Variability in nitrate-reducing oral bacteria and nitric oxide metabolites in biological fluids following dietary nitrate administration: An assessment of the critical difference

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Abstract

There is conflicting evidence on whether dietary nitrate supplementation can improve exercise performance. This may arise from the complex nature of nitric oxide (NO) metabolism which causes substantial inter-individual variability, within-person biological variation (CV_B), and analytical imprecision (CV_A) in experimental endpoints. However, no study has quantified the CV_A and CV_B of NO metabolites or the factors that influence their production. These data are important to calculate the critical difference (CD), defined as the smallest difference between sequential measurements required to signify a true change. The main aim of the study was to evaluate the CV_B, CV_A, and CD for markers of NO availability (nitrate and nitrite) in plasma and saliva before and after the ingestion of nitrate-rich beetroot juice (BR). We also assessed the CV_B of nitrate-reducing bacteria from the dorsal surface of the tongue. It was hypothesised that there would be substantial CV_B in markers of NO availability and the abundance of nitrate-reducing bacteria. Ten healthy male participants (age 25 ± 5 years) completed three identical trials at least 6 days apart. Blood and saliva were collected before and after (2, 2.5 and 3 h) ingestion of 140 ml of BR (~12.4 mmol nitrate) and analysed for [nitrate] and [nitrite]. The tongue was scraped and the abundance of nitrate-reducing bacterial species were analysed using 16S rRNA next generation sequencing. There was substantial CV_B for baseline concentrations of plasma (nitrate 11.9%, nitrite 9.0%) and salivary (nitrate 15.3%, nitrite 32.5%) NO markers. Following BR ingestion, the CV_B for nitrate (plasma 3.8%, saliva 12.0%) and salivary nitrite (24.5%) were lower than baseline, but higher for plasma nitrite (18.6%). The CD thresholds that need to be exceeded to ensure a meaningful change from baseline are 25, 19, 37, and 87% for plasma nitrate, plasma nitrite, salivary nitrate, and salivary nitrite, respectively. The CV_B for selected nitrate-reducing bacteria detected were: *Prevotella melaninogenica* (37%), *Veillonella dispar* (35%), *Haemophilus parainfluenzae* (79%), *Neisseria subflava* (70%), *Veillonella parvula* (43%),

Rothia mucilaginosa (60%), and *Rothia dentocariosa* (132%). There is profound CV_B in the abundance of nitrate-reducing bacteria on the tongue and the concentration of NO markers in human saliva and plasma. Where these parameters are of interest following experimental intervention, the CD values presented in this study will allow researchers to interpret the meaningfulness of the magnitude of the change from baseline.

Key Words: beetroot juice; nitrite; microbiome

Highlights

- Concentration of nitric oxide markers varies considerably between individuals
- Nitric oxide markers are subject to substantial biological variation
- Pharmacokinetics following nitrate supplementation can vary within individuals
- Variation in bacteria only partly account for variability in nitric oxide markers
- Critical difference values presented herein will aid interpretation of nitric oxide data

1. Introduction

Dietary nitrate (NO_3^-) supplementation increases the concentration of nitric oxide (NO) metabolites within the blood (Kapil et al. 2010). Crucial to this process is the reduction of concentrated NO_3^- in saliva (Lundberg and Govoni 2004) to nitrite (NO_2^-) by facultative anaerobic bacteria in the oral cavity (Duncan et al. 1995). The importance of this mechanism to cardiovascular health is evident in the breadth of research showing that ingestion of inorganic NO_3^- acutely lowers blood pressure (Webb et al. 2008; Siervo and Lara 2013). Elevations in plasma NO_2^- have been associated with decreased cardiovascular risks factors and increased exercise capacity in healthy and chronically diseased cohorts (Kleinbongard et al. 2006; Allen et al. 2010; Totzeck et al. 2012). Dietary NO_3^- supplementation has also been shown to improve time trial (Lansley et al. 2011; Muggeridge et al. 2014) and intermittent (Wylie et al. 2013) exercise performance. However, some studies report no ergogenic effects (Peacock et al. 2012; MacLeod et al. 2015) and, taken as a whole, the effects of dietary NO_3^- supplementation on exercise performance outcomes appear to be equivocal (McMahon et al. 2017). One hypothesis that may account for the lack of consensus across the literature is that individuals respond differently to NO_3^- supplementation (Porcelli et al. 2015). Indeed, there appears to be substantial inter-individual variability in plasma [NO_3^-] and [NO_2^-] pharmacokinetics before and after NO_3^- administration (James et al. 2015). For example, we have previously shown that the increase in plasma [NO_2^-] can range from 80 to 400 nM with a time-to-peak ranging from 1.5 to 6 h following ingestion of NO_3^- supplements (McIlvenna et al. 2017).

Surprisingly, the within-individual variability in NO metabolites, either at basal concentrations or following ingestion of NO_3^-, has not been reported in the literature. This is

important as there are several potential factors that could affect both the intra- and inter-individual variability of circulating [NO_3^-] and [NO_2^-]. These factors include, but are not limited to: posture during blood collection (Liddle et al. 2018), prior sunlight exposure (Monaghan et al. 2018), the NO_3^- and NO_2^- content of the diet (Bryan et al. 2007), the rate of endogenous NO synthesis, NO_3^- transport in the salivary glands (Lundberg 2012; Qin et al. 2012), the abundance of NO_3^--reducing bacteria in the mouth (Burleigh et al. 2018), salivary flow-rate (Webb et al. 2008), the rate of NO_3^- and NO_2^- reduction in the gut (Lundberg et al. 1994), urinary excretion rates (Pannala et al. 2003), and training status (Porcelli et al. 2015). Whilst it is impossible to control all of the factors that influence the concentration of circulating NO metabolites, it is important to understand the extent to which they can vary within the same individual and the analytical error (CV_A) associated with their measurement.

The within-individual or biological variation (CV_B) establishes the inherent fluctuations around a homeostatic set-point of a measured variable (Harris 1970). The CV_B can be used in combination with the CV_A to calculate the critical difference (CD) which is defined as the change from baseline that must occur before a meaningful biological difference can be claimed (Fraser and Fogarty 1989). In short, a researcher is able to use the CV_B and the CV_A to determine the typical "noise" in the variable of interest. The CD provides a single criterion threshold which, if exceeded, they can conclude a true change has occurred in response to any intervention. For reference, it has been previously reported that serum cholesterol has a CV_B of 7.6% and a CD of 17.2% (Fraser 2001). Blood glucose has been shown to have a CV_B and CD of 7.2% and 14.9%, respectively (Widjaja et al. 1999). In the context of dietary NO_3^- supplementation researchers must first be confident that the intervention results in a true increase in NO availability if there is to be potential for any ergogenic effect.

To our knowledge, the CD values of NO_3^- and NO_2^- in plasma, saliva, and urine at baseline and in response to NO_3^- have not been previously reported. Likewise, despite recognition of the importance of NO_3^--reducing bacteria for the generation of NO through the NO_3^--NO_2^--NO pathway, no study has quantified the CV_B in the abundance of these bacteria in the oral cavity. Therefore, the primary aim was to quantify the CV_B and CD of the abundance of NO_3^--reducing bacteria, blood pressure, and plasma, saliva, and urine [NO_3^-] and [NO_2^-] before and after ingestion of NO_3^--rich beetroot juice (BR). A secondary aim was to determine whether the variation in these NO metabolites was associated with the abundance of NO_3^--reducing bacteria. It was hypothesised that there would be substantial CV_B of the abundance of NO_3^--reducing bacteria and the concentration of NO metabolites in plasma, saliva, and urine. Further, it was hypothesised that the variations in plasma and salivary [NO_3^-] and [NO_2^-] would be positively associated with the abundance NO_3^--reducing bacteria.

2. Methods

2.1. Participants

Ten healthy and recreationally active male participants (age 25 ± 5 years, stature 177 ± 5 cm, and body mass 81 ± 11 kg) volunteered to participate in the study and provided written informed consent. The study was approved by the School of Science and Sport Ethics Committee at The University of the West of Scotland and all procedures were performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

2.2. Study design

Each participant attended the laboratory on three separate occasions with 6-10 days between each visit. Each trial comprised a 3.5 h period where participants lay supine and repeated samples of biological fluids were collected and blood pressure was measured. The experimental conditions were identical in each visit. Following the collection of baseline measurements, participants immediately ingested 2 x 70 ml of BR (Beet It SPORT, James White Drinks, UK; total of ~12.4 mmol NO_3^-). Participants were instructed to avoid caffeine, foods high in NO_2^- and NO_3^- (e.g. green leafy vegetables and cured meats), alcohol, and strenuous exercise in the 24 h prior to the experiment. Participants were also asked to avoid mouthwash 7 days prior to the first trial and for the duration of the study. All participants confirmed that they were not using medication of any kind for a month before the first trial or at any point during the study period. Participants were also asked to refrain from brushing their teeth and tongue on the morning of each lab visit. Participants recorded dietary intake and the modality, frequency, and intensity of exercise undertaken 72 h prior to the first experimental trial and replicated this for the subsequent visits. Participants were provided access to bottled water (Strathrowan Scottish Mountain water, Aldi Stores Ltd, Ireland) to consume *ad libitum* during the first visit. The volume of water and the time of ingestion was recorded during the first visit and matched for subsequent trials.

2.3. Procedures

A schematic of the experimental procedures is provided in Figure 1. Following standard anthropometric measurements (stature and body mass), participants lay in a supine position to allow the insertion of a cannula into the antecubital vein. Following cannulation, participants continued to lay in a supine position for a total of 30 min before baseline samples of venous

blood and saliva were collected. Baseline blood pressure was then recorded in triplicate by using an automated oscillometric device (Omron 705IT, Omron Global. Hoofddorp, Netherlands). Mean arterial pressure (MAP) was calculated using the following equation:

MAP = (2 x diastolic blood pressure + systolic blood pressure) / 3

Venous blood (4 ml) was collected in EDTA vacutainers (BD vacutainer K2E 7.2mg, Plymouth, U.K.) and the cannula flushed with sterile 0.9% saline solution between samples to keep the line patent. The vacutainer was centrifuged (Harrier 18/80, Henderson Biomedical, UK) at 4000 rpm for 10 min at 4^{o}C immediately after collection (Pelletier et al. 2006). Plasma was then separated, frozen at −80°C, and analysed within 4 months (Pinder et al. 2009) of initial collection for determination of [NO_3^-] and [NO_2^-]. Samples of unstimulated saliva were collected via a non-cotton polymer oral swab (Saliva Bio Oral Swab (SOS) Salimetrics, Pennsylvania, USA) placed under the tongue for 2 min. Swabs were then transferred to a collection tube (Sarstedt, Aktiengesellschaft & Co, Numbrecht, Germany) and centrifuged at 4000 rpm for 10 min at 4°C. Samples were separated into two cryovials and immediately stored at −80^{0}C for later analysis of [NO_3^-] and [NO_2^-]. Swabs were used to collect saliva samples in preference to the “passive drool” technique in an attempt to improve the consistency of saliva collection within and between participants.

Participants were then instructed to sit up to allow for the collection of a bacterial sample from the posterior dorsal surface of the tongue using a sterile stainless-steel metal tongue cleaner (Soul Genie, Health Pathways LLP, India). The tongue cleaner was scraped over the dorsal surface of the tongue 3-5 times or until there was a visible coating on the instrument. A

sterile collection swab (Deltalab, S.L. Barcelona, Spain) was then used to collect the bacteria from the tongue cleaner before being placed into a PowerSoil Bead Tube (MoBio Laboratories Inc., West Carlsbad, California) and immediately frozen at −80ºC for later isolation of DNA, as per the manufacturer's instructions. Participants were then requested to void their bladder and a sample of urine was frozen at −80 °C for later analysis of [NO_3^-]. The volume of all further bladder voids were recorded following ingestion of BR to allow for the calculation of total NO_3^- excretion using the following equation:

Total NO_3^- excretion (g) = NO_3^- (M) * urine volume (L)

Repeated measurements of blood pressure and collection of saliva, blood, and urine samples were collected at various subsequent time points as detailed in Figure 1. All blood samples were collected when participants were supine to allow plasma [NO_2^-] to stabilise following postural alterations. Blood pressure was also measured when participants were supine to ensure measurements were time-aligned with plasma [NO_2^-] and [NO_2^-].

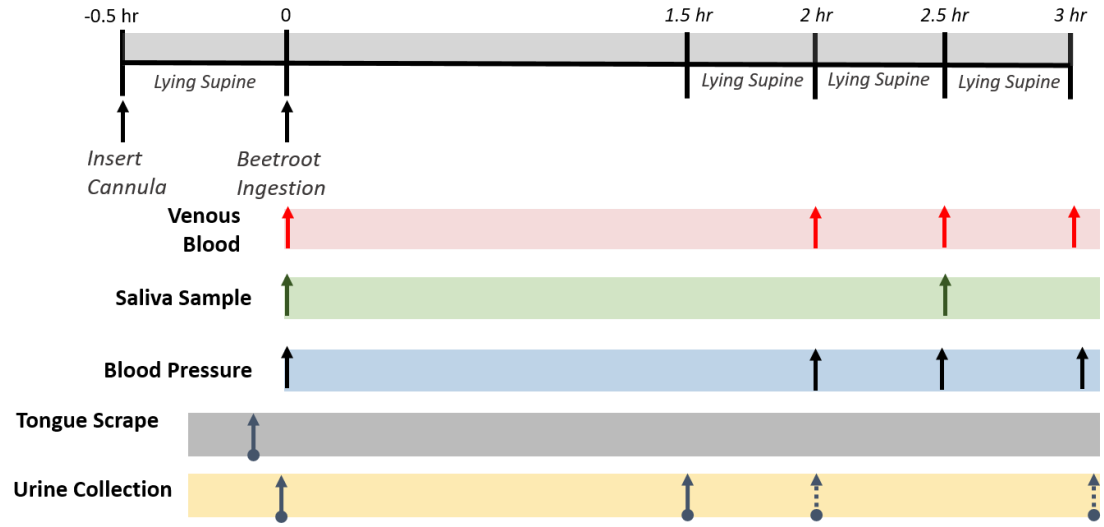


Figure. 1. Schematic of measurement time points for all trials. Dashed arrows depict optional urine collection.

2.4. Plasma nitrate and nitrite analysis

Measurements of [NO_3^-] and [NO_2^-] were conducted using ozone-based chemiluminescence (Rogers et al. 2005). For the measurement of plasma [NO_3^-], vanadium reagent (24 mg of vanadium tri-chloride and 3 ml of 1M Hydrochloric acid) and 100 µL of anti-foaming agent were placed into a customised glass purge vessel infused with nitrogen and heated to 95°C. This purge vessel was connected to an NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was produced by injecting 25 µL of NO_3^- solutions (100 µM, 50 µM, 25 µM, 12.5 µM, and 6.25 µM) and a control sample containing deionised water. The area under the curve (AUC) for the latter was subtracted from the NO_3^- solutions to account for NO_3^- in the water used for dilutions. Plasma samples were thawed in a water bath at 37°C for 3 min and de-proteinised using zinc sulphate/sodium hydroxide solution (200 µL of plasma, 400 µL of zinc sulphate in deionised water at 10% w/v and 400 µL of 0.5M sodium hydroxide). The

samples were then vortexed for 30 s and remained at room temperature for 15 min before being spun at 4000 rpm for 5 min. Subsequently, 15-25 μL of the sample was injected into the purge vessel in duplicate. The concentration of NO cleaved during the reaction was then measured by the NO analyser. The AUC was calculated using Origin software (version 7) and divided by the gradient of the slope.

For the measurement of plasma [NO_2^-], tri-iodide reagent (2.5 ml glacial acetic acid, 0.5 ml of 18 Ω deionised water and 25 mg sodium iodide) and 100 μL of anti-foaming agent were placed into the glass purge vessel and heated to 50°C. A standard curve was produced by injecting 100 μL of NO_2^- solutions (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and a control sample of deionised water. The AUC for the latter was subtracted from the NO_2^- solutions to account for NO_2^- in the water used for dilutions. Following this, plasma samples were thawed in a water bath and 100 μL of the sample was injected into the purge vessel in duplicate and [NO_2^-] was determined via the AUC, as previously described.

2.5. Salivary nitrite and nitrate analysis

The same reagents used for plasma [NO_3^-] and [NO_2^-] analyses were used for the analysis of salivary metabolites. The standard curve for salivary [NO_3^-] was the same as described for plasma [NO_3^-]. The standard curve for salivary [NO_2^-] was produced by injecting 100 μL NO_2^- solutions up to 5 μM. For both metabolites, saliva samples were thawed as previously described and then diluted at a ratio of 1:100 with deionised water. Subsequently, 100 μL of the sample was injected for the measurement of [NO_2^-] and 10-25 μL for [NO_3^-]. Samples were injected into the purge vessel in duplicate and calculated as previously described before being corrected for the dilution factor.

243 2.6. Urinary nitrate analysis

244 The same reagent and standard curve used for plasma [NO_3^-] analysis was used for the
245 measurement of urinary [NO_3^-]. Urine samples were thawed and diluted at a ratio of 1:100
246 with deionised water. Following this, 15-25 μL of the sample was injected to the purge vessel
247 in duplicate and [NO_3^-] calculated as previously described.

248

249 2.7. Human Oral Microbe Identification using Next Generation Sequencing (HOMINGS)

250 DNA samples were transported to a commercial centre (HOMMINGS, The Forsyth Institute,
251 Boston MA, USA) for sequencing analysis. A full description of the protocol is described by
252 Caporaso et al. (2011). In brief, the V3-V4 region of the bacterial genomic DNA was
253 amplified using barcoded primers; ~341F (forward [oligonucleotide] primer)
254 AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTCCTACGGGAGGCA
255 GCAG and ~806R (reverse primer)
256 CAAGCAGAAGACGGCATACGAGATNNNNNNNNNNNNAGTCAGTCAGCCGGACT
257 ACHVGGGTWTCTAAT. Samples (10 – 50 ng) of DNA were amplified by polymerase
258 chain reaction using V3-V4 primers and 5 PrimeHotMaster Mix and purified using AMPure
259 beads. A small volume (100 ng) of each library was pooled, gel-purified, and quantified using
260 a bioanalyser and qPCR. Finally, 12pM of the library mixture, spiked with 20% Phix, was
261 analysed on the Illumina MiSeq (Illumina, San Diego, CA).

262

263 2.8. 16s rRNA gene data analysis

264 Quality filtered data received from the sequencing centre was further analysed for taxonomic
265 classification and bacterial abundance using Qiime 1.8 (Caporaso et al. 2010). One sample with
266 less than 5000 reads was discarded from further analysis. Sequences were clustered *de novo*

and binned into operational taxonomic units (OTU) 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 14870 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. Alpha diversity metrics were calculated using the Shannon diversity equation, which accounts for the richness and evenness of species in a sample. The smallest number of reads associated with any one sample was 14870 reads. These analyses enabled the calculation of the abundance of bacteria at the specific genus and species level that have been previously reported to reduce NO_3^- in the oral cavity (Doel et al. 2005; Hyde et al. 2014a). The sum of the abundance of NO_3^--reducing bacteria was also calculated and used in further analysis.

2.9. Statistical analysis

All analyses were carried out using the Statistical Package for Social Sciences, Version 22 (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 7 (GraphPad Software Inc., San Diego, USA) was used to create the figures. Data are expressed as the mean ± standard deviation (SD). The distribution of the data were tested using the Shapiro-Wilk test. A two-way repeated-measures ANOVA was used to assess the main effects of time and visit and the time x visit interaction for [NO_3^-], [NO_2^-], and blood pressure variables. A one-way repeated measures ANOVA was used to determine whether there were differences in the abundance of each genus of bacteria across the three trials. The between trial differences in the Shannon diversity index was assessed using a Friedman's rank test. *Post-hoc* analysis was conducted following a significant main effect or interaction using paired samples t-tests with Bonferroni

correction for multiple pairwise comparisons. Correlation coefficients (Pearson's for normally distributed data and Spearman's Rho for non-normally distributed data) were used to assess the association between the concentration of NO metabolites and the abundance of species specific NO_3^--reducing bacteria. Using the same analyses, associations of between-trial differences (Δ) in these parameters were also analysed. Statistical significance was declared when $P<0.05$.

2.9.1. *Inter-individual variation*

The inter-individual coefficient of variation (CV_I) was calculated using the pooled mean ± SD of the three-trial average using the following equation:

CV_I (%) = 100 – (SD/mean)

Where SD = the between participant standard deviation

Where mean = the average of all participant

2.9.2. *Analytical variation*

The CV_A was calculated using the pooled mean ± SD of each duplicate/triplicate measure using the following equation:

CV_A (%) = 100 − (SD/mean)

Where SD and mean are the standard deviation and the mean duplicate/triplicate measures of the same time point, respectively.

2.9.3. *Biological variation*

The CV_B for all measured variables was calculated using the mean ± SD of three samples from each participant at each time point of the experiment using the following equation:

CV_B (%) = 100 − (SD/mean)

Where SD and mean are the standard deviation and mean of repeated measures of the same time point of separate laboratory visits.

2.9.4. *Intra-individual variation*

The within subject coefficient of variation (CV_W) was calculated using the following equation:

CV_W (%) = CV_B – CV_A

2.9.5. *Critical difference*

The CD was assessed using the equation of Fraser and Fogarty (1989):

$CD = k\sqrt{CV_A^2 + CV_W^2}$

Where k = Constant determined by the probability level (2.77 at $P<0.05$)

3. Results

3.1. Nitrate and nitrite in biological fluids

The three-trial mean ± SD, CV_I, CD, and residuals (CV_A and CV_B) for each measurement are displayed in Tables 1 and 2. Inter-individual data and group mean ± SD are presented in

Figure 2 and 3 for plasma and saliva, respectively. The CV_A for the measurement of [NO_3^-] (range 1.0 – 4.1%) and [NO_2^-] (range 1.2 – 3.9%) indicates good precision for these analyses. There was a significant main effect of 'time' (P<0.01) but no effect of 'visit' or a 'time x visit' interaction (P>0.05) for plasma and salivary [NO_3^-] and [NO_2^-]. *Post-hoc* analyses showed that baseline values were significantly lower (all P<0.01) than at all other time points that followed the ingestion of BR. Plasma [NO_3^-] was significantly higher at the 2 h measurement point compared to 2.5 and 3 h post ingestion (both P<0.05).

Within-participant comparisons demonstrate that total urinary NO_3^- excretion did not differ between the three laboratory visits (P>0.05) (Table 1). The CV_B for salivary, plasma, and urinary [NO_3^-] variables ranged from 3.8 to 15.3% (Table 1). There was a greater degree of heterogeneity in saliva and plasma [NO_2^-] which ranged from 9 to 32.5 % (Table 2). The CD values were also considerable for [NO_3^-] variables (8.4 – 37.9%) and [NO_2^-] variables (19.3 – 86.5%). Between-participant comparisons reveal that, as expected, the CV_I was substantial, with [NO_3^-] variables ranging from 18.6 to 49.1% and [NO_2^-] from 29.9 to 73.5%.

3.2. Abundance of nitrate-reducing bacteria

After quality filtering the data and removal of singleton reads, tongue scrapings of 9 participants over three separate trials were included in the analysis. Alpha diversity metrics revealed that the Shannon diversity index for the whole group across all three visits was 5.4 ± 0.4 with 1356 ± 171 observed species. The Shannon diversity index did not differ between trials (P=0.50). There were 117 genera of bacteria detected in the samples. The only genera of bacteria where the abundance changed significantly was *Peptostreptoccocus* which was

more abundant in visit one compared to visit two (P=0.03). Previous research has shown that *Peptostreptoccocus* species do not have NO_3^- reductase activity (Smith et al. 1999).

All of the genera that have previously been implicated in NO_3^- reduction (Hyde et al. 2014a) were detected in our analyses (Table 3). *Prevotella* was the most abundant genera and had the lowest CV_B (22.7%) whilst *Haemophilus*, the fourth most abundant NO_3^--reducing genera, had the highest CV_B (77.6%). Seven of the bacterial species previously implicated in NO_3^- reduction (Doel et al. 2005; Hyde et al. 2014a) were detected in the samples and the variation in the relative abundance of these species were analysed across the three visits (Fig. 4). Further analyses at the species level showed that the sum of the NO_3^--reducing bacteria had a CV_B of 19.5%. The CV_B of individual species showed that *Rothia dentocariosa* and *Haemophilus parainfluenzae* were the most variable (132.1 and 78.6%, respectively, Table 4). The two most abundant species, *Prevotella melaninogenica* and *Veillonella dispar*, had the lowest CV_B of 37 and 35.1 %, respectively.

3.3. Blood pressure

Blood pressure data are presented alongside the variability metrics in Table 5. The CV_A for the measurement of systolic blood pressure (range 1.3 – 3.8%), diastolic blood pressure (range 2.5 – 3.6%), and MAP (range 2.2 – 3.7%) indicates good precision for these parameters. There was a significant main effect of 'time' for systolic blood pressure (P<0.01), diastolic blood pressure (P=0.04), and MAP (P<0.01) but no 'time x visit' interaction (all P>0.05). There was no main effect of 'visit' for systolic blood pressure or MAP (P>0.05) but there was an effect of 'visit' on diastolic blood pressure (P=0.02). *Post-hoc* analyses showed that systolic blood pressure was significantly lower at all measurement points following BR ingestion (all P<0.05). Diastolic blood pressure was not different

between measurement points or individual visits (all $P>0.05$). MAP was not different to baseline after 2 h ($P=0.08$) but was lower than baseline at 2.5 and 3 h post BR ingestion (both $P<0.05$). Measurements of systolic blood pressure (range 2.0 – 3.4%) and MAP (range 2.9 – 3.9%) had minimal CV_B. The CV_B for diastolic blood pressure was greater, ranging from 4.2 to 6.0%. Values of CD ranged from 5.3 to 11.9% for all blood pressure markers and values of CV_I ranged from 4.7 to 8.1%.

3.4. Association between nitrate and nitrite in biological fluids and the abundance of nitrate-reducing bacteria

The sum of the NO_3^--reducing bacteria was not associated with measurements of $[NO_2^-]$ at any time point (all $P>0.2$). Individual species analysis showed that the abundance of *Neisseria subflava* was negatively associated with peak salivary $[NO_2^-]$ (R=-0.43, $P=0.03$, Fig. 5) and plasma $[NO_2^-]$ (R=-0.43, $P=0.03$, Fig. 5). There were no other associations between the concentration of NO metabolites and the abundance of all other individual species of NO_3^--reducing bacteria (all $P>0.07$). The between-trial Δ in salivary $[NO_2^-]$ following BR and the between-trial Δ *Rothia mucilaginosa* abundance were significantly associated (R=0.49, $P=0.01$, Fig. 6). The between-trial Δ *Haemophilus parainfluenzae* abundance was negatively associated with the between-trial Δ plasma $[NO_2^-]$ at 3 h post BR ingestion (R=-0.4, $P=0.04$, Fig. 6). There were no other relationships between the variation in $[NO_2^-]$ variables and the abundance of NO_3^- reducing species (all, $P>0.09$).

Table 1. Three-trial mean ± SD, analytical variation (CV_A), biological variation (CV_B), critical difference (CD), and inter-individual variability (CV_I) for plasma, salivary and urinary [NO_3^-] at each measurement point. * denotes significant difference compared to baseline (P<0.001).

Parameter	Mean ± SD	CV_A (%)	CV_B (%)	CD (%)	CV_I (%)
Plasma baseline	33.2 ± 7.6 μM	4.1	11.9	24.4	22.8
Plasma 2 h	452.1 ± 83.9 μM*	1.0	3.8	8.4	18.5
Plasma 2.5 h	415.0 ± 92.2 μM*	1.2	4.7	10.3	22.2
Plasma 3 h	391.6 ± 99.2 μM*	1.8	8.8	19.9	25.3
Saliva baseline	0.5 ± 0.2 mM	2.1	15.3	37.1	30.7
Saliva 2.5 h	8.5 ± 2.1 mM*	1.4	12.0	29.7	24.1
Urine total	1.7 ± 0.3 g ($x10^{-4}$)	1.7	15.3	37.9	49.1

Table 2. Three-trial mean ± SD, analytical variation (CV_A), biological variation (CV_B), critical difference (CD), and inter-individual variability (CV_I) for plasma and salivary [NO_2^-] at each measurement point. * denotes significant difference compared to baseline (P<0.001).

Parameter	Mean ± SD	CV_A (%)	CV_B (%)	CD (%)	CV_I (%)
Plasma baseline	124.2 ± 48.8 nM	2.5	9.0	19.3	39.3
Plasma 2 h	284.9 ± 83.5 nM*	2.1	19.3	47.9	29.3
Plasma 2.5 h	278.6 ± 73.9 nM*	2.4	18.6	45.4	26.5
Plasma 3 h	323.9 ± 94.1 nM*	2.2	20.6	51.3	29.0
Saliva baseline	135.7 ± 99.8 μM	1.2	32.5	86.5	73.5
Saliva 2.5 h	903.6 ± 267.6 μM*	3.9	24.5	58.1	29.6

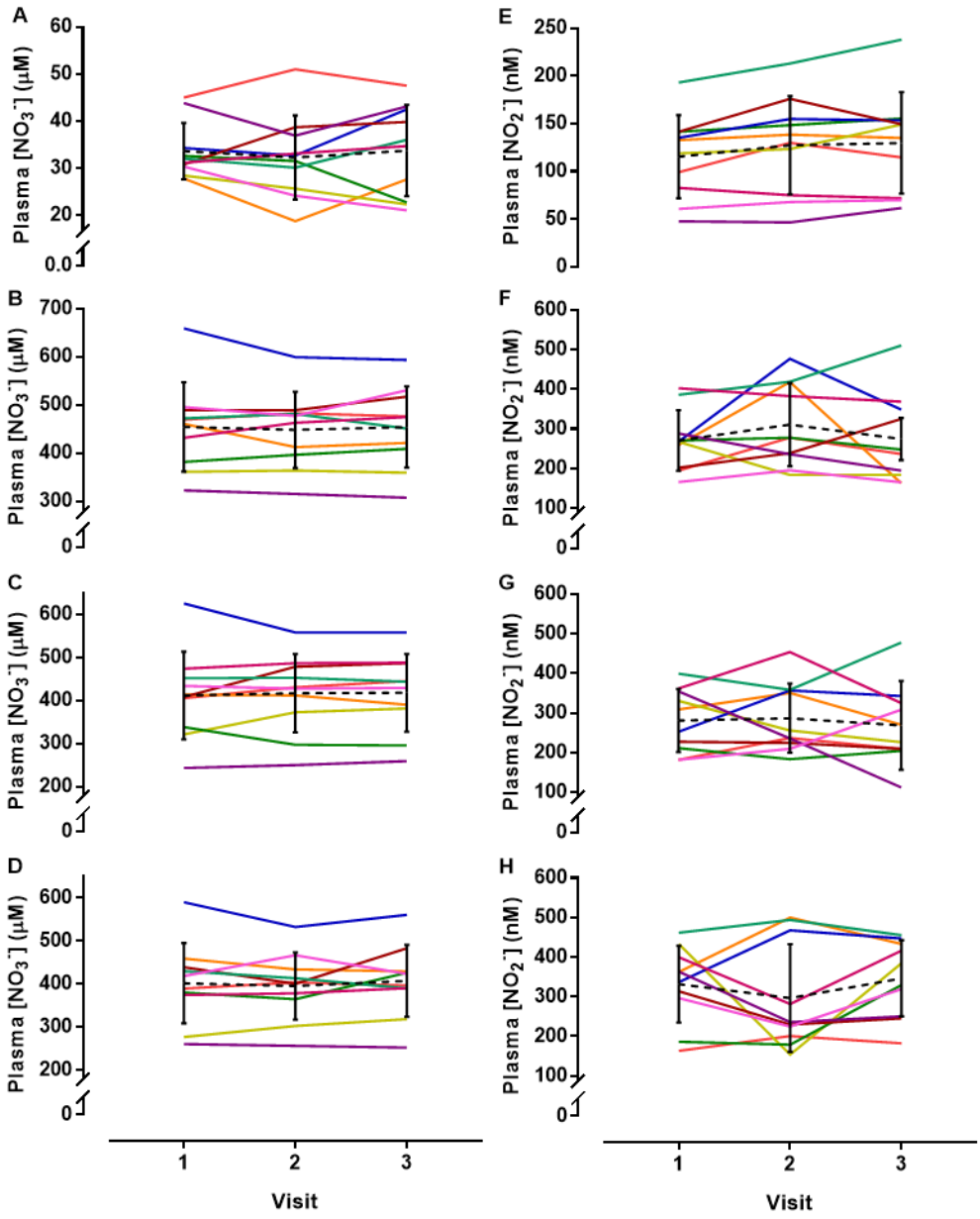
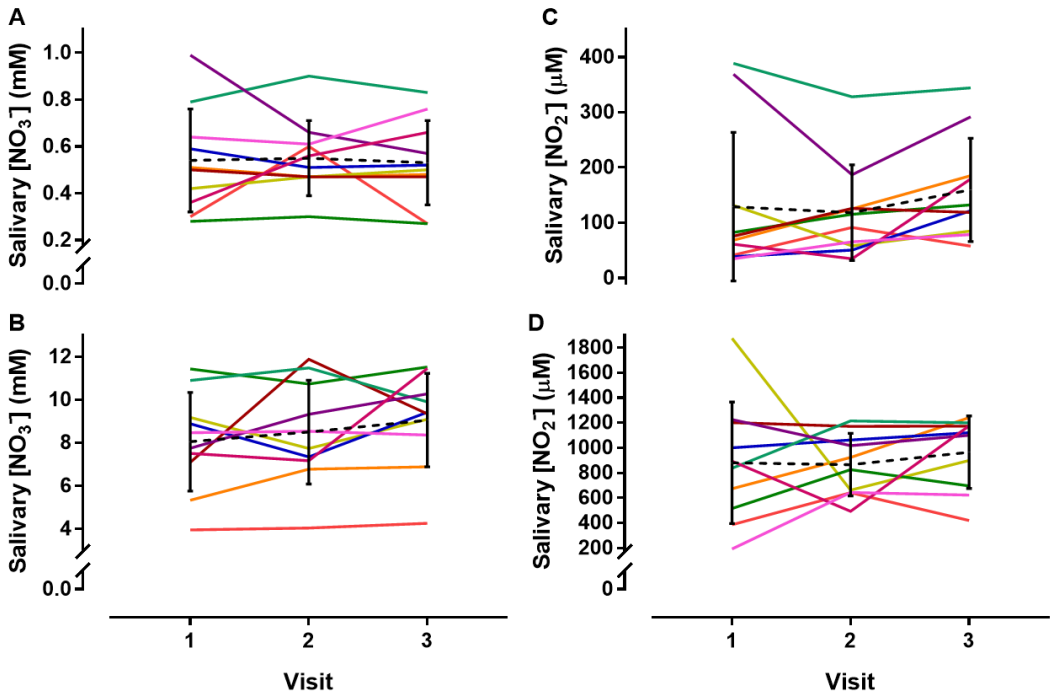


Figure 2. Group mean ± SD and inter-individual variation across the three identical trials for plasma [NO_3^-] at baseline (A), 2 h (B), 2.5 h (C), and 3 h (D), and for plasma [NO_2^-] at baseline (E), 2 h (F), 2.5 h (G), and 3 h (H). All post supplementation time points for plasma [NO_3^-] and [NO_2^-] were significantly elevated compared to baseline concentrations (all $P < 0.01$).



422

423 **Figure 3**. Group mean ± SD and inter-individual variation across the three identical trials for
424 salivary [NO_3^-] at baseline (A), and 2.5 h (B), and for salivary [NO_2^-] at baseline (C), and 2 h
425 (D). Following supplementation salivary [NO_3^-] and [NO_2^-] were significantly elevated
426 compared to baseline concentrations (all $P < 0.01$).

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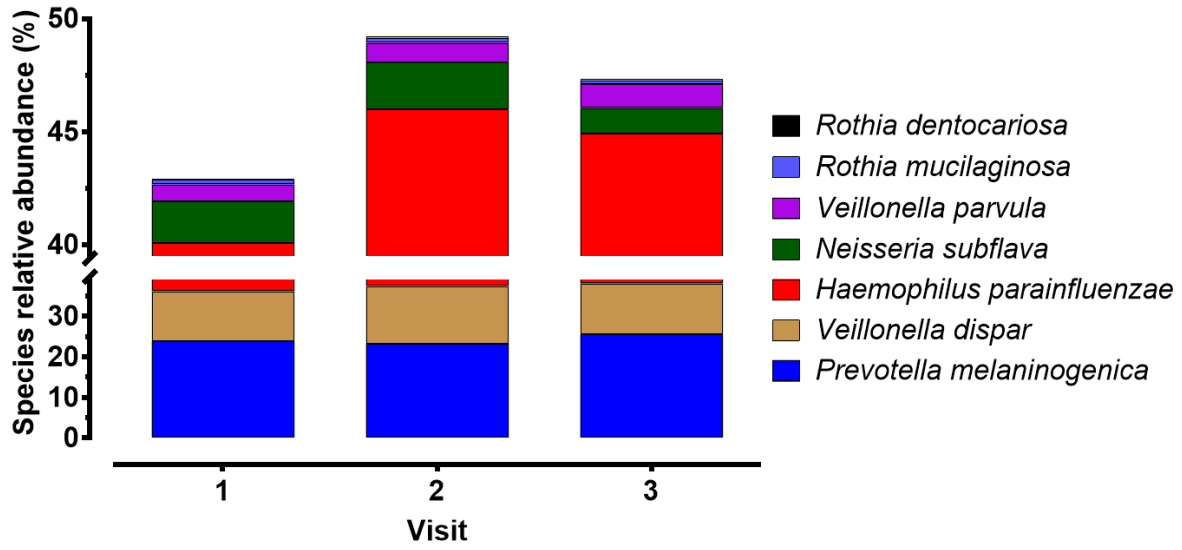
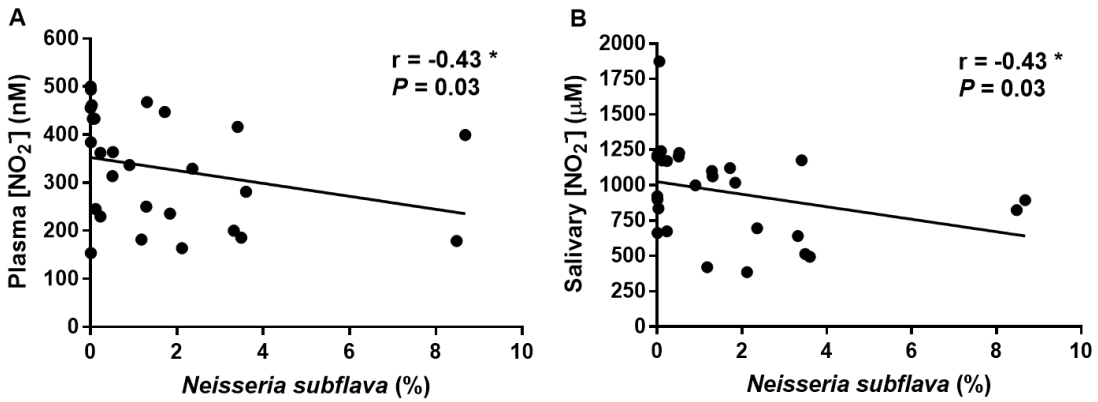


430

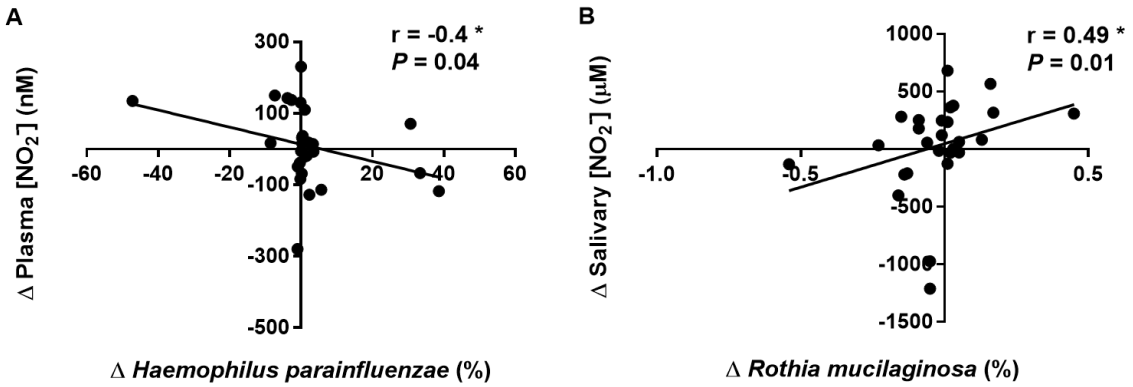
431 **Figure 4.** A comparison of the relative abundance of NO_3^--reducing species between three
432 identical trials taken at baseline during each laboratory visit. Data are presented as group
433 means with SD excluded for clarity.

434

435



436
437 **Figure 5.** Correlations between *Neisseria subflava* and peak concentration plasma $[NO_2^-]$ (A)
438 and salivary $[NO_2^-]$ (B). * denotes significant difference.



439

440 **Figure 6.** Correlations between Δ plasma [NO_2^-]/Δ *Haemophilus parainfluenzae* (A) and Δ
441 salivary [NO_2^-]/Δ *Rothia mucilaginosa* (B). * denotes significant difference.

442

443 **Table 3.** Relative abundance of genera previously implicated in NO_3^-
444 reduction and the corresponding biological variation (CV_B) and inter-
445 individual variability (CV_I).

OTU ID	Mean ± SD (%)	CV_B (%)	CV_I (%)
Prevotella	35.6 ± 13.5	22.7	38.6
Veillonella	14.7 ± 7.2	33.4	50.1
Fusobacterium	9.5 ± 9.3	54.5	97.8
Haemophilus	6.5 ± 11.1	77.6	145.0
Leptotrichia	6.4 ± 3.6	52.7	56.1
Streptococcus	2.0 ± 1.9	45.7	96.8
Neisseria	1.8 ± 2.5	67.9	130.7
Porphyromonas	1.6 ± 1.8	76.1	119.4
Actinomyces	1.0 ± 0.8	64.5	82.8
Rothia	0.2 ± 0.2	57.7	108.6
Granulicatella	0.1 ± 0.2	72.0	122.9

446

447 **Table 4.** Relative abundance of species previously implicated in NO_3^- reduction and

448 the corresponding biological variation (CV_B) and inter-individual variability (CV_I).

Species	Mean ± SD (%)	CV_B (%)	CV_I (%)
Prevotella melaninogenica	23.8 ± 6.4	37.0	26.9
Veillonella dispar	13.0 ± 4.0	35.1	30.7
Haemophilus parainfluenzae	6.5 ± 5.9	78.6	90.7
Neisseria subflava	1.7 ± 1.0	70.0	57..7
Veillonella parvula	0.9 ± 0.4	43.2	44.3
Rothia mucilaginosa	0.2 ± 0.1	60.0	41.0
Rothia dentocariosa	<0.01 ± <0.01	132.1	118.4

449

450

Table 5. Three-trial mean ± SD, analytical variation (CV_A), biological variation (CV_B), critical difference (CD), and inter-individual variability (CV_I) for blood pressure parameters at each measurement point. * denotes significant difference compared to baseline (P<0.05).

Blood Pressure	Mean ± SD (mmHg)	CV_A (%)	CV_B (%)	CD (%)	CV_I (%)
Systolic baseline	126 ± 7	1.9	2.0	5.3	5.9
Systolic 2 h	121 ± 7*	1.3	3.1	6.1	6.1
Systolic 2.5 h	120 ± 7*	3.8	3.4	10.6	6.4
Systolic 3 h	122 ± 7*	3.3	3.2	10.1	5.8
Diastolic baseline	70 ± 5	3.4	4.8	10.2	7.7
Diastolic 2 h	67 ± 5	3.0	4.9	9.9	8.1
Diastolic 2.5 h	67 ± 4	3.6	4.2	10.2	5.4
Diastolic 3 h	67 ± 4	2.5	6.0	11.9	6.2
MAP baseline	88 ± 5	2.7	3.9	8.1	5.4
MAP 2 h	85 ± 5	2.2	3.4	7.0	5.9
MAP 2.5 h	85 ± 4*	3.7	3.1	10.4	5.0
MAP 3 h	85 ± 4*	3.1	2.9	8.5	4.7

4. Discussion

The present study demonstrates that, as hypothesised, the concentration of NO_3^- and conversion to NO_2^- in biological fluids varies substantially within individuals across repeated laboratory visits under the same conditions. Likewise, the CV_B for the abundance of NO_3^--reducing bacteria were also profound, suggesting substantial heterogeneity in these measurements. The CD values for NO metabolites at baseline suggest that large relative changes in these parameters are required before a meaningful difference can be concluded following an intervention. On the other hand, measurements of blood pressure at baseline

demonstrated much lower CV_B across repeated trials. The relative abundance of *Neisseria subflava* on the tongue was negatively associated with [NO_2^-] in the saliva and plasma following ingestion of BR. The variation in salivary [NO_2^-] following BR between repeated trials was also associated with the variation in the abundance of *Rothia mucilaginosa* and the between-trial variation in peak plasma [NO_2^-] was negatively associated with the variation in the abundance of *Haemophilus parainfluenzae*. These data suggest that, contrary to our hypothesis, the CV_B of NO metabolites is only partly accounted for by the CV_B in the abundance of NO_3^--reducing bacterial species.

4.1. Variability of the tongue microbiome of healthy humans

There were 1356 ± 171 observed species of bacteria in the tongue scrape samples across the three trials which is comparable with some (Li et al. 2014; Burleigh et al. 2018) and considerably higher than others (Hyde et al. 2014a). The Shannon Diversity Index, which accounts for both richness and evenness of OTUs, was also similar to previous reports in healthy humans (Zaura et al. 2009; Hyde et al. 2014a; Burleigh et al. 2018). *Veillonella* is commonly reported to be the most abundant of the taxa that are specifically implicated in NO_3^- reduction (Doel et al. 2005; Hyde et al. 2014a). In the present study, however, *Prevotella* were found to be more than twice as abundant as *Veillonella*. These dissimilarities are likely explained by inter-individual differences in study cohorts as corroborated by the profound CV_I across all genera previously implicated in NO_3^- reduction (Table 3). In line with our previous work (Burleigh et al. 2018), *Prevotella melaninogenica and Veillonella dispar* were the most abundant species of NO_3^--reducing bacteria in all three trials.

The inter-individual diversity and temporal dynamics of tongue microbiota in the oral cavity has previously been investigated by Hall and colleagues (2017) who collected samples daily, weekly, and monthly from 10 healthy participants. There was significant drift in the composition of the microbiome over both short and long time scales, the magnitude of which varied between subjects. Nevertheless, several species were consistently observed (≥ 95% samples) at all measurement points, including several species that have been implicated in NO_3^- reduction (*Haemophilus parainfluenzae, Neisseria subflava, and Rothia dentocariosa*). In the present study, the CV_B for seven of the bacteria previously implicated in NO_3^- reduction are reported for the first time. Here, we show that there is profound within-participant variation at both the level of genera (23 – 78%) and species (35 – 132%) at three controlled measurement points over a 15-21 day period. This may be reasonably expected given that the mouth is exposed to the external environment and regularly subjected to brushing, flossing, and nutrient intake (Hall et al. 2017) which may consequently influence pH (Krulwich et al. 2011). It has been shown previously that 7 days of sodium NO_3^- supplementation (Hyde et al. 2014b) and 10 days (Vanhatalo et al. 2018) or 6 weeks (Velmurugan et al. 2016) of BR supplementation results in significant alterations to the oral microbiome, including species of NO_3^--reducing bacteria. Our study demonstrates that despite standardising diet, physical activity, mouthwash, teeth brushing, and tongue cleaning before each trial, the abundance of these bacteria vary considerably. Quantifying the magnitude of this variation provides useful metrics which will aid researchers to interpret the meaningfulness of changes to the oral microbiome following an intervention.

520

521 4.2. Variability in the measurements of nitric oxide metabolites

522 Values of plasma and salivary [NO_2^-] and [NO_3^-] at baseline and following the ingestion of
523 BR are broadly in line with those reported in the literature (e.g. James et al. 2015; Liddle et
524 al. 2018; Woessner et al. 2016). Some of the subtle differences between studies may be partly
525 explained by dissimilarities in methodology and study control (Bryan et al. 2007; Feelisch et
526 al. 2010; Liddle et al. 2018). Inter-individual differences between participants in each cohort
527 will also likely underpin some of the variation in basal NO metabolite concentration and NO
528 pharmacokinetics following the ingestion of BR (Muggeridge et al. 2014; James et al. 2015;
529 McIlvenna et al. 2017). This is highlighted profoundly by the CV_I values in the current data
530 set which were 19 – 31% for salivary and plasma NO_3^- and 27 – 74% for NO_2^-. Porcelli and
531 colleagues (2015) have demonstrated that physical fitness appears to affect the response to
532 NO_3^- supplementation whereby the increase in plasma [NO_2^-] is suppressed in individuals
533 with better aerobic fitness. Alternatively, other factors which may influence endogenous
534 production of NO (Luiking et al. 2010) or differences in the oral (Burleigh et al. 2018) and
535 gut microbiota (Flint et al. 2012) may also account for some of the inter-cohort variations.
536 For example, we have recently demonstrated that individuals with a higher abundance of
537 NO_3^--reducing bacteria generate more NO_2^- in the saliva and at a faster rate (Burleigh et al.
538 2018).

539

540 Given the exponential rise in research exploring the health promoting and ergogenic effects
541 of BR it is perhaps surprising that the CV_B for the physiological responses to this
542 supplementation regimen have not previously been reported. Particularly where it is argued
543 that changes in any outcome should be interpreted within the boundaries of CD in order to
544 quantify a meaningful difference (Fraser and Fogarty 1989). At baseline, there was moderate

CV_B in plasma markers (9 and 12% for NO_2^- and NO_3^-, respectively) although the variation was more substantial in salivary measures (33 and 15% for NO_2^- and NO_3^-, respectively). Following the ingestion of BR, the CV_B of NO_3^- ranged from 4 – 9% in plasma and 12 – 15% in saliva which was considerably lower than the CV_B of NO_2^- markers (19 – 21% in plasma and 25 – 33% in saliva). Urinary excretion of NO_3^- was also shown to have a large CV_B (15%) and CV_I (49%). The CD values demonstrate that substantial changes in NO markers in biological fluids are required at baseline or following the ingestion of BR to be deemed biologically meaningful (Fraser and Fogarty 1989).

4.3. Association between nitrate-reducing bacteria and nitric oxide metabolites

The oral microbiome is known to be a crucial component of the NO_3^--NO_2^--NO pathway. Abolishing oral bacterial species with anti-bacterial mouthwash, for example, has been shown to substantially interrupt oral reductase capacity (Kapil et al. 2013; Bondonno et al. 2015; McDonagh et al. 2015; Woessner et al. 2016). Given the oral microbiome is exceptionally sensitive and modifiable within individuals, it is plausible that intra-individual variations in the abundance of NO_3^--reducing bacteria would influence circulating levels of NO_2^- and NO metabolite pharmacokinetics following the ingestion of BR. A large CV_B in $[NO_2^-]$ values would, therefore, be reasonably expected given the large CV_B in the abundance of NO_3^--reducing bacteria. Further analyses of our data reveals that variation in oral microbiota do influence the CV_B of the NO metabolites, at least to some extent. The relative abundance of *Neisseria subflava* on the tongue was negatively associated with the peak $[NO_2^-]$ in the saliva and plasma following ingestion of BR. The Δ in salivary $[NO_2^-]$ following BR between repeated trials was also positively associated with the between-trial Δ in *Rothia mucilaginosa.* Additionally, the between-trial Δ in plasma $[NO_2^-]$ at 3 h post BR ingestion was negatively associated with the between-trial Δ in *Haemophilus parainfluenzae*. Whilst it

570 is possible that these species may be particularly important for NO_3^- reduction, it must be
571 acknowledged that all statistically significant associations were only "moderate" in strength
572 (R = 0.40 – 0.49), are likely underpowered, and do not necessarily imply "cause-effect".
573 Furthermore, while the dorsal surface of tongue is the area of the oral cavity in which the
574 majority of NO_3^- reduction activity occurs (Doel et al. 2005), our sampling of the oral
575 microbiome was not comprehensive. For example, NO_3^- reduction is also reported to occur
576 directly in the saliva (Goaz and Biswell 1961) and in other areas of the mouth. It is also
577 recognised that some species of bacteria are capable of reducing NO_2^- to NO in the saliva and
578 the abundance of these microbiota may be considered to influence plasma [NO_2^-]. However,
579 NO_2^- reduction via bacterial enzymatic activity is a slow process (Doel et al. 2005) and,
580 given the rapid extrusion of NO_2^- through continuous swallowing, the abundance of these
581 microbiota are likely to be less relevant.
582
583 While the relevant abundance of the oral microbiome seems to contribute towards the
584 regulation of NO bioavailability (Burleigh et al. 2018), it does not fully account for the large
585 CV_B in basal [NO_2^-] and [NO_3^-] and the variable response to ingested inorganic NO_3^-. Indeed,
586 the metabolic activity of the NO_3^--reducing bacteria may be more important than the relevant
587 abundance (Hyde et al. 2014a). Alternatively, CV_B of other factors including the
588 aforementioned abundance and activity of gut bacteria, stomach pH (Lundberg et al. 1994;
589 Montenegro et al. 2017), rates of gastric emptying and intestinal absorption (Leiper 2015), or
590 the availability of sialin, a NO_3^- transporter in the saliva (Qin et al. 2012), may also contribute
591 towards a high CV_B in NO metabolism. There also seems to be circadian variation in
592 endogenous NO production (Antosova et al. 2009). Furthermore, while participants were
593 requested to replicate their diet prior to each trial, the NO_3^- content of regularly consumed
594 vegetables is known to vary considerably (Lidder and Webb 2013). Non-compliance with

these instructions also cannot be ruled out although all participants gave verbal assurances on this point. Exposure to different doses of sunlight has also been shown to influence circulating levels of NO_2^- (Monaghan et al. 2018). However, the latter mechanism may have had minimal influence in the present study as data were collected in the autumn/winter months. Establishing the independent contribution of each of these factors to NO bioavailability will be a difficult task due to a lack of gold-standard measurements or challenges in isolating each as an independent variable rather than a covariate.

4.4. Variability in the blood pressure response to nitrate supplementation

Ingestion of BR resulted in significant reductions in systolic blood pressure and MAP which supports findings from a recent meta-analysis showing a mean reduction in systolic blood pressure of 4.4 mmHg (Siervo and Lara 2013). Novel data in this study shows that the reduction in blood pressure markers is consistently observed in response to NO_3^- supplementation and, in contrast to NO metabolites, the CV_B for these measurements are relatively low (all <5%). This contrasts with previous research which reports the visit-to-visit variation is larger (>8%) for systolic and diastolic blood pressure in various clinical cohorts (Marshall 2004; Howard and Rothwell 2009). In absolute terms, baseline systolic blood pressure (mean 126 ± 7 mmHg) varied by 2.5 mmHg across the three trials of the present study compared to 14.7 mmHg (mean 147 ± 18.4 mmHg) in patients who had suffered a minor transient ischemic attach or minor ischemic stroke (Howard and Rothwell 2009). This suggests that cohorts with a higher blood pressure will also have an increased CV_B for this metric. Indeed, an increased variability CV_B may also have some prognostic value as it has been associated with the development, progression, and severity of cardiac, vascular, and renal damage and with an increased risk of cardiovascular events and mortality (Parati et al. 2013). It is important to highlight that the participants in the present study were all

from a homogenous cohort; namely they were all healthy Caucasian males from a relatively narrow age range. It is likely that CV_B and CD for all measured outcomes would increase in a more heterogonous group of healthy participants which included females and older adults.

Webb and colleagues (2008) have previously reported that ingestion of BR reduces systolic blood pressure by up to ~10 mmHg in healthy participants. Notably, the magnitude of this reduction in systolic blood pressure exceeds the baseline CD reported here (6.7 mmHg, 5.3 %) which confirms that this is a meaningful change in this parameter. In contrast, the BR-induced reduction in blood pressure reported in this study and more widely across the literature in healthy normotensive participants (Siervo and Lara 2013) are typically smaller and do not exceed the CD threshold. In patients with stage 1 hypertension, a single dose of NO_3^--rich BR reduced systolic blood pressure by 11 mmHg (7.3%) (Ghosh et al. 2013) suggesting the effects of BR are more pronounced in those with an elevated blood pressure. However, given that a high blood pressure will also elevate the CV_B, researchers should be cautious about using CD values generated from healthy participants to interpret data in hypertensive or diseased cohorts. While this does not rule out a therapeutic effect of inorganic NO_3^- supplementation in hypertensive patients, the potential influence of CV_A and CV_B on experimental outcomes should be duly considered when interpreting the data.

5. Conclusion

The data in the current study demonstrates that there is profound intra-individual variability in the measurement of NO metabolites in plasma and saliva, both at basal levels and when elevated following ingestion of BR. While the change in the abundance of certain species of NO_3^--reducing bacteria appears to account for some of this variation, other biological and

experimental factors are also likely to contribute. Markers of blood pressure were consistently reduced on three separate occasions following the ingestion of BR but the magnitude of the change was small and did not exceed the CD. The data presented in this manuscript presents metrics which facilitate a more meaningful interpretation of changes in key physiological variables following dietary NO_3^- supplementation.

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Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interests.

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