivary pH, NO metabolites, and blood pressure

3.2.1. Salivary pH

There was a significant arm*time interaction for salivary pH ($P = 0.022$). There were no differences in salivary pH at baseline (day 0) between the supplementation arms ($P > 0.05$). In the NO_3^- supplementation arm, salivary pH increased from baseline ($P = 0.043$, 95% CI 0.1 – 0.48) but did not change in the placebo arm ($P = 0.20$, Fig. 2). The post-NO_3^- supplementation salivary pH was also higher than the equivalent value in the placebo arm ($P = 0.05$, 95% CI 0.0 – 0.7).

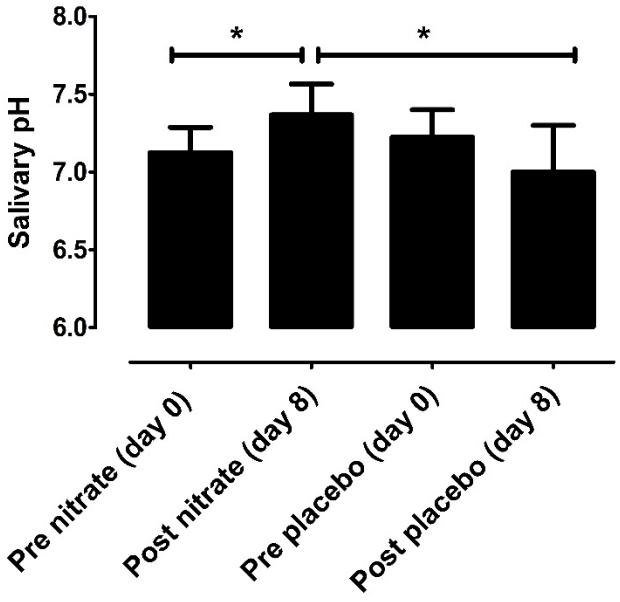

Fig. 2. Salivary pH pre- and post-supplementation with NO_3^- and placebo. * denotes a significant difference between measurement points ($P \leq 0.05$).

3.3. Nitrate and nitrite levels in plasma and saliva

There were no differences in baseline measurements of plasma and saliva NO metabolites between the NO_3^- and placebo arms of the study (Table 2). Supplementation with NO_3^- increased salivary [NO_2^-] ($P = 0.012$, 95% CI 263 – 1701 μM), plasma [NO_2^-] ($P = 0.01$,

95% CI 30 – 175 nM), salivary [NO_3^-] ($P = 0.001$, 95% CI 3228 – 8694 μM) and plasma [NO_3^-] ($P < 0.001$, 95% CI 90 – 208 μM). In the placebo arm of the study, none of the metabolites changed from baseline (all $P > 0.05$). The post-supplementation levels of salivary NO_3^-, plasma NO_3^-, and salivary NO_2^- were higher in the NO_3^- arm compared to the placebo (all $P < 0.001$). Conversely, the post-supplementation levels of plasma NO_2^- did not differ between supplementation arms ($P > 0.05$).

3.4. Blood Pressure, flow mediated dilation, and resting heart rate

There were no differences in SBP, DBP, MAP, flow mediated dilation, or resting heart rate between supplementation arms at baseline (all $P > 0.05$, Table 3). There was a main effect of study arm on MAP, but further interrogation with *post hoc* analyses revealed no differences between study arms at either measurement point. None of the cardiovascular variables were altered following supplementation with either NO_3^- or placebo (all $P > 0.05$).

3.5. Acute nitrate response test

3.5.1. Nitrate and nitrite metabolism

The levels of NO metabolites in the saliva and plasma are presented in Figure 3. For salivary [NO_2^-] and [NO_3^-] there was a main effect of 'time' (all $P \leq 0.01$), 'measurement' (all $P \leq 0.002$) and a 'time * measurement' interaction (all $P \leq 0.015$). Salivary [NO_2^-] increased following the acute administration of NO_3^- in both the pre-supplementation (day 0) ($P = 0.002$, 95% CI 968 - 3331 μM) and post-supplementation (day 8) acute response tests ($P = 0.043$, 95% CI 50 – 2582 μM). Likewise, salivary NO_3^- was significantly elevated in the acute tests on day 0 ($P < 0.001$, 95% CI 7107 – 16725 μM) and day 8 ($P = 0.039$, 95% CI 400 – 13262 μM). The magnitude of the increase in both salivary [NO_2^-] and [NO_3^-] was similar on days 0 and 8 (both $P > 0.05$). There were significant main effects of 'time' ($P < 0.001$), 'measurement' ($P < 0.001$), and a 'time x measurement' interaction ($P = 0.001$) on plasma [NO_3^-]. For plasma [NO_2^-], only the 'measurement" main effect was significant ($P = 0.01$). Plasma [NO_2^-] and [NO_3^-] increased in the acute response tests on both day 0 (NO_2^- $P < 0.001$, 95% CI 214 – 415

nM, NO_3^- $P < 0.001$, 278 – 428 μM) and day 8 (NO_2^- $P = 0.004$, 95% CI 72 - 275 nM, NO_3^- $P < 0.001$, 95% CI 220 – 337 μM). The magnitude of the increase in both plasma $[NO_2^-]$ and $[NO_3^-]$ was similar on each day (both $P > 0.05$).

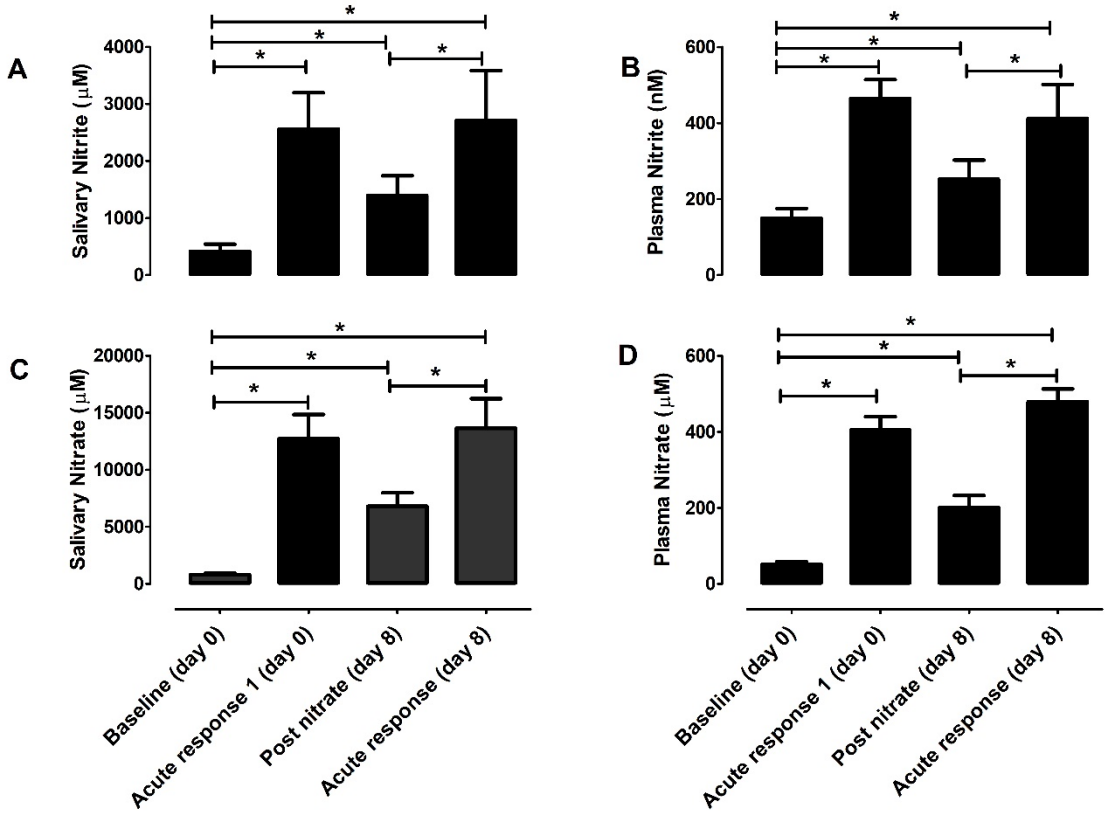

Fig. 3. Salivary and plasma nitrate and nitrite concentration measured at baseline (day 0), following the acute administration of nitrate (day 0), after 7 days of nitrate supplementation (day 8), and following further acute administration of nitrate (day 8). In the acute response measurements, saliva and plasma were measured 1.5 h and 2.5 h, respectively, after the ingestion of nitrate-rich beetroot juice. (A) Salivary NO_2^-, (B) plasma NO_2^-, (C) salivary NO_3^-, and (D) plasma NO_3^-. * denotes significant change from baseline ($P < 0.05$).

3.5.2. Blood pressure

BP data in the acute response tests are presented in Figure 4. There was a significant main effect of 'measurement' on SBP ($P = 0.004$) but no 'time' effect or 'time * measurement' interaction. SBP was significantly reduced from baseline in the acute NO_3^- response test

on day 0 ($P = 0.05$, 95% CI 0 – 4 mmHg) and on day 8 ($P = 0.031$, 95% CI 0 – 6 mmHg, Fig. 5). The magnitude of the decline in SBP did not differ between days 0 and 8 ($P > 0.05$). DBP and MAP did not differ between any measurements (all $P > 0.05$).

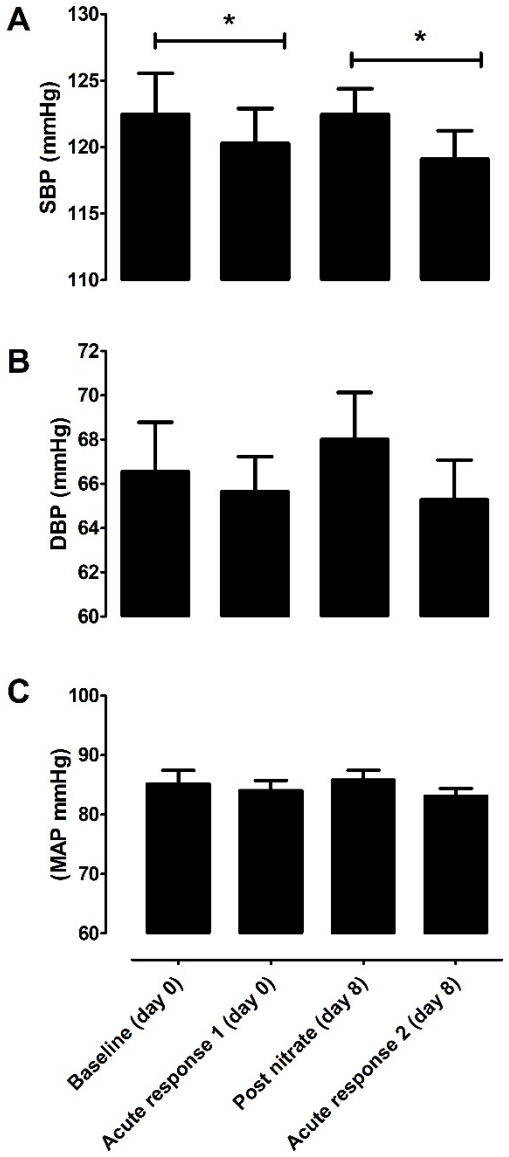

Fig.4. Blood pressure measured at baseline (day 0), following the acute administration of nitrate (day 0), after 7 days of nitrate supplementation (day 8), and following further acute administration of nitrate (day 8). In the acute response measurements blood pressure was measured 2.5 h after the ingestion of nitrate-rich beetroot juice. (A) Systolic blood pressure, (B) Diastolic blood pressure, (C) Mean arterial blood pressure. * denotes significant change from baseline ($P < 0.05$).

3.5.3. Flow Mediated Dilation

There was a significant main effect of 'measurement' on FMD % ($P = 0.021$). The FMD response increased from baseline in the acute NO_3^- response tests on both day 0 ($P = 0.014$, 95% CI 0.5% – 3.2%) and day 8 ($P = 0.042$, 95% CI 0.1% – 3.8%, Fig.5). The magnitude of the FMD response was similar between days 0 and day 8 ($P > 0.05$). The acute administration of NO_3^- did not alter the baseline or peak diameter of the brachial artery (all $P > 0.05$).

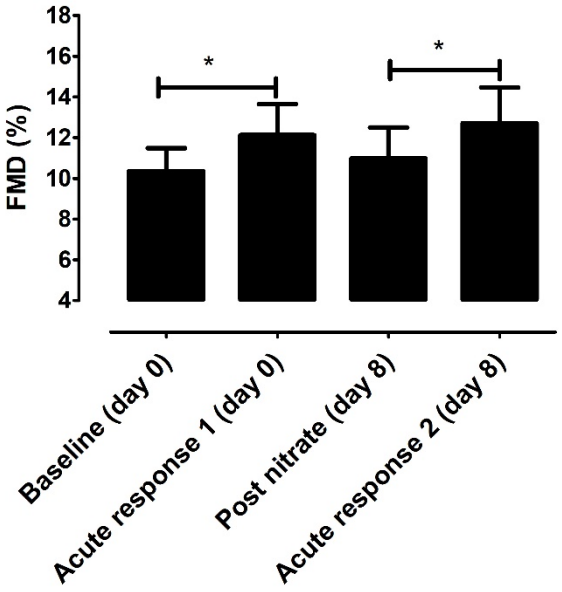

Fig. 5. The flow mediated dilation response measured at baseline (day 0), following the acute administration of nitrate (day 0), after 7 days of nitrate supplementation (day 8), and following further acute administration of nitrate (day 8). In the acute response measurements flow mediated dilation was measured 2.5 h after the ingestion of nitrate-rich beetroot juice. * denotes significant change from baseline ($P < 0.05$).

4. Discussion

This study demonstrates that, as expected, 7 days of dietary NO_3^- supplementation in healthy adults increases the levels of circulating NO metabolites and alters the abundance of oral bacteria that have been previously implicated in the enterosalivary NO_3^--NO_2^--NO pathway. Importantly, the magnitude of the change we observed in the altered bacterial populations exceeds that of the typical biological variation [35] suggesting dietary NO_3^- supplementation results in meaningful alterations to the oral microbiome. Contrary to our hypothesis, however, the adaptations to the oral environment did not enhance the plasma and salivary responses to a NO_3^- dose. Furthermore, whilst the ingestion of NO_3^--rich beetroot juice transiently increased the FMD response and reduced SBP in the hours immediately following a NO_3^- dose, these effects were not augmented following a period of chronic supplementation and had dissipated 10 h following the final NO_3^- dose. These data suggest that frequent daily doses of NO_3^- would be necessary to result in a sustained reduction in BP, at least in this healthy population.

4.1. Impact of 7 days of nitrate supplementation on tongue bacteria and salivary pH

Our samples had a high number of sequences (964,418) with a median of 21918 sequences per sample indicating that our sequencing coverage was at a sufficient depth to detect meaningful changes in the dataset. This is further confirmed by the high Shannon diversity index of 6.2 ± 0.6 and observed OTU value of 337 ± 81. In concordance with previous findings [30], NO_3^- supplementation did not change the Alpha diversity metric demonstrating that this dietary intervention does not alter the community evenness of bacterial species. However, 7 days of NO_3^- supplementation doubled the abundance of the phylum Proteobacteria. These changes were predominantly due to an increase in the abundance of the genus *Neisseria* and specifically the species *Neisseria subflava.*

Salivary pH increased in ten out of our eleven participants (from 7.13 ± 0.54 to 7.39 ± 0.68) following dietary supplementation with NO_3^-. These data are in agreement with previous work [36] which found that regular ingestion of beetroot juice increased salivary pH from 7.0 to 7.5. We show further that supplementation with NO_3^--rich beetroot juice

445 reduced the abundance of *Prevotella melaninogeni*ca, an acidogenic species of bacteria
446 which thrive in environments with a pH between 5.5 and 6 and are thought to contribute
447 to dental caries [37,38]. This species are suggested to be important to NO_3^- reduction by
448 some [3] but not others [39]. One week of NO_3^- supplementation also reduced the
449 abundance of the genera *Streptococcus* and *Actinomyces* and the species *Actinomyces*
450 *hyovaginalis.* In support of these findings, Doel and colleagues [40] observed lower
451 counts of *Streptococcus mutans* in children with higher levels of NO_3^- and NO_2^- in their
452 saliva. While we did not detect this particular species in any of our samples, this is not
453 unusual in a healthy mouth [41]. Of note, both *Prevotella melaninogenica* and
454 *Streptococcus mutans* have been detected in atherosclerotic plaques and diseased heart
455 valve tissue suggesting these species may also be involved in the pathogenesis of
456 cardiovascular disease [42,43], whilst *Actinomyces* species can produce organic acid
457 leading to the accumulation of intracellular polysaccharides causing dysbiosis in the
458 biofilm leading to caries [44].

459

460 *Prevotella* was recently identified as the most abundant species in periodontal plaque
461 samples followed by *Streptococcus*, with *Actinomyces* identified as the fourth most
462 abundant and it is suggested that these bacteria are involved in the pathogenesis of oral
463 disease [45]. The reduction in the abundance of *Prevotella, Actinomyces,* and
464 *Streptococci* are likely due to the antimicrobial effects arising from elevated salivary NO_2^-
465 levels. Studies conducted *in-vitro* have shown that NO formed from NO_2^- can exert
466 bactericidal effects [46,47]. When present in the mouth, these pathogenic species of
467 bacteria ferment carbohydrates from the diet with strong acids produced as bi-products
468 [47]. A reduction in the number of these bacteria, therefore, will reduce the amount of
469 acid in the mouth and increase the pH of the saliva. These findings are important given
470 that a salivary pH sustained below 5.5 will result in de-mineralisation of the teeth [48]
471 and oral acidosis and acidogenic bacteria are the primary drivers behind dental caries and
472 periodontitis [49].

473

474 Dietary NO_3^- supplementation also increased the abundance of *Neisseria subflava* on the
475 tongue. This species of bacteria are able to use oxidised nitrogen compounds as

alternative electron acceptors for energy production [50] and can reduce NO_3^- in the mouth [3]. *Neisseria subflava* are generally considered to be non-pathogenic and are associated with good oral health [51]. *Neisseria subflava* favour a pH of between 7 – 7.5 and this species will replicate via binary fission when conditions and resources are optimal [51,52]. The increase in salivary pH resulting from the ingestion of NO_3^--rich beetroot juice coupled with the concomitant reduction of other species within the oral community, likely created an optimal environment for *Neisseria subflava* to propagate.

While the main outcomes of this study are broadly in agreement with two previous studies [29,30], there are some notable differences. Firstly, both of the earlier studies reported that NO_3^--rich beetroot juice supplementation increased the abundance of *Rothia mucilaginosa.* Secondly, Vanhatalo and colleagues [29] reported that NO_3^- supplementation reduced the relative abundance of *Veillonella* whereas we did not. We did, however, observe significant reductions in *Actinomyces* and *Streptococcus*. Although the reasons for these conflicting findings are unclear, inter-individual differences between participants and variations in oral bacteria sampling methodologies provide the most likely explanations. Participants in the present study were a heterogeneous group of healthy males (age 21 – 44 years). The earlier studies used hypercholesteremia patients [29] or separate groups of younger (age 18-22 years) and older (70 – 79 years) adults [30]. Furthermore, both previous studies analysed the abundance of bacteria in saliva samples whereas we collected bacteria directly from the tongue dorsum. While saliva samples will likely provide a more representative composition of bacteria from all areas of the mouth, the dorsal surface of the tongue has been shown to have the highest NO_3^- reduction capacity of all oral sites [39]. The deep clefts of the tongue provide a protective and stable anaerobic environment that is more conducive to the production of biofilms where bacterial NO_3^- reduction can easily occur [2]. In addition, the bacteria in saliva include those shed from biofilms [53] which may be less metabolically active than those found on the tongue [13]. Given that we aimed to relate bacterial presence to NO_3^--reduction capacity it was considered more appropriate to sample the tongue in this instance.

An unexpected finding of the study was the increase in the abundance of *Neisseria subflava* following ingestion of the placebo, albeit to a lesser extent than in the NO_3^- supplementation arm. This is all the more surprising given there was a small but non-significant reduction in salivary pH after 7 days of NO_3^--depleted beetroot juice (from 7.22 ± 0.61 to 6.99 ± 1.00); an environment which may be expected to suppress *Neisseria subflava.* Of note is that both NO_3^--rich and NO_3^--depleted versions of the beetroot juice contained a considerable amount of sugar (~15g total carbohydrate per 70 ml bottle). In the absence of an elevation in salivary NO_2^-, cariogenic bacteria will increase acid production in response to an increased availability of carbohydrate. However, beetroot juice also has a high total antioxidant capacity and polyphenol content and is rich in several compounds including phenolic acids, flavonoids, and betalains [54]. It is possible that that *Neisseria subflava* responded positively to some of these components although the effects are clearly augmented by NO_3^-. Conversely, a previous study [29] did not report alterations to the oral microbiome after placebo. It is not possible to elucidate whether the placebo altered the microbiome of participants in similar work [30] as samples were not collected at baseline. While our data require corroboration, they do suggest that the NO_3^--depleted beetroot juice is not completely inert; a point that should be carefully considered by researchers during study design.

4.2. Consequences of changes in the oral microbiome on nitrate and nitrite levels

Recent work [30] showed that individuals with a high abundance of *Prevotella melaninogenica* and *Campylobacter concisus* on the tongue at baseline had less NO_2^- in the plasma and smaller reductions in BP in response to chronic NO_3^- supplementation. The authors suggested that the NO_2^- reduction genes encoded by these bacteria impair downstream NO_2^- accumulation via bacterial reduction of NO_2^- in the oral cavity before it enters the circulation. In the present study, seven days of NO_3^- supplementation reduced the abundance of *Prevotella melaninogenica* and increased *Neisseria subflava*. As expected, both saliva and plasma NO_2^- were elevated from baseline in the NO_3^- arm of the study. However, it is not possible to isolate the influence of the altered microbiome on basal levels of NO_2^- as these parameters were almost certainly increased directly by the ingestion of beetroot juice on the previous day. Nevertheless, previous data from our

laboratory has demonstrated that the capacity to generate NO_2^- in the mouth is associated with the abundance of NO_3^--reducing bacteria on the tongue [20]. As a consequence, we also expected that saliva and plasma NO_2^- levels would be augmented post-NO_3^- supplementation following ingestion of a NO_3^--rich beetroot juice bolus. Data from the acute response component of this study, however, provides evidence to the contrary. Firstly; the peak levels of saliva and plasma in response to the beetroot juice bolus were similar before and after the NO_3^- supplementation period. This is particularly intriguing given baseline levels were elevated in the post-supplementation test. This suggests that when "excess" NO_2^- is produced it is excreted, perhaps to avoid excessive drops in BP. Secondly; the magnitude of increase in salivary NO_2^- during the acute response test did not change following 7 days of NO_3^- supplementation. The lack of changes to NO_2^- generation may be due to the fact that *Prevotella* and *Actinomyces,* although antagonistic to oral health, have also been identified as important to NO_3^- reduction either directly or through bacterial community interactions [3]. Therefore, an increase in the abundance of one species of bacteria thought to be important to the NO_3^- reduction process (*Neisseria subflava)* has been offset by reductions in others. An enhanced reduction of NO_2^- to NO in the oral cavity to prevent accumulation of NO_2^- in the saliva [30] seems unlikely in this instance as the abundance of these bacterial species were not altered by NO_3^- supplementation. Furthermore, NO_2^- reduction is a slow reaction and it is questionable whether there would be time for this to occur in the open in vivo salivary system [55].

It should also be acknowledged that the participants in the present study were all in good oral health meaning their oral microbiome was already capable of efficient NO_3^- reduction. Alternatively, there may be other rate limiting steps in the NO_3^- reduction process including gastric emptying and absorption rates, the availability of sialin (NO_3^- transporter in saliva), and salivary flow rates. Further mechanistic insight would also be provided by a direct test of NO_3^- reduction in the mouth, metatranscriptomic analysis to determine NO_2^- and NO_3^- reductase gene expression of the oral bacteria and collecting data from patients with oral diseases such as periodontitis.

4.3. Consequences of changes in the oral microbiome on vascular function

In the present study, there was a transient reduction in SBP and increase in the FMD response during the acute NO_3^- response tests before and after NO_3^- supplementation. These effects were likely mediated by the increased production of NO resulting in vasodilation [56]. Likewise, it has been previously observed that SBP was similarly reduced after acute (2.5 h after ingestion) and chronic (15 d) supplementation with beetroot juice [57]. Our data extends these findings and demonstrates that adaptations to the oral microbiome arising from sustained NO_3^- supplementation did not in this instance alter vascular responsiveness to a NO_3^- dose. This is not surprising given that the increase in plasma [NO_2^-] was not augmented in the post-supplementation acute response test. It should be noted, however, that our participants were a group of normotensive healthy volunteers and results may be different in populations with compromised vascular responsiveness.

It should be highlighted that SBP was only reduced during the acute NO_3^- response tests but not following 7 days of NO_3^- supplementation. This was likely due to the 10 h gap between the ingestion of the last NO_3^- dose and the collection of measurements on day 8. While plasma NO_2^- was elevated from baseline, the magnitude of this increase was small (102 nM) and was seemingly insufficient to reduce BP in this healthy population. Therefore, larger or more frequent doses of NO_3^- may be needed to elicit sustained improvements in vascular function.

5. Conclusions

Seven days of supplementation with NO_3^--rich beetroot juice significantly increased the levels of circulating NO metabolites, increased the pH of saliva, and caused meaningful alterations to the oral microbiome in favour of oral health. These data are significant given that a high abundance of pathogenic bacteria can cause periodontitis and sustained oral acidosis will result in dental caries. For the first time, our data shows that the aforementioned adaptions to the oral microbiome do not alter the capacity to produce salivary NO_2^- or enhance vascular responsiveness following a dose of beetroot juice, at least in a healthy adult population.

Additional Information

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

The study was conceived by MB and CE and all authors contributed towards the experimental design. Data were collected by MB, LL, CM, NS, and CE. Analysis of FMD data were performed by MB and NS. Analysis of plasma and saliva samples were performed by MB, LL, and CM. Bacterial samples were prepared for analyses by MB and JB. Bioinformatical analysis of bacteria were performed by MB. Statistical analyses were completed by MB and CE. MB prepared the first draft of the manuscript. All authors have critically revised and approved the final version of the manuscript submitted for publication.

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Table 1: Pre- and post-supplementation bacterial diversity metrics and relative abundance of the bacteria that were altered by dietary nitrate supplementation.

Diversity Metric	Time (Day)	Nitrate	Placebo	ANOVA (*P* Value)
Shannon Diversity Index	Pre (0)	6.3 ± 0.6	5.9 ± 0.7	Time = 0.707 Arm = 0.858 Interaction = 0.122
	Post (8)	6 ± 0.9	6.6 ± 0.3	
Observed OTUs	Pre (0)	312 ± 89	349 ± 97	Time = 0.876 Arm = 0.856 Interaction = 0.07
	Post (8)	383 ± 56	304 ± 83	
Bacteria				
Prevotella *(% relative abundance)*	Pre (0)	34 ± 17	26 ± 16	Time = 0.283 Arm = 0.993 Interaction = 0.053
	Post (8)	23 ± 11*†	31 ± 14	
Neisseria *(% relative abundance)*	Pre (0)	2 ± 3	1 ± 1	Time = 0.001 Arm < 0.001 Interaction = 0.008
	Post (8)	9 ± 5*†	4 ± 3*	
Streptococcus *(% relative abundance)*	Pre (0)	9 ± 6	6 ± 4	Time = 0.404 Arm = 0.816 Interaction = 0.006
	Post (8)	6 ± 4*†	8 ± 3	
Actinomyces *(% relative abundance)*	Pre (0)	1.1 ± 0.7	0.9 ± 0.6	Time = 0.376 Arm = 0.014 Interaction = 0.164
	Post (8)	0.5 ± 0.4*†	0.7 ± 0.2	

* denotes a significant difference from the pre-supplementation (day 0).

† denotes a greater change from the pre-supplementation value compared to the placebo arm.

624 **Table 2:** Levels of nitric oxide metabolites pre- and post-supplementation in each study arm.

Parameter	Time (Day)	Nitrate	Placebo	ANOVA (*P* Value)
Plasma nitrite (nM)	Pre (0)	150 ± 84	174 ± 111	Time = 0.001
	Post (8)	252 ± 165*	220 ± 112	Arm = 0.898
				Interaction = 0.290
Plasma nitrate (μM)	Pre (0)	52 ± 24	69 ± 64	Time < 0.001
	Post (8)	201 ± 104*†	57 ± 36	Arm < 0.001
				Interaction = 0.001
Salivary nitrite (μM)	Pre (0)	415 ± 420	365 ± 301	Time = 0.01
	Post (8)	1397 ± 1151*†	367 ± 297	Arm = 0.002
				Interaction = 0.015
Salivary nitrate (μM)	Pre (0)	810 ± 404	746 ± 388	Time = 0.001
	Post (8)	6801 ± 3956*†	875 ± 589	Arm < 0.001
				Interaction = 0.001

625 * denotes a significant difference from the pre-supplementation (day 0).

626 † denotes a greater change from the pre-supplementation value compared to the placebo arm.

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628

629 **Table 3:** Cardiovascular variables pre- and post-supplementation in each study arm.

Parameter	**Time (Day)**	**Nitrate**	**Placebo**	**ANOVA (*P* Value)**
Systolic blood pressure (mmHg)	Pre (0) Post (8)	122 ± 10 122 ± 6	124 ± 6 127 ± 8	Time = 0.196 Arm = 0.325 Interaction = 0.290
Diastolic blood pressure (mmHg)	Pre (0) Post (8)	67 ± 7 66 ± 5	68 ± 7 65 ± 6	Time = 0.141 Arm = 0.771 Interaction = 0.215
Mean arterial pressure (mmHg)	Pre (0) Post (8)	85 ± 8 86 ± 5	89 ± 6 91 ± 6	Time = 0.311 Arm = 0.043 Interaction = 0.581
Resting heart rate (beat·min^{-1})	Pre (0) Post (8)	55 ± 7 56 ± 8	55 ± 5 55 ± 6	Time = 0.973 Arm = 0.631 Interaction = 0.459
Flow mediated dilation (%)	Pre (0) Post (8)	10.46 ± 3.76 12.03 ± 5.09	12.1 ± 5.25 14.05 ± 6.18	Time = 0.021 Arm = 0.221 Interaction = 0.854

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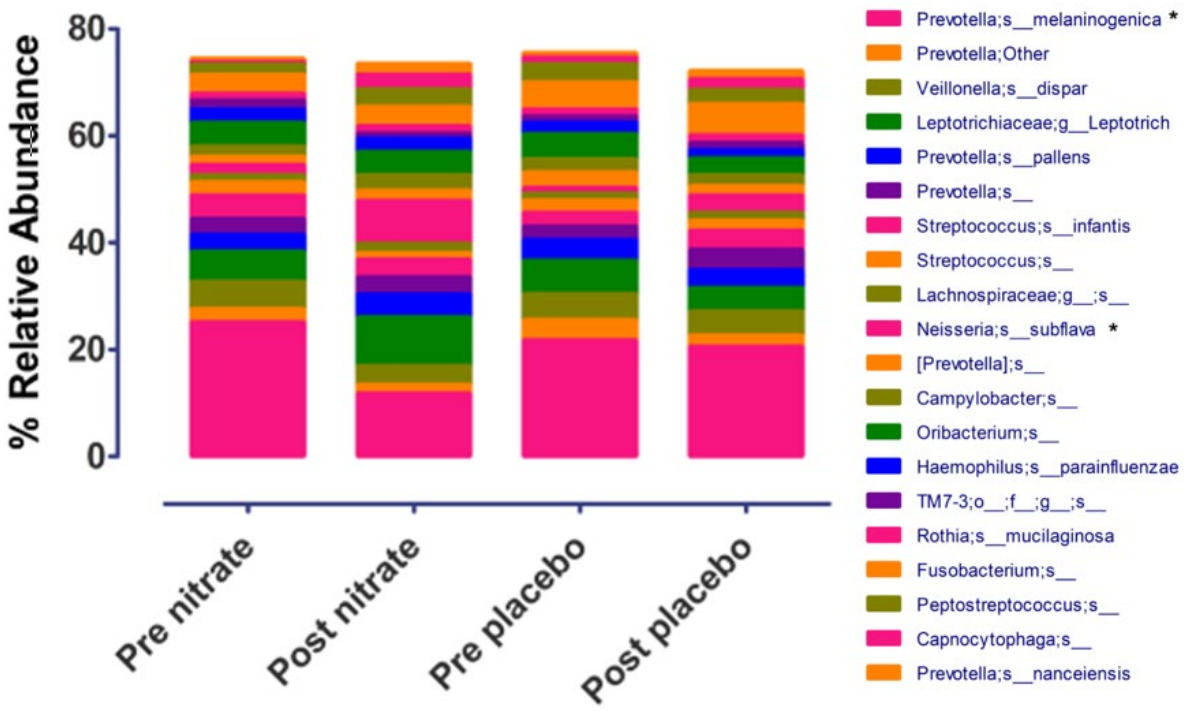

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828 *Supplementary Fig. 1* Bacterial species that made up > 1% of the dataset displayed in ascending order of abundance.
829 *Prevotella melaninogenica* was the most abundant species. * indicates those which were significantly altered.

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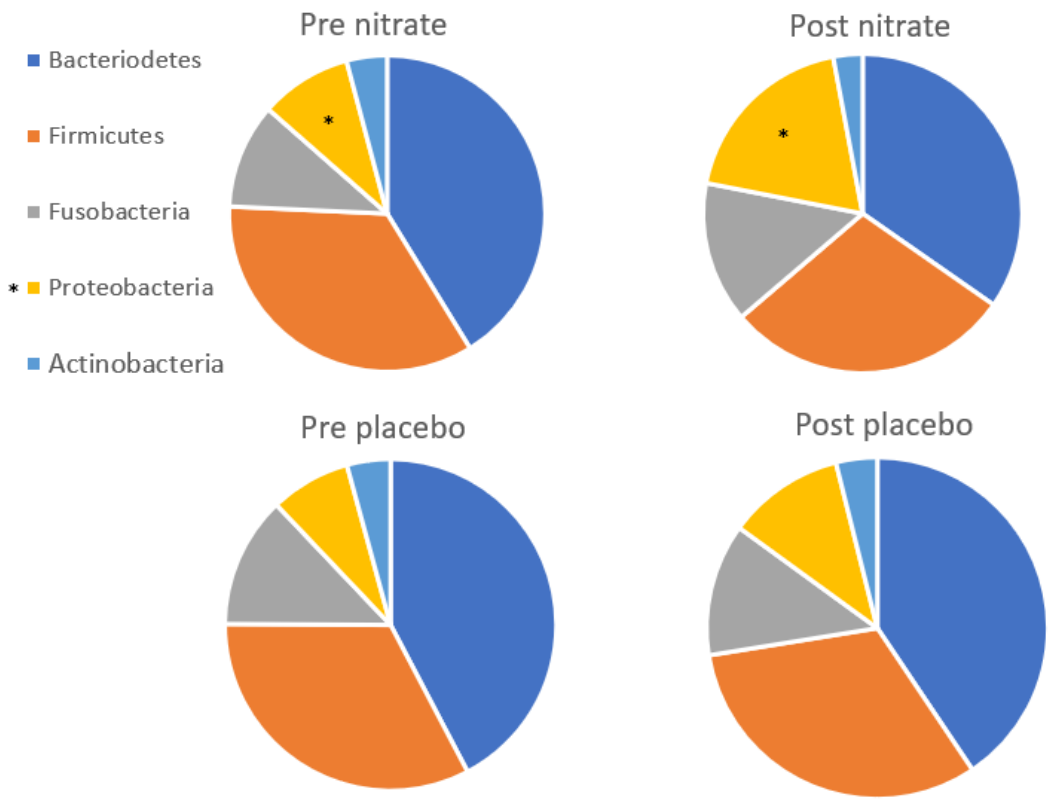

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833 *Supplementary Fig. 2* The proportions of the five main phyla on the tongue dorsum at the four measurement time
834 points, Pre nitrate, Post nitrate, Pre placebo, and Post placebo. * indicates a significant increase in Proteobacteria
835 from Pre to Post nitrate.