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*Published in:*  
Nitric Oxide

*DOI:*  
[10.1016/j.niox.2018.12.003](https://doi.org/10.1016/j.niox.2018.12.003)

E-pub ahead of print: 05/12/2018

*Document Version*  
Peer reviewed version

[Link to publication on the UWS Academic Portal](#)

*Citation for published version (APA):*

Liddle, L., Burleigh, M. C., Monaghan, C., Muggeridge, D. J., Sculthorpe, N., Pedlar, C. R., Butcher, J., Henriquez, F. L., & Easton, C. (2018). Variability in nitrate-reducing oral bacteria and nitric oxide metabolites in biological fluids following dietary nitrate administration: an assessment of the critical difference. *Nitric Oxide*, 83, 1-10. <https://doi.org/10.1016/j.niox.2018.12.003>

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

3

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## 29 Abstract

30 There is conflicting evidence on whether dietary nitrate supplementation can improve exercise  
31 performance. This may arise from the complex nature of nitric oxide (NO) metabolism which  
32 causes substantial inter-individual variability, within-person biological variation ( $CV_B$ ), and  
33 analytical imprecision ( $CV_A$ ) in experimental endpoints. However, no study has quantified the  
34  $CV_A$  and  $CV_B$  of NO metabolites or the factors that influence their production. These data are  
35 important to calculate the critical difference (CD), defined as the smallest difference between  
36 sequential measurements required to signify a true change. The main aim of the study was to  
37 evaluate the  $CV_B$ ,  $CV_A$ , and CD for markers of NO availability (nitrate and nitrite) in plasma  
38 and saliva before and after the ingestion of nitrate-rich beetroot juice (BR). We also assessed  
39 the  $CV_B$  of nitrate-reducing bacteria from the dorsal surface of the tongue. It was hypothesised  
40 that there would be substantial  $CV_B$  in markers of NO availability and the abundance of nitrate-  
41 reducing bacteria. Ten healthy male participants (age  $25 \pm 5$  years) completed three identical  
42 trials at least 6 days apart. Blood and saliva were collected before and after (2, 2.5 and 3 h)  
43 ingestion of 140 ml of BR (~12.4 mmol nitrate) and analysed for [nitrate] and [nitrite]. The  
44 tongue was scraped and the abundance of nitrate-reducing bacterial species were analysed  
45 using 16S rRNA next generation sequencing. There was substantial  $CV_B$  for baseline  
46 concentrations of plasma (nitrate 11.9%, nitrite 9.0%) and salivary (nitrate 15.3%, nitrite  
47 32.5%) NO markers. Following BR ingestion, the  $CV_B$  for nitrate (plasma 3.8%, saliva 12.0%)  
48 and salivary nitrite (24.5%) were lower than baseline, but higher for plasma nitrite (18.6%).  
49 The CD thresholds that need to be exceeded to ensure a meaningful change from baseline are  
50 25, 19, 37, and 87% for plasma nitrate, plasma nitrite, salivary nitrate, and salivary nitrite,  
51 respectively. The  $CV_B$  for selected nitrate-reducing bacteria detected were: *Prevotella*  
52 *melaninogenica* (37%), *Veillonella dispar* (35%), *Haemophilus parainfluenzae* (79%),  
53 *Neisseria subflava* (70%), *Veillonella parvula* (43%), *Rothia mucilaginosa* (60%), and *Rothia*

54 *dentocariosa* (132%). There is profound CV<sub>B</sub> in the abundance of nitrate-reducing bacteria on  
55 the tongue and the concentration of NO markers in human saliva and plasma. Where these  
56 parameters are of interest following experimental intervention, the CD values presented in  
57 this study will allow researchers to interpret the meaningfulness of the magnitude of the  
58 change from baseline.

59 **Key Words:** beetroot juice; nitrite; microbiome

60

61 **Highlights**

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

67

## 68 **1. Introduction**

69 Dietary nitrate ( $\text{NO}_3^-$ ) supplementation increases the concentration of nitric oxide (NO)  
70 metabolites within the blood (Kapil et al. 2010). Crucial to this process is the reduction of  
71 concentrated  $\text{NO}_3^-$  in saliva (Lundberg and Govoni 2004) to nitrite ( $\text{NO}_2^-$ ) by facultative  
72 anaerobic bacteria in the oral cavity (Duncan et al. 1995). The importance of this mechanism  
73 to cardiovascular health is evident in the breadth of research showing that ingestion of  
74 inorganic  $\text{NO}_3^-$  acutely lowers blood pressure (Webb et al. 2008; Siervo and Lara 2013).  
75 Elevations in plasma  $\text{NO}_2^-$  have been associated with decreased cardiovascular risks factors  
76 and increased exercise capacity in healthy and chronically diseased cohorts (Kleinbongard et  
77 al. 2006; Allen et al. 2010; Totzeck et al. 2012). Dietary  $\text{NO}_3^-$  supplementation has also been  
78 shown to improve time trial (Lansley et al. 2011; Muggeridge et al. 2014) and intermittent  
79 (Wylie et al. 2013) exercise performance. However, some studies report no ergogenic effects  
80 (Peacock et al. 2012; MacLeod et al. 2015) and, taken as a whole, the effects of dietary  $\text{NO}_3^-$   
81 supplementation on exercise performance outcomes appear to be equivocal (McMahon et al.  
82 2017). One hypothesis that may account for the lack of consensus across the literature is that  
83 individuals respond differently to  $\text{NO}_3^-$  supplementation (Porcelli et al. 2015). Indeed, there  
84 appears to be substantial inter-individual variability in plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$   
85 pharmacokinetics before and after  $\text{NO}_3^-$  administration (James et al. 2015). For example, we  
86 have previously shown that the increase in plasma  $[\text{NO}_2^-]$  can range from 80 to 400 nM with a  
87 time-to-peak ranging from 1.5 to 6 h following ingestion of  $\text{NO}_3^-$  supplements (McIlvenna et  
88 al. 2017).

89

90 Surprisingly, the within-individual variability in NO metabolites, either at basal concentrations  
91 or following ingestion of  $\text{NO}_3^-$ , 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  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$ . 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  $\text{NO}_3^-$   
95 and  $\text{NO}_2^-$  content of the diet (Bryan et al. 2007), the rate of endogenous NO synthesis,  $\text{NO}_3^-$   
96 transport in the salivary glands (Lundberg 2012; Qin et al. 2012), the abundance of  $\text{NO}_3^-$ -  
97 reducing bacteria in the mouth (Burleigh et al. 2018), salivary flow-rate (Webb et al. 2008),  
98 the rate of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  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 ( $\text{CV}_A$ ) associated with their measurement.

103

104 The within-individual or biological variation ( $\text{CV}_B$ ) establishes the inherent fluctuations around  
105 a homeostatic set-point of a measured variable (Harris 1970). The  $\text{CV}_B$  can be used in  
106 combination with the  $\text{CV}_A$  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  $\text{CV}_B$  and the  $\text{CV}_A$  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  $\text{CV}_B$   
112 of 7.6% and a CD of 17.2% (Fraser 2001). Blood glucose has been shown to have a  $\text{CV}_B$  and  
113 CD of 7.2% and 14.9%, respectively (Widjaja et al. 1999). In the context of dietary  $\text{NO}_3^-$   
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.

116 To our knowledge, the CD values of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in plasma, saliva, and urine at baseline and  
117 in response to  $\text{NO}_3^-$  have not been previously reported. Likewise, despite recognition of the  
118 importance of  $\text{NO}_3^-$ -reducing bacteria for the generation of NO through the  $\text{NO}_3^-$ - $\text{NO}_2^-$ -NO  
119 pathway, no study has quantified the  $\text{CV}_B$  in the abundance of these bacteria in the oral cavity.  
120 Therefore, the primary aim was to quantify the  $\text{CV}_B$  and CD of the abundance of  $\text{NO}_3^-$ -reducing  
121 bacteria, blood pressure, and plasma, saliva, and urine [ $\text{NO}_3^-$ ] and [ $\text{NO}_2^-$ ] before and after  
122 ingestion of  $\text{NO}_3^-$ -rich beetroot juice (BR). A secondary aim was to determine whether the  
123 variation in these NO metabolites was associated with the abundance of  $\text{NO}_3^-$ -reducing  
124 bacteria. It was hypothesised that there would be substantial  $\text{CV}_B$  of the abundance of  $\text{NO}_3^-$ -  
125 reducing bacteria and the concentration of NO metabolites in plasma, saliva, and urine. Further,  
126 it was hypothesised that the variations in plasma and salivary [ $\text{NO}_3^-$ ] and [ $\text{NO}_2^-$ ] would be  
127 positively associated with the abundance  $\text{NO}_3^-$ -reducing bacteria.

128

## 129 **2. Methods**

### 130 2.1. Participants

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

136

137

138

139 2.2. Study design

140 Each participant attended the laboratory on three separate occasions with 6-10 days between  
141 each visit. Each trial comprised a 3.5 h period where participants lay supine and repeated  
142 samples of biological fluids were collected and blood pressure was measured. The experimental  
143 conditions were identical in each visit. Following the collection of baseline measurements,  
144 participants immediately ingested 2 x 70 ml of BR (Beet It SPORT, James White Drinks, UK;  
145 total of ~12.4 mmol NO<sub>3</sub><sup>-</sup>). Participants were instructed to avoid caffeine, foods high in NO<sub>2</sub><sup>-</sup>  
146 and NO<sub>3</sub><sup>-</sup> (e.g. green leafy vegetables and cured meats), alcohol, and strenuous exercise in the  
147 24 h prior to the experiment. Participants were also asked to avoid mouthwash 7 days prior to  
148 the first trial and for the duration of the study. All participants confirmed that they were not  
149 using medication of any kind for a month before the first trial or at any point during the study  
150 period. Participants were also asked to refrain from brushing their teeth and tongue on the  
151 morning of each lab visit. Participants recorded dietary intake and the modality, frequency, and  
152 intensity of exercise undertaken 72 h prior to the first experimental trial and replicated this for  
153 the subsequent visits. Participants were provided access to bottled water (Strathrowan Scottish  
154 Mountain water, Aldi Stores Ltd, Ireland) to consume *ad libitum* during the first visit. The  
155 volume of water and the time of ingestion was recorded during the first visit and matched for  
156 subsequent trials.

157

158 2.3. Procedures

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



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

166

$$167 \text{ MAP} = (2 \times \text{diastolic blood pressure} + \text{systolic blood pressure}) / 3$$

168

169 Venous blood (4 ml) was collected in EDTA vacutainers (BD vacutainer K2E 7.2mg,  
170 Plymouth, U.K.) and the cannula flushed with sterile 0.9% saline solution between samples to  
171 keep the line patent. The vacutainer was centrifuged (Harrier 18/80, Henderson Biomedical,  
172 UK) at 4000 rpm for 10 min at 4°C immediately after collection (Pelletier et al. 2006). Plasma  
173 was then separated, frozen at -80°C, and analysed within 4 months (Pinder et al. 2009) of  
174 initial collection for determination of [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>]. Samples of unstimulated saliva were  
175 collected via a non-cotton polymer oral swab (Saliva Bio Oral Swab (SOS) Salimetrics,  
176 Pennsylvania, USA) placed under the tongue for 2 min. Swabs were then transferred to a  
177 collection tube (Sarstedt, Aktiengesellschaft & Co, Numbrecht, Germany) and centrifuged at  
178 4000 rpm for 10 min at 4°C. Samples were separated into two cryovials and immediately stored  
179 at -80°C for later analysis of [NO<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>]. Swabs were used to collect saliva samples in  
180 preference to the “passive drool” technique in an attempt to improve the consistency of saliva  
181 collection within and between participants.

182

183 Participants were then instructed to sit up to allow for the collection of a bacterial sample from  
184 the posterior dorsal surface of the tongue using a sterile stainless-steel metal tongue cleaner  
185 (Soul Genie, Health Pathways LLP, India). The tongue cleaner was scraped over the dorsal  
186 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  $-80^{\circ}\text{C}$  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  $-80^{\circ}\text{C}$  for later analysis of  $[\text{NO}_3^-]$ . The volume of all further  
192 bladder voids were recorded following ingestion of BR to allow for the calculation of total  
193  $\text{NO}_3^-$  excretion using the following equation:

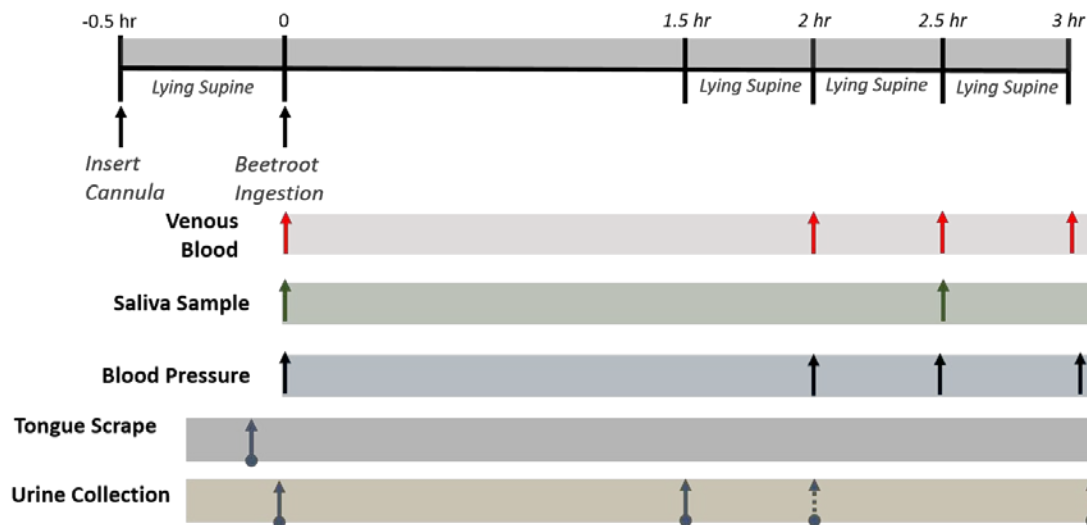
194

$$195 \text{ Total } \text{NO}_3^- \text{ excretion (g)} = \text{NO}_3^- \text{ (M)} * \text{urine volume (L)}$$

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  $[\text{NO}_2^-]$  to stabilise following  
200 postural alterations. Blood pressure was also measured when participants were supine to  
201 ensure measurements were time-aligned with plasma  $[\text{NO}_2^-]$  and  $[\text{NO}_2^-]$ .

202



203

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

206

#### 207 2.4. Plasma nitrate and nitrite analysis

208 Measurements of  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  were conducted using ozone-based chemiluminescence  
209 (Rogers et al. 2005). For the measurement of plasma  $[\text{NO}_3^-]$ , vanadium reagent (24 mg of  
210 vanadium tri-chloride and 3 ml of 1M Hydrochloric acid) and 100  $\mu\text{L}$  of anti-foaming agent  
211 were placed into a customised glass purge vessel infused with nitrogen and heated to  $95^\circ\text{C}$ .  
212 This purge vessel was connected to an NO analyser (Sievers NOA 280i, Analytix, UK). A  
213 standard curve was produced by injecting 25  $\mu\text{L}$  of  $\text{NO}_3^-$  solutions (100  $\mu\text{M}$ , 50  $\mu\text{M}$ , 25  $\mu\text{M}$ ,  
214 12.5  $\mu\text{M}$ , and 6.25  $\mu\text{M}$ ) and a control sample containing deionised water. The area under the  
215 curve (AUC) for the latter was subtracted from the  $\text{NO}_3^-$  solutions to account for  $\text{NO}_3^-$  in the  
216 water used for dilutions. Plasma samples were thawed in a water bath at  $37^\circ\text{C}$  for 3 min and  
217 de-proteinised using zinc sulphate/sodium hydroxide solution (200  $\mu\text{L}$  of plasma, 400  $\mu\text{L}$  of  
218 zinc sulphate in deionised water at 10% w/v and 400  $\mu\text{L}$  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  $\mu\text{L}$  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  $[\text{NO}_2^-]$ , tri-iodide reagent (2.5 ml glacial acetic acid, 0.5 ml of  
226 18  $\Omega$  deionised water and 25 mg sodium iodide) and 100  $\mu\text{L}$  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  $\mu\text{L}$  of  $\text{NO}_2^-$  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  $\text{NO}_2^-$  solutions to  
230 account for  $\text{NO}_2^-$  in the water used for dilutions. Following this, plasma samples were thawed  
231 in a water bath and 100  $\mu\text{L}$  of the sample was injected into the purge vessel in duplicate and  
232  $[\text{NO}_2^-]$  was determined via the AUC, as previously described.

233

#### 234 2.5. Salivary nitrite and nitrate analysis

235 The same reagents used for plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  analyses were used for the analysis of  
236 salivary metabolites. The standard curve for salivary  $[\text{NO}_3^-]$  was the same as described for  
237 plasma  $[\text{NO}_3^-]$ . The standard curve for salivary  $[\text{NO}_2^-]$  was produced by injecting 100  $\mu\text{L}$   $\text{NO}_2^-$   
238 solutions up to 5  $\mu\text{M}$ . 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  $\mu\text{L}$  of the sample  
240 was injected for the measurement of  $[\text{NO}_2^-]$  and 10-25  $\mu\text{L}$  for  $[\text{NO}_3^-]$ . 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<sub>3</sub><sup>-</sup>] analysis was used for the  
245 measurement of urinary [NO<sub>3</sub><sup>-</sup>]. 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<sub>3</sub><sup>-</sup>] 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 (HOMINGS, 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 CAAGCAGAAGACGGCATAACGAGATNNNNNNNNNNNNNAGTCAGTCAGCCGGACT  
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*

267 and binned into operational taxonomic units (OTU) based on 97% identity. Taxonomy was  
268 assigned using RDP classifier trained to the GreenGenes database (October 2013 release).  
269 Singleton reads were removed from the dataset. In order to calculate alpha diversity metrics,  
270 the OTU table was sub-sampled to 14870 reads per sample and repeated 5 times. The mean  
271 values were then calculated across the 5 sub-sampled OTU tables and used to calculate alpha  
272 diversity metrics. Alpha diversity metrics were calculated using the Shannon diversity  
273 equation, which accounts for the richness and evenness of species in a sample. The smallest  
274 number of reads associated with any one sample was 14870 reads. These analyses enabled the  
275 calculation of the abundance of bacteria at the specific genus and species level that have been  
276 previously reported to reduce NO<sub>3</sub><sup>-</sup> in the oral cavity (Doel et al. 2005; Hyde et al. 2014a). The  
277 sum of the abundance of NO<sub>3</sub><sup>-</sup>-reducing bacteria was also calculated and used in further  
278 analysis.

279

## 280 2.9. Statistical analysis

281 All analyses were carried out using the Statistical Package for Social Sciences, Version 22  
282 (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 7 (GraphPad Software Inc., San  
283 Diego, USA) was used to create the figures. Data are expressed as the mean ± standard  
284 deviation (SD). The distribution of the data were tested using the Shapiro-Wilk test. A two-  
285 way repeated-measures ANOVA was used to assess the main effects of time and visit and the  
286 time x visit interaction for [NO<sub>3</sub><sup>-</sup>], [NO<sub>2</sub><sup>-</sup>], and blood pressure variables. A one-way repeated  
287 measures ANOVA was used to determine whether there were differences in the abundance of  
288 each genus of bacteria across the three trials. The between trial differences in the Shannon  
289 diversity index was assessed using a Friedman's rank test. *Post-hoc* analysis was conducted  
290 following a significant main effect or interaction using paired samples t-tests with Bonferroni

291 correction for multiple pairwise comparisons. Correlation coefficients (Pearson's for normally  
292 distributed data and Spearman's Rho for non-normally distributed data) were used to assess  
293 the association between the concentration of NO metabolites and the abundance of species  
294 specific NO<sub>3</sub><sup>-</sup>-reducing bacteria. Using the same analyses, associations of between-trial  
295 differences ( $\Delta$ ) in these parameters were also analysed. Statistical significance was declared  
296 when  $P < 0.05$ .

297

### 298 2.9.1. *Inter-individual variation*

299 The inter-individual coefficient of variation ( $CV_I$ ) was calculated using the pooled mean  $\pm$  SD  
300 of the three-trial average using the following equation:

$$301 \quad CV_I (\%) = 100 - (SD/\text{mean})$$

302 Where SD = the between participant standard deviation

303 Where mean = the average of all participant

304

### 305 2.9.2. *Analytical variation*

306 The  $CV_A$  was calculated using the pooled mean  $\pm$  SD of each duplicate/triplicate measure using  
307 the following equation:

$$308 \quad CV_A (\%) = 100 - (SD/\text{mean})$$

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

311

312

313 2.9.3. *Biological variation*

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

316  $CV_B (\%) = 100 - (SD/\text{mean})$

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  $\pm$  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  $\pm$  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<sub>2</sub><sup>-</sup>] (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<sub>3</sub><sup>-</sup>] and [NO<sub>2</sub><sup>-</sup>]. *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<sub>3</sub><sup>-</sup>] 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<sub>3</sub><sup>-</sup> excretion did not differ  
342 between the three laboratory visits ( $P>0.05$ ) (Table 1). The CV<sub>B</sub> for salivary, plasma, and  
343 urinary [NO<sub>3</sub><sup>-</sup>] variables ranged from 3.8 to 15.3% (Table 1). There was a greater degree of  
344 heterogeneity in saliva and plasma [NO<sub>2</sub><sup>-</sup>] which ranged from 9 to 32.5 % (Table 2). The CD  
345 values were also considerable for [NO<sub>3</sub><sup>-</sup>] variables (8.4 – 37.9%) and [NO<sub>2</sub><sup>-</sup>] variables (19.3 –  
346 86.5%). Between-participant comparisons reveal that, as expected, the CV<sub>I</sub> was substantial,  
347 with [NO<sub>3</sub><sup>-</sup>] variables ranging from 18.6 to 49.1% and [NO<sub>2</sub><sup>-</sup>] 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  $5.4 \pm$   
353  $0.4$  with  $1356 \pm 171$  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 *Peptostreptococcus* which was more  
356 abundant in visit one compared to visit two ( $P=0.03$ ). Previous research has shown that  
357 *Peptostreptococcus* species do not have NO<sub>3</sub><sup>-</sup> reductase activity (Smith et al. 1999).

358 All of the genera that have previously been implicated in  $\text{NO}_3^-$  reduction (Hyde et al. 2014a)  
359 were detected in our analyses (Table 3). *Prevotella* was the most abundant genera and had the  
360 lowest  $\text{CV}_B$  (22.7%) whilst *Haemophilus*, the fourth most abundant  $\text{NO}_3^-$ -reducing genera, had  
361 the highest  $\text{CV}_B$  (77.6%). Seven of the bacterial species previously implicated in  $\text{NO}_3^-$   
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  $\text{NO}_3^-$ -reducing bacteria had a  $\text{CV}_B$  of  
365 19.5%. The  $\text{CV}_B$  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  $\text{CV}_B$  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  $\text{CV}_A$  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

382 2.0 – 3.4%) and MAP (range 2.9 – 3.9%) had minimal CV<sub>B</sub>. The CV<sub>B</sub> for diastolic blood  
383 pressure was greater, ranging from 4.2 to 6.0%. Values of CD ranged from 5.3 to 11.9% for all  
384 blood pressure markers and values of CV<sub>I</sub> ranged from 4.7 to 8.1%.

385

386 3.4. Association between nitrate and nitrite in biological fluids and the abundance of nitrate-  
387 reducing bacteria

388 The sum of the NO<sub>3</sub><sup>-</sup>-reducing bacteria was not associated with measurements of [NO<sub>2</sub><sup>-</sup>] at any  
389 time point (all  $P > 0.2$ ). Individual species analysis showed that the abundance of *Neisseria*  
390 *subflava* was negatively associated with peak salivary [NO<sub>2</sub><sup>-</sup>] ( $R = -0.43$ ,  $P = 0.03$ , Fig. 5) and  
391 plasma [NO<sub>2</sub><sup>-</sup>] ( $R = -0.43$ ,  $P = 0.03$ , Fig. 5). There were no other associations between the  
392 concentration of NO metabolites and the abundance of all other individual species of NO<sub>3</sub><sup>-</sup>-  
393 reducing bacteria (all  $P > 0.07$ ). The between-trial  $\Delta$  in salivary [NO<sub>2</sub><sup>-</sup>] following BR and the  
394 between-trial  $\Delta$  *Rothia mucilaginosa* abundance were significantly associated ( $R = 0.49$ ,  
395  $P = 0.01$ , Fig. 6). The between-trial  $\Delta$  *Haemophilus parainfluenzae* abundance was negatively  
396 associated with the between-trial  $\Delta$  plasma [NO<sub>2</sub><sup>-</sup>] at 3 h post BR ingestion ( $R = -0.4$ ,  $P = 0.04$ ,  
397 Fig. 6). There were no other relationships between the variation in [NO<sub>2</sub><sup>-</sup>] variables and the  
398 abundance of NO<sub>3</sub><sup>-</sup> reducing species (all,  $P > 0.09$ ).

399

400

401

402

403

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

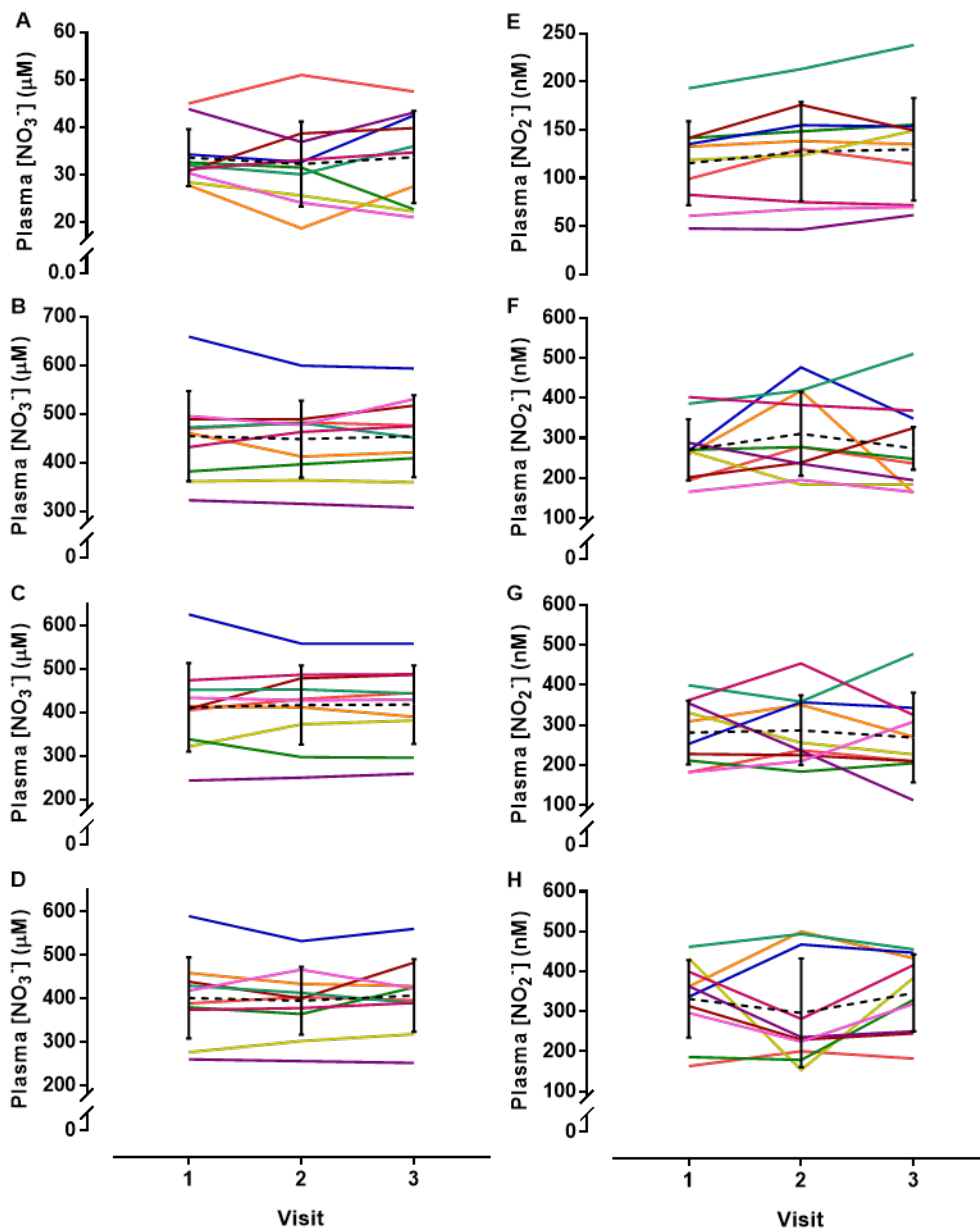
Parameter	Mean $\pm$ SD	$CV_A$ (%)	$CV_B$ (%)	CD (%)	$CV_I$ (%)
Plasma baseline	33.2 $\pm$ 7.6 $\mu$ M	4.1	11.9	24.4	22.8
Plasma 2 h	452.1 $\pm$ 83.9 $\mu$ M*	1.0	3.8	8.4	18.5
Plasma 2.5 h	415.0 $\pm$ 92.2 $\mu$ M*	1.2	4.7	10.3	22.2
Plasma 3 h	391.6 $\pm$ 99.2 $\mu$ M*	1.8	8.8	19.9	25.3
Saliva baseline	0.5 $\pm$ 0.2 mM	2.1	15.3	37.1	30.7
Saliva 2.5 h	8.5 $\pm$ 2.1 mM*	1.4	12.0	29.7	24.1
Urine total	1.7 $\pm$ 0.3 g ( $\times 10^{-4}$ )	1.7	15.3	37.9	49.1

408

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

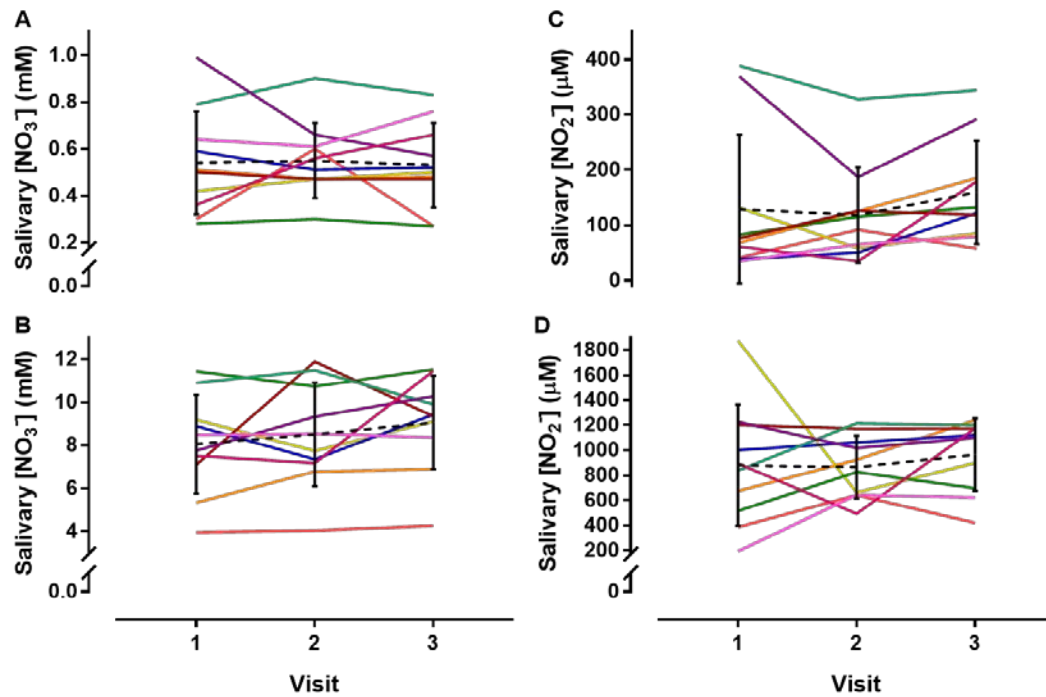
Parameter	Mean $\pm$ SD	$CV_A$ (%)	$CV_B$ (%)	CD (%)	$CV_I$ (%)
Plasma baseline	124.2 $\pm$ 48.8 nM	2.5	9.0	19.3	39.3
Plasma 2 h	284.9 $\pm$ 83.5 nM*	2.1	19.3	47.9	29.3
Plasma 2.5 h	278.6 $\pm$ 73.9 nM*	2.4	18.6	45.4	26.5
Plasma 3 h	323.9 $\pm$ 94.1 nM*	2.2	20.6	51.3	29.0
Saliva baseline	135.7 $\pm$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

413



414

415 **Figure 2.** Group mean  $\pm$  SD and inter-individual variation across the three identical trials for  
 416 plasma  $[\text{NO}_3^-]$  at baseline (A), 2 h (B), 2.5 h (C), and 3 h (D), and for plasma  $[\text{NO}_2^-]$  at baseline  
 417 (E), 2 h (F), 2.5 h (G), and 3 h (H). All post supplementation time points for plasma  $[\text{NO}_3^-]$  and  
 418  $[\text{NO}_2^-]$  were significantly elevated compared to baseline concentrations (all  $P < 0.01$ ).



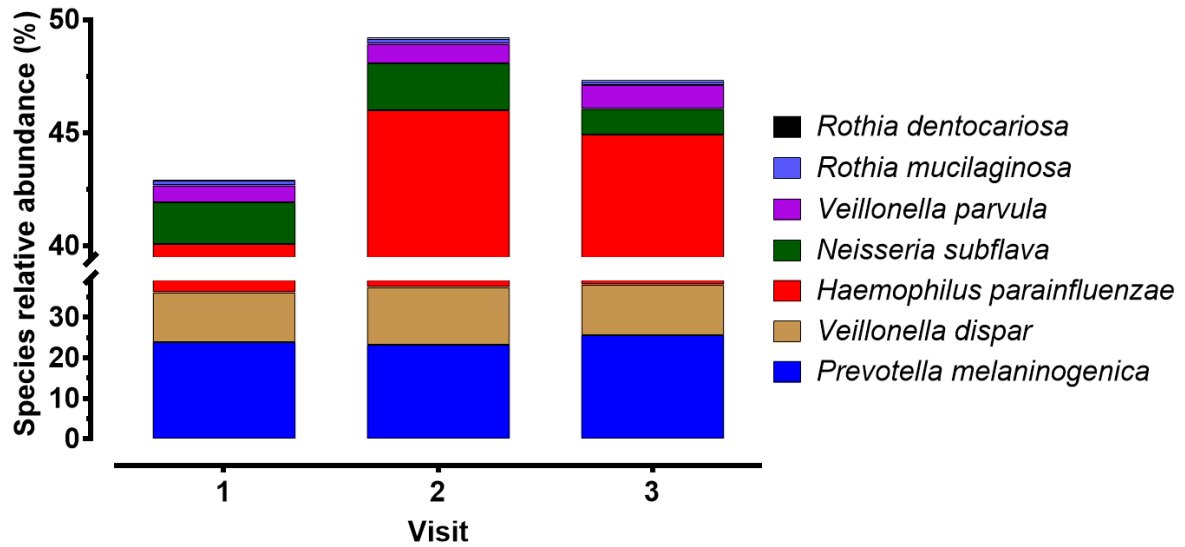
419

420 **Figure 3.** Group mean  $\pm$  SD and inter-individual variation across the three identical trials for  
 421 salivary  $[\text{NO}_3^-]$  at baseline (A), and 2.5 h (B), and for salivary  $[\text{NO}_2^-]$  at baseline (C), and 2 h  
 422 (D). Following supplementation salivary  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  were significantly elevated  
 423 compared to baseline concentrations (all  $P < 0.01$ ).

424

425

426

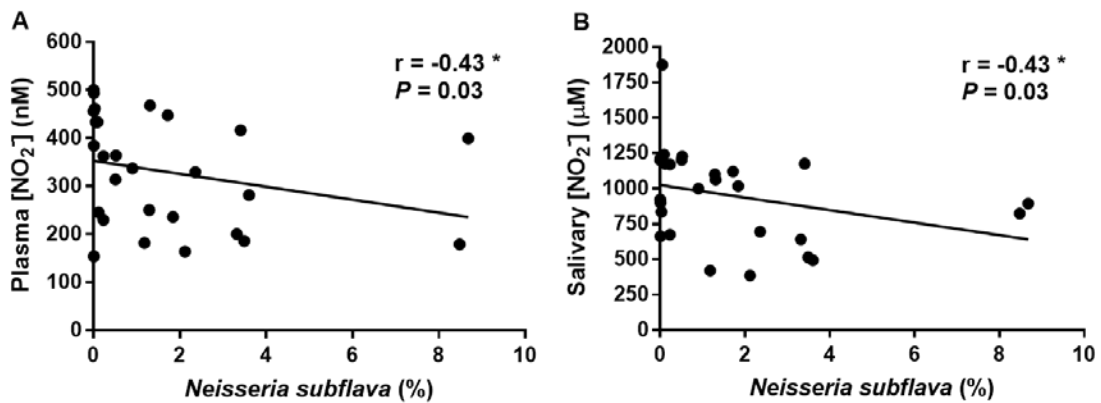


427

428 **Figure 4.** A comparison of the relative abundance of  $\text{NO}_3^-$ -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.

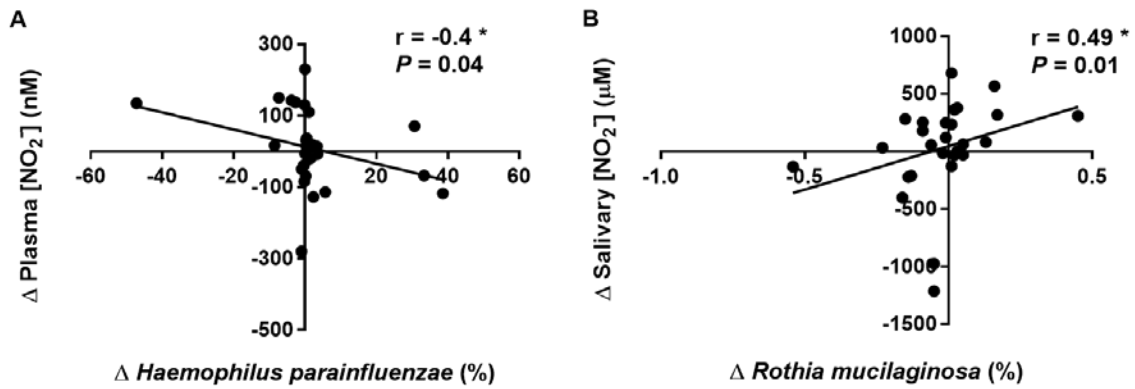
431

432



433

434 **Figure 5.** Correlations between *Neisseria subflava* and peak concentration plasma  $[\text{NO}_2^-]$  (A)  
 435 and salivary  $[\text{NO}_2^-]$  (B). \* denotes significant difference.



436

437 **Figure 6.** Correlations between  $\Delta$  plasma  $[\text{NO}_2^-]$ / $\Delta$  *Haemophilus parainfluenzae* (A) and  $\Delta$   
 438 salivary  $[\text{NO}_2^-]$ / $\Delta$  *Rothia mucilaginosa* (B). \* denotes significant difference.

439

440 **Table 3.** Relative abundance of genera previously implicated in  $\text{NO}_3^-$   
 441 reduction and the corresponding biological variation ( $\text{CV}_B$ ) and inter-  
 442 individual variability ( $\text{CV}_I$ ).

OTU ID	Mean $\pm$ SD (%)	$\text{CV}_B$ (%)	$\text{CV}_I$ (%)
<i>Prevotella</i>	35.6 $\pm$ 13.5	22.7	38.6
<i>Veillonella</i>	14.7 $\pm$ 7.2	33.4	50.1
<i>Fusobacterium</i>	9.5 $\pm$ 9.3	54.5	97.8
<i>Haemophilus</i>	6.5 $\pm$ 11.1	77.6	145.0
<i>Leptotrichia</i>	6.4 $\pm$ 3.6	52.7	56.1
<i>Streptococcus</i>	2.0 $\pm$ 1.9	45.7	96.8
<i>Neisseria</i>	1.8 $\pm$ 2.5	67.9	130.7
<i>Porphyromonas</i>	1.6 $\pm$ 1.8	76.1	119.4
<i>Actinomyces</i>	1.0 $\pm$ 0.8	64.5	82.8
<i>Rothia</i>	0.2 $\pm$ 0.2	57.7	108.6
<i>Granulicatella</i>	0.1 $\pm$ 0.2	72.0	122.9

443



444 **Table 4.** Relative abundance of species previously implicated in NO<sub>3</sub><sup>-</sup> reduction and  
 445 the corresponding biological variation (CV<sub>B</sub>) and inter-individual variability (CV<sub>I</sub>).

Species	Mean ± SD (%)	CV <sub>B</sub> (%)	CV <sub>I</sub> (%)
<i>Prevotella melaninogenica</i>	23.8 ± 6.4	37.0	26.9
<i>Veillonella dispar</i>	13.0 ± 4.0	35.1	30.7
<i>Haemophilus parainfluenzae</i>	6.5 ± 5.9	78.6	90.7
<i>Neisseria subflava</i>	1.7 ± 1.0	70.0	57.7
<i>Veillonella parvula</i>	0.9 ± 0.4	43.2	44.3
<i>Rothia mucilaginosa</i>	0.2 ± 0.1	60.0	41.0
<i>Rothia dentocariosa</i>	<0.01 ± <0.01	132.1	118.4

446

447

448 **Table 5.** Three-trial mean  $\pm$  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 $\pm$ SD (mmHg)	$CV_A$ (%)	$CV_B$ (%)	CD (%)	$CV_I$ (%)
Systolic baseline	126 $\pm$ 7	1.9	2.0	5.3	5.9 <sup>453</sup>
Systolic 2 h	121 $\pm$ 7*	1.3	3.1	6.1	6.1 <sub>454</sub>
Systolic 2.5 h	120 $\pm$ 7*	3.8	3.4	10.6	6.4
Systolic 3 h	122 $\pm$ 7*	3.3	3.2	10.1	5.8
Diastolic baseline	70 $\pm$ 5	3.4	4.8	10.2	7.7
Diastolic 2 h	67 $\pm$ 5	3.0	4.9	9.9	8.1
Diastolic 2.5 h	67 $\pm$ 4	3.6	4.2	10.2	5.4
Diastolic 3 h	67 $\pm$ 4	2.5	6.0	11.9	6.2
MAP baseline	88 $\pm$ 5	2.7	3.9	8.1	5.4
MAP 2 h	85 $\pm$ 5	2.2	3.4	7.0	5.9
MAP 2.5 h	85 $\pm$ 4*	3.7	3.1	10.4	5.0
MAP 3 h	85 $\pm$ 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

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

478

#### 479 4.1. Variability of the tongue microbiome of healthy humans

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

492

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 ( $\geq 95\%$   
498 samples) at all measurement points, including several species that have been implicated in  
499  $\text{NO}_3^-$  reduction (*Haemophilus parainfluenzae*, *Neisseria subflava*, and *Rothia dentocariosa*).  
500 In the present study, the  $\text{CV}_B$  for seven of the bacteria previously implicated in  $\text{NO}_3^-$   
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  $\text{NO}_3^-$   
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  $\text{NO}_3^-$ -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  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  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  $\text{CV}_I$  values in the current data set  
526 which were 19 – 31% for salivary and plasma  $\text{NO}_3^-$  and 27 – 74% for  $\text{NO}_2^-$ . Porcelli and  
527 colleagues (2015) have demonstrated that physical fitness appears to affect the response to  
528  $\text{NO}_3^-$  supplementation whereby the increase in plasma  $[\text{NO}_2^-]$  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  $\text{NO}_3^-$ -reducing bacteria  
533 generate more  $\text{NO}_2^-$  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  $\text{CV}_B$  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  $\text{CV}_B$  in plasma markers  
540 (9 and 12% for  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , respectively) although the variation was more substantial in  
541 salivary measures (33 and 15% for  $\text{NO}_2^-$  and  $\text{NO}_3^-$ , 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  $\Delta$  in salivary [ $NO_2^-$ ] following BR between  
562 repeated trials was also positively associated with the between-trial  $\Delta$  in *Rothia mucilaginosa*.  
563 Additionally, the between-trial  $\Delta$  in plasma [ $NO_2^-$ ] at 3 h post BR ingestion was negatively  
564 associated with the between-trial  $\Delta$  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

567 likely underpowered, and do not necessarily imply “cause-effect”. Furthermore, while the  
568 dorsal surface of tongue is the area of the oral cavity in which the majority of  $\text{NO}_3^-$  reduction  
569 activity occurs (Doel et al. 2005), our sampling of the oral microbiome was not comprehensive.  
570 For example,  $\text{NO}_3^-$  reduction is also reported to occur directly in the saliva (Goaz and Biswell  
571 1961) and in other areas of the mouth. It is also recognised that some species of bacteria are  
572 capable of reducing  $\text{NO}_2^-$  to NO in the saliva and the abundance of these microbiota may be  
573 considered to influence plasma  $[\text{NO}_2^-]$ . However,  $\text{NO}_2^-$  reduction via bacterial enzymatic  
574 activity is a slow process (Doel et al. 2005) and, given the rapid extrusion of  $\text{NO}_2^-$  through  
575 continuous swallowing, the abundance of these microbiota are likely to be less relevant.

576

577 While the relevant abundance of the oral microbiome seems to contribute towards the  
578 regulation of NO bioavailability (Burleigh et al. 2018), it does not fully account for the large  
579  $\text{CV}_B$  in basal  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  and the variable response to ingested inorganic  $\text{NO}_3^-$ . Indeed,  
580 the metabolic activity of the  $\text{NO}_3^-$ -reducing bacteria may be more important than the relevant  
581 abundance (Hyde et al. 2014a). Alternatively,  $\text{CV}_B$  of other factors including the  
582 aforementioned abundance and activity of gut bacteria, stomach pH (Lundberg et al. 1994;  
583 Montenegro et al. 2017), rates of gastric emptying and intestinal absorption (Leiper 2015), or  
584 the availability of sialin, a  $\text{NO}_3^-$  transporter in the saliva (Qin et al. 2012), may also contribute  
585 towards a high  $\text{CV}_B$  in NO metabolism. There also seems to be circadian variation in  
586 endogenous NO production (Antosova et al. 2009). Furthermore, while participants were  
587 requested to replicate their diet prior to each trial, the  $\text{NO}_3^-$  content of regularly consumed  
588 vegetables is known to vary considerably (Lidder and Webb 2013). Non-compliance with these  
589 instructions also cannot be ruled out although all participants gave verbal assurances on this  
590 point. Exposure to different doses of sunlight has also been shown to influence circulating  
591 levels of  $\text{NO}_2^-$  (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<sub>3</sub><sup>-</sup> supplementation and,  
602 in contrast to NO metabolites, the CV<sub>B</sub> 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 attack 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<sub>B</sub> for this metric. Indeed, an increased variability CV<sub>B</sub>  
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<sub>B</sub> and CD  
615 for all measured outcomes would increase in a more heterogenous group of healthy  
616 participants which included females and older adults.



617 Webb and colleagues (2008) have previously reported that ingestion of BR reduces systolic  
618 blood pressure by up to ~10 mmHg in healthy participants. Notably, the magnitude of this  
619 reduction in systolic blood pressure exceeds the baseline CD reported here (6.7 mmHg, 5.3 %)  
620 which confirms that this is a meaningful change in this parameter. In contrast, the BR-induced  
621 reduction in blood pressure reported in this study and more widely across the literature in  
622 healthy normotensive participants (Siervo and Lara 2013) are typically smaller and do not  
623 exceed the CD threshold. In patients with stage 1 hypertension, a single dose of NO<sub>3</sub><sup>-</sup>-rich BR  
624 reduced systolic blood pressure by 11 mmHg (7.3%) (Ghosh et al. 2013) suggesting the effects  
625 of BR are more pronounced in those with an elevated blood pressure. However, given that a  
626 high blood pressure will also elevate the CV<sub>B</sub>, researchers should be cautious about using CD  
627 values generated from healthy participants to interpret data in hypertensive or diseased cohorts.  
628 While this does not rule out a therapeutic effect of inorganic NO<sub>3</sub><sup>-</sup> supplementation in  
629 hypertensive patients, the potential influence of CV<sub>A</sub> and CV<sub>B</sub> on experimental outcomes  
630 should be duly considered when interpreting the data.

631

## 632 **5. Conclusion**

633 The data in the current study demonstrates that there is profound intra-individual variability in  
634 the measurement of NO metabolites in plasma and saliva, both at basal levels and when  
635 elevated following ingestion of BR. While the change in the abundance of certain species of  
636 NO<sub>3</sub><sup>-</sup>-reducing bacteria appears to account for some of this variation, other biological and  
637 experimental factors are also likely to contribute. Markers of blood pressure were consistently  
638 reduced on three separate occasions following the ingestion of BR but the magnitude of the  
639 change was small and did not exceed the CD. The data presented in this manuscript presents

640 metrics which facilitate a more meaningful interpretation of changes in key physiological  
641 variables following dietary NO<sub>3</sub><sup>-</sup> supplementation.

642

### 643 **Acknowledgments**

644 The authors wish to acknowledge the financial support of the University of the West of  
645 Scotland for the PhD studentship for the first author and the project costs. Dr David  
646 Muggeridge is supported by the European Union's INTERREG VA Programme, managed by  
647 the Special EU Programmes Body (SEUPB).

648

649

### 650 **Compliance with ethical standards**

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

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