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

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

103

104 The within-individual or biological variation (CV_B) establishes the inherent fluctuations around
105 a homeostatic set-point of a measured variable (Harris 1970). The CV_B can be used in
106 combination with the CV_A to calculate the critical difference (CD) which is defined as the
107 change from baseline that must occur before a meaningful biological difference can be claimed
108 (Fraser and Fogarty 1989). In short, a researcher is able to use the CV_B and the CV_A to
109 determine the typical "noise" in the variable of interest. The CD provides a single criterion
110 threshold which, if exceeded, they can conclude a true change has occurred in response to any
111 intervention. For reference, it has been previously reported that serum cholesterol has a CV_B
112 of 7.6% and a CD of 17.2% (Fraser 2001). Blood glucose has been shown to have a CV_B and
113 CD of 7.2% and 14.9%, respectively (Widjaja et al. 1999). In the context of dietary NO_3^-
114 supplementation researchers must first be confident that the intervention results in a true
115 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 4ºC 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 -80^0C 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

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

194

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

196

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

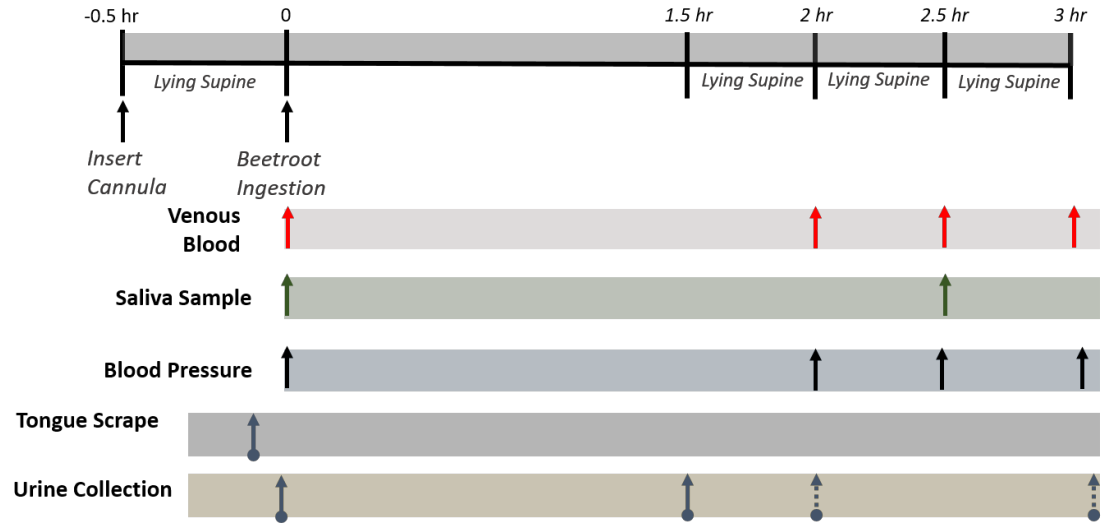


Figure. 1. Schematic of measurement time points for all trials. Dashed arrows depict optional urine collection.

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

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

224

225 For the measurement of plasma [NO_2^-], tri-iodide reagent (2.5 ml glacial acetic acid, 0.5 ml of
226 18 Ω deionised water and 25 mg sodium iodide) and 100 μL of anti-foaming agent were placed
227 into the glass purge vessel and heated to 50°C. A standard curve was produced by injecting
228 100 μL of NO_2^- solutions (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and a control
229 sample of deionised water. The AUC for the latter was subtracted from the NO_2^- solutions to
230 account for NO_2^- in the water used for dilutions. Following this, plasma samples were thawed
231 in a water bath and 100 μL of the sample was injected into the purge vessel in duplicate and
232 [NO_2^-] was determined via the AUC, as previously described.

233

234 2.5. Salivary nitrite and nitrate analysis

235 The same reagents used for plasma [NO_3^-] and [NO_2^-] analyses were used for the analysis of
236 salivary metabolites. The standard curve for salivary [NO_3^-] was the same as described for
237 plasma [NO_3^-]. The standard curve for salivary [NO_2^-] was produced by injecting 100 μL NO_2^-
238 solutions up to 5 μM. For both metabolites, saliva samples were thawed as previously described
239 and then diluted at a ratio of 1:100 with deionised water. Subsequently, 100 μL of the sample
240 was injected for the measurement of [NO_2^-] and 10-25 μL for [NO_3^-]. Samples were injected
241 into the purge vessel in duplicate and calculated as previously described before being corrected
242 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 with
246 deionised water. Following this, 15-25 μL of the sample was injected to the purge vessel in
247 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 amplified
253 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 chain
258 reaction using V3-V4 primers and 5 PrimeHotMaster Mix and purified using AMPure beads.
259 A small volume (100 ng) of each library was pooled, gel-purified, and quantified using a
260 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.

313 2.9.3. *Biological variation*

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

316 CV_B (%) = 100 − (SD/mean)

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

319

320 2.9.4. *Intra-individual variation*

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

322 CV_W (%) = CV_B – CV_A

323

324 2.9.5. *Critical difference*

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

326 $CD = k\sqrt{CV_A^2 + CV_W^2}$

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

328

329 **3. Results**

330 3.1. Nitrate and nitrite in biological fluids

331 The three-trial mean ± SD, CV_I, CD, and residuals (CV_A and CV_B) for each measurement are
332 displayed in Tables 1 and 2. Inter-individual data and group mean ± SD are presented in Figure
333 2 and 3 for plasma and saliva, respectively. The CV_A for the measurement of [NO_3^-] (range 1.0

334 – 4.1%) and [NO_2^-] (range 1.2 – 3.9%) indicates good precision for these analyses. There was
335 a significant main effect of 'time' (P<0.01) but no effect of 'visit' or a 'time x visit' interaction
336 (P>0.05) for plasma and salivary [NO_3^-] and [NO_2^-]. *Post-hoc* analyses showed that baseline
337 values were significantly lower (all P<0.01) than at all other time points that followed the
338 ingestion of BR. Plasma [NO_3^-] was significantly higher at the 2 h measurement point
339 compared to 2.5 and 3 h post ingestion (both P<0.05).

340

341 Within-participant comparisons demonstrate that total urinary NO_3^- excretion did not differ
342 between the three laboratory visits (P>0.05) (Table 1). The CV_B for salivary, plasma, and
343 urinary [NO_3^-] variables ranged from 3.8 to 15.3% (Table 1). There was a greater degree of
344 heterogeneity in saliva and plasma [NO_2^-] which ranged from 9 to 32.5 % (Table 2). The CD
345 values were also considerable for [NO_3^-] variables (8.4 – 37.9%) and [NO_2^-] variables (19.3 –
346 86.5%). Between-participant comparisons reveal that, as expected, the CV_I was substantial,
347 with [NO_3^-] variables ranging from 18.6 to 49.1% and [NO_2^-] from 29.9 to 73.5%.

348

349 3.2. Abundance of nitrate-reducing bacteria

350 After quality filtering the data and removal of singleton reads, tongue scrapings of 9
351 participants over three separate trials were included in the analysis. Alpha diversity metrics
352 revealed that the Shannon diversity index for the whole group across all three visits was 5.4 ±
353 0.4 with 1356 ± 171 observed species. The Shannon diversity index did not differ between
354 trials (P=0.50). There were 117 genera of bacteria detected in the samples. The only genera of
355 bacteria where the abundance changed significantly was *Peptostreptoccocus* which was more
356 abundant in visit one compared to visit two (P=0.03). Previous research has shown that
357 *Peptostreptoccocus* species do not have NO_3^- reductase activity (Smith et al. 1999).

358 All of the genera that have previously been implicated in NO_3^- reduction (Hyde et al. 2014a)
359 were detected in our analyses (Table 3). *Prevotella* was the most abundant genera and had the
360 lowest CV_B (22.7%) whilst *Haemophilus*, the fourth most abundant NO_3^--reducing genera, had
361 the highest CV_B (77.6%). Seven of the bacterial species previously implicated in NO_3^-
362 reduction (Doel et al. 2005; Hyde et al. 2014a) were detected in the samples and the variation
363 in the relative abundance of these species were analysed across the three visits (Fig. 4). Further
364 analyses at the species level showed that the sum of the NO_3^--reducing bacteria had a CV_B of
365 19.5%. The CV_B of individual species showed that *Rothia dentocariosa* and *Haemophilus*
366 *parainfluenzae* were the most variable (132.1 and 78.6%, respectively, Table 4). The two most
367 abundant species, *Prevotella melaninogenica* and *Veillonella dispar*, had the lowest CV_B of 37
368 and 35.1 %, respectively.

369

370 3.3. Blood pressure

371 Blood pressure data are presented alongside the variability metrics in Table 5. The CV_A for the
372 measurement of systolic blood pressure (range 1.3 – 3.8%), diastolic blood pressure (range 2.5
373 – 3.6%), and MAP (range 2.2 – 3.7%) indicates good precision for these parameters. There was
374 a significant main effect of 'time' for systolic blood pressure (P<0.01), diastolic blood pressure
375 (P=0.04), and MAP (P<0.01) but no 'time x visit' interaction (all P>0.05). There was no main
376 effect of 'visit' for systolic blood pressure or MAP (P>0.05) but there was an effect of 'visit'
377 on diastolic blood pressure (P=0.02). *Post-hoc* analyses showed that systolic blood pressure
378 was significantly lower at all measurement points following BR ingestion (all P<0.05).
379 Diastolic blood pressure was not different between measurement points or individual visits (all
380 P>0.05). MAP was not different to baseline after 2 h (P=0.08) but was lower than baseline at
381 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

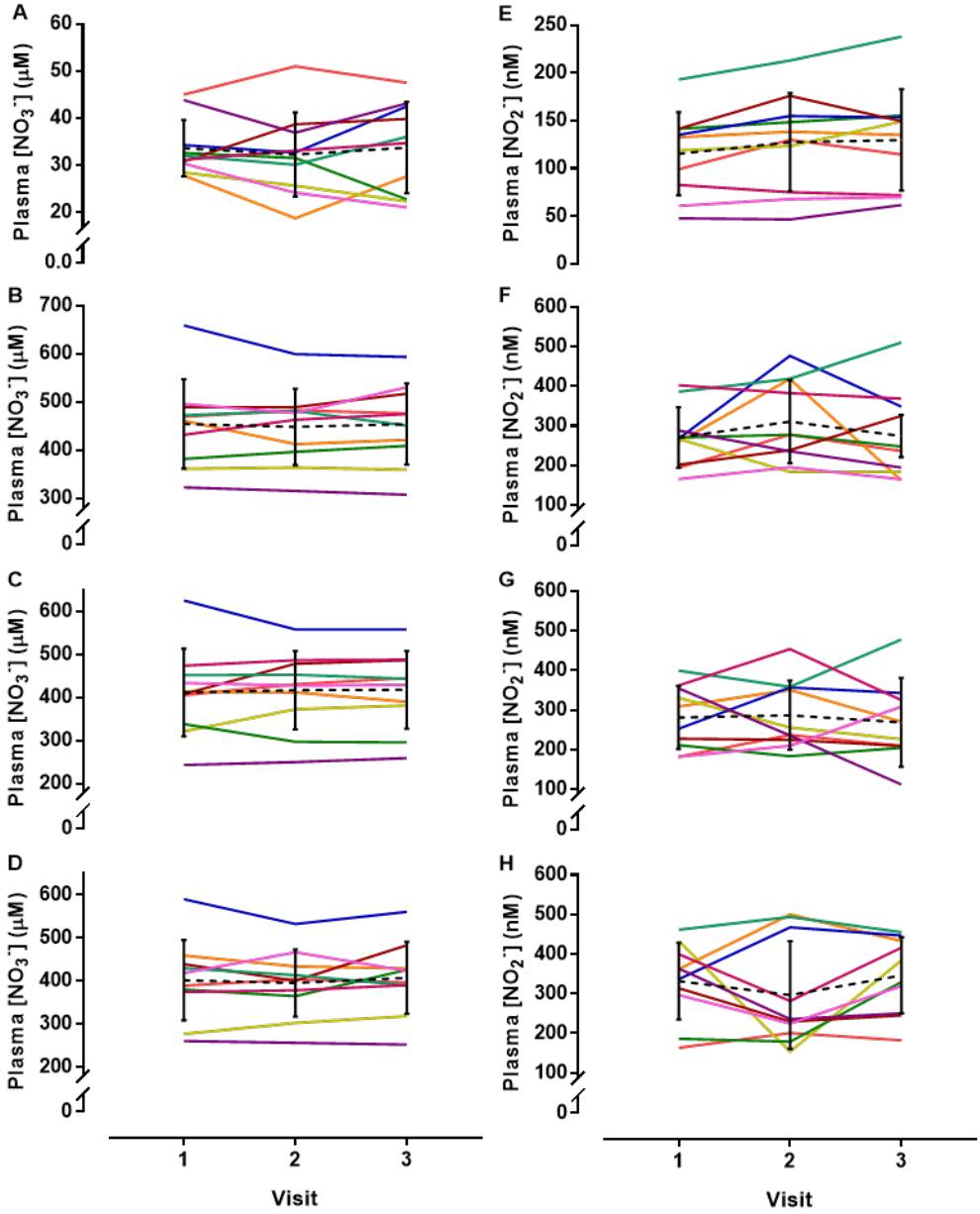
The sum of the NO_3^--reducing bacteria was not associated with measurements of $[NO_2^-]$ at any time point (all $P>0.2$). Individual species analysis showed that the abundance of *Neisseria subflava* was negatively associated with peak salivary $[NO_2^-]$ (R=-0.43, $P=0.03$, Fig. 5) and plasma $[NO_2^-]$ (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



414

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

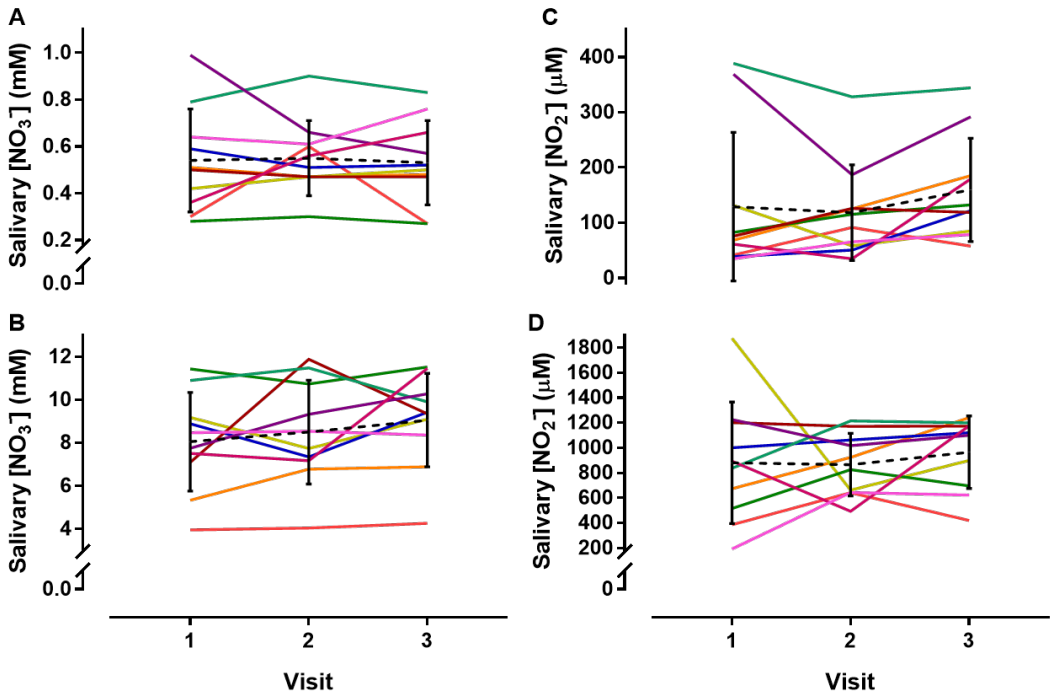
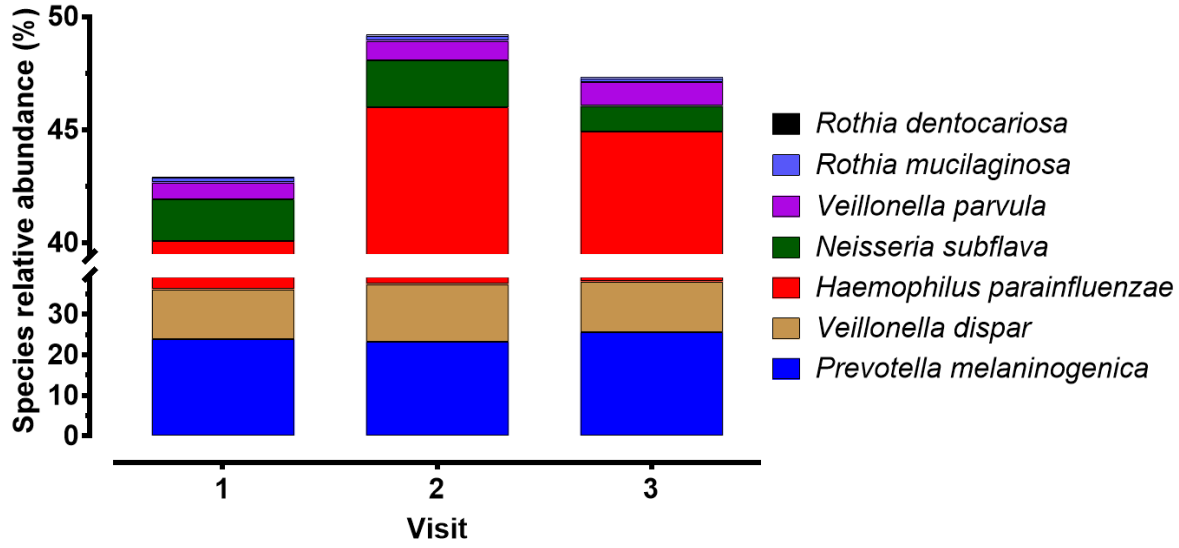


Figure 3. Group mean ± SD and inter-individual variation across the three identical trials for salivary [NO_3^-] at baseline (A), and 2.5 h (B), and for salivary [NO_2^-] at baseline (C), and 2 h (D). Following supplementation salivary [NO_3^-] and [NO_2^-] were significantly elevated compared to baseline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

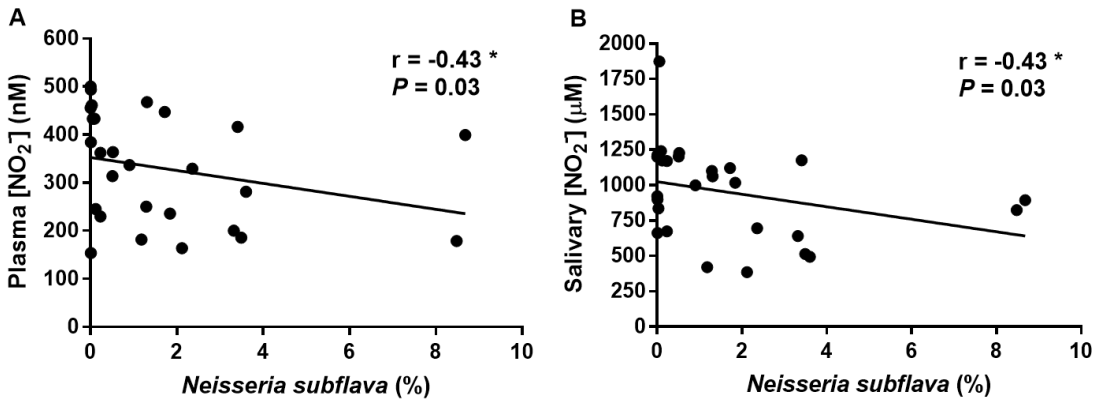


427

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

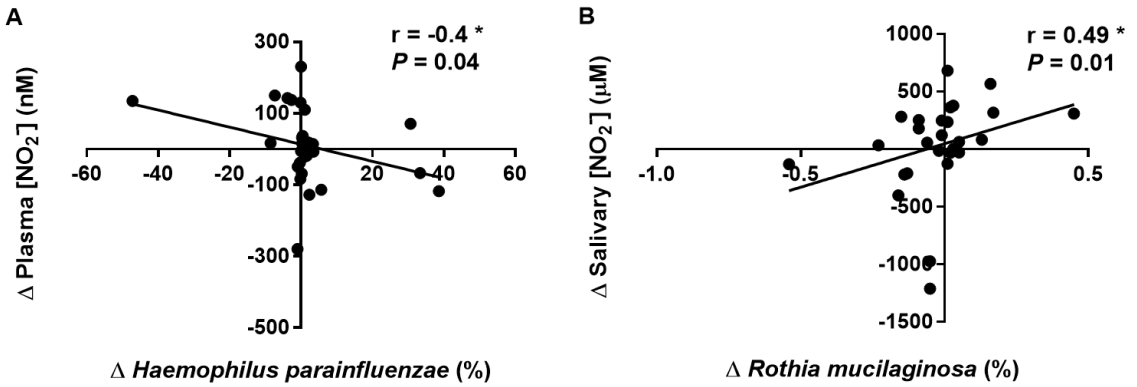
431

432



433

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



436

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

439

440 **Table 3.** Relative abundance of genera previously implicated in NO_3^-
441 reduction and the corresponding biological variation (CV_B) and inter-
442 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

443

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

445 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

446

447

448 **Table 5.** Three-trial mean ± SD, analytical variation (CV_A), biological
449 variation (CV_B), critical difference (CD), and inter-individual variability
450 (CV_I) for blood pressure parameters at each measurement point. *
451 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

461

462 **4. Discussion**

463 The present study demonstrates that, as hypothesised, the concentration of NO_3^- and conversion
464 to NO_2^- in biological fluids varies substantially within individuals across repeated laboratory
465 visits under the same conditions. Likewise, the CV_B for the abundance of NO_3^--reducing
466 bacteria were also profound, suggesting substantial heterogeneity in these measurements. The
467 CD values for NO metabolites at baseline suggest that large relative changes in these
468 parameters are required before a meaningful difference can be concluded following an
469 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.

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

514

515

516

517 4.2. Variability in the measurements of nitric oxide metabolites

518 Values of plasma and salivary [NO_2^-] and [NO_3^-] at baseline and following the ingestion of BR
519 are broadly in line with those reported in the literature (e.g. James et al. 2015; Liddle et al.
520 2018; Woessner et al. 2016). Some of the subtle differences between studies may be partly
521 explained by dissimilarities in methodology and study control (Bryan et al. 2007; Feelisch et
522 al. 2010; Liddle et al. 2018). Inter-individual differences between participants in each cohort
523 will also likely underpin some of the variation in basal NO metabolite concentration and NO
524 pharmacokinetics following the ingestion of BR (Muggeridge et al. 2014; James et al. 2015;
525 McIlvenna et al. 2017). This is highlighted profoundly by the CV_I values in the current data set
526 which were 19 – 31% for salivary and plasma NO_3^- and 27 – 74% for NO_2^-. Porcelli and
527 colleagues (2015) have demonstrated that physical fitness appears to affect the response to
528 NO_3^- supplementation whereby the increase in plasma [NO_2^-] is suppressed in individuals with
529 better aerobic fitness. Alternatively, other factors which may influence endogenous production
530 of NO (Luiking et al. 2010) or differences in the oral (Burleigh et al. 2018) and gut microbiota
531 (Flint et al. 2012) may also account for some of the inter-cohort variations. For example, we
532 have recently demonstrated that individuals with a higher abundance of NO_3^--reducing bacteria
533 generate more NO_2^- in the saliva and at a faster rate (Burleigh et al. 2018).
534
535 Given the exponential rise in research exploring the health promoting and ergogenic effects of
536 BR it is perhaps surprising that the CV_B for the physiological responses to this supplementation
537 regimen have not previously been reported. Particularly where it is argued that changes in any
538 outcome should be interpreted within the boundaries of CD in order to quantify a meaningful
539 difference (Fraser and Fogarty 1989). At baseline, there was moderate CV_B in plasma markers
540 (9 and 12% for NO_2^- and NO_3^-, respectively) although the variation was more substantial in
541 salivary measures (33 and 15% for NO_2^- and NO_3^-, respectively). Following the ingestion of

542 BR, the CV_B of NO_3^- ranged from 4 – 9% in plasma and 12 – 15% in saliva which was
543 considerably lower than the CV_B of NO_2^- markers (19 – 21% in plasma and 25 – 33% in saliva).
544 Urinary excretion of NO_3^- was also shown to have a large CV_B (15%) and CV_I (49%). The CD
545 values demonstrate that substantial changes in NO markers in biological fluids are required at
546 baseline or following the ingestion of BR to be deemed biologically meaningful (Fraser and
547 Fogarty 1989).
548

549 4.3. Association between nitrate-reducing bacteria and nitric oxide metabolites

550 The oral microbiome is known to be a crucial component of the NO_3^--NO_2^--NO pathway.
551 Abolishing oral bacterial species with anti-bacterial mouthwash, for example, has been shown
552 to substantially interrupt oral reductase capacity (Kapil et al. 2013; Bondonno et al. 2015;
553 McDonagh et al. 2015; Woessner et al. 2016). Given the oral microbiome is exceptionally
554 sensitive and modifiable within individuals, it is plausible that intra-individual variations in the
555 abundance of NO_3^--reducing bacteria would influence circulating levels of NO_2^- and NO
556 metabolite pharmacokinetics following the ingestion of BR. A large CV_B in [NO_2^-] values
557 would, therefore, be reasonably expected given the large CV_B in the abundance of NO_3^--
558 reducing bacteria. Further analyses of our data reveals that variation in oral microbiota do
559 influence the CV_B of the NO metabolites, at least to some extent. The relative abundance of
560 *Neisseria subflava* on the tongue was negatively associated with the peak [NO_2^-] in the saliva
561 and plasma following ingestion of BR. The Δ in salivary [NO_2^-] following BR between
562 repeated trials was also positively associated with the between-trial Δ in *Rothia mucilaginosa.*
563 Additionally, the between-trial Δ in plasma [NO_2^-] at 3 h post BR ingestion was negatively
564 associated with the between-trial Δ in *Haemophilus parainfluenzae*. Whilst it is possible that
565 these species may be particularly important for NO_3^- reduction, it must be acknowledged that
566 all statistically significant associations were only "moderate" in strength (R = 0.40 – 0.49), are

likely underpowered, and do not necessarily imply "cause-effect". Furthermore, while the dorsal surface of tongue is the area of the oral cavity in which the majority of NO_3^- reduction activity occurs (Doel et al. 2005), our sampling of the oral microbiome was not comprehensive. For example, NO_3^- reduction is also reported to occur directly in the saliva (Goaz and Biswell 1961) and in other areas of the mouth. It is also recognised that some species of bacteria are capable of reducing NO_2^- to NO in the saliva and the abundance of these microbiota may be considered to influence plasma [NO_2^-]. However, NO_2^- reduction via bacterial enzymatic activity is a slow process (Doel et al. 2005) and, given the rapid extrusion of NO_2^- through continuous swallowing, the abundance of these microbiota are likely to be less relevant.

While the relevant abundance of the oral microbiome seems to contribute towards the regulation of NO bioavailability (Burleigh et al. 2018), it does not fully account for the large CV_B in basal [NO_2^-] and [NO_3^-] and the variable response to ingested inorganic NO_3^-. Indeed, the metabolic activity of the NO_3^--reducing bacteria may be more important than the relevant abundance (Hyde et al. 2014a). Alternatively, CV_B of other factors including the aforementioned abundance and activity of gut bacteria, stomach pH (Lundberg et al. 1994; Montenegro et al. 2017), rates of gastric emptying and intestinal absorption (Leiper 2015), or the availability of sialin, a NO_3^- transporter in the saliva (Qin et al. 2012), may also contribute towards a high CV_B in NO metabolism. There also seems to be circadian variation in endogenous NO production (Antosova et al. 2009). Furthermore, while participants were requested to replicate their diet prior to each trial, the NO_3^- content of regularly consumed 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

592 influence in the present study as data were collected in the autumn/winter months. Establishing
593 the independent contribution of each of these factors to NO bioavailability will be a difficult
594 task due to a lack of gold-standard measurements or challenges in isolating each as an
595 independent variable rather than a covariate.
596
597 4.4. Variability in the blood pressure response to nitrate supplementation
598 Ingestion of BR resulted in significant reductions in systolic blood pressure and MAP which
599 supports findings from a recent meta-analysis showing a mean reduction in systolic blood
600 pressure of 4.4 mmHg (Siervo and Lara 2013). Novel data in this study shows that the reduction
601 in blood pressure markers is consistently observed in response to NO_3^- supplementation and,
602 in contrast to NO metabolites, the CV_B for these measurements are relatively low (all <5%).
603 This contrasts with previous research which reports the visit-to-visit variation is larger (>8%)
604 for systolic and diastolic blood pressure in various clinical cohorts (Marshall 2004; Howard
605 and Rothwell 2009). In absolute terms, baseline systolic blood pressure (mean 126 ± 7 mmHg)
606 varied by 2.5 mmHg across the three trials of the present study compared to 14.7 mmHg (mean
607 147 ± 18.4 mmHg) in patients who had suffered a minor transient ischemic attach or minor
608 ischemic stroke (Howard and Rothwell 2009). This suggests that cohorts with a higher blood
609 pressure will also have an increased CV_B for this metric. Indeed, an increased variability CV_B
610 may also have some prognostic value as it has been associated with the development,
611 progression, and severity of cardiac, vascular, and renal damage and with an increased risk
612 of cardiovascular events and mortality (Parati et al. 2013). It is important to highlight that
613 the participants in the present study were all from a homogenous cohort; namely they were
614 all healthy Caucasian males from a relatively narrow age range. It is likely that CV_B and CD
615 for all measured outcomes would increase in a more heterogonous group of healthy
616 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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