Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria

Mia C. Burleigh[1], Luke Liddle[1], Chris Monaghan[1], David J. Muggeridge[2], Nicholas Sculthorpe[1], John P. Butcher [3,5], Fiona L. Henriquez [3], Jason D. Allen [4], Chris Easton[1*],

[1]Institute for Clinical Exercise and Health Science, University of the West of Scotland, Hamilton, UK

[2]Physical Activity and Health Group, School of Psychological Science and Health, University of Strathclyde, Glasgow, UK.

[3]Institute of Biomedical and Environmental Health Research, University of the West of Scotland, Paisley, UK

[4]Department of Kinesiology, Curry School of Education, University of Virginia, Charlottesville, VA 22904, USA.

[5]Department of Life Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, UK

*Correspondence to: University of the West of Scotland, Almada Street, Hamilton, ML3 0JB, UK. Tel: (+44) 1698 283100x8282. Fax: N/A. chris.easton@uws.ac.uk

Abstract

Nitric oxide (NO) can be generated endogenously via NO synthases or via the diet following the action of symbiotic nitrate-reducing bacteria in the oral cavity. Given the important role of NO in smooth muscle control there is an intriguing suggestion that cardiovascular homeostasis may be intertwined with the presence of these bacteria. Here, we measured the abundance of nitrate-reducing bacteria in the oral cavity of 25 healthy humans using 16S rRNA sequencing and observed, for 3.5 hours, the physiological responses to dietary nitrate ingestion via measurement of blood pressure, and salivary and plasma NO metabolites. We identified 7 species of bacteria previously known to contribute to nitrate-reduction, the most prevalent of which were *Prevotella melaninogenica* and *Veillonella dispar*. Following dietary

nitrate supplementation, blood pressure was reduced and salivary and plasma nitrate and nitrite increased substantially. We found that the abundance of nitrate-reducing bacteria was associated with the generation of salivary nitrite but not with any other measured variable. To examine the impact of bacterial abundance on pharmacokinetics we also categorised our participants into two groups; those with a higher abundance of nitrate reducing bacteria (>50%), and those with a lower abundance (<50%). Salivary nitrite production was lower in participants with lower abundance of bacteria and these individuals also exhibited slower salivary nitrite pharmacokinetics. We therefore show that the rate of nitrate to nitrite reduction in the oral cavity is associated with the abundance of nitrate-reducing bacteria. Nevertheless, higher abundance of these bacteria did not result in an exaggerated plasma nitrite response, the best known marker of NO bioavailability. These data from healthy young adults suggest that the abundance of oral nitrate-reducing bacteria does not influence the generation of NO through the diet, at least when the host has a functional minimum threshold of these microorganisms.

Keywords: Nitric oxide; microbiome; beetroot juice

Introduction

NO is a multifunctional signalling molecule which is involved in various biological processes such as; host defence [1], regulation of mucosal blood flow and mucus generation [2], regulation of smooth muscle contraction [3], cerebral blood flow [4], glucose homeostasis [5], and mitochondrial function [6]. Ingestion of inorganic NO_3^- from sources such as green leafy vegetables and roots has been consistently shown to increase plasma and salivary [NO_3^-] [7] and augment NO bioavailability [8]. In this pathway, NO_3^- is rapidly absorbed in the upper gastrointestinal tract and enters the circulation [9] before it is subsequently concentrated in the saliva [10], [11] and a proportion reduced to NO_2^-. Salivary NO_2^- can be further reduced to

nitric oxide (NO) in certain physiological conditions such as hypoxia or stored in the blood and tissues for use when endogenous production of NO via NO synthases (NOS) is limited [12]. As a consequence, ingestion of inorganic NO_3^- may elicit a myriad of positive biological effects likely mediated by an increased NO bioavailability. Some studies have demonstrated that ingestion of NO_3^-–rich beetroot juice can reduce blood pressure (BP) [13], enhance endothelial function [14], protect against ischaemic injury [15], and improve exercise performance [16] although these effects are not consistently observed [17], [18], [19].

The reduction of NO_3^- to NO_2^- in saliva is achieved through the action of certain microbes which reside in the oral cavity [20], [21]. The whole human microbiome is characterised by body site-specific microbial ecosystems capable of exerting effects on their host through production of metabolites, immune responses, and gene expression [22]. Some microbes live in symbiosis with their host and can significantly contribute to health [23], [24]. Conversely, low diversity of microbial species resulting in dysbiotic states, have been linked to a number of adverse health conditions including; metabolic syndrome, allergies, asthma, obesity, and cardiovascular disease amongst others [25]. The oral cavity is heavily colonised by microbes and is one site where a symbiotic relationship between humans and bacteria is clearly evident.

A series of studies have confirmed the importance of commensal bacteria to the mammalian enterosalivary cycle, and NO bioavailability. These studies show consistently that rinsing the oral cavity with chlorhexidine anti-bacterial mouthwash disrupts bacterial enzymatic activity and abolishes the BP lowering effects associated with dietary NO_3^- ingestion [26]–[28]. Hyde and colleagues [21] recently analysed oral microflora from a small sample of healthy human participants (n = 6) and identified 14 bacterial candidate species that are thought to contribute

to NO_3^- reduction. The majority of operational taxonomic units (OTUs) with NO_3^- reducing capability originated from the genera *Granulicatella, Actinomyces, Veillonella, Prevotella, Neisseria,* and *Haemophilus*. Other studies have also associated OTUs from the genera *Rothia and Staphylococcus* with NO_3^- reduction [20], [29]. Despite emerging evidence linking NO_3^- reducing bacteria with cardiovascular homeostasis no study has explored the relationship between the abundance of NO_3^- reducing bacteria in the oral cavity and the capacity to process dietary NO_3^- in vivo. This is important because the conversion of NO_3^- from the diet to NO_2^- is known to be profoundly variable [30] and the abundance of NO_3^- reducing bacteria may be a rate-limiting step in this process.

Therefore, our primary objective was to perform descriptive analysis of the abundance and diversity of oral NO_3^- reducing bacteria in a larger cohort than previously utilised [21]. The secondary objective was to determine the association between the abundance of known NO_3^- reducing bacteria with cardiovascular variables and NO biomarkers in blood and saliva. A further objective was to determine whether participants with a higher abundance of NO_3^- reducing bacteria had different salivary and plasma NO pharmacokinetics following ingestion of dietary NO_3^- compared to those with a lower abundance.

Methods

Participants

Twenty five healthy adults (age 27 ± 7 years, stature 172 ± 9 cm, body mass 75 ± 15 kg, 11 female) volunteered and provided written informed consent prior to participating in the study. Ethical approval was provided by the School of Science Ethics Committee at The University of the West of Scotland. All participants were in good cardiovascular and oral health, did not

report any habitual use of antibacterial mouthwash, were free from non-prescription medicines known to interfere with stomach acid production, and were not taking any prescribed medication. Cardiovascular 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. All procedures were conducted in accordance with the Declaration of Helsinki 1974 and its later amendments.

Experimental Design

Each participant attended the laboratory on one occasion for this cross-sectional study. Prior to the trial, participants were briefed on procedures and provided with an adapted version of the National Institutes of Health daily food list. The questionnaire was adapted to differentiate between high, medium, and low NO_3^- containing foods [31]. Participants were asked to record their diet for seven days prior to arrival at the laboratory and instructed to maintain a normal dietary routine. Participants arrived at the laboratory on the morning of the trial in a fasted and euhydrated state after consuming 500 ml of water. Prior to the trial, participants were instructed to avoid strenuous exercise for 24 h and caffeine for 12 h. On the morning of the trial participants were requested not to brush their teeth or tongue and not to use mouthwash or chew gum. Participants provided verbal assurance of their compliance with these instructions.

Procedures

On arrival at the laboratory, stature and body mass were recorded. Participants then lay supine for the remainder of the experiment. During the first 30 min a cannula was inserted

into one of the forearm veins and a tongue scrape sample collected. No other physiological measurements were collected for 30 min to ensure plasma [NO_2^-] had stabilised following the change in body posture [32]. Following this initial phase, baseline measurements of BP and heart rate (HR) were recorded and samples of blood and saliva were collected. Participants then ingested 2 x 70 ml of NO_3^--rich beetroot juice (~12.4 mmol NO_3^-) (Pro-Elite Shot, James White Drinks Ltd., Suffolk, England) and physiological measurements were collected at regular intervals for the next 3.5 h (Fig. 1).

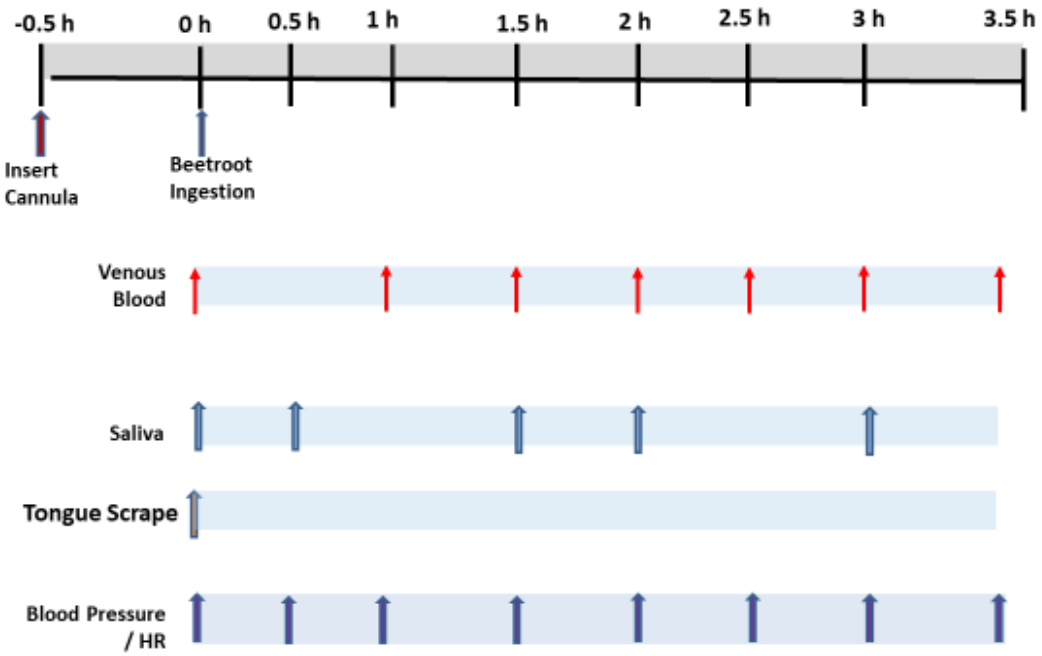

Figure 1: Schematic diagram depicting time-course of data collection from 0 h to 3.5 h following the consumption of NO_3^--rich beetroot juice

Blood Collection

Venous blood was collected in 4 ml aliquots in tubes containing ethylenediaminetetraacetic acid (BD vacutainer K2E 7.2mg, Plymouth, U.K.). Plasma NO_2^- has been shown to peak, on

average, at 2.5 h after ingestion of beetroot juice [33] so multiple blood samples were taken before and after this point. Samples of whole blood were immediately centrifuged for 10 min at 4000 rpm at 4°C (Harrier 18/80, Henderson Biomedical. UK) immediately following collection. The plasma was then separated into two cryovials and immediately stored at -80^{0}C for later analysis of NO_3^- and NO_2^- content via ozone-based chemiluminescence. The cannula was flushed with 2 ml sterile 0.9% saline immediately following blood draws to keep the line patent.

Saliva Collection

Samples of unstimulated saliva were collected via an oral swab (Saliva Bio Oral Swab (SOS) Salimetrics, Pennsylvania, USA) placed under the tongue for 3 min. Samples of saliva were collected from 0.5 h onwards as previous data has shown salivary [NO_2^-] and [NO_3^-] may peak earlier than 1 h [28]. Swabs were then 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). Samples were then separated into two cryovials and immediately stored at -80^{0}C for later analysis of NO_3^- and NO_2^-.

Measurement of Salivary and Plasma [NO_3^-] and [NO_2^-]

For the analysis of plasma NO_2^-, tri-iodide reagent comprised of 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 the NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was created by injecting 100 µL of NO_2^- solutions at concentrations up to 1000 nM. Plasma and saliva samples were thawed in a water bath at 37°C and 100µL of the thawed sample was injected immediately into the purge vessel, in duplicate. Saliva samples

were diluted with deionised water at a ratio of 1:100 before injection. 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 consisting of 24 mg of vanadium tri-chloride and 3 ml of 1 M hydrochloric acid was placed into the purge vessel and was heated to 90°C. A standard curve was created by injecting 25-50 µL NO_3^- solutions at concentrations up to 100 µM. Plasma samples were 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. Supernatant was then injected into the purge vessel and concentration calculated as described for NO_2^-.

Heart Rate and Blood Pressure

HR was continually monitored via telemetry (Polar Electro, Oy, Finland). Measurements of BP were recorded in triplicate by standard auscultation using an automated device (Orman M6, Intelli-Sense. Hoofdorp, the Netherlands). Mean arterial pressure (MAP) was calculated using the following equation;

$$MAP = (2 \times \text{diastolic BP} + \text{systolic BP}) / 3$$

Tongue Scrape and Bacteria Collection

Bacteria were collected from the posterior dorsal surface of the tongue using a sterile metal tongue cleaner (Soul Genie, Health Pathways LLP, Uttar Pradesh, India). This area of the

mouth has previously been shown to contain a high abundance of NO_3^- reducing bacteria as they favour the anaerobic environment provided by the deep crypts of the tongue [20]. The tongue cleaner was gently glided from the back to the front of the tongue until there was a visible coating on the instrument [21]. Tongue scrape samples were subsequently transferred via a sterile sample collection swab (Deltalab, S.L. Barcelona, Spain) to a MoBio Powersoil DNA Isolation Kit (MoBio Laboratories Inc. West Carlsbad, California) and immediately frozen at -80^0C. Bacterial DNA was subsequently extracted using the MoBio Powersoil Isolation Kit according to the manufacturer's guidelines.

Bacterial Analysis

DNA was transported to a commercial centre (HOMMINGS, The Forsyth Institute, Boston MA, USA) for sequencing analysis. A full description of the protocol is described in previous research [34]. In brief, the V3-V4 region of the bacterial genomic DNA was amplified using barcoded primers; ~341F (forward primer) (AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTCCTACGGGAGGCAGCAG) and ~806R (reverse primer) CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNNNAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT).

Samples *(*10 – 50 ng) of DNA were PCR-amplified using V3-V4 primers and 5 PrimeHotMaster Mix and purified using AMPure beads. A small volume (100 ng) of each library was pooled, gel-purified, and quantified using a bioanalyser and qPCR. Finally, 12pM of the library mixture, spiked with 20% Phix, was analysed on the Illumina MiSeq (Illumina, San Diego, CA).

16s rRNA gene data analysis

Quality filtered data received from the sequencing centre was further analysed for taxonomic classification and bacterial abundance using Qiime 1.8 [35]. One sample with less than 5000 reads was discarded from further analysis. Sequences were clustered *de novo* and binned into OTUs based on 97% identity. Taxonomy was assigned using RDP classifier trained to the GreenGenes database (October 2013 release). Singleton reads were removed from the dataset. In order to calculate alpha diversity metrics, the OTU table was sub-sampled to 20090 reads per sample and repeated 5 times. The mean values were then calculated across the 5 sub-sampled OTU tables and used to calculate alpha diversity metrics. The smallest number of reads associated with any one sample was 20094 reads. To analyse the effect of bacterial abundance on pharmacokinetic changes in response to NO_3^-, participants were split into two groups; those with a higher overall abundance (>50%) of NO_3^- reducing bacteria (High) and those with a lower abundance (<50%) (Low).

Statistical Analysis

Statistical Package for the Social Sciences (SPSS Version 22.0. Armonk, NY: IBM Corp) was used for statistical analysis. GraphPad Prism version 7 (GraphPad Software Inc., San Diego, USA) was used to create the figures. The distributions of data were assessed using the Shapiro Wilk test and non-parametric tests were used where data were not normally distributed. A one-way repeated measures ANOVA was used to assess changes in plasma and salivary NO_3^- and NO_2^-, and BP measurements.

The association between the abundance of NO_3^- reducing bacteria and peak values of plasma and salivary NO variables was analysed using the Spearman's rank correlation co-efficient. Peak delta values were defined as the value with the biggest change from baseline. The association between the abundance of NO_3^- reducing bacteria and the area under the curve for salivary nitrite across the experiment was calculated using the same method.

Differences in bacterial abundances between Low and High groups were assessed using an independent t test. A two-factor mixed model ANOVA (group and time) was used to compare differences in BP and NO metabolites between groups and gender. Data are presented as mean ± standard deviation unless otherwise stated. Statistical significance was declared when $P \leq 0.05$. Probability values are expressed with 95% confidence intervals (95% CI) where appropriate**.**

Results

Ingestion of dietary NO_3^- raises plasma and salivary NO metabolites and lowers blood pressure

Salivary and plasma NO_2^- and NO_3^- were increased at all time points compared to baseline (all $P < 0.001$), with the exception of plasma NO_2^- at 1 h ($P = 0.1$). Ingestion of beetroot juice significantly reduced SBP ($P = 0.018$, 95% CI 1 - 6 mmHg) and DBP ($P = 0.045$, 95% CI 1 - 4 mmHg) at 2.5 h. MAP was significantly lower at 1.5 h ($P = 0.01$, 95% CI 1 - 5 mmHg), 2 h ($P = 0.03$, 95% CI 1 - 4 mmHg), and 2.5 h ($P = 0.05$, 95% CI 1 - 4 mmHg) (Figure 2). Mean HR tended to be lower overall after NO_3^- ingestion ($P = 0.07$) but there was no significant

main effect for HR at any specific time point (all $P > 0.05$). There were no significant differences between males and females for any variable (all $P > 0.05$).

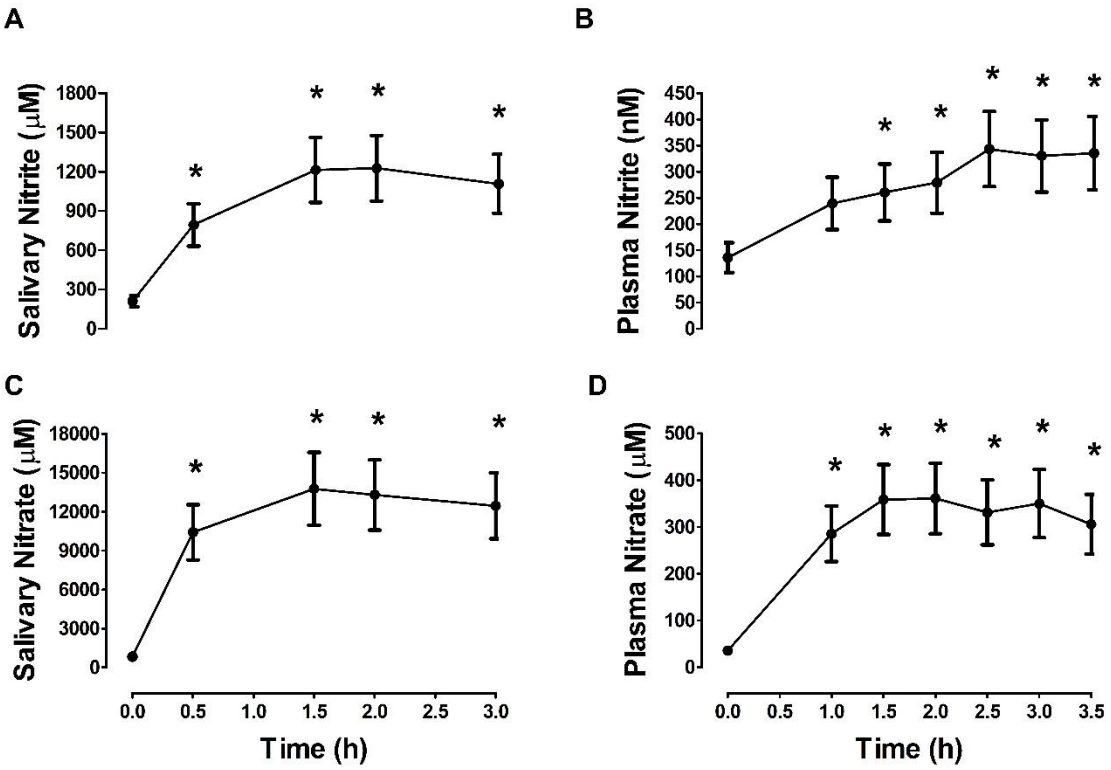

Figure 2. Graphs show change in NO metabolites from baseline after ingestion of beetroot juice. Salivary nitrite (A), plasma nitrite (B), salivary nitrate (C) and plasma nitrate (D). * denotes significant increase from baseline ($P<0.05$).

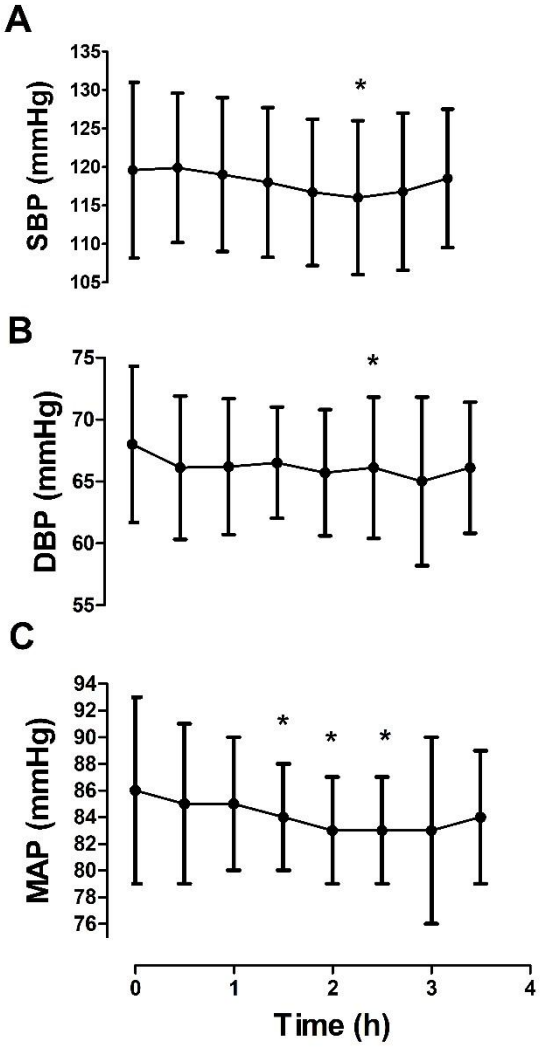

Figure 3. Graphs show change in BP from baseline to 3.5 h after ingestion of beetroot juice. SBP (A), DBP (B) and MAP (C). Value shown are mean ± SD, * denotes significant decrease from baseline, ($P < 0.05$).

Comparison of nitrate reducing communities of healthy human tongues

After quality filtering of the data and removal of singleton reads, tongue scrapings of 24 subjects were included in the analysis. Alpha diversity metrics revealed that samples were diverse with an average of 1165 ± 157 OTUs. The Shannon diversity index was 5.2 ± 0.6, however, there was notable variation in relative abundance. Previous in vitro work [21] suggests that the genera displayed in Table 1 contribute to NO_3^- reduction. Some of these

were amongst the top five most abundant genera as indicated by the blue shaded area (Table 1).

Table 1, % relative abundance of genera present in our samples which have previously been implicated in nitrate reduction. The blue shaded area indicates the top five most abundant genera identified overall.

OTU ID	Mean ± SD (%)	Max (%)	Min (%)
Prevotella	42.12 ± 10.09	63.43	19.92
Veillonella	20.55 ± 12.31	45.5	6.07
Leptotrichia	4.13 ± 4.11	13.91	0.02
Fusobacterium	3.60 ± 3.89	13.56	0.01
Haemophilus	2.84 ± 1.63	6.06	0.00
Neisseria	2.60 ± 5.50	20.54	0.00
Actinomyces	0.84 ± 3.77	14.52	0.05
Porphyromonas	0.47 ± 0.81	2.6	0.00
Rothia	0.41 ± 0.53	20.54	0.00
Granulicatella	0.14 ± 0.14	0.014	0.00

We found seven of fourteen known species which have previously been identified as having a NO_3^- reduction gene (Table 2). It has been suggested that bacteria do not work independently but as consortium. To reflect this, we calculated the total % relative abundances of the seven NO_3^- reducing bacteria shown in Table 2. We assessed if gender influenced the abundance of nitrate reducing bacteria finding that there were no significant differences ($P > 0.05$).

Table 2, % relative abundance of NO_3^- reducing species

Species	Mean ± (%)
Prevotella melaninogenica	31.43 ± 10.33
Veillonella dispar	19.30 ± 11.97
Haemophilus parainfluenzae	2.78 ± 3.83
Neisseria subflava	2.57 ± 5.52
Veillonella parvula	0.24 ± 0.46

Rothia mucilaginosa	0.37 ± 0.49
Rothia dentocariosa	0.003 ± 0.004

High abundance of nitrate reducing bacteria correlates with high salivary nitrite response

The correlation analysis between the sum of the NO_3^- reducing species (identified in Table 2) and the peak delta change in relevant physiological measurements are displayed in Figure 4. The abundance of NO_3^- reducing bacteria was significantly correlated with the change in salivary NO_2^- ($P = 0.03$, r = 0.44, Fig. 3A) but not with any other variable (all $P > 0.05$). The area under the curve for salivary NO_2^- (3010 ± 614.52 µM) was also significantly correlated with the sum of the NO_3^- reducing species ($P = 0.05$, r = 0.40).

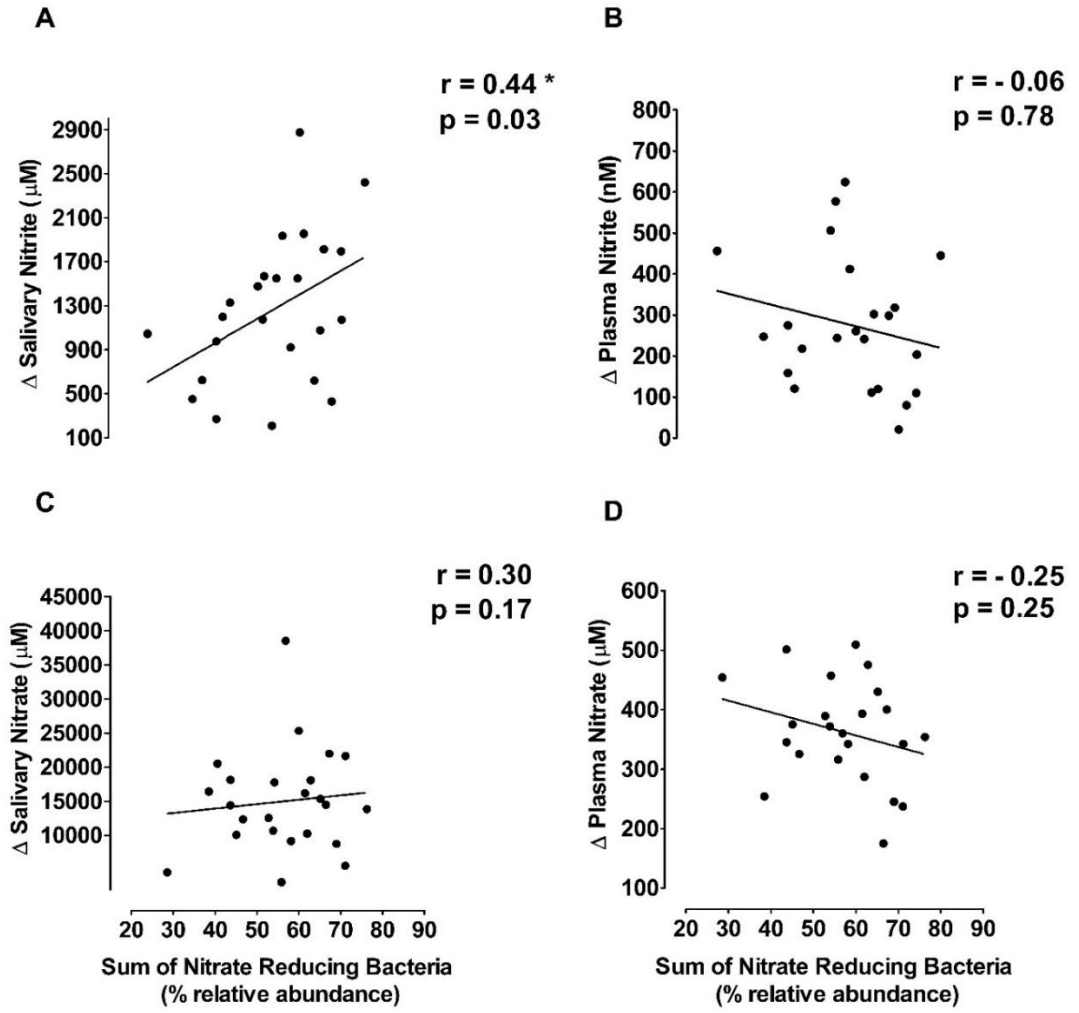

Figure 4. Correlations between the sum of NO_3^- reducing bacteria and peak change in salivary NO_2^- (A) plasma NO_2^- (B), salivary NO_3^- (C), plasma NO_3^- (D). * denotes significance, ($P<0.05$).

Impact of bacterial abundance on pharmacokinetics and pharmacodynamics following acute NO_3^- ingestion

Seven participants were identified as having less than 50% total relative abundance of the NO_3^- reducing species identified in their tongue scrapes and were classified to the Low group. The remaining participants were classified as the High group. At the OTU level, 40.99% ± 6.11% NO_3^- reducing species were observed in the Low group compared with 62.64% ± 6.92% in the High group (Figure 5).

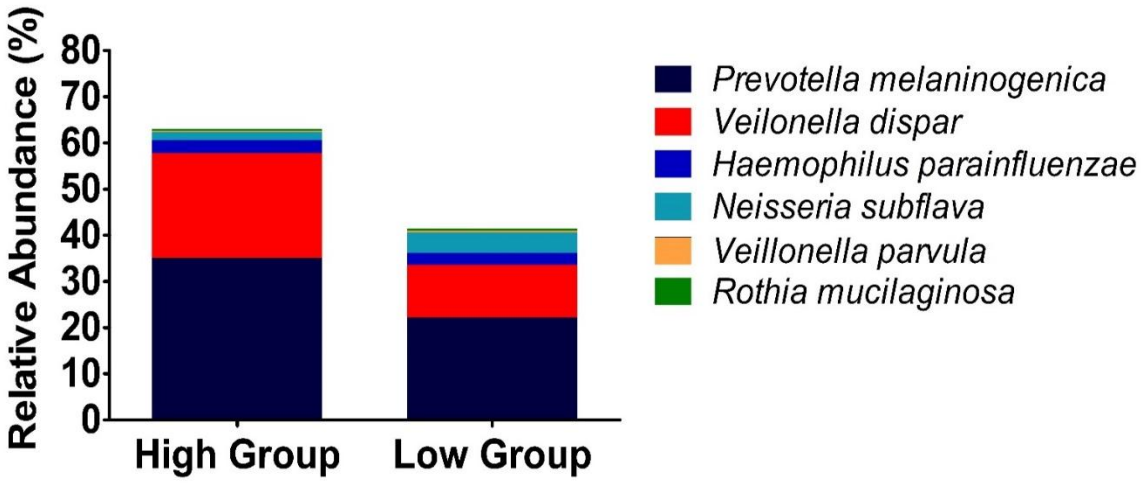

Figure 5. A comparison of the relative abundance of NO_3^- reducing species between those classified as having a high (>50%) or low (<50%) overall abundance of NO_3^- reducing bacteria. Data are presented as group means with S.D. excluded for clarity. *Rothia dentocariosa* is not shown due to low abundance (high group 0.003 ± 0.001 %, low group 0.002 ± 0.001 %).

At both species and genera level, the sum of NO_3^- reducing bacteria was significantly higher in the High group compared to the Low (species level: $P < 0.05$, 95% CI 15 – 28%; genus level $P < 0.05$, 95% CI 11 – 21%). Alpha diversity metrics revealed that bacterial species in the tongue scrape samples of the Low group were more diverse than the high group ($P < 0.001$, 1279 ± 136 vs. 1098 ± 129 OTUs, respectively). The Shannon diversity index was also higher in the Low group compared to the High group ($P = 0.002$, 5.9 ± 0.0 vs. 4.9 ± 0.6, respectively). There were no differences in the consumption of high, medium, and low NO_3^- vegetables or cured meats between groups. Nor was there any difference in baseline values for any physiological variable (all $P > 0.05$, Table 3).

Table 3, baseline values for the high and low groups. Values are mean ± standard error of the mean

	High Group Mean ± SEM	Low Group Mean ± SEM
SBP (mmHg)	120 ± 3	123 ± 4
DBP (mmHg)	68 ± 16	71 ± 27
MAP (mmHg)	85 ± 2	88 ± 3
Salivary Nitrite (µM)	227 ± 43	168 ± 97
Salivary Nitrate (µM)	933 ± 226	549 ± 207
Plasma Nitrite (nM)	131 ± 32	151 ± 61
Plasma Nitrate (µM)	39 ± 10	27 ± 11

Salivary NO_2^- peaked earlier in the High group (1.6 ± 1 h) compared to the Low (3 ± 0.6 h, $P = 0.04$). Salivary NO_2^- was significantly higher in the High group compared to Low at 1.5 h ($P = 0.02$, 95% CI 130 – 1320 µM) and 2 h ($P = 0.01$, 95% CI 182 – 1375 µM) after ingestion of beetroot juice. There were no other differences between groups for salivary NO_3^- or plasma NO metabolites (all $P > 0.05$) (Figure 6). The time to peak for salivary NO_3^-, plasma NO metabolites, and BP measurements were also not different between groups (all $P > 0.05$).

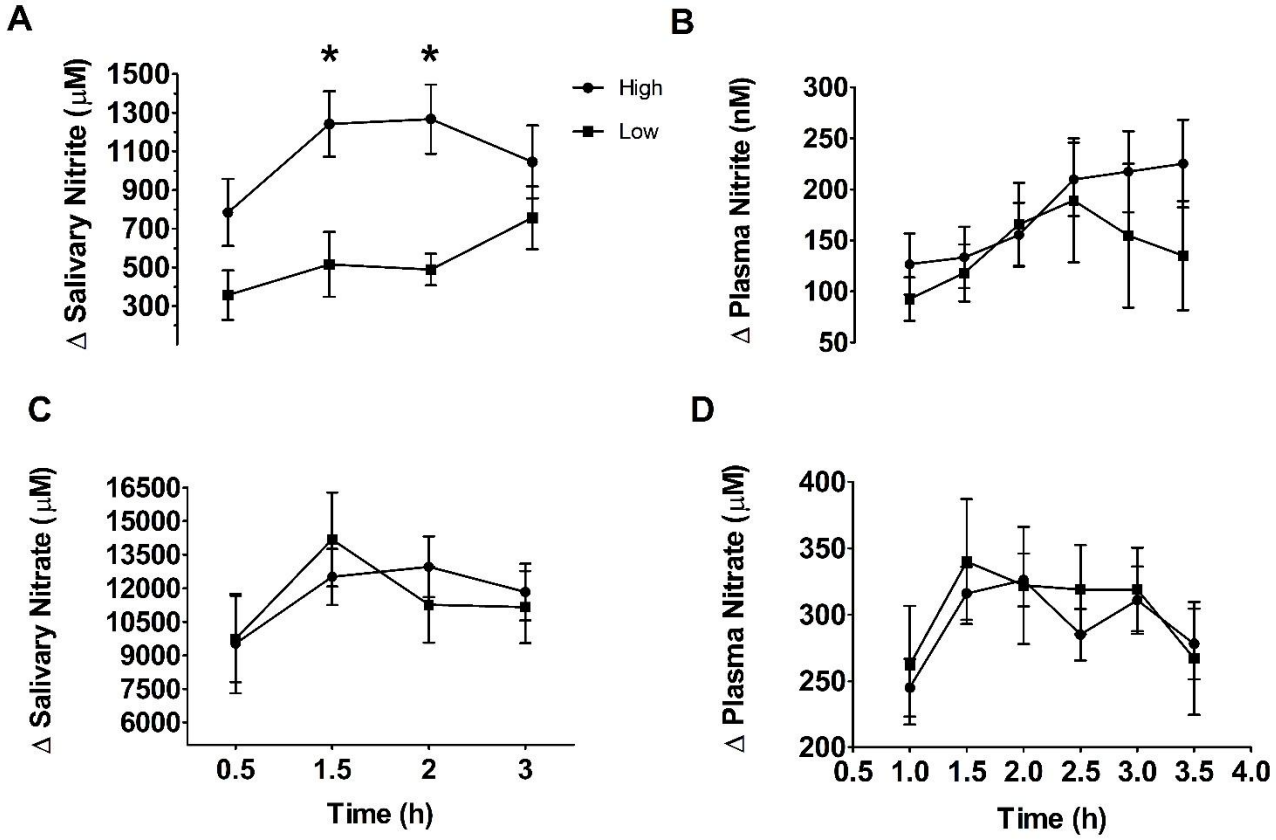

Figure 6. Change relative to baseline in salivary NO_2^- (A), plasma NO_2^- (B), salivary NO_3^- (C), plasma NO_3^- (D). Data are displayed as means and standard error of the mean. * denotes significant differences between groups, ($P < 0.05$).

Discussion

Despite the emergent importance of the enterosalivary NO_3^-, NO_2^- to NO pathway for cardiovascular health, no study has directly investigated the association between the abundance of NO_3^- reducing bacteria in the oral cavity and the capacity to reduce exogenous NO_3^- to NO_2^- in vivo. Guided by previous work [20], [21], [36], we first investigated the abundance of known NO_3^- reducing bacteria through 16s rRNA gene sequencing. We provide descriptive data at both genus and species level in a much larger sample size than has been reported previously in healthy humans. In addition, this is the first description of sequencing

data in conjunction with in vivo measurements to demonstrate that the abundance of NO_3^- reducing bacteria on the dorsal surface of the tongue significantly correlates with salivary NO_2^- generation following the ingestion of NO_3^- rich beetroot juice. A higher abundance of these bacteria also results in a more rapid reduction of salivary NO_3^- to NO_2^-. Despite this, higher abundance of oral NO_3^- reducing bacteria does not appear to exaggerate changes in plasma NO_2^- or BP following ingestion of beetroot juice, at least in this young healthy cohort.

16S rRNA gene sequencing analysis of the healthy human tongue microbiome

Our samples were similar in bacterial diversity to those reported previously [21], [37]. At the genus level, all genera previously implicated in NO_3^- reduction [20], [21] were detected. *Prevotella* and *Veillonella* were found to be the first and second most abundant genera in our samples, respectively. In contrast, previous research has typically identified *Veillonella* as the most abundant taxa found on the tongue dorsum [21]. Although direct comparison cannot be made between studies due to differences in sequencing platforms and culturing methods, these findings support the notion that the composition of the microbiome may differ profoundly, even in healthy individuals [38].

Through 16s RNA sequencing we identified only seven of fourteen known species which have previously been demonstrated to reduce NO_3^- in vitro (Table 2) [21]. In this previous work, three tongue scrape samples were analysed using whole genome shotgun sequencing (WGS) to identify bacterial species followed by metabolic pathway reconstruction to determine NO_3^- reduction capacity. Given that WGS sequences all genes rather than the more targeted approach of 16s RNA sequencing, this method allows for a more accurate taxonomic

assignment at species level and likely explains the disparity in the experimental outcomes. Nevertheless, we analysed a far greater number of samples (n=24) than has been reported in previous research [21] which seems necessary given the aforementioned variability in the abundance of bacterial species within the oral microbiome.

Impact of bacterial abundance on the reduction of salivary NO_3^- to NO_2^-

Next, we examined how the abundance of NO_3^- reducing bacteria influenced pharmacokinetics and pharmacodynamics following ingestion of a standardised dietary NO_3^- dose. In line with previous research [26], [27], [39] , the ingestion of NO_3^- rich beetroot juice resulted in a marked elevation of NO metabolites in the plasma and saliva. A novel finding of this study is that the abundance of known oral NO_3^- reducing bacteria was associated with the peak increase in salivary NO_2^- concentration following ingestion of dietary NO_3^-. Furthermore, NO_2^- peaks earlier in the saliva following ingestion of beetroot juice in individuals who have a higher abundance of these bacteria. These data are perhaps unsurprising given that oral bacteria are known to play a crucial role in the reduction of salivary NO_3^- to NO_2^- [26]–[28]. Nevertheless, where previous research has established that the *presence* of NO_3^- reducing bacteria is essential, we show that the abundance of these bacteria seems to impact the magnitude of salivary NO_2^- accumulation in the presence of elevated salivary NO_3^-. It is, however, important to acknowledge that these analyses do not necessarily imply "cause-effect" relationship between bacterial abundance and salivary NO_2^- generation. Other factors, including the efficiency of NO_3^- transport via sialin in the salivary glands [9], [40], inhibition of stomach acid production [41], and the metabolic activities of bacteria [21], may also influence this process. Our findings contrast with previous in vitro analysis of three isolated samples which suggested that the NO_3^- reducing capacity of oral

bacterial species was not influenced by the metabolic pathway coverage or the abundance of these bacteria [21]. It is evident, therefore, that whilst computational and in vitro methods are useful in determining characteristics of microbes in a controlled environment, there is a further challenge in determining the functional capacity of a microbial community, especially when attempting to relate outcomes to the dynamic in vivo environment.

Impact of bacterial abundance on plasma pharmacokinetics and BP

Despite the association with salivary NO_2^-, the abundance of NO_3^- reducing bacteria was not related to the change in plasma NO_2^- or BP markers. Nor did a higher abundance of these bacteria alter plasma pharmacokinetics following the ingestion of beetroot juice. This has important implications since plasma NO_2^- is considered to provide the best approximation of circulating NO bioavailability [42], [43] and is suggested to be a marker of endothelial function [44] and cardiovascular risk [45]. While some have proposed that salivary NO_2^- may be a useful point of care diagnostic for assessing total body NO bioavailability [46], the discordance between salivary and plasma changes in NO_2^- observed in the present study would seem to refute this suggestion for healthy young subjects.

Our data suggests that, at least in this homogenous sample, higher abundance of NO_3^- reducing bacteria does not seem to further increase circulating NO bioavailability. Whilst it is useful to characterise the healthy human microbiome in this context, it is necessary to further explore these data in populations with compromised NO bioavailability, including older adults [47], patients with endothelial dysfunction [48], and those treated with antibiotics [49] . Furthermore, it should be acknowledged that while some participants were classified as

having a "low" abundance of NO_3^- reducing bacteria, this cohort still had 41 ± 6% of taxa which possess a NO_3^- reductase gene (with the lowest abundance being 29%) and all experienced a substantial increase in plasma NO metabolites. Previous work by Woessner and colleagues [28] demonstrated a stepwise reduction in salivary NO_2^- and BP responses when differing strengths of mouthwash were administered which further supports the notion that the magnitude of NO_3^- conversion is related to the abundance of NO_3^- reducing bacteria. The apparent consequence of the lower abundance of these bacteria is that salivary NO_3^- reduction occurs at a slower rate than those in the high group but the appearance of salivary NO_2^- can continue to accelerate at least up to 3 h after ingestion of a NO_3^- dose. It would, therefore, be of interest to collect further data from individuals with an altered microbiome such as that which might occur with ageing [23][22] or periodontal disease [50].

There are a number of reasons why an augmented salivary NO_2^- concentration was not paralleled by the expected additional increase in plasma NO_2^- but these remain speculative until further experimental data is collected. Firstly, it may be that "excess" NO_2^- from the saliva is excreted via the urinary system. NO_3^- and NO_2^-, originating from either exogenous and endogenous sources, have been shown to be excreted in the urine [51]. This suggests that there may be a saturation threshold for circulating NO_2^- over which the excess is either stored or excreted. This may be to prevent excessive drops in BP which would be detrimental to homeostasis [52]. Future studies could include urine collection and analysis to verify this hypothesis. Alternatively, the lack of accordance between oral bacterial abundance and plasma NO_2^- may be due to the generation of NO_2^- at sites outside the oral cavity. NO_3^- reduction is thought to occur in the gastrointestinal tract, for example, through conversion to bioactive nitrogen oxides via hydrogen chloride [1]. Vermerien and colleagues [53] also showed that, in conditions simulating the colon, faecal microbiota can reduce NO_3^- to NO via

dissimilatory reduction to ammonia. Given that NO possesses a very short in vivo half-life it may then be rapidly oxidised back to NO_2^- and NO_3^- [43][42][41][40]. It must also be acknowledged that there are many storage forms of NO in the red blood cells and plasma that may exert physiological effects, including s-nitrosothiols [41] and nitrated lipids [54] It is possible, therefore, that plasma NO_2^- may simply be a marker of NO availability [41] and not the intermediate directly responsible to the reduction in BP resulting from NO_3^- administration.

Conclusions

We show in vivo in healthy adults that there is a positive linear relationship between the total relative abundance of commensal NO_3^- reducing oral bacteria and the generation of salivary NO_2^- following a dose of dietary NO_3^-. While these data are cross-sectional and correlative in nature, these findings are significant given the supposed therapeutic benefits of dietary NO_3^- supplementation. Nevertheless, a higher relative abundance of NO_3^- reducing bacteria did not result in further increases in plasma NO_2^- concentration (a marker of vascular NO bioavailability) and nor did it influence the extent by which BP was reduced following ingestion of NO_3^--rich beetroot juice. This suggests that where sufficient quantities of these bacteria are present on the tongue, dietary NO_3^- supplementation will consistently increase circulating NO with potentially meaningful biological consequences. Further work should explore these phenomena in populations with compromised endogenous NO generation capacity or with an altered oral microbiome to better understand the link between commensal bacteria and cardiovascular health.

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References

[1] J. O. Lundberg, E. Weitzberg, and M. T. Gladwin, 'The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics', *Nat. Rev. Drug Discov.*, vol. 7, no. 2, pp. 156–167, Feb. 2008.

[2] J. Petersson, M. Phillipson, E. Å. Jansson, A. Patzak, J. O. Lundberg, and L. Holm, 'Dietary nitrate increases gastric mucosal blood flow and mucosal defense', *Am. J. Physiol. - Gastrointest. Liver Physiol.*, vol. 292, no. 3, pp. G718–G724, Mar. 2007.

[3] P. Kleinbongard *et al.*, 'Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans', *Free Radic. Biol. Med.*, vol. 40, no. 2, pp. 295–302, Jan. 2006.

[4] E. L. Wightman *et al.*, 'Dietary nitrate modulates cerebral blood flow parameters and cognitive performance in humans: A double-blind, placebo-controlled, crossover investigation', *Physiol. Behav.*, vol. 149, no. Supplement C, pp. 149–158, Oct. 2015.

[5] Z. Bahadoran, A. Ghasemi, P. Mirmiran, F. Azizi, and F. Hadaegh, 'Beneficial effects of inorganic nitrate/nitrite in type 2 diabetes and its complications', *Nutr. Metab.*, vol. 12, p. 16, May 2015.

[6] F. J. Larsen *et al.*, 'Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans', *Cell Metab.*, vol. 13, no. 2, pp. 149–159, Feb. 2011.

[7] G. Eisenbrand, B. Spiegelhalder, and R. Preussmann, 'Nitrate and nitrite in saliva', *Oncology*, vol. 37, no. 4, pp. 227–231, 1980.

[8] M. Golzarand, Z. Bahadoran, P. Mirmiran, A. Zadeh-Vakili, and F. Azizi, 'Consumption of nitrate-containing vegetables is inversely associated with hypertension in adults: a prospective investigation from the Tehran Lipid and Glucose Study', *J. Nephrol.*, vol. 29, no. 3, pp. 377–384, Jun. 2016.

[9] J. O. Lundberg, 'Nitrate transport in salivary glands with implications for NO homeostasis', *Proc. Natl. Acad. Sci.*, vol. 109, no. 33, pp. 13144–13145, Aug. 2012.

[10] S. R. Tannenbaum, A. J. Sinskey, M. Weisman, and W. Bishop, 'Nitrite in human saliva. Its possible relationship to nitrosamine formation', *J. Natl. Cancer Inst.*, vol. 53, no. 1, pp. 79–84, Jul. 1974.

[11] C. D. Koch, M. T. Gladwin, B. A. Freeman, J. O. Lundberg, E. Weitzberg, and A. Morris, 'Enterosalivary nitrate metabolism and the microbiome: Intersection of microbial metabolism, nitric oxide and diet in cardiac and pulmonary vascular health', *Free Radic. Biol. Med.*, vol. 105, pp. 48–67, Apr. 2017.

[12] A. Kocher and J. Loscalzo, Eds., *Nitrite and Nitrate in Human Health and Disease*, 2011 edition. New York: Humana Press, 2011.

[13] V. Kapil, R. S. Khambata, A. Robertson, M. J. Caulfield, and A. Ahluwalia, 'Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study', *Hypertension*, vol. 65, no. 2, pp. 320–327, Feb. 2015.

[14] J. Lara, A. W. Ashor, C. Oggioni, A. Ahluwalia, J. C. Mathers, and M. Siervo, 'Effects of inorganic nitrate and beetroot supplementation on endothelial function: a systematic review and meta-analysis', *Eur. J. Nutr.*, vol. 55, no. 2, pp. 451–459, Mar. 2016.

[15] M. Lavu, S. Gundewar, and D. J. Lefer, 'Nitrite and Nitrate in Ischemia-Reperfusion Injury', in *Nitrite and Nitrate in Human Health and Disease*, Humana Press, 2011, pp. 225–246.

[16] D. J. Muggeridge, C. C. F. Howe, O. Spendiff, C. Pedlar, P. E. James, and C. Easton, 'A single dose of beetroot juice enhances cycling performance in simulated altitude', *Med. Sci. Sports Exerc.*, vol. 46, no. 1, pp. 143–150, Jan. 2014.

[17] D. J. Muggeridge, C. C. F. Howe, O. Spendiff, C. Pedlar, P. E. James, and C. Easton, 'The effects of a single dose of concentrated beetroot juice on performance in trained flatwater kayakers', *Int. J. Sport Nutr. Exerc. Metab.*, vol. 23, no. 5, pp. 498–506, 2013.

[18] J. Puype, M. Ramaekers, R. Van Thienen, L. Deldicque, and P. Hespel, 'No effect of dietary nitrate supplementation on endurance training in hypoxia', *Scand. J. Med. Sci. Sports*, vol. 25, Apr. 2014.

[19] E. Marsch *et al.*, 'The effect of prolonged dietary nitrate supplementation on atherosclerosis development', *Atherosclerosis*, vol. 245, pp. 212–221, Jan. 2016.

[20] J. J. Doel, N. Benjamin, M. P. Hector, M. Rogers, and R. P. Allaker, 'Evaluation of bacterial nitrate reduction in the human oral cavity', *Eur. J. Oral Sci.*, vol. 113, no. 1, pp. 14–19, Feb. 2005.

[21] E. R. Hyde *et al.*, 'Metagenomic Analysis of Nitrate-Reducing Bacteria in the Oral Cavity: Implications for Nitric Oxide Homeostasis', *PLoS ONE*, vol. 9, no. 3, p. e88645, Mar. 2014.

[22] V. B. Young, 'The role of the microbiome in human health and disease: an introduction for clinicians', *BMJ*, vol. 356, p. j831, Mar. 2017.

[23] E. Biagi, M. Candela, S. Turroni, P. Garagnani, C. Franceschi, and P. Brigidi, 'Ageing and gut microbes: Perspectives for health maintenance and longevity', *Pharmacol. Res.*, vol. 69, no. 1, pp. 11–20, Mar. 2013.

[24] V. Robles Alonso and F. Guarner, 'Linking the gut microbiota to human health', *Br. J. Nutr.*, vol. 109 Suppl 2, pp. S21-26, Jan. 2013.

[25] M. Mazidi, P. Rezaie, A. P. Kengne, M. G. Mobarhan, and G. A. Ferns, 'Gut microbiome and metabolic syndrome', *Diabetes Metab. Syndr. Clin. Res. Rev.*, vol. 10, no. 2, pp. S150–S157, Apr. 2016.

[26] M. Govoni, E. A. Jansson, E. Weitzberg, and J. O. Lundberg, 'The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash', *Nitric Oxide Biol. Chem.*, vol. 19, no. 4, pp. 333–337, Dec. 2008.

[27] C. P. Bondonno *et al.*, 'Antibacterial mouthwash blunts oral nitrate reduction and increases blood pressure in treated hypertensive men and women', *Am. J. Hypertens.*, vol. 28, no. 5, pp. 572–575, May 2015.

[28] M. Woessner, J. M. Smoliga, B. Tarzia, T. Stabler, M. Van Bruggen, and J. D. Allen, 'A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load', *Nitric Oxide*, vol. 54, pp. 1–7, Apr. 2016.

[29] H. Li *et al.*, 'Nitrate-reducing bacteria on rat tongues., Nitrate-reducing bacteria on rat tongues.', *Appl. Environ. Microbiol. Appl. Environ. Microbiol.*, vol. 63, 63, no. 3, 3, pp. 924, 924–930, Mar. 1997.

[30] P. E. James, G. R. Willis, J. D. Allen, P. G. Winyard, and A. M. Jones, 'Nitrate pharmacokinetics: Taking note of the difference', *Nitric Oxide*, vol. 48, no. Supplement C, pp. 44–50, Aug. 2015.

[31] S. Lidder and A. J. Webb, 'Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway', *Br. J. Clin. Pharmacol.*, vol. 75, no. 3, pp. 677–696, Mar. 2013.

[32] L. Liddle, C. Monaghan, M. C. Burleigh, L. C. McIlvenna, D. J. Muggeridge, and C. Easton, 'Changes in body posture alter plasma nitrite but not nitrate concentration in humans', *Nitric Oxide Biol. Chem.*, vol. 72, no. 10.1016/j.niox.2017.11.008, pp. 59–65, Jan. 2018.

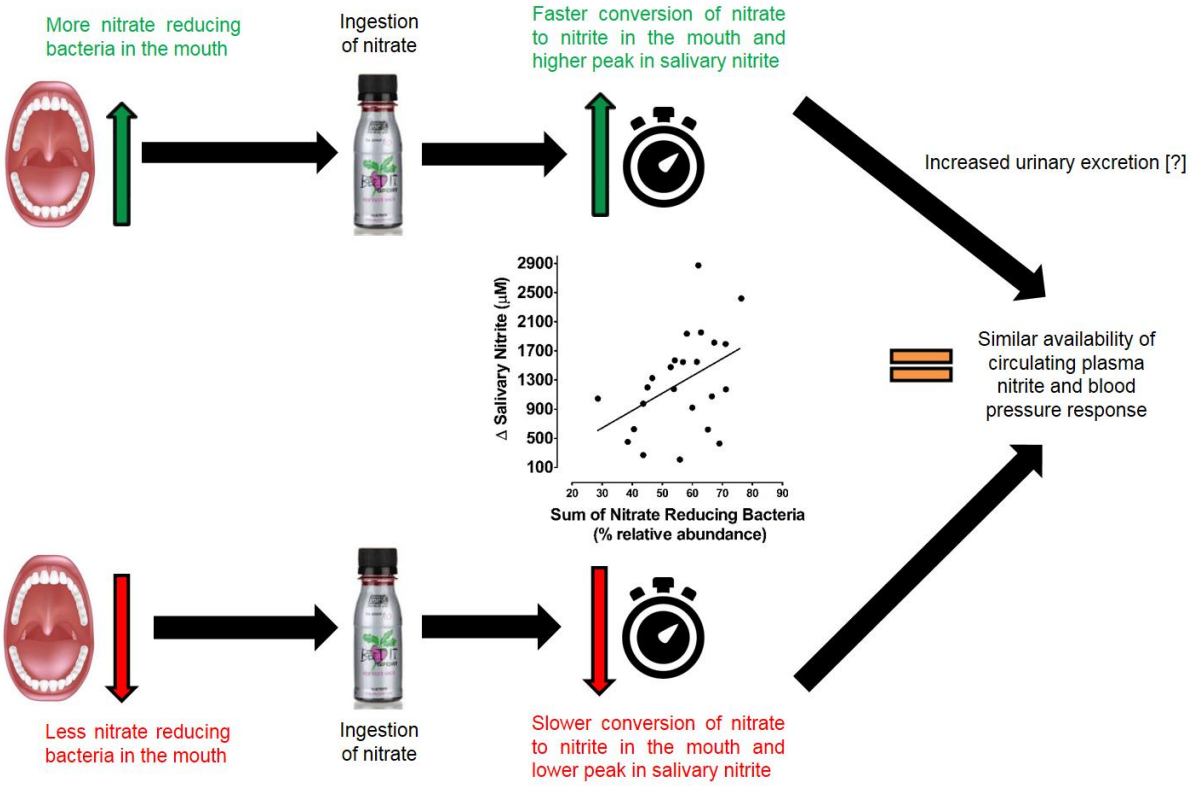
[33] L. J. Wylie *et al.*, 'Beetroot juice and exercise: pharmacodynamic and dose-response relationships', *J. Appl. Physiol. Bethesda Md 1985*, vol. 115, no. 3, pp. 325–336, Aug. 2013.

[34] J. G. Caporaso *et al.*, 'Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample', *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108 Suppl 1, pp. 4516–4522, Mar. 2011.

[35] J. G. Caporaso *et al.*, 'QIIME allows analysis of high-throughput community sequencing data', *Nat. Methods*, vol. 7, no. 5, p. 335, May 2010.
[36] E. R. Hyde *et al.*, 'Characterization of the rat oral microbiome and the effects of dietary nitrate', *Free Radic. Biol. Med.*, vol. 77, pp. 249–257, Dec. 2014.
[37] E. Zaura, B. J. Keijser, S. M. Huse, and W. Crielaard, 'Defining the healthy "core microbiome" of oral microbial communities', *BMC Microbiol.*, vol. 9, no. 1, p. 259, Dec. 2009.
[38] Human Microbiome Project Consortium, 'Structure, function and diversity of the healthy human microbiome', *Nature*, vol. 486, no. 7402, pp. 207–214, Jun. 2012.
[39] F. J. Larsen, B. Ekblom, K. Sahlin, J. O. Lundberg, and E. Weitzberg, 'Effects of Dietary Nitrate on Blood Pressure in Healthy Volunteers', *N. Engl. J. Med.*, vol. 355, no. 26, pp. 2792–2793, Dec. 2006.
[40] L. Qin *et al.*, 'Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane', *Proc. Natl. Acad. Sci.*, vol. 109, no. 33, pp. 13434–13439, Aug. 2012.
[41] M. F. Montenegro *et al.*, 'Blood Pressure–Lowering Effect of Orally Ingested Nitrite Is Abolished by a Proton Pump InhibitorNovelty and Significance', *Hypertension*, vol. 69, no. 1, pp. 23–31, Jan. 2017.
[42] T. Lauer *et al.*, 'Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action.', *Proc. Natl. Acad. Sci. U. S. A.*, vol. 98, no. 22, pp. 12814–12819, 2001.
[43] M. Kelm, 'Nitric oxide metabolism and breakdown', *Biochim. Biophys. Acta - Bioenerg.*, vol. 1411, no. 2–3, pp. 273–289, 1999.
[44] P. Kleinbongard *et al.*, 'Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans', *Free Radic. Biol. Med.*, vol. 40, no. 2, pp. 295–302, 2006.
[45] J. D. Allen, E. M. Miller, E. Schwark, J. L. Robbins, B. D. Duscha, and B. H. Annex, 'Plasma nitrite response and arterial reactivity differentiate vascular health and performance', *Nitric Oxide - Biol. Chem.*, vol. 20, no. 4, pp. 231–237, 2009.
[46] N. S. Bryan and J. Loscalzo, Eds., *Nitrite and Nitrate in Human Health and Disease*. Cham: Springer International Publishing, 2017.
[47] E. Biagi, M. Candela, S. Turroni, P. Garagnani, C. Franceschi, and P. Brigidi, 'Ageing and gut microbes: Perspectives for health maintenance and longevity', *Pharmacol. Res.*, vol. 69, no. 1, pp. 11–20, Mar. 2013.
[48] M. Kina-Tanada *et al.*, 'Long-term dietary nitrite and nitrate deficiency causes the metabolic syndrome, endothelial dysfunction and cardiovascular death in mice', *Diabetologia*, vol. 60, no. 6, pp. 1138–1151, Jun. 2017.
[49] N. S. Bryan, G. Tribble, and N. Angelov, 'Oral Microbiome and Nitric Oxide: the Missing Link in the Management of Blood Pressure', *Curr. Hypertens. Rep.*, vol. 19, no. 4, p. 33, Apr. 2017.
[50] M. Nagarajan, V. R. Prabhu, and R. Kamalakkannan, 'Chapter 9 - Metagenomics: Implications in Oral Health and Disease', in *Metagenomics*, Academic Press, 2018, pp. 179–195.
[51] D. Tsikas, M.-T. Suchy, A. Mitschke, B. Beckmann, and F.-M. Gutzki, 'Measurement of Nitrite in Urine by Gas Chromatography-Mass Spectrometry', in *Leucocytes*, Humana Press, 2012, pp. 277–293.
[52] N. Ashton, 'Neurological and humoral control of blood pressure', *Anaesth. Intensive Care Med.*, vol. 8, no. 6, pp. 221–226, Jun. 2007.
[53] J. Vermeiren, T. Van de Wiele, W. Verstraete, P. Boeckx, and N. Boon, 'Nitric Oxide Production by the Human Intestinal Microbiota by Dissimilatory Nitrate Reduction to Ammonium', *BioMed Research International*, 2009. [Online]. Available: https://www.hindawi.com/journals/bmri/2009/284718/. [Accessed: 18-Sep-2017].
[54] E. S. Lima, M. G. Bonini, O. Augusto, H. V. Barbeiro, H. P. Souza, and D. S. P. Abdalla, 'Nitrated lipids decompose to nitric oxide and lipid radicals and cause vasorelaxation', *Free Radic. Biol. Med.*, vol. 39, no. 4, pp. 532–539, Aug. 2005.

Highlights

- Prevotella are the most abundant genera of nitrate-reducing bacteria in the mouth
- Abundance of nitrate-reducing bacteria is associated with the generation of nitrite
- Abundance of these bacteria is not associated with plasma nitrite concentration



Graphical abstract