Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans

Anni Vanhatalo[1], Jamie R. Blackwell[1], Joanna L'Heureux[1], David W. Williams[2], Ann Smith[3], Mark van der Giezen[1], Paul G. Winyard[4], James Kelly[1], Andrew M. Jones[1]

[1] College of Life and Environmental Sciences, University of Exeter, Exeter EX1 1TE, UK; [2] School of Dentistry and [3] Division of Population Medicine, Cardiff University, Cardiff CF14 4XY UK; [4] University of Exeter Medical School, University of Exeter, Exeter EX1 1TE, UK.

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Correspondence:
Anni Vanhatalo, Ph.D.
E-mail: a.vanhatalo@exeter.ac.uk
Tel: 01392 722886
Fax: 01392 264726

Abstract

Imbalances in the oral microbial community have been associated with reduced cardiovascular and metabolic health. A possible mechanism linking the oral microbiota to health is the nitrate (NO_3^-)-nitrite (NO_2^-)-nitric oxide (NO) pathway, which relies on oral bacteria to reduce NO_3^- to NO_2^-. NO (generated from both NO_2^- and L-arginine) regulates vascular endothelial function and therefore blood pressure (BP). By sequencing bacterial 16S rRNA genes we examined the relationships between the oral microbiome and physiological indices of NO bioavailability and possible changes in these variables following 10 days of NO_3^- (12 mmol/d) and placebo supplementation in young (18-22 yrs) and old (70-79 yrs) normotensive humans (n=18). NO_3^- supplementation altered the salivary microbiome compared to placebo by increasing the relative abundance of Proteobacteria (+225%) and decreasing the relative abundance of Bacteroidetes (-46%; $P<0.05$). After NO_3^- supplementation the relative abundances of *Rothia* (+127%) and *Neisseria* (+351%) were greater, and *Prevotella* (-60%) and *Veillonella* (-65%) were lower than in the placebo condition (all $P<0.05$). NO_3^- supplementation increased plasma concentration of NO_2^- and reduced systemic blood pressure in old (70-79 yrs), but not young (18-22 yrs), participants. High abundances of *Rothia* and *Neisseria* and low abundances of *Prevotella* and *Veillonella* were correlated with greater increases in plasma [NO_2^-] in response to NO_3^- supplementation. The current findings indicate that the oral microbiome is malleable to change with increased dietary intake of inorganic NO_3^-, and that diet-induced changes in the oral microbial community are related to indices of NO homeostasis and vascular health *in vivo*.

Keywords: Ageing; cardiovascular health; oral nitrate reduction; nitrite; 16S rRNA sequencing; host-microbe symbiosis; prebiotic.

Introduction

Inorganic nitrate (NO_3^-) is a natural part of the human diet that is found in high concentrations in many vegetables. NO_3^- itself is biologically inert and human cells are believed to lack NO_3^--reductase capability. However, commensal bacteria in the oral cavity can use nitrate as a terminal electron acceptor for ATP synthesis, reducing NO_3^- to nitrite (NO_2^-; which can be vasoactive in low-oxygen and low-pH conditions) and this NO_2^- can be further reduced to the potent vasodilator, nitric oxide (NO) (Dejam et al. 2004). The NO_3^--NO_2^--NO reduction pathway underpins the discovery that dietary NO_3^- supplementation through consumption of NO_3^- salts (Larsen et al. 2006) or vegetable products such as beetroot juice (Kelly et al. 2013a; Webb et al. 2008) reduces blood pressure (BP) in healthy young and old humans.

The importance of a functioning oral microbiome for the NO_3^-- NO_2^--NO reduction pathway is highlighted in cases where use of antibacterial mouthwash markedly blunts the increase in plasma and saliva NO_2^- concentrations and associated decrease in BP following ingestion of a standardised NO_3^- dose (Govoni et al. 2008; Kapil et al. 2013; McDonagh et al. 2015). Epidemiological studies also indicate that dysbiosis of the oral microbial community is associated with poor cardiovascular health (Briskey et al. 2016). Conversely, a diet rich in vegetables, which contain high concentrations of inorganic NO_3^-, significantly protects against both coronary heart disease and stroke (Bondonno et al. 2017; Hung et al. 2004; Joshipura et al. 2009). Ageing has been associated with reduced salivary flow rate ('dry mouth') and altered oral bacterial colonisation (Percival et al. 1991; Xu et al. 2015), but it is not known whether the abundances of NO_3^- reducing oral bacteria decline with age. Dietary NO_3^- intake, and the abundance of NO_3^--reducing oral bacteria, therefore represent routes to

lower blood pressure and maintain and improve cardiovascular health across the human lifespan.

It is possible that dietary NO_3^- as a prebiotic treatment might promote proliferation of NO_3^- reducing bacteria. In a rodent model, a NO_3^--rich diet over 7 days increased abundance of oral bacteria (*Streptococcus* and *Haemophilus*) that contain NO_3^- reductase genes (Hyde et al. 2014a). In saliva samples of hypercholesterolaemic humans, 6 weeks of NO_3^- supplementation with beetroot juice significantly increased the abundance of *Neisseria flavescens* and tended to increase *Rothia mucilaginosa* which are known NO_3^- reducers (Velmurugan et al. 2016). These studies indicate that increased dietary NO_3^- intake may alter the oral microbiome in a way which enhances an individual's ability to reduce ingested NO_3^-, resulting in greater plasma NO_2^- concentration and a greater reduction in systemic blood pressure. However, characterisation of potential changes in the oral microbiome of healthy young and old humans in response to NO_3^- supplementation is lacking.

In the present study, we used 16S rRNA gene sequencing to investigate whether abundances of NO_3^--reducing bacteria on the surface of the human tongue modulate an individual's response to NO_3^- supplementation in young (18-22 years) and old (70-79 years) normotensive adults. We hypothesised that at baseline, abundances of known NO_3^--reducing bacteria (including *Neisseria, Prevotella, Rothia, Veillonella* and Actinomycetales) would be greater in young compared to old participants, and that high abundances of these bacteria at baseline would be associated with higher plasma NO_2^- concentrations, and greater changes in blood pressure and arterial stiffness in response to NO_3^- supplementation. Secondly, we investigated whether 10 days of regular dietary NO_3^- ingestion altered the oral microbiome compared with placebo supplementation. It was hypothesised that oral microbiomes would be different

between placebo and NO_3^- conditions, and specifically that the relative abundances of bacteria capable of NO_3^- reduction would be greater after NO_3^- compared to placebo supplementation.

Methods

Ethical approval

The study was approved by the institutional Ethics Committee (Sport and Health Sciences, University of Exeter) and conducted in accordance with the code of the ethical principles of the World Medical Association (*Declaration of Helsinki*). All participants gave their written, informed consent before the commencement of the study, once the experimental procedures, associated risks, and potential benefits of participation had been explained.

Study participants

Nine old adults including six females (mean ± SD, age 75 ± 3 yrs, age range 70-79 yrs, height 162 ± 6 cm, body mass 61.8 ± 14.0 kg) and three males (age 73 ± 5 yrs, age range 70-78 yrs, height 172 ± 4 cm, body mass 77.7 ± 11.6 kg) and nine young adults including five females (age 20 ± 1 yrs, age range 19-22 yrs, height 168 ± 7 cm, body mass 67.9 ± 10.3 kg) and four males (age 20 ± 2 yrs, age range 18-22 yrs, height 180 ± 4 cm, body mass 73.4 ± 12.9 kg) volunteered to participate in this study (Table 1). All participants were of Caucasian ethnicity. The nine old adults represented a subsample of a larger cohort tested for a Dunhill Medical Trust funded project (R269/1112) from which the microbiome of the tongue and saliva were retrospectively analysed. Participants were screened prior to participation to ensure suitability for the study. All participants were ostensibly healthy and were not taking medication or dietary supplements. None of the participants were tobacco smokers and all

reported having no oral diseases. Participants were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous physical exertion in the 24 h preceding each laboratory visit. Participants were also asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively. All tests were performed at approximately the same time of day (± 2 h) for each participant.

Experimental design

Prior to commencing dietary supplementation, participants visited the laboratory for health screening and familiarisation to test protocols. Participants completed a salivary flow rate questionnaire (SFR-Q; Fox et al. 1987). The SFR-Q included eleven questions asking the participant to rate the frequency of various symptoms of low salivary flow rate on a scale of 1 ('never') to 5 ('very often'). Participants then underwent two 10-day dietary supplementation periods with NO_3^- and placebo in a randomised, double-blind, cross-over design (Figure 1). On days 8, 9 and 10 of each supplementation period, participants returned to the laboratory. Upon arrival at the laboratory on days 8, 9 and 10 venous blood samples were collected for the measurement of plasma [NO_2^-] and [NO_3^-] and resting BP was measured. The mean values of three measurements of [NO_2^-], [NO_3^-] and BP were used for further analyses. Saliva samples were also collected on each visit and three samples were pooled for analysis of the salivary microbiome. Arterial stiffness was assessed on one occasion (day 8, 9 or 10) using radial-femoral pulse wave velocity (PWV).

Supplementation

The supplements were NO_3^--rich concentrated beetroot juice (BR) (2 x 70 ml·d^{-1}, each 70 ml containing ~6.2 mmol NO_3^-; Beet It, James White Drinks, Ipswich, UK) and NO_3^--depleted concentrated beetroot juice placebo (PL) (2 x 70 ml·d^{-1}, each 70 ml containing ~0.01 mmol

NO_3^-; Beet It, James White Drinks, Ipswich, UK). The PL was indistinguishable from the BR supplement in appearance, taste and smell. Participants were instructed to consume one 70-ml beverage in the morning and one in the afternoon. On testing days, participants were asked to ingest one 70-ml beverage in the morning and one 2.5 h prior to their laboratory visit. A washout period of at least three days and up to 47 days separated the supplementation periods. Participants were instructed to maintain their normal daily activities, food intake and oral hygiene regime throughout the study. However, participants were instructed to refrain from using antibacterial mouthwash during the study period. Participants were advised that supplementation may cause beeturia (red urine) and red stools temporarily, but that such side effects were harmless.

Oral bacteria

Oral swabs of the tongue dorsum were collected at baseline. Saliva samples (~ 1 mL) were collected by expectoration, without stimulation, over a period of 5 min on three occasions following PL and BR supplementation periods. Oral swab and saliva samples were stored at -80°C until analysis. Genomic DNA was isolated from tongue swabs using a Gentra Puregene Buccal Cell Kit (Qiagen, Germantown, MD), and from saliva samples following the methods of Goode et al. (2014). Double-stranded DNA concentration was fluorometrically quantified (Qubit 3.0 high-sensitivity fluorescence detection, ThermoFisher Scientific, Waltham, MA). Library preparation employed a NEXTflex 16S V1-V3 Amplicon-Seq Kit (Bioo Scientific, Austin, USA). The 16S V1-V3 rDNA region was amplified using 5ng of dsDNA and subjected to 8 thermal cycles of 30 s at 98°, 30 s at 60° and 30 s at 72° with primers A and B (Table S1). Following AMPure® XP bead cleanup (Becton Dickinson, Franklin Lakes, NJ), a subsequent PCR with indexing primers to identify individual samples, containing Illumina flow cell binding sites, was performed.

The samples were sequenced using paired-end 300 base pair (bp) MiSeq Illumina platform (Illumina, San Diego, CA) using v3 MiSeq reagents. For each sample, the nucleotide sequence data in FASTQ format was trimmed using Trim-Galore! (Krueger F. Trim-Galore!, accessible at http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Quality trimming was performed by removing low-quality bases from the 3' read ends. The adapter sequences were subsequently removed from the 3' end (the first 13 base pairs). Trim-Galore! paired-end validation was performed to remove short sequences once the trimming was complete, where the minimum specified length was 20 bp. The bacterial taxonomies and abundance were assigned using Kraken standard build, which uses the genomes in Refseq and NCBI taxonomic information (Kraken manual, accessible at: http://ccb.jhu.edu/software/kraken/MANUAL.html#kraken-databases). The paired read sequences were classified and processed by the Kraken Taxonomic Sequence Classification System (Woods and Salzberg 2014). Variations in the V1-V3 regions enabled NCBI taxonomic identification, and kraken-translate was used to translate the NCBI Identifiers to taxonomy identifiers. A Kraken report was generated for each sample, which was visualised using Krona bioinformatics pie charts (Ondov et al. 2011).

Plasma [NO_3^-] and [NO_2^-]

Blood samples for determination of plasma [NO_2^-] and [NO_3^-] were collected from an antecubital vein into lithium heparin tubes and centrifuged for 8 min at 3000 *g* and 4 °C within 2 min of collection. Plasma was extracted and samples stored at -80°C for later determination of [NO_3^-] and [NO_2^-] using a modified chemiluminescence technique as previously described (Kelly et al. 2013a).

Blood pressure and arterial stiffness

Blood pressure of the brachial artery was measured following 10 min of seated rest in a quiet room using an automated sphygmomanometer (Dinamap Pro, GE Medical Systems, Tampa, USA). A total of four measurements were taken, with the mean of the final three measurements recorded. The mean of the systolic (SBP), diastolic (DBP) and mean arterial pressure (MAP) measurements made over three laboratory visits in each condition were calculated for each individual and used for subsequent analyses. Arterial stiffness was estimated via pulse-wave velocity (PWV) (Complior SP; Alam Medical, Vincennes, Paris, France). Electrodes were placed on the carotid, femoral, and radial arteries, and the pulse transit time was calculated and recorded. A mean of three measurements was calculated and used for subsequent analyses. The position of each electrode was measured in relation to the nearest bony landmark to enable precise reproduction of the position of the electrodes in each condition.

Statistical analyses

The Kraken raw data output of phylogenetic data were analysed using R-script (R Development Core Team 2008), SPSS V20 and Microsoft Excel. Non-metric multidimensional scaling (NMDS) was used to assess the level of microbiome similarity between young and old, and PL and BR conditions using non-parametric relationships, and analysed using ADONIS (Vegan R Software). Differences between PL and BR conditions were assessed using paired t-tests with Bonferroni-Hoechberg correction on bacteria that made up >0.01% of bacteria (R statistical software). The Shannon-Wiener diversity index (H') was used to explore differences in diversity (Vegan R Software). Paired-samples t-tests were used to assess differences between BR and PL conditions in plasma [NO_2^-] and [NO_3^-], BP and arterial stiffness. Relationships between plasma NO biomarkers, oral microbiome and

physiological responses to supplementation were assessed using Pearson's correlation coefficients. Statistical significance was accepted when $P<0.05$ and statistical trend was defined as $P<0.10$. Data were expressed as mean ± SD.

Results

The young and old participants were similar in terms of body mass and BMI (Table 1). The young participants had a greater mean score in SFR-Q than the old participants (Table 1), indicative of more frequent self-reported symptoms of low salivary flow rate. The young participants reported greater frequency of sensations associated with dry mouth (old 1.6 ± 1.0, young 2.7 ± 0.5; $P<0.05$) and having difficulty eating dry foods (old 1.1 ± 0.3, young 1.8 ± 0.4; $P<0.05$).

NO biomarkers, blood pressure and arterial stiffness

The NO_3^- dose relative to body mass was not different between young and old participants, but the latter had a greater increase in plasma [NO_2^-] in response to BR supplementation (Table 1). Plasma [NO_3^-] and [NO_2^-] were significantly higher following BR supplementation compared with PL (all $P<0.05$; Figure 2, panels A and B). BR supplementation increased plasma [NO_3^-] relative to PL by a similar amount in old (1509 ± 744%) and young participants (1481 ± 909%) (Figure 2C), but the increase in [NO_2^-] was greater in old (648 ± 477%) compared to young participants (365 ± 249%, $P<0.05$; Figure 2D). There were no differences between young and old participants in plasma [NO_3^-] in PL (old: 28 ± 12 μM, young: 28 ± 14 μM; $P>0.05$) or BR conditions (old: 379 ± 55 μM, young: 366 ± 101 μM; $P>0.05$). Plasma [NO_2^-] tended to be greater in the old in PL (old: 173 ± 97 nM, young: 104 ±

63 nM; P=0.09) and was significantly greater in the old than young participants in the BR condition (old: 1029 ± 393 nM, young: 380 ± 175 nM; P<0.05).

MAP, SPB, DBP and PWV were not different between PL and BR conditions across all participants (n=18, P>0.05; Figure 3, panels A, D, G and J). In old participants (n=9), SBP and MAP were significantly lower following BR supplementation compared with PL (Figure 3, panels B and E). Changes between PL and BR (Δ) in MAP, SBP, and DBP between PL and BR conditions inversely correlated with the change in plasma [NO_2^-] relative to NO_3^- dose per kg body mass (Δ[NO_2^-]/NO_3 dose; Figure 3, panels C, F and I). BR supplementation did not significantly alter radial-femoral PWV (Figure 3, panels J and K) across all participants, but ΔPWV between BR and PL in the old participants (increase of 4.3 ± 10.9 $m \cdot s^{-1}$; n=9) was different (P<0.05) from ΔPWV in the young participants (decrease of -6.8 ± 9.8 $m \cdot s^{-1}$; n=9). ΔPWV positively correlated with Δ[NO_2^-]/NO_3 dose (Figure 3L). Absolute plasma [NO_2^-] or [NO_3^-] measured in the PL condition were not correlated with Δ[NO_2^-], ΔDBP, ΔSBP, ΔMAP or Δ PWV. One old male participant did not wish to undertake PWV measurement and therefore all PWV data are derived from 17 participants.

Tongue microbiome at baseline

Relative abundances of the five main oral bacterial phyla in tongue swab samples collected at baseline were Bacteroidetes 32 ± 9%, Fusobacteriales 27 ± 11%, Proteobacteria 20 ± 7%, Firmicutes 17 ± 5% and Actinobacteria 1 ± 1 %. The most abundant bacterial species found on the tongue were *Fusobacterium nucleatum subsp. nucleatum* (16 ± 8%), *Prevotella melaninogenica* (14 ± 7 %), *Campylobacter concisus* (13 ± 8%), *Leptorichia buccalis*, (7 ± 6%) *Veillonella parvula* (5 ± 3%), *Prevotella intermedia* (4 ± 2%), *Fusobacterium nucleatum subsp. vincentii* (3 ± 3%) and *Neisseria meningitidis* (3 ± 3%). Correlations between relative

abundances of selected taxonomic units of tongue bacteria and physiological responses to BR supplementation are shown in Table 2. The greatest decreases in BP were associated with high abundances at baseline of *Fusobacterium nucleatum* subsp. *vincentii* and *nucleatum,* and order Actinomycetales, whereas a high relative abundance of *Prevotella melaninogenica* was associated with a greater mean score in SFR-Q, and smaller changes in plasma [NO_2^-], SBP and PWV in response to BR supplementation (Table 2).

Saliva microbiome after PL and BR supplementation

Relative abundances of the main phyla of oral bacteria differed between PL and BR conditions (Figure 4). Relative abundance of Proteobacteria was greater and Bacteroidetes was lower following BR compared with PL ($P<0.05$), while abundances of Firmicutes and Fusobacteria tended to be lower following BR supplementation compared with PL ($P<0.10$). NMDS plots revealed that oral microbial communities differed significantly between PL and BR supplemented conditions (Figure 5A), but there were no differences between young and old participants (Figure 5B). A Shannon diversity index revealed no significant differences in species diversity between PL and BR conditions (Figure 6). Overall, 52 taxonomic units were significantly different between BR and PL conditions. Figure 7 illustrates statistically significant differences at genera and species levels between PL and BR. The trend for reduction in the relative abundance of Firmicutes following BR was primarily due to a decrease in *Veillonella* (-65%), including a 65% decrease in *Veillonella parvula* species (both $P<0.05$), while the order Lactobacilliales and genus *Streptococcus* were not affected by BR ($P>0.05$). Within the phylum Bacteroidetes, BR resulted in a reduction in the genus *Prevotella* (-60%), and specifically *P. melaninogenica* (-67%) compared to PL (both $P<0.05$). The increase in Proteobacteria after BR stemmed from an increase in the order Neisseriales (+348%), containing the genus *Neisseria* (+351%) and *N. meningitidis* (+439%)

294 (all $P<0.05$), while there were no statistically significant changes in the genera
295 *Campylobacter* or *Haemophilus*. Proportions of the phylum Actinobacteria were not
296 significantly different between PL and BR, but there was an increase in the genus *Rothia*
297 (+127%, $P<0.05$) and *R. mucilaginosa* (+234%, $P<0.05$) after BR supplementation relative to
298 PL. There was insufficient microbial DNA in a saliva sample of one young male participant
299 and therefore the saliva microbiome data were for 17 participants.
300
301 Correlations across PL and BR conditions showed that plasma [NO_3^-] and [NO_2^-] positively
302 correlated with relative abundances of *Rothia* and *Neisseria* and inversely correlated with
303 *Prevotella* and *Veillonella* (Table 3). PWV positively correlated with relative abundance of
304 *Rothia* and *R. mucilaginosa* (Table 3).
305
306 **Discussion**
307
308 We used an *in vivo* experimental model and bacterial 16S rRNA gene sequencing to examine
309 relationships between the oral microbiome and physiological indices of NO bioavailability in
310 humans and changes in these variables following NO_3^- supplementation. The principal
311 finding of this study was that dietary NO_3^- supplementation altered the salivary microbiome
312 in young (~20 yrs) and old (~74 yrs) normotensive humans, such that it increased relative
313 abundances of some bacteria capable of NO_3^-reduction (*Rothia* and *Neisseria*) while reducing
314 the abundances of other NO_3^- reducers (*Prevotella* and *Veillonella*). NO_3^- supplementation
315 increased NO bioavailability in all participants, as indicated by plasma concentrations of
316 NO_3^- and NO_2^-, and reduced systemic blood pressure in the old, but not young, participants.
317 Across placebo and NO_3^- supplemented conditions, high abundances of *Rothia* and *Neisseria*
318 and low abundances of *Prevotella* and *Veillonella* were associated with high NO

bioavailability. The current findings indicate that the oral microbial community was malleable to change with increased dietary intake of inorganic NO_3^-, and, importantly, that the oral microbiome was related to indices of NO homeostasis and vascular health *in vivo*.

Relationships between the tongue microbiome at baseline and responsiveness to NO_3^- supplementation

It has been proposed that the oral microbiome modulates the magnitude of plasma [NO_2^-] increase, and changes in associated physiological indices, in response to NO_3^- supplementation (Bryan et al. 2017; Hyde et al. 2014ab; Koch et al. 2016). A recent study indicated that a composite relative abundance of seven species (including *Prevotella melaninogenica*, *Veillonella parvula*, and *Rothia mucilaginosa*) was positively correlated with the rise in salivary [NO_2^-], but not plasma [NO_2^-], in response to acute ingestion of a single NO_3^- bolus (Burleigh et al. 2018). We found that individuals who had high proportions of *P. melaninogenica* and *Campylobacter concisus* at baseline were less responsive to chronic NO_3^- supplementation, *i.e.* had smaller increases in plasma [NO_2^-] and smaller or no reductions in BP, than those individuals who had low abundances of *P. melaninogenica* and *C. concisus*. In addition, a high mean SFR-Q score at baseline, which indicated more frequent self-reported symptoms of dry mouth, was associated with greater abundance of *Prevotella* and a smaller increase in plasma [NO_2^-] in response to NO_3^- supplementation. *Campylobacter concisus* is believed to express dissimilatory NO_3^- reduction to NH_3 and its main physiological function is NO_2^- reduction (Simon & Klotz 2013), while *P. melaninogenica* has been shown to encode NO_2^-, but not NO_3^-, reductase genes (Hyde et al. 2014b). It may, therefore, be speculated that during subsequent NO_3^- supplementation both *C. concisus* and *P. melaninogenica,* which were dominant species in tongue swab samples at baseline, may have acted as net consumers of NO_2^- in the oral cavity. In contrast, high abundances of

Fusobacterium nucleatum subspecies and Actinomycetales at baseline were associated with greater increases in plasma [NO_2^-] and greater reductions in blood pressure in response to NO_3^- supplementation (Table 2). Actinomycetales are generally obligate anaerobes, and includes several species such as *Actinomyces odontolyticus* and *Actinomyces naeslundii* that have been identified as effective NO_3^- reducers (Doel et al. 2005). *Fusobacterium nucleatum* can reduce NO_2^-, but does not possess NO_3^--reductase genes. However, *F. nucleatum* provides 'scaffolding' in biofilms, enabling microbial attachments (Kolenbrander et al. 2002), and it is possible that these bacteria may have facilitated attachment and proliferation of key NO_3^- reducing bacteria during subsequent dietary interventions.

Changes in the salivary microbiome after NO_3^- supplementation

We showed that, relative to placebo, NO_3^- supplementation altered the proportions of the main oral microbial phyla by decreasing the Bacteroidetes and increasing the Proteobacteria. At the genus level, NO_3^- supplementation significantly increased relative abundances of the previously identified NO_3^- reducers *Neisseria* (Hyde et al. 2014b) and *Rothia* (Doel et al. 2005) in saliva. This was consistent with a report of a significant increase in *N. flavescens* and a trend for an increase in *R. mucilaginosa* in saliva samples of hypercholesterolaemic patients after 6 weeks of NO_3^- supplementation (Velmurugan et al. 2015). A novel finding in the present study was that a high abundance of the facultative anaerobe *R. mucilaginosa* was associated with faster pulse wave velocity, indicative of lower arterial stiffness. These data suggest that high relative abundances of bacteria belonging to *Neisseria* and *Rothia* were related to high NO bioavailability and may promote vascular health.

We also found that compared to placebo, NO_3^- supplementation decreased relative abundances of the obligate anaerobic bacteria *Prevotella* and *Veillonella* in saliva. This

finding appears to contradict studies that have used *in vitro* approaches to identify key oral NO_3^- reducing taxa. Hyde et al. (2014b) categorised biofilms prepared from tongue swab samples of six healthy humans as best, intermediate and worst NO_3^- reducers and found greater abundances of both *Prevotella* and *Veillonella* in the best *versus* worst NO_3^- reducing biofilms. Using tongue swab samples from ten healthy humans, which were incubated on solid medium under aerobic and anaerobic conditions and analysed by 16S rDNA sequencing, Doel et al. (2005) concluded that *Veillonella* were the most prevalent oral NO_3^- reducers and were major contributors to net NO_2^- production. Intricate metabolic interactions among the oral microbiota might mean that increased NO_3^- availability in the oral cavity may not facilitate the growth of all bacteria capable of NO_3^- reduction. It is not directly apparent why NO_3^- supplementation resulted in a decline in *Veillonella*. One factor that may have contributed to proliferation of some taxa and inhibition of others is the oral pH, which is a powerful modulator of the oral microbial community. Beetroot juice supplementation has been shown to increase oral pH from 7.0 to 7.5 (Hohensinn et al. 2016), and notably, a pH of 8 is optimal for NO_3^- reductase activity (van Maanen et al. 1996). Monitoring of salivary pH alongside alterations in the oral microbiome during NO_3^- supplementation should be undertaken in future studies to address the possible effects of pH.

Given that many oral bacteria with ability to reduce NO_3^- are also capable of downstream metabolism of the produced NO_2^-, it is important to differentiate between NO_3^-reducing bacteria in general and NO_2^- accumulating bacteria specifically. NO_3^- reducing oral bacteria have been identified *in vitro* from human samples, including *Veillonella, Actinomyces, Rothia, Staphylococcus* and *Propionibacterium* (Dole et al. 2005). More recently Hyde et al. (2014b) added *Neisseria*, *Haemophilus parainfluenzae*, *Prevotalla* (including *P. melaninogenica*) and *Granulicatella* to the list of candidate species for most potent

394 contributors to oral NO_2^- production. We showed that some of the oral bacteria that have been
395 proposed as key NO_2^- accumulators on the basis of *in vitro* experiments, such as *Veillonella*
396 and *Prevotella*, do not thrive under high NO_3^- availability *in vivo*. Indeed, we found no
397 significant changes in relative abundances of *Actinomyces, Staphylococcus*,
398 *Propionibacterium, Granulicatella* or *Haemophilus* after NO_3^- supplementation. In order to
399 contribute significantly to the amount of NO_2^- that is swallowed from the oral cavity, the
400 bacteria need to reduce NO_3^- at a faster rate than they reduce NO_2^-, or not undertake
401 downstream metabolism of NO_2^- at all. Overall, to maximise NO bioavailability from a NO_3^-
402 -rich diet, the composition of the oral microbial community needs to be such that it contains a
403 greater quantity of net NO_2^- accumulators than net NO_2^- consumers. Further research is
404 needed to establish whether the observed microbiome changes following chronic NO_3^-
405 supplementation in the present cohort, are replicated in different populations and whether
406 such changes are associated with an increased capacity for acute NO_3^- reduction in the oral
407 cavity.
408
409 The present data suggest that the chronic (10-day) NO_3^- supplementation serves to change the
410 relative abundance of a few, but not all, NO_3^- reducing taxa and that these changes are
411 correlated with beneficial changes in NO bioavailability and indices of cardiovascular health.
412 It should be noted that elevated NO bioavailability may have further beneficial effects on
413 aspects of healthy ageing, including maintenance of a strong immune response. A recent
414 study showed that tongue microbiomes that had high abundances of *Prevotella* and
415 *Veillonella* species were associated with elevated risks of all-cause mortality and mortality
416 from pneumonia in frail elderly nursing home residents (Kageyama et al. 2017). NO_3^-
417 supplementation in older people, which reduces the relative abundances of *Prevotella* and

418 *Veillonella*, may therefore have potential to enhance the NO-mediated immune response in
419 this high-risk population.
420
421 *Differences in [NO_2^-] and blood pressure responses between young and old participants*
422 Previous studies have shown appreciable inter-individual variability in the plasma [NO_2^-] and
423 blood pressure responses to NO_3^- ingestion in both young and older populations (*e.g.* Casey et
424 al. 2015; Jaija et al. 2014; Kelly et al. 2013). We found that despite ingesting the same dose
425 of NO_3^- the old adults showed a greater plasma [NO_2^-] increase than the young adults, and
426 also exhibited a reduction in blood pressure which was absent in the young participants. The
427 magnitude of the BP response to NO_3^-supplementation is correlated with baseline blood
428 pressure (Kapil et al. 2013; Webb et al. 2008), such that in the present study the scope for a
429 BP decrease in the young adults, who had a BP of ~112/63 mmHg in placebo, was likely
430 small. Although the balance of evidence indicates that NO_3^- represents an potent dietary
431 means for reducing systemic BP (Bryan et al. 2017; Kapil et al. 2013 Koch et al. 2017;
432 Larsen et al. 2006; Webb et al. 2008), it is important to note that there are several studies that
433 concur with the present finding of not showing a significant change in resting systolic or
434 diastolic BP (or both) in young, healthy adults (*e.g.* Kelly et al. 2013b; Larsen et al. 2010;
435 McDonagh et al. 2015; Vanhatalo et al. 2014).
436
437 Since the human vascular response to NO_3^- supplementation is dependent on efficient
438 bacterial reduction of NO_3^- to NO_2^-, it is possible that at least some of this variability is linked
439 to differences between individuals in the oral microbiota and therefore oral NO_3^- reducing
440 capacity. NMDS analysis revealed no overall differences in the salivary microbiomes
441 between young and old participants, or following placebo or NO_3^- supplementation. The
442 greater responsiveness to supplementation in the old compared to young participants was

surprising, given that ageing is typically associated with reduced salivary flow rate and altered oral bacterial colonisation (Percival et al. 1991). It is important to note that although the self-reported frequency of dry mouth symptoms was greater in the young adults, we did not directly quantify salivary flow rate in this study, and agreement between self-reported xerostomia questionnaire data and measured saliva secretion rate varies (Fox et al. 1987; Lima et al. 2017; Malicka et al. 2014). We are therefore unable to determine whether the higher occurrence of self-reported symptoms of dry mouth in the young adults was in fact attributed to significantly lower salivary flow in comparison to the old adults. Whether possible differences in salivary flow rate and/or NO_3^- uptake into the enterosalivary circulation via sialin $2NO_3^-/H^+$ transporters (Qu et al. 2016) contribute to inter-individual variability in responsiveness to NO_3^- supplementation irrespective of age warrants further investigation with large cross-sectional cohorts across the human lifespan.

Physical exercise and diet are emerging as powerful modulators of the gut microbiota (Barton et al. 2017) and it is intuitive that the microbiota in the oral cavity, the uppermost section of the alimentary canal, may also vary according to age, diet, and with physical activity levels. There is evidence to suggest that the gut microbiome of healthy older people may be remarkably similar to that of young adults (Bian et al. 2017), and that age-related alterations in the gut microbiome may be related to advancing frailty and development of disease (Biagi et al. 2010). Whether the maintenance of a 'young' gut microbiome into older age is a cause or consequence of healthy ageing is unknown. Our data suggest that the oral microbiome of individuals who have reached their 8^{th} decade of life without chronic disease is indistinguishable from oral microbiome of young adults. The inclusion criteria in the present study meant that the enrolled old participants were in exceptionally good health for their age and therefore not representative of an ageing population with poor cardiovascular and/or

metabolic health, who may be less responsive to NO_3^- supplementation due to impairments in NO bioactivity (Siervo et al. 2015). Indeed, we found that there was no difference in blood pressure between young and old participants following NO_3^- supplementation. Longitudinal studies are necessary to identify possible changes that may occur in the oral microbiome, and NO bioavailability, at the onset of chronic disease.

The dietary interventions in the present study consisted of twice daily ingestion of NO_3^--rich and NO_3^--depleted beetroot juice concentrate that were matched for carbohydrate and polyphenol content (Gilchrist et al. 2013). It should be noted that the alterations we observed in the salivary microbiome following NO_3^--rich beetroot juice supplementation may be due to NO_3^- alone or an additive effect of NO_3^- and other nutritional components of the beetroot supplement. Elevated carbohydrate intake may have favoured the growth of those microbes that use carbohydrate as an energy substrate at the expense of proteolytic bacteria, such as *Prevotella*. Further studies using supplementation with salts of NO_3^- (KNO_3, $NaNO_3$) are warranted to ascertain the effect of NO_3^- alone on the oral microbiome.

Methodological considerations

Microbial analysis was performed on tongue swab samples at baseline and on saliva samples following placebo and NO_3^- supplementation periods. Saliva samples represent a composite of bacteria from all oral sites, while tongue swab samples target the tongue dorsum which was shown to have the highest NO_3^- reductase activity by Doel et al. (2005). The quantities and relative abundances of bacteria vary between different oral sites, such that potential changes in microbiome assessed from tongue swabs following NO_3^- supplementation may differ from those observed in the saliva samples in the present study. However, given the similarities of findings on *Neisseria* and *Rothia* between the present study and that of

493 Velmurugan et al. (2016), we are confident that the salivary microbiome analyses in the
494 present study captured representative and meaningful differences between conditions in the
495 NO_3^- reducing oral microbiome. A limitation of the current study was that saliva samples
496 could not be included at the beginning of each supplementation period, and the wash-out
497 period required for NO_3^- -induced changes in the oral microbiome to return to baseline
498 following cessation of supplementation is not known. Future work should include *in vivo*
499 tests of oral NO_3^- reduction capacity to ascertain whether the changes in oral microbiome
500 following chronic NO_3^- supplementation are associated with enhanced oral NO_3^- reduction. In
501 the present study, we inferred enhanced oral NO_3^- reduction from plasma [NO_2^-]. Finally,
502 further investigation should target the optimisation of the dose and duration of prebiotic NO_3^-
503 supplementation, and its possible interactions with the macronutrient content of the diet, to
504 provide maximal functional effects of NO_3^- supplementation.
505
506 *Conclusions*
507 Imbalances in the oral microbial community and poor dental health have been associated with
508 reduced cardiovascular and metabolic health. We showed that ageing, *per se,* in the absence
509 of chronic disease, does not impair an individual's ability to reduce dietary NO_3^- and increase
510 plasma [NO_2^-] in response to NO_3^- supplementation. Using 16S rRNA gene sequencing of
511 oral bacteria in an *in vivo* experimental model, we showed that high abundances of oral
512 bacteria belonging to genera *Prevotella* and *Veillonella* were likely detrimental, while high
513 abundances of the genera *Rothia* and *Neisseria* were likely beneficial for the maintenance of
514 NO homeostasis and associated indices of cardiovascular health. The symbiotic relationship
515 between the oral microbiome and its human host is a fast evolving field of research with
516 significant implications for development of prebiotic and probiotic interventions to improve
517 cardiovascular and metabolic health. Our results identify dietary NO_3^- as a modulator of the

518 oral NO_2^--producing microbiome in healthy humans and highlight the potential of oral
519 microbiota-targeted therapies for ameliorating conditions related to low NO bioavailability.
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695 **Figure legends**

696

697 **Figure 1** Participants underwent 10-day supplementation periods with nitrate (~12.4 mmol/d)
698 and placebo in a balanced cross-over design. Screening, protocol familiarisation and Salivary
699 Flow Rate Questionnaires (SFR-Q) were completed at baseline. Measurements of plasma
700 nitrite (NO_2^-) and nitrate (NO_3^-) concentrations, blood pressure (BP) of the brachial artery,
701 arterial stiffness as carotid-femoral pulse wave velocity (PWV), and the collection of saliva
702 samples for microbiome analysis were undertaken on days 8, 9 and 10 of each
703 supplementation period.

704

705 **Figure 2** Plasma [NO_3^-] (panel A) and [NO_2^-] (panel B) were significantly greater after
706 nitrate supplementation (white bars) compared to placebo (black bars) (n=18). The change
707 (Δ) in plasma [NO_3^-] between nitrate and placebo conditions was similar in young (n=9) and
708 old participants (n=9) (panel C), but Δ[NO_2^-] was significantly greater in the old compared to
709 young participants. Error bars indicate standard deviations and black squares (panels C and
710 D) indicate means for young and old participants. * P<0.05.

711

712 **Figure 3** Mean arterial pressure (MAP; panel A), systolic blood pressure (SBP; panel D),
713 diastolic blood pressure (DBP; panel G) and pulse wave velocity (PWV; panel J) were not
714 different between placebo and nitrate conditions across all participants (n=18). The old
715 participants (n=9) showed greater reductions (Δ) in MAP, SBP and DBP between placebo
716 and nitrate conditions than the young participants (n=9; panels B, E and H), as well as a
717 greater increase in PWV (young n=9, old n=8; panel K). ΔMAP, ΔSBP and ΔDBP inversely
718 correlated with the change in plasma [NO_2^-] relative to nitrate dose (Δ[NO_2^-]/NO_3^- dose)

(panels C, F and I) and ΔPWV was positively correlated with $\Delta[NO_2^-]/NO_3^-$ dose (panel L). *P<0.05.

Figure 4 The proportions of five main phyla of oral bacteria identified in the saliva samples following placebo (PL) and NO_3^- supplementation (BR). *Difference between PL and BR (P<0.05).

Figure 5 Overall salivary microbiome composition illustrated by non-metric multidimensional scaling (NMDS) analysis. The salivary microbiome composition was different between nitrate (BR) and placebo (PL) conditions (P<0.05; panel A) but not between young and old participants (P>0.05; panel B).

Figure 6 The Shannon diversity index indicated no statistically significant difference in species diversity between nitrate (BR) and placebo (PL) conditions.

Figure 7 The genera (panels A and B) and species (panels C and D) that comprised >0.01% of all bacteria and showed significant differences (P<0.05) between nitrate (BR) and placebo (PL) conditions.

Table 1. Participant characteristics, nitrate (NO_3^-) dose and plasma $[NO_2^-]$ responsiveness to supplementation, and salivary flow rate questionnaire (SFR-Q) results. The young and old participants were similar in terms of body mass and BMI. The NO_3^- dose was similar in both groups but old participants had a greater increase than the young in plasma $[NO_2^-]$ in response to supplementation. The young reported feeling more frequent symptoms of low salivary flow rate than the old participants.

744

745 **Table 2**. Correlation coefficients for relationships between selected taxonomic units of the
746 tongue microbiome (% of total bacteria) at baseline and subsequent changes between placebo
747 and NO_3^- supplementation in plasma [NO_2^-], blood pressure and arterial stiffness. PWV data
748 were not available for one old male participant, such that ΔPWV correlations are for n=17.
749 Δ= change between placebo and NO_3^-; [NO_2^-]/NO_3^- dose = plasma [nitrite] relative to nitrate
750 dose per kg body mass ingested; DBP=diastolic blood pressure; SBP= systolic blood
751 pressure; MAP= mean arterial pressure; PWV= pulse wave velocity; SFR-Q= salivary flow
752 rate questionnaire; ***P*<0.01, **P*<0.05, #*P*<0.10.

753

754 **Table 3**. Correlation coefficients for relationships between relative abundances of selected
755 taxonomic units of the salivary microbiome (% of total bacteria) and plasma [NO_3^-] and
756 [NO_2^-]; diastolic (DBP), systolic (SBP) and mean arterial (MAP) blood pressure; and pulse
757 wave velocity (PWV) across placebo and nitrate conditions. Saliva microbiome data were not
758 available for one young male participant and PWV data were not available for one old male
759 participant, such that [NO_3^-], [NO_2^-] and BP correlations are for n=34 and PWV correlations
760 are for n=32.

Table

Table 1. Participant characteristics, nitrate (NO_3^-) dose and plasma [NO_2^-] responsiveness to supplementation, and salivary flow rate questionnaire (SFR-Q) results. The young and old participants were similar in terms of body mass and BMI. The NO_3^- dose was similar in both groups but old participants had a greater increase than the young in plasma [NO_2^-] in response to supplementation. The young reported feeling more frequent symptoms of low salivary flow rate than the old participants.

	Sex	Age (yrs)	Body mass (kg)	BMI (kg/m^2)	NO_3^- dose (mmol/kg/d)	$\Delta[NO_2^-]/NO_3^-$ dose (nM/mmol/kg/d)	SFR-Q mean score
OLD							
1	F	77	88.0	33.1	0.14	7543	1.2
2	F	79	66.2	22.1	0.19	2325	1.3
3	F	70	60.0	24.3	0.21	2962	1.7
4	F	76	53.1	20.7	0.23	3942	1.1
5	F	72	51.4	20.1	0.24	4819	1.7
6	F	74	52.3	20.2	0.24	5428	1.9
7	M	78	88.8	31.1	0.14	812	2.0
8	M	70	78.6	25.4	0.16	5461	1.0
9	M	70	65.6	22.7	0.19	4829	1.5
Mean		74.0	67.1	24.4	0.19	4236	1.4
SD		3.6	14.8	4.7	0.04	1988	0.4
YOUNG							
10	F	22	71.5	29.4	0.17	803	1.9
11	F	19	60.8	22.9	0.20	2202	2.5
12	F	19	64.5	22.6	0.19	880	1.6
13	F	20	69.7	24.4	0.18	1604	2.7
14	F	19	85.2	28.5	0.15	999	1.5
15	M	18	55.7	18.0	0.22	2371	1.5
16	M	22	81.2	23.7	0.15	3254	1.6
17	M	19	72.3	22.3	0.17	1127	2.1
18	M	19	84.4	26.0	0.15	70	2.1
Mean		19.7*	71.7	24.2	0.18	1479 *	2.0 *
SD		1.4	10.4	3.5	0.03	977	0.4
OVERALL (OLD + YOUNG)							
Mean		46.8	69.4	24.3	0.18	2857	1.7
SD		28.1	12.6	4.0	0.03	2079	0.5

F, female; M, male; BMI, body mass index; $\Delta[NO_2^-]/NO_3^-$ dose, change in plasma [NO_2^-] relative to dose of NO_3^- ingested per kg body mass; SFR-Q, salivary flow rate questionnaire; *Different from old, $P<0.05$.

Table

Table 2. Correlation coefficients for relationships between selected taxonomic units of the tongue microbiome (% of total bacteria) at baseline and subsequent changes between placebo and NO_3^- supplementation in plasma [NO_2^-], blood pressure and arterial stiffness. PWV data were not available for one old male participant, such that ΔPWV correlations are for n=17.

		Δ[NO_2^-]/NO_3^- dose (nM/mmol/kg/D)	ΔDBP (mmHg)	ΔSBP (mmHg)	ΔMAP (mmHg)	ΔPWV (m/s)	SFR-Q mean score
	Δ[NO_2^-]/NO_3^- dose		-0.57*	-0.73**	-0.65**	0.65**	-0.52*
Actinobacteria	Actinomycetales (order)	0.40	-0.39	-0.46 #	-0.46 #	0.47 #	-0.004
	Micrococcales (order)	-0.41	0.25	0.36	0.40	-0.21	-0.04
	Rothia (genus)	-0.20	0.10	0.32	0.37	-0.07	0.20
	Rothia mucilaginosa	-0.22	0.17	0.38	0.44 #	-0.08	0.18
Proteobacteria	*Neisseria* (genus)	0.21	-0.06	0.06	0.29	0.59*	-0.39
	Neisseria meningitidis	-0.09	-0.08	0.044	0.36	0.13	-0.19
	Campylobacter concisus	-0.55*	0.43 #	0.65**	0.34	-0.62**	0.30
Bacteroi-detes	*Prevotella* (genus)	-0.49*	0.19	0.43 #	0.41 #	-0.52*	0.51*
	Prevotella melaninogenica	-0.57*	0.16	0.53*	0.37	-0.68**	0.49*
Firmicutes	*Veillonella* (genus)	-0.22	0.22	0.35	0.09	-0.02	0.46 #
	Veillonella parvula	-0.19	0.22	0.35	0.10	0.02	0.41 #
Fuso-bacteria	*Fusobacterium nucleatum* subsp. *nucleatum*	0.44 #	-0.17	-0.56*	-0.30	0.32	-0.29
	Fusobacterium nucleatum subsp. *vincentii*	0.55*	-0.43 #	-0.60**	-0.63**	0.45 #	-0.36

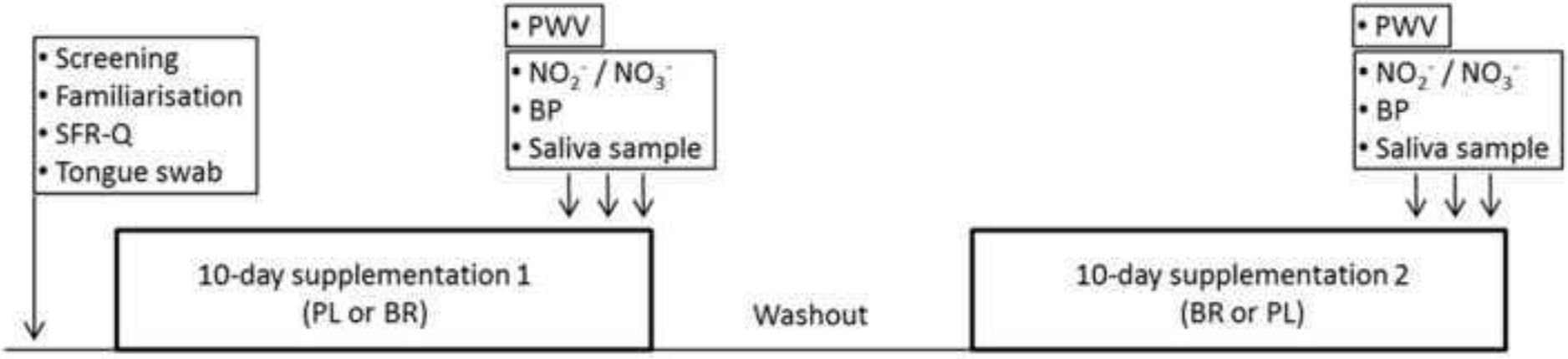
Δ= change between placebo and NO_3^-; [NO_2^-]/NO_3^- dose = plasma [nitrite] relative to nitrate dose per kg body mass ingested; DBP=diastolic blood pressure; SBP= systolic blood pressure; MAP= mean arterial pressure; PWV= pulse wave velocity; SFR-Q= salivary flow rate questionnaire; **P<0.01, *P<0.05, #P<0.10.

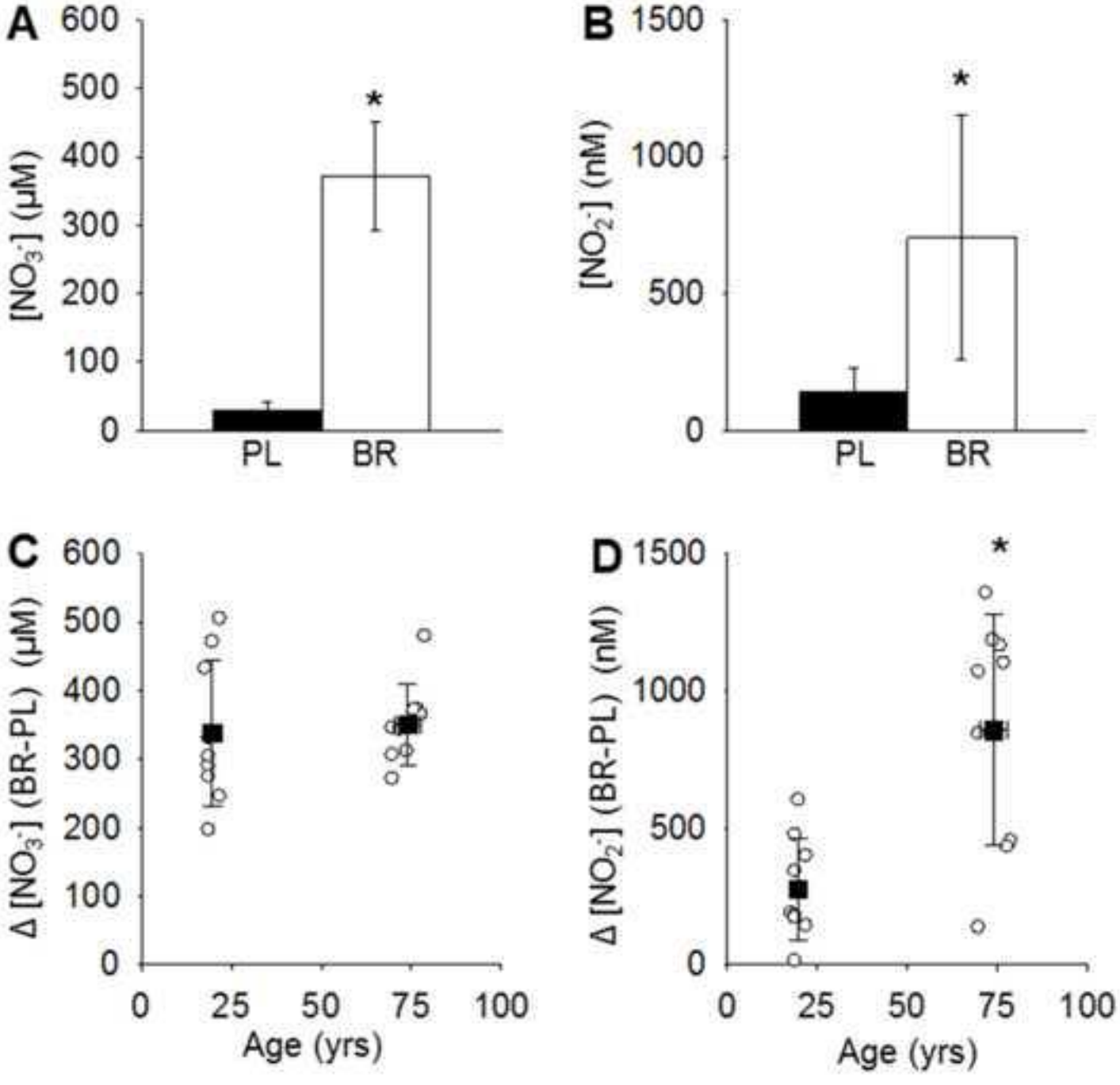
Table

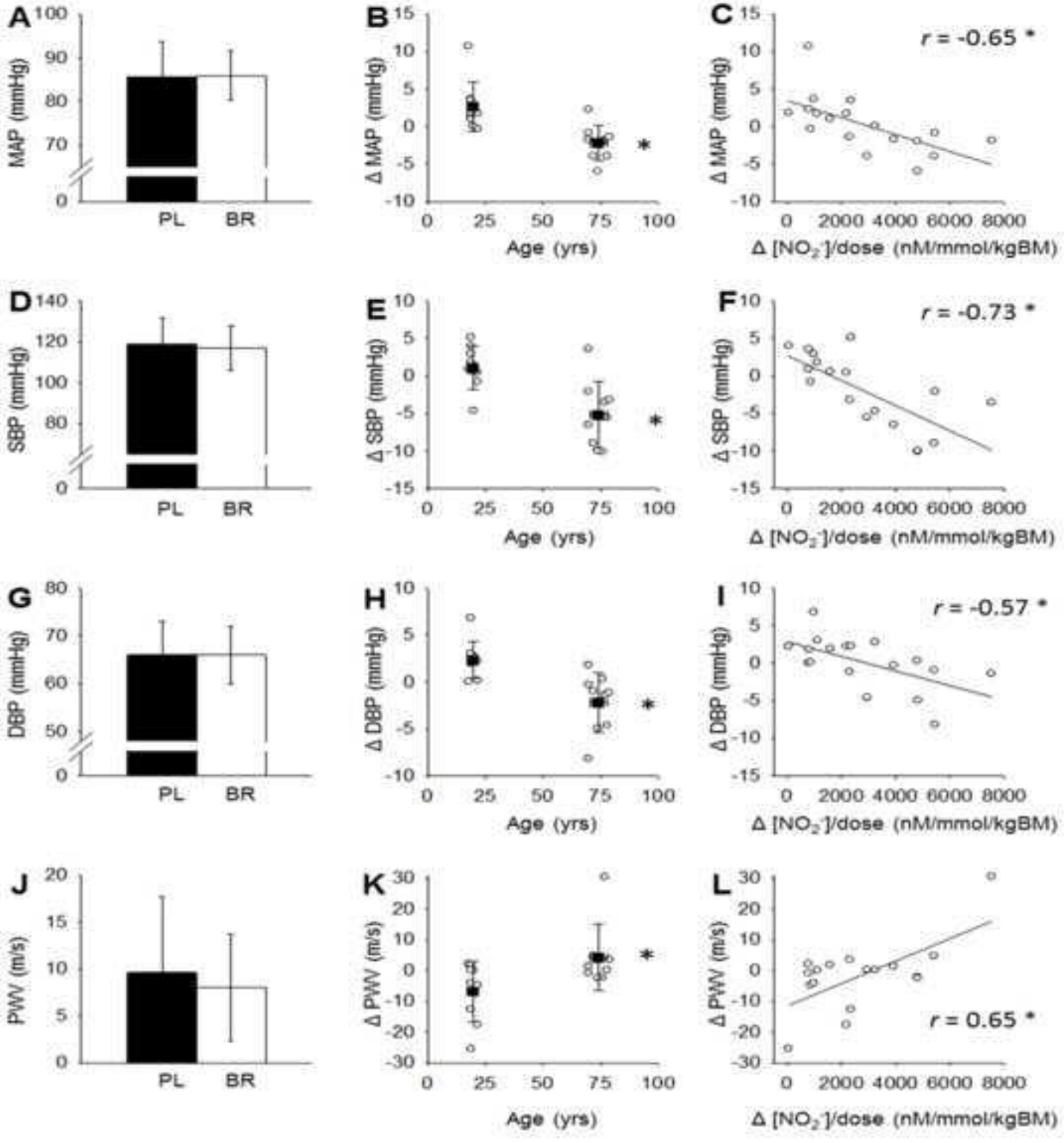
Table 3. Correlation coefficients (*r*) for relationships between relative abundances of selected taxonomic units of saliva microbiome (% of total bacteria) and plasma nitrate ($[NO_3^-]$) and nitrite ($[NO_2^-]$); diastolic (DBP), systolic (SBP) and mean arterial (MAP) blood pressure; and pulse wave velocity (PWV) across placebo and nitrate conditions. Microbiome data were not available for one young male subject and PWV data were not available for one older male subject, such that $[NO_3^-]$, $[NO_2^-]$ and BP correlations are for n=34 and PWV correlations are for n=32.

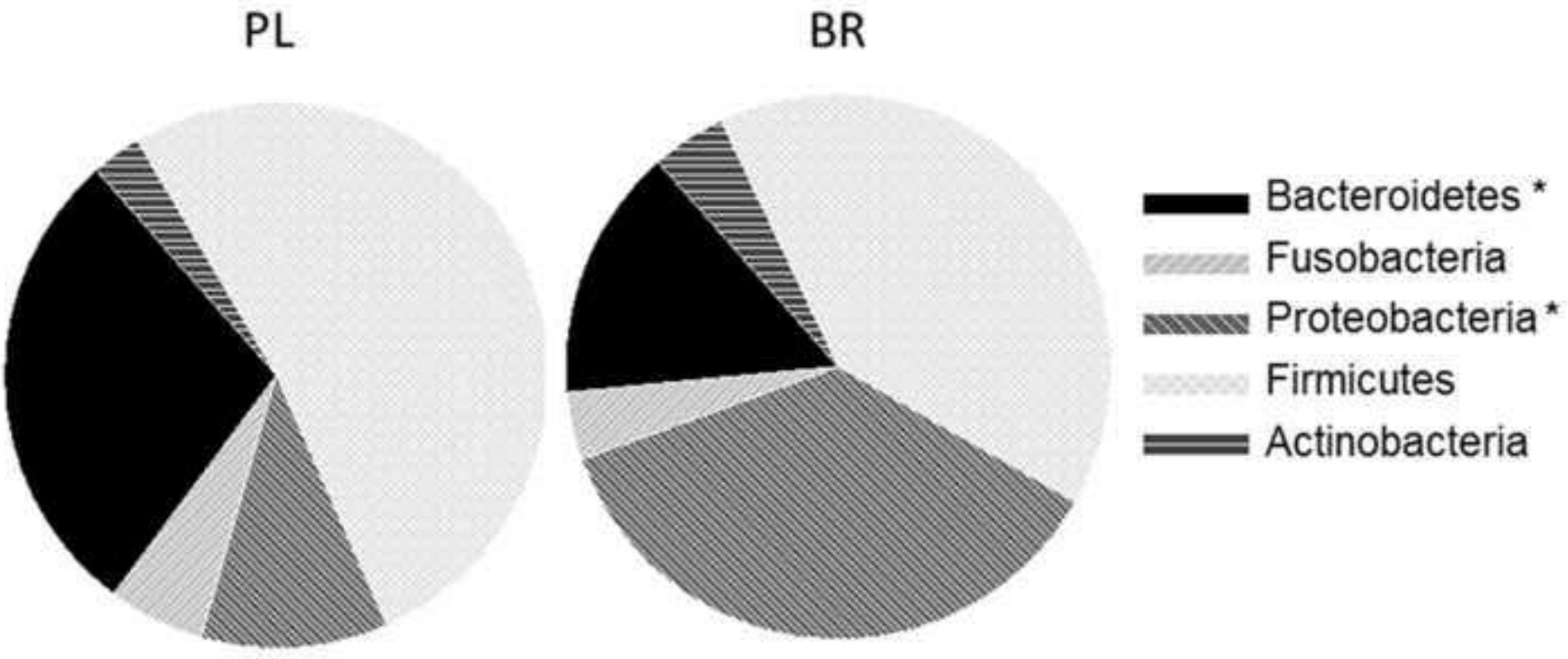
		$[NO_3^-]$ (μM)	$[NO_2^-]$ (nM)	DBP (mmHg)	SBP (mmHg)	MAP (mmHg)	PWV (m/s)
Actinobacteria	Actinomycetales (order)	0.25	0.09	-0.01	-0.09	-0.05	0.06
	Micrococcales (order)	0.37 *	0.23	-0.03	0.02	0.00	0.48 **
	Rothia (genus)	0.45 **	0.30 #	0.04	0.03	0.04	0.45 *
	Rothia mucilaginosa	0.46 **	0.37 *	0.06	-0.03	-0.01	0.41 *
Proteobacteria	*Neisseria* (genus)	0.61 **	0.64 **	0.02	0.15	0.14	-0.13
	Neisseria meningitidis	0.54 **	0.53 **	-0.00	0.07	0.08	-0.23
	Campylobacter concisus	-0.26	-0.17	-0.17	0.08	0.02	0.42 *
Bacteroi-detes	*Prevotella* (genus)	-0.47 **	-0.35 *	0.11	0.21	0.18	-0.18
	Prevotella melaninogenica	-0.47 **	-0.35 *	0.06	0.17	0.12	-0.09
Firmicutes	*Veillonella* (genus)	-0.62 **	-0.50 **	-0.10	-0.01	-0.22	-0.07
	Veillonella parvula	-0.60 **	-0.49 **	-0.10	-0.03	-0.23	-0.08
Fuso-bacteria	*Fusobacterium nucleatum* subsp. *nucleatum*	-0.28	-0.18	0.00	0.08	0.05	-0.10
	Fusobacterium nucleatum subsp. *vincentii*	-0.16	-0.01	-0.03	0.12	0.07	-0.17

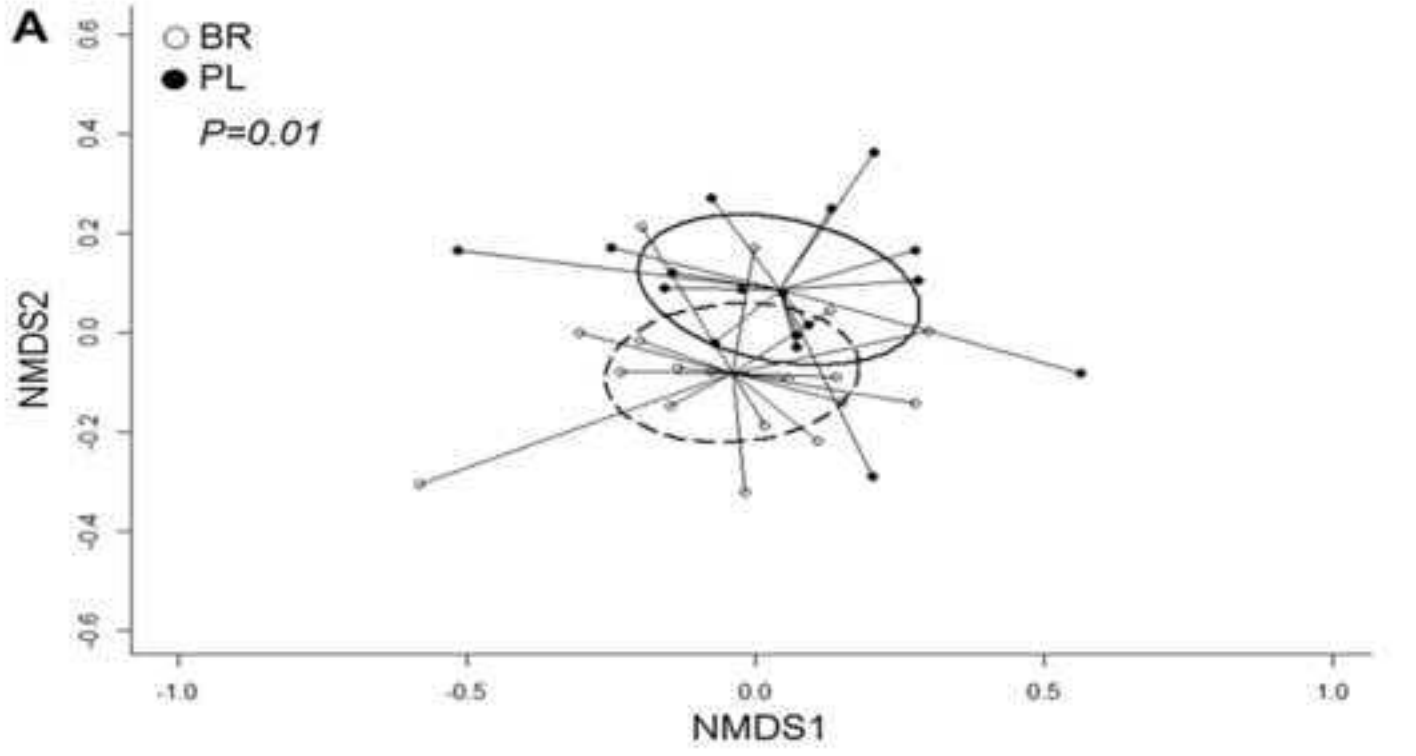
DBP=diastolic blood pressure; SBP= systolic blood pressure; MAP= mean arterial pressure; PWV= pulse wave velocity; ***P*<0.01, **P*<0.05, #*P*<0.10.

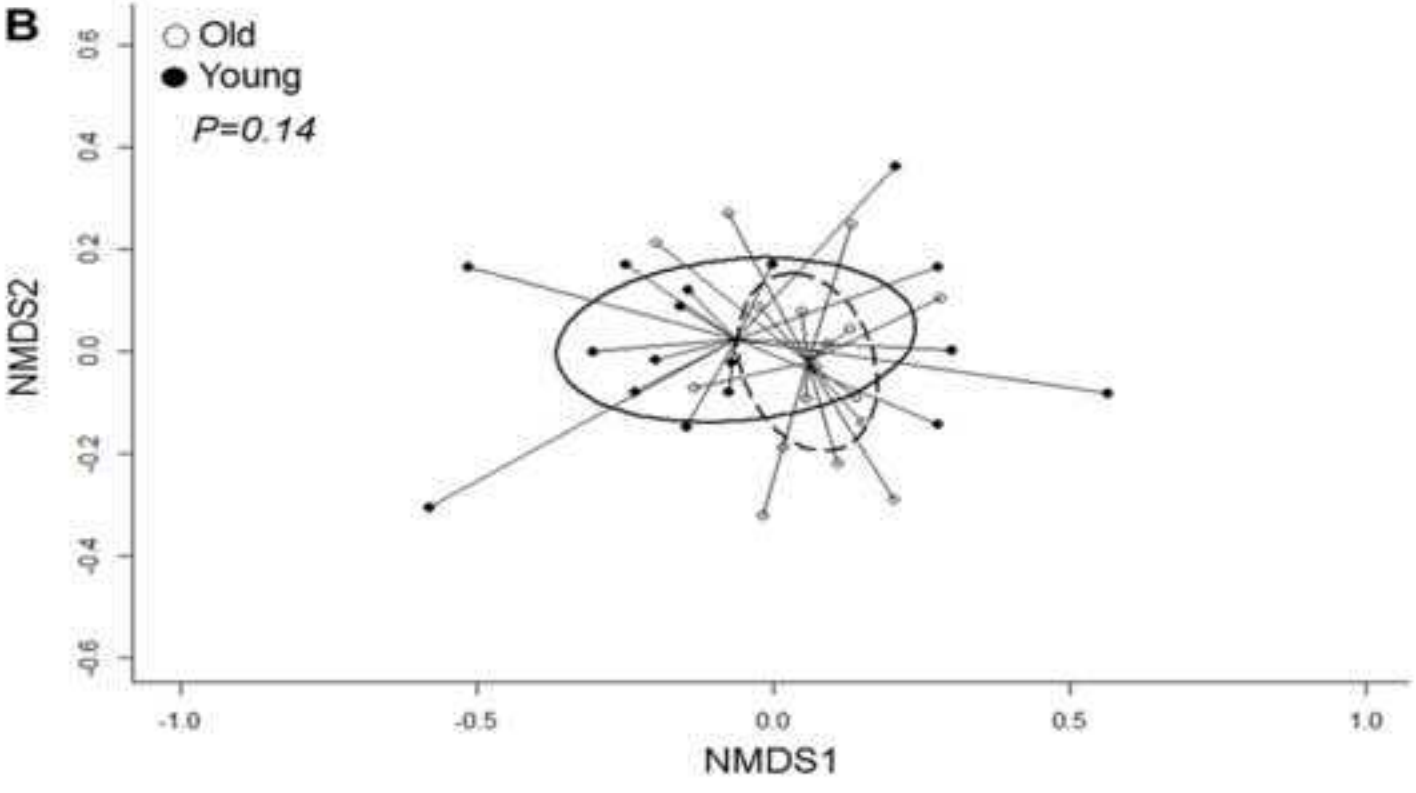

• Screening
• Familiarisation
• SFR-Q
• Tongue swab
• PWV
• NO_2^- / NO_3^-
• BP
• Saliva sample
10-day supplementation 1
(PL or BR)
Washout
• PWV
• NO_2^- / NO_3^-
• BP
• Saliva sample
10-day supplementation 2
(BR or PL)

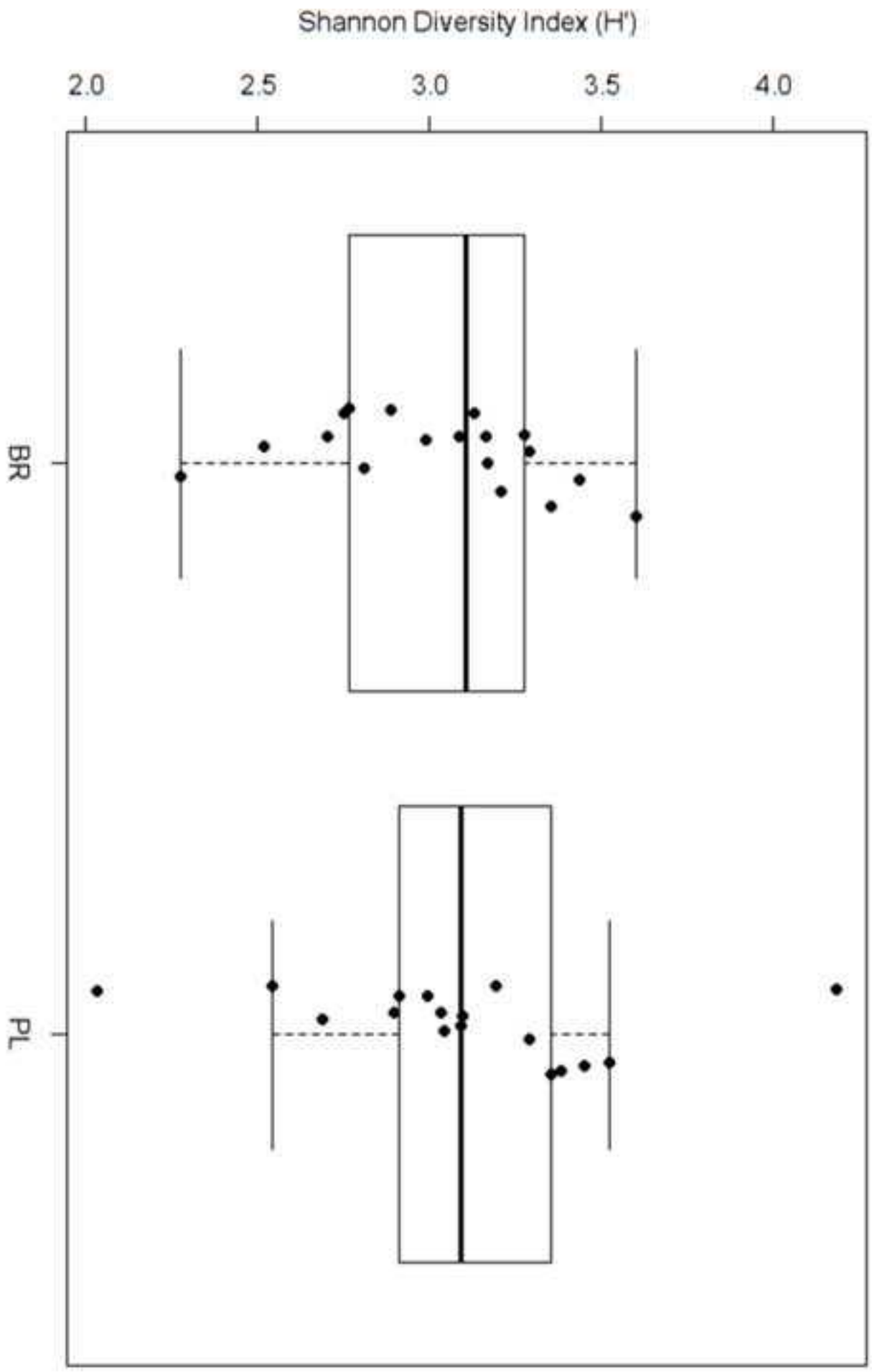

A
600
500
400
300
200
100
0
[NO3-] (μM)
*
PL
BR
B
1500
1000
500
0
[NO2-] (nM)
*
PL
BR
C
600
500
400
300
200
100
0
Δ [NO3-] (BR-PL) (μM)
0
25
50
75
100
Age (yrs)
D
1500
1000
500
0
Δ [NO2-] (BR-PL) (nM)
*
0
25
50
75
100
Age (yrs)


A
MAP (mmHg)
PL
BR
B
Δ MAP (mmHg)
Age (yrs)
C
r = -0.65 *
Δ MAP (mmHg)
Δ [NO2-]/dose (nM/mmol/kgBM)
D
SBP (mmHg)
E
Δ SBP (mmHg)
F
r = -0.73 *
G
DBP (mmHg)
H
Δ DBP (mmHg)
I
r = -0.57 *
J
PWV (m/s)
K
Δ PWV (m/s)
L
r = 0.65 *


PL
BR
Bacteroidetes *
Fusobacteria
Proteobacteria *
Firmicutes
Actinobacteria


A
BR
PL
P=0.01
NMDS2
NMDS1
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-1.0
-0.5
0.0
0.5
1.0


B
Old
Young
P=0.14
NMDS2
NMDS1
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-1.0
-0.5
0.0
0.5
1.0


Shannon Diversity Index (H')
2.0
2.5
3.0
3.5
4.0
BR
PL

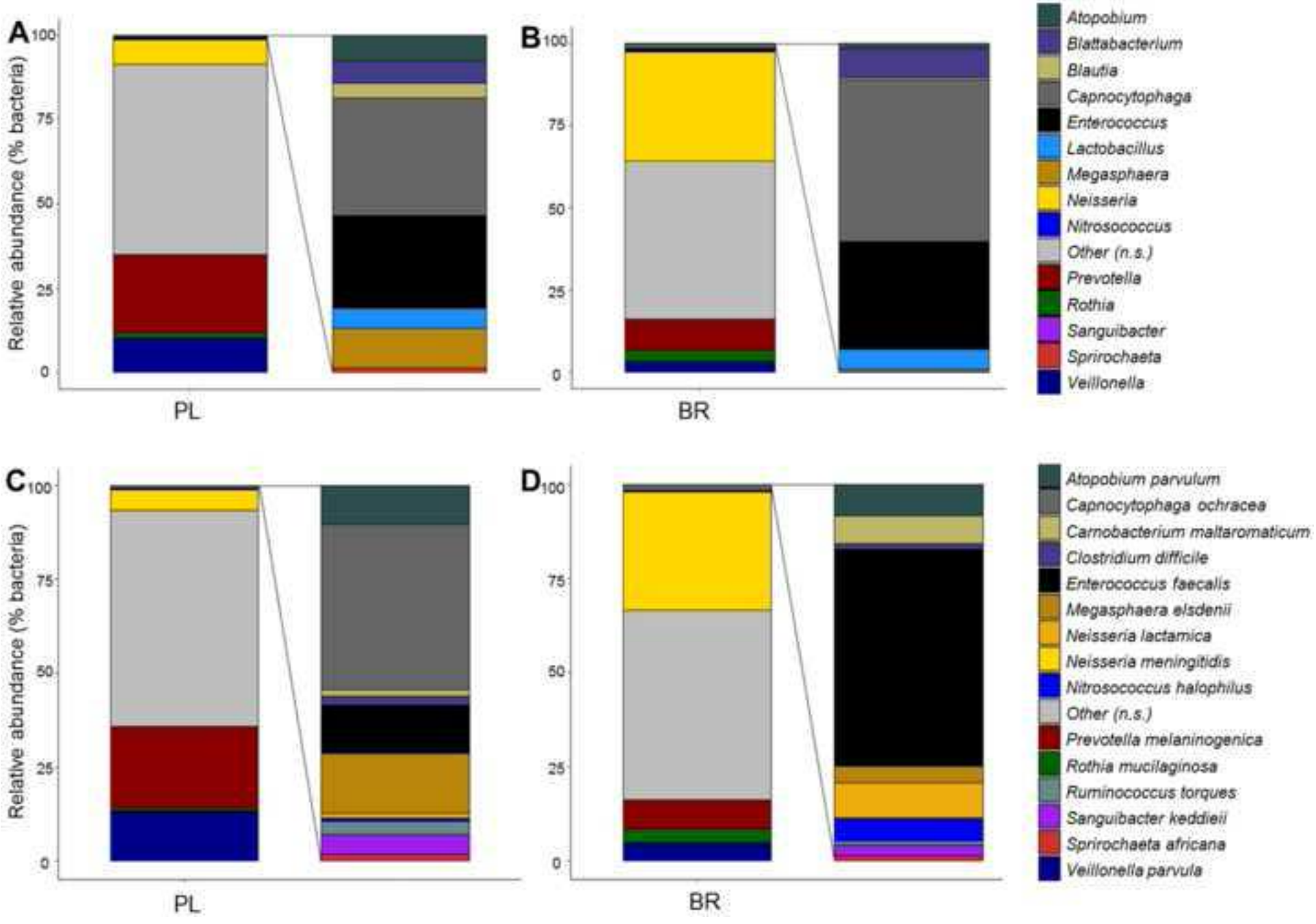

A
B
C
D
Relative abundance (% bacteria)
100
75
50
25
0
PL
BR
Atopobium
Blattabacterium
Blautia
Capnocytophaga
Enterococcus
Lactobacillus
Megasphaera
Neisseria
Nitrosococcus
Other (n.s.)
Prevotella
Rothia
Sanguibacter
Sprirochaeta
Veillonella
Atopobium parvulum
Capnocytophaga ochracea
Carnobacterium maltaromaticum
Clostridium difficile
Enterococcus faecalis
Megasphaera elsdenii
Neisseria lactamica
Neisseria meningitidis
Nitrosococcus halophilus
Other (n.s.)
Prevotella melaninogenica
Rothia mucilaginosa
Ruminococcus torques
Sanguibacter keddieii
Sprirochaeta africana
Veillonella parvula

Table S1. Barcode sequences for primers A and B.

Name	Description	Sequence (5′ to 3′)
A_FW1	PCR to amplify 16s v1-v3 amplicon (forward sequence)	CTCTTTCCCTACACGACGCTCTTCCG ATCTAGAGTTTGATCCTGGCTCAG
A_RV1	PCR to amplify 16s v1-v3 amplicon (reverse sequence)	CTGGAGTTCAGACGTGTGCTCTTCCG ATCTGTATTACCGCGGCTGCTGG
B_FW1	PCR to attach Illumina flowcell binding nucleotides and identifying barcodes (forward sequence)	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTT CCGATCT
B_RV1	PCR to attach Illumina flowcell binding nucleotides and identifying barcodes (reverse sequence)	CAAGCAGAAGACGGCATACGAGATX XXXXXXXXXXXXGTGACTGGAGTTCA GACGTGTGCTCTTCCGATCT *
*** Adapter bar codes for tongue swab samples:**		
		GGCCGGCTAGAT
		GGACGGCATCTA
		AAGGAAGGAGCG
		GGACGGCGCTCG
		CCGGACTCTCGA
		GGCCGGCCGAGC
		CCGGACTGAGCT
		GGACGCGGCAGT
		CCGGAGAAGTAA
		GGCCGCGCGTCA
		CCGGAGATCATT
		CCGGATCCTTAT
		CCGGATCGAATA
		GGACCGGCCATG
		GGACCGGTTGCA
		GGACCAAGGCGG
		CCGGTTGGTAGT
		GGACCAATTATT
*** Adapter bar codes for saliva samples:**		
		CCCAGAGAGCAA
		TCAGCATGCTGG
		AGGAGTCAGCAA
		AGTCGATGCTGG
		GAAGATCAGCAA

	GACTAATGCTGG
	CTTCTTCAGCAA
	CTGATATGCTGG
	CTAGGTTGGCAA
	CTCTGACACTGG
	TCGAATTGGCAA
	AGAGTACACTGG
	GATCCTTGGCAA
	TTCACTGACGGG
	CCGTTAAGGCAA
	AAGTGTGACTGG
	TTACCAAGGCAA
	ATCCAGTACGGG
	TATTTCCGGCAA
	TAGGTGTACCGG
	ATAAACCGGTAA
	CTAGAAGTCCGG
	GAGATTACGTAA
	GATCTAGTCCGG
	AGAGCTACGGAA
	TTGACCATGTGG
	CCCTTGGCCCAA
	GCATGACTGTGG
	ATTAATTCCCAA
	CGTACACTGTGG
	GCCGGTTCCCAA
	TTTCTCTAGTGG
	CCAGCGCGCCAA
	AAAGACTAGTGG