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Ageing modifies the oral microbiome, nitric oxide bioavailability and vascular responses to dietary nitrate supplementation

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ABSTRACT

This study evaluated whether changes in the oral microbiome in response to dietary nitrate and antiseptic mouthwash treatments were related to changes in nitric oxide bioavailability and vascular function. Thirty-nine young (18–30 years) and thirty-six older (67–79 years) males and females completed a placebo-controlled, double-blind cross-over intervention including three 2-week conditions separated by 2-week washouts: placebo beetroot juice (PL), nitrate-rich beetroot juice (BR) and antiseptic mouthwash (MW). The oral microbiomes of young and older adults responded differently to BR (post BR non-metric multidimensional scaling $P = 0.01$), while the oral microbiomes of both age groups were unaffected by PL and MW interventions. Older people, who had elevated baseline mean arterial pressure (MAP; 95 ± 9 mmHg) compared to young adults (87 ± 7 mmHg, $P < 0.001$), showed decreased brachial MAP (−4±4 mmHg, $P = 0.003$) after BR while this effect was absent in the young. Flow mediated dilatation (FMD) variables were not affected by the interventions in older adults, while in the young there was a difference in changes (from pre to post) in ΔFMD% between the MW and BR conditions ($P = 0.04$). Decreased blood pressure in older adults correlated with increased plasma nitrite concentration (change in central MAP vs. $[NO_2^-]$ $r = -0.41$, $P = 0.02$), which in turn correlated with decreases within the co-occurring module of bacteria dominated by the genus *Prevotella* (*P. intermedia* $r = -0.72$, $P = 0.001$; *P. dentalis* $r = -0.88$, $P < 0.0001$; *Crassaminicella* sp. *SY095* $r = -0.81$, $P < 0.0001$). Greater blood pressure benefits from supplemental dietary nitrate in older compared to younger people are mediated primarily by the suppression of potentially harmful oral bacteria, that have been associated with ammonia production.

1. Introduction

Imbalances in the oral microbial community, and poor dental health, have been associated with impaired cardiovascular health [1]. One mechanism that may link the oral microbiome to systemic health is its integral role in the nitrate-nitrite-nitric oxide ($^{\bullet}NO$) reduction pathway. This pathway crucially relies on the commensal oral bacteria to reduce nitrate, which can be obtained through a vegetable-rich diet, to nitrite which serves as an $^{\bullet}NO$ precursor in circulation and tissues [2,3]. Among many other physiological roles, $^{\bullet}NO$ regulates vascular endothelial function, and therefore blood pressure [4]. The ability to produce $^{\bullet}NO$ in the body through the classical pathway involving $^{\bullet}NO$ synthases (NOS) is impaired during ageing, and therefore the nitrate-nitrite-$^{\bullet}NO$ pathway has significant potential to compensate for dysfunctional NOS in older age [5].

Hypertension is the main modifiable risk factor for cardiovascular diseases. Epidemiological studies indicate that increased consumption of foodstuffs that naturally contain high amounts of inorganic nitrate (green leafy vegetables and some fruits, such as in the 'Mediterranean' or 5-a-day diets) may protect against adverse cardiovascular events [6,7]. Supplemental dietary nitrate has been shown to reduce blood pressure in young and older adults [8–10], while the use of chlorhexidine

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containing mouthwash, which suppresses oral bacteria, elevated blood pressure in healthy adults (mean age 24 years [11]) and in older people with treated hypertension (mean age 65 years [12]). An individual's ability to benefit from ingested nitrate may be affected not only by the number of oral bacteria but also by the composition of their oral microbiome. We showed that healthy older adults who had reached the seventh decade of life with no chronic disease exhibited a significantly greater increase in plasma nitrite concentration and a greater reduction in blood pressure in response to nitrate ingestion compared to young adults [13]. These findings suggested that the symbiotic relationship between the human host and the nitrite-producing oral microbiome might change with ageing, and that healthy ageing may be associated with a high capacity for nitrite production from dietary nitrate.

We previously explored causal relationships between the changes in oral microbiome and physiological and cognitive function in healthy older people in response to dietary nitrate supplementation [14]. Microbiome co-occurrence network analysis revealed two distinct microbiome modules of co-occurring bacteria, that benefitted from nitrate supplementation, and that showed stable relationships with cardiovascular (module dominated by *Rothia-Streptococcus*) and cognitive indices of health (module dominated by *Neisseria-Haemophilus)* across high-nitrate and placebo conditions [14]. Conversely, a microbiome module dominated by *Prevotella-Veillonella,* that has been associated with inflammatory metabolism [15] and has been found to be dominant in systemic diseases including rheumatoid arthritis [16], pneumonia [17], COVID-19 [18] and hypertension [19], was diminished in healthy older people after nitrate supplementation [14]. These findings indicate that dietary nitrate is a powerful modulator of the oral microbiome in older people with significant implications for systemic health.

In the present study, we investigated the influence of the oral microbiome as a mediator of the effects on vascular function and $^{\bullet}$NO bioavailability that may occur following the consumption of nitrate-rich beetroot juice, nitrate-depleted beetroot juice placebo, and antiseptic mouthwash, in young and older adults. The primary objective was to assess whether changes in oral microbiome modules were related to changes in $^{\bullet}$NO bioavailability (plasma nitrate and nitrite concentrations) and indices of vascular function (brachial and central blood pressure; brachial artery flow mediated dilatation, FMD; pulse wave velocity, PWV) in response to dietary interventions, and whether these relationships differ between young and older healthy adults. The secondary objective was to establish whether changes in the oral microbiome, $^{\bullet}$NO bioavailability and vascular function following dietary nitrate supplementation or antiseptic mouthwash use differ between young and older healthy adults.

2. Results

2.1. Comparison of oral microbiomes between placebo, nitrate and mouthwash conditions

We assessed the oral microbiome from tongue swabs of 39 young adults and 36 older adults before and after 14 days of dietary inorganic nitrate (BR; nitrate-rich beetroot juice, ~750 mg NO_3^-/d) and placebo (PL; nitrate-depleted beetroot juice, ~1 mg NO_3^-/d) supplementation in a randomised, placebo-controlled cross-over design. A CONSORT chart indicating participant screening, randomization and allocation, and cases lost to follow-up during the trial, is shown in Supplemental Fig. S1. Following the completion of the nitrate and placebo conditions, microbiome samples were also collected before and after 14 days of twice-daily antiseptic mouthwash (MW) use. Baseline participant characteristics are shown in Table 1. There were no serious adverse events in response to the intervention and participants' self-reported adherence to supplementation and mouthwash use was 100 %. Amplicons of the hypervariable V1-V3 region of bacterial 16S rRNA gene were amplified and sequenced by synthesis [20]. Sequences were aligned with 1819 bacterial operational taxonomic units (OTU) in 446 tongue swab samples. There was an average of 205,497 reads mapped to the Bacteria domain. For the weighted gene co-expression network analysis (WGCNA), the raw OTU counts were log transformed and rare OTUs removed if there were less than 10 log transformed counts. OTUs were also removed if there were over 36 missing (or 0) counts in each group. There were 486 bacterial counts remaining for analysis. Species-level taxonomic units were assigned using a 97 % gene similarity cut-off for pairwise-identity comparisons. Participants were excluded from the final analyses if there was incomplete microbiome data across all three conditions. Thus, in total seven samples were excluded, so the final data set included microbiome data for 36 young adults and 35 older adults.

Table 1
Participant baseline characteristics presented as mean ± SD (range). BMI, body mass index; SBP, brachial systolic blood pressure; DBP, brachial diastolic blood pressure; MAP, brachial mean arterial pressure; SFR-Q, salivary flow rate questionnaire.

	Young (n = 39)	Older (n = 36)	*P*
Sex, M/F	20/19	12/24	0.120
Age, y	24 ± 3 (18–30)	71 ± 3 (67–79)	<0.001
Height, m	1.73 ± 0.09 (1.56–1.89)	1.66 ± 0.09 (1.50–1.82)	0.001
Body mass, kg	71.5 ± 11.9 (48.8–98.0)	68.3 ± 11.2 (48.3–92.0)	0.224
BMI, kg/m^2	23.8 ± 2.9 (18.0–31.2)	24.5 ± 2.8 (19.5–31.2)	0.261
SBP (mmHg)	117 ± 9 (96–135)	126 ± 14 (90–151)	<0.001
DBP (mmHg)	72 ± 8 (57–91)	80 ± 7 (62–92)	<0.001
MAP (mmHg)	87 ± 7 (73–104)	95 ± 9 (71–110)	<0.001
SFR-Q mean score	1.9 ± 0.4 (1.1–2.7)	1.0 ± 2.9 (1.8–0.5)	0.250

PL and BR conditions did not alter the oral microbiome alpha diversity in either the young or the older group, while the 2-week MW intervention reduced the Shannon Diversity index in the young (P = 0.004), but not the older adults (P = 0.78) (Fig. 1A). There were no differences in species richness (Chao-1 index, number of OTUs per sample) between conditions within the young (P = 0.65) or older groups (P = 0.70), or between age groups (Fig. 1B). The tongue microbiome compositions were compared across time and between conditions using non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity (ADONIS, with FDR post hoc correction). There were no differences in oral microbiomes at baseline (pre-PL, pre-BR or pre-MW) between young and older groups (Fig. 2A, B and 2C). The PL and MW interventions did not alter the overall microbiome compositions in young or older groups, but in BR, NMDS revealed a significant change from pre to post supplementation within both young (P = 0.0015) and older (P = 0.0015) groups (Fig. 2D and E). NMDS also showed that post-BR tongue microbiomes differed between young and older groups (P = 0.01) with no significant differences post-MW or post-PL (Fig. 2F, G, 2H) indicating profound, but different, responses to supplemental nitrate across age groups. Post-BR, 144 taxonomic units differed between young and older groups: 68 taxonomic units had a greater abundance in the older group, with 33 of these belonging in Proteobacteria (including *Neisseria*), while 76 taxonomic units had a lower abundance in the older group, with 38 of these belonging in Firmicutes (Supplemental Fig. S2).

Significant species level changes occurred according to linear discriminant analysis effect size (LEfSe) analysis from pre to post BR in young and older groups (Fig. 3). Common responses to BR shared by the young and older groups included significant increases in *Capnocytophaga (C. gingivalis, C. leadbetteri, C.* sp *FDAARGOS 737), Lautropia (L. mirabilis), Neisseria* (*N. bacilliformis, N. flavescens, N. elongata*) and *Ottowia (O.* sp. *oral taxon 894)*; however, each of these OTUs was more abundant in the older group compared to the young group post-BR (P < 0.05 for all; Fig. 3). Significant decreases common for young and older groups included *Butyrivibrio (B. fibrisolvens), Gemella (G. sanguinis), Lachnospira (L. eligens), Lancefieldella (L. parvula), Megasphaera, Mogibacterium (M. diversum)* and *Olsenella* (Fig. 3), with *Lachnospira* less abundant in the older group compared to the young group post-BR (P = 0.004;

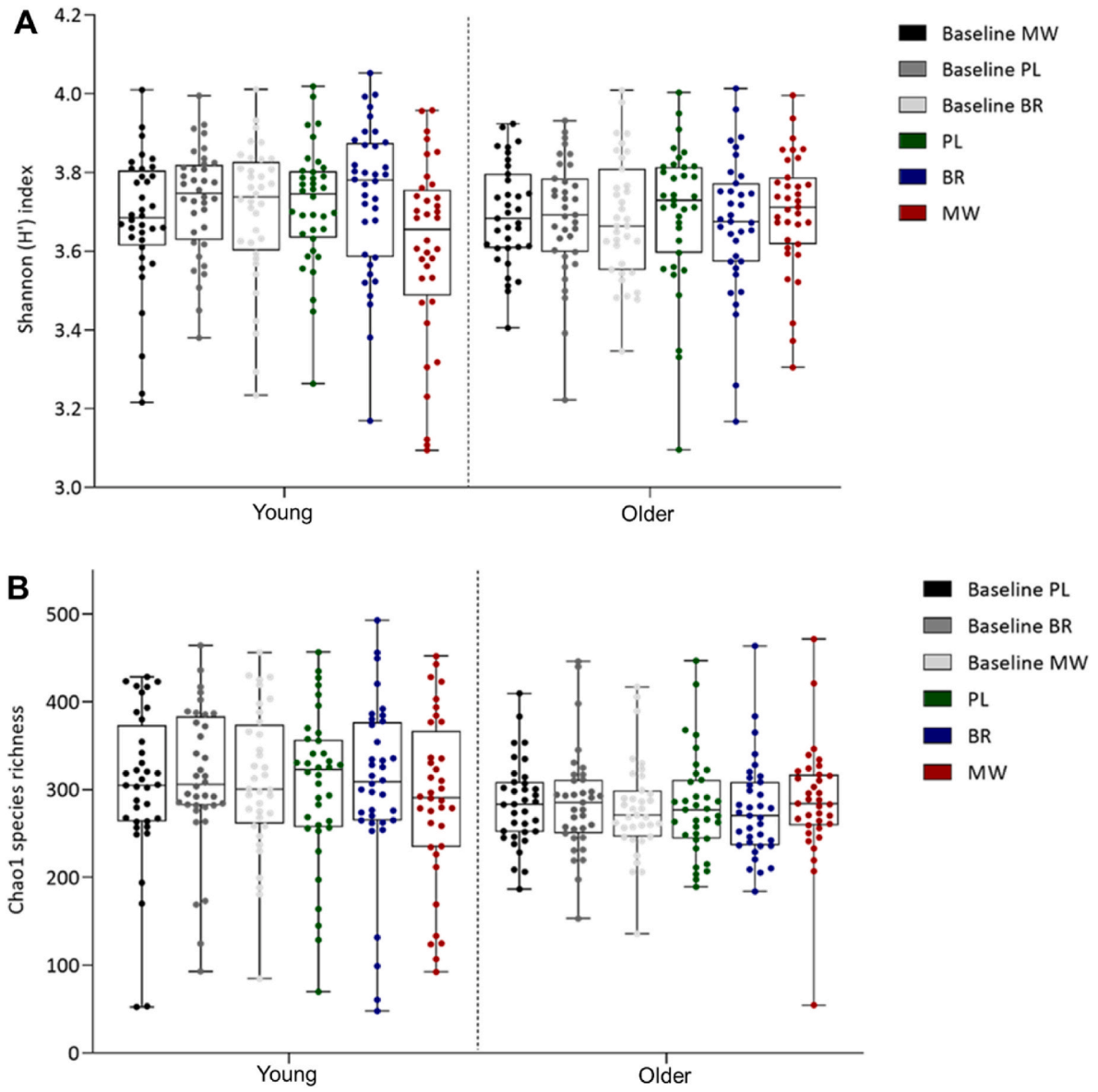

Fig. 1. Shannon-Wiener diversity index (A) and Chao 1 index of species richness (B) pre and post 2 weeks of placebo (PL), nitrate (BR) and mouthwash (MW) interventions in young and older healthy adults. * = The MW intervention reduced the Shannon Diversity index in young ($P = 0.004$) but not in the older adults ($P = 0.78$), while PL and BR interventions did not alter diversity in either the young or the older group. There were no differences between or within conditions in Chao-1 index.

Supplemental Tables S1 and S2). Discrete changes seen in the old but not in the young group included increases in *Cardiobacterium (C. hominis), Eikenella (E. corrodens), Enterococcus, Haemophilus* and *Rothia (R. aeria, R. mucilaginosa)*, and decreases in *Crassaminicella (C.* sp. *SY095), Dialister* and *Tannerella (T.* sp. *oral taxon HOT 286)* (Supplemental Table S1 and S2). Changes in the young group that were absent in the older group included increases in *Aggregatibacter* and *Tenacibaculum (T. dicentrarchi)*, and decreases in *Actinomyces (A.* sp. *oral taxon 169)*, *Anaerotignum (A. propionicum), Lachnoclostridium (L. phytofermentans)* and *Stackebrandtia* (*S. nassauensis*) (Supplemental Tables S1 and S2), with *Anaerotignum* and *Stackebrandtia* less abundant in the older group compared to the young group post-BR.

2.2. Physiological responses to placebo, nitrate and mouthwash conditions within young and older age groups

Before comparing responses *between* age groups, changes in $^{\bullet}$NO biomarker, supine blood pressure, PWV and FMD variables from pre-to post-intervention *within* young and older groups were analysed using ANOVAs across time and condition with Bonferroni *post hoc* corrections. Plasma [NO_3^-] and [NO_2^-] were significantly elevated in BR compared to PL and MW conditions in both the young (Table 2) and the older group (Table 3). [NO_3^-] and [NO_2^-] were ~10-fold greater following BR compared to both PL and MW conditions. Notably, however, plasma [NO_3^-] (but not [NO_2^-]) also showed a significant increase from pre to post PL condition in the young and older group (Table 2, Table 3). In the older group, there were significant reductions in both brachial and central supine blood pressure variables from pre-to post-BR, and the post-BR blood pressure was lower than post-MW, but not lower compared to post-PL (Table 3). There were no significant effects on FMD variables in the older group. Conversely, there were no effects on supine blood pressure variables in the young group but there was a significant decrease in ΔFMD (mm) from pre to post MW condition and a difference between pre-MW and pre-PL (Table 2). Plasma [NO_2^-] correlated with peak diameter ($r = 0.24$, $P = 0.009$, n = 117) and plasma [NO_3^-] correlated with ΔFMD (mm) ($r = 0.24$, $P = 0.01$, n = 114) across MW

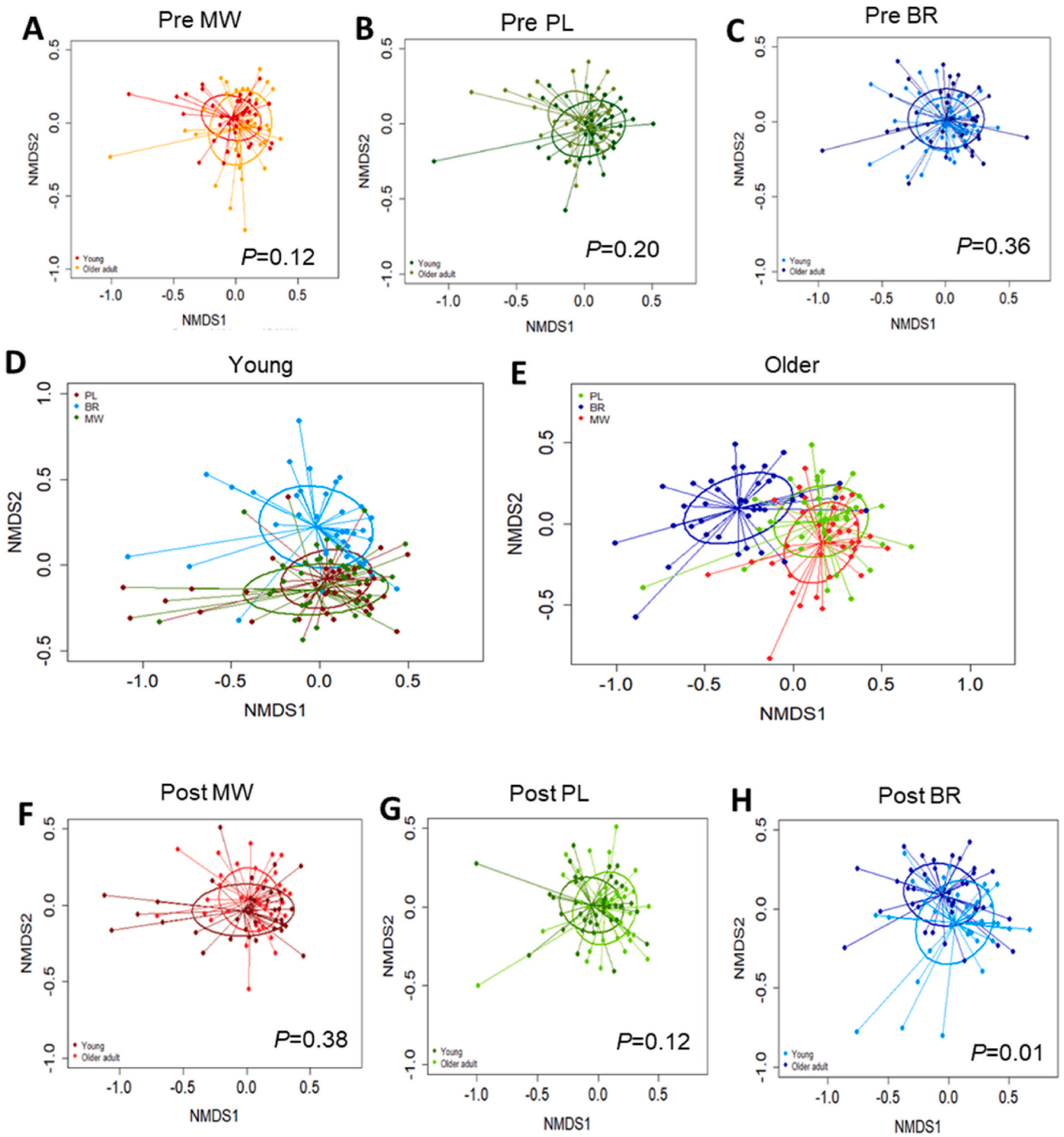

Fig. 2. Non-metric multidimensional scaling (NMDS) indicated no differences in oral microbiomes at baseline (pre-PL, pre-BR or pre-MW) between young and older groups (panels A, B and C). Post-BR oral microbiome differed from post-PL and post-MW in both young and older age groups (panels D and E). There were no differences between young and older groups post MW and post-PL (panels F and G) but post-BR oral microbiomes differed between the age groups (panel H).

and BR conditions when young and older groups were combined. Carotid-femoral and carotid-radial PWV were unaffected by PL, BR and MW conditions in both age groups (Table 2, Table 3). The older group showed an increase in heart rate from pre-to post-BR (Table 3) which correlated with the decrease in SBP ($r = -0.53$, $P < 0.05$).

2.3. Between age group comparisons of changes in NO bioavailability and physiological function in response to placebo, nitrate and mouthwash treatments

The change (Δ) from pre-to post-condition in plasma [NO_3^-] was greater in BR compared to PL and MW in the young and older groups (Fig. 4B and C). Δ [NO_2^-] was greater in BR compared to PL and MW in the older, but not in the young group (Fig. 4E and F). Both Δ[NO_3^-] and

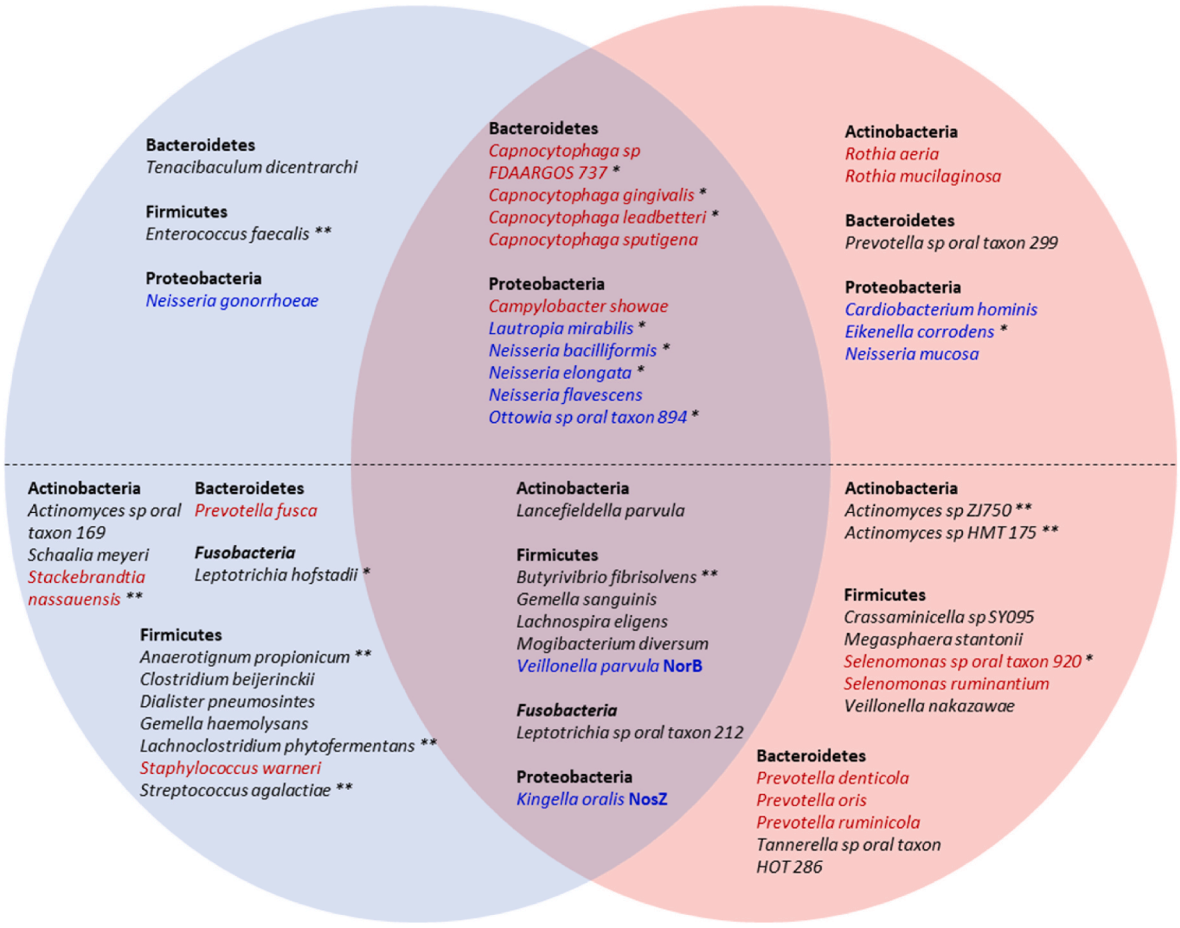

Fig. 3. Species level changes in relative abundances of oral bacteria from pre to post dietary nitrate supplementation in young (blue sphere) and older adults (red sphere). Species above the horizontal dashed line increased and species below the dashed line decreased. The overlapping area (purple) indicates the species that changed in both young and older groups. * Indicates greater relative abundance in older group than young post-BR, ** indicates greater relative abundance in young than older group post-BR. Significance was determined as logarithmic LDA score threshold of 2 in linear discriminant analysis (LDA) effect size (LEfSe). Red font indicates bacteria that have been shown to encode proteins related to the DNRA pathway (NrfA or NirB) and blue font those bacteria with denitrification pathway genes (NirK, NirS, NorB or NosZ) based on a search performed on Uniprot (https://www.uniprot.org/). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$\Delta[NO_2^-]$ in BR were greater in the older group compared to young (Fig. 4C and F).

At baseline, the older group had a significantly higher supine blood pressure than the young group (Table 1). There were no significant differences between PL, BR and MW conditions in Δ brachial (Fig. 5A–F) or Δ central (Fig. 6A–F) supine blood pressure from pre-to post-intervention within the young group. In the older group, there was a greater decrease in brachial supine systolic blood pressure (SBP) in BR compared to MW and PL in (Fig. 5B), and greater decreases in brachial and central diastolic blood pressure (DBP) in BR compared to MW. There was a greater decrease in both brachial and central supine SBP in the older group compared to the young group in the BR condition (Figs. 5B and 6B). Baseline central supine SBP correlated with ΔSBP from pre-to post-BR across young and older groups ($r = -0.45$, $P < 0.001$). In the older group, changes in central supine blood pressure from pre-to post-BR condition correlated with the change in plasma $[NO_2^-]$ (DBP $r = -0.38$, $P = 0.035$; MAP $r = -0.41$, $P = 0.021$). There were no significant correlations between Δ plasma $[NO_2^-]$ and changes in any vascular variables in the young group.

There were no differences between conditions or age groups in ΔFMD diameter expressed in mm (Fig. 7A and B). There was a difference in changes (from pre to post) in ΔFMD% between the MW and BR conditions within the young group only ($P = 0.04$, Fig. 7C and D). The decrease in FMD% from pre-to post-MW intervention was 12 % ($P = 0.03$) in the young group and 23 % ($P = 0.07$) in the older group.

2.3.1. *Microbiome modules of co-occurring bacteria*

Using a signed network where modules represented positively correlated taxonomic units (OTUs) and a thresholding value of >0.8, a total of seven microbiome modules (MM) were identified in the young group and eight different microbiome modules were found in the older group (Supplemental Fig. 3). The size of the modules ranged from 6 to 214 taxonomic units in the young, and 20 to 102 in the older group. In the older group, most of the OTUs that increased with nitrate supplementation clustered together in $MM2_O$ and $MM7_O$ ($_O$ indicating the older group), while the OTUs that decreased with nitrate supplementation clustered in $MM6_O$ (Supplemental Table S1). In the young group, OTUs that thrived during nitrate supplementation clustered in $MM6_Y$ ($_Y$ indicating the young group), while those that decreased were predominantly in $MM5_Y$ (Supplemental Table S2).

A WGCNA consensus network was applied on 8 modules in the older and 7 in the young, against 22 physiological variables across 4 time points (pre and post PL and BR), a total of 704 eigengene correlations in older and 616 in the young group. There were no significant consensus correlations in the young group, but in the older group the WGCNA four-layer consensus indicated a tendency for correlation between central supine mean arterial pressure (cMAP) and $MM2_O$ ($r = 0.50$, $P = 0.05$), a module containing 16 different genera (Supplemental Table S1) and

Table 2
Physiological variables (mean ± standard deviation) measured pre and post placebo (PL), nitrate (BR) and antibacterial mouthwash (MW) conditions in the young group. *P*-values are given for time-by-condition interaction effect for each variable. * = different from pre within same condition, a = different from same time point in PL, b = different from same time point in BR, c = different from same time point in MW. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PWV, pulse wave velocity; WSR, wall shear stress; aucttp, area-under-curve for time-to-peak dilation.

	Placebo		Nitrate		Mouthwash		*P*-value
	Pre	Post	Pre	Post	Pre	Post	
Body mass (kg)	71.9 ± 11.7	71.9 ± 11.0	71.5 ± 11.3	71.7 ± 11.2	71.5 ± 10.9	71.2 ± 10.7 *	**0.01**
Saliva flow rate (g/min)	0.45 ± 0.25	0.48 ± 0.29	0.48 ± 0.26	0.52 ± 0.29	0.54 ± 0.34	0.54 ± 0.34	0.48
Plasma [NO_3^-] (μM)	51 ± 28	76 ± 60 *bc	58 ± 42	1034 ± 274 *ac	45 ± 19	45 ± 17 ab	**<0.001**
Plasma [NO_2^-] (nM)	125 ± 54	170 ± 145 b	163 ± 98	448 ± 300 *ac	126 ± 35	120 ± 46 b	**<0.001**
Saliva [NO_3^-] (μM)	1576 ± 1446	1916 ± 1507 *b	1857 ± 1497	17724 ± 5620 *	1365 ± 941	1520 ± 1059 b	**<0.001**
Saliva [NO_2^-] (nM)	167 ± 132	289 ± 372 *b	173 ± 102	1667 ± 1492 *	170 ± 155	209 ± 172 b	**<0.001**
Heart rate (bpm)	69 ± 13	71 ± 12	70 ± 14	71 ± 13	68 ± 13	69 ± 11	0.67
Brachial SBP (mmHg)	115 ± 9	113 ± 9	115 ± 7	114 ± 8	114 ± 8	114 ± 9	0.11
Brachial DBP (mmHg)	64 ± 7	63 ± 6	63 ± 7	63 ± 6	63 ± 7	63 ± 7	0.57
Brachial MAP (mmHg)	80 ± 7	78 ± 7	79 ± 7	78 ± 6	79 ± 7	79 ± 7	0.18
Brachial pulse pressure (mmHg)	52 ± 7	51 ± 9	51 ± 7	50 ± 7	51 ± 6	51 ± 6	0.45
Central SBP (mmHg)	95 ± 8	93 ± 8	94 ± 7	93 ± 7	94 ± 8	95 ± 8	0.06
Central DBP (mmHg)	64 ± 7	63 ± 6	64 ± 8	63 ± 6	63 ± 7	63 ± 7	0.40
Central MAP (mmHg)	78 ± 8	76 ± 7	77 ± 8	76 ± 7	77 ± 7	77 ± 8	0.16
Central pulse pressure (mmHg)	32 ± 4	31 ± 5	31 ± 4	30 ± 4	31 ± 4	32 ± 4	0.15
Carotid-radial PWV (cm/s)	7.7 ± 1.0	7.4 ± 0.8	7.5 ± 1.0	7.4 ± 1.1	7.3 ± 0.9	7.2 ± 0.8	0.70
Carotid-femoral PWV (cm/s)	5.8 ± 0.6	5.7 ± 0.7	5.9 ± 0.7	5.7 ± 0.7	5.9 ± 0.8	5.7 ± 0.8	0.89
FMD variables							
WSR baseline (1/s)	43 ± 19	46 ± 18	57 ± 34	55 ± 35	52 ± 23	51 ± 26	0.89
WSR peak (1/s)	558 ± 152	568 ± 149	573 ± 166	556 ± 141	572 ± 144	564 ± 128	0.65
Δ WSR peak-baseline (1/s)	515 ± 146	523 ± 143	516 ± 157	501 ± 126	520 ± 137	513 ± 125	0.69
WSR aucttp (au)	12024 ± 3219	11964 ± 3053	11713 ± 4027	11461 ± 3239	12390 ± 3272	11925 ± 2596	0.91
Diameter baseline (mm)	3.3 ± 0.5	3.3 ± 0.5	3.3 ± 0.6	3.2 ± 0.6	3.3 ± 0.5	3.3 ± 0.5	0.50
Diameter peak (mm)	3.4 ± 0.5	3.5 ± 0.5	3.4 ± 0.6	3.4 ± 0.6	3.5 ± 0.5	3.5 ± 0.5	0.88
Δ Diameter (mm)	0.17 ± 0.09c	0.18 ± 0.09	0.17 ± 0.08	0.18 ± 0.09	0.21 ± 0.11a	0.18 ± 0.09 *	**0.022**
Δ Diameter (%)	5.5 ± 3.2c	5.7 ± 3.4	5.3 ± 2.9	5.9 ± 3.5	6.6 ± 3.9a	5.8 ± 3.2	**0.025**
Time to peak diameter (s)	45 ± 15	42 ± 10	44 ± 12	50 ± 27	44 ± 11	44 ± 11	0.24

including *Actinomyces, Capnocytophaga, Gemella, Rothia, Streptococcus* and *Tannerella* species.

We also correlated the changes (Δ) in •NO bioavailability and physiological function against the changes in OTUs in the BR condition. These Δ correlations revealed that in the older group, there were correlations between changes in plasma [NO_2^-] and OTUs in $MM3_O$ (*Selenomonas* oral taxon 126, $r = -0.84$), $MM5_O$ (*Neisseria meningitidis, r=-0.71)*, $MM6_O$ *Crassaminicella* sp. *SY095,* $r = -0.81$, FDR<0.0001; *Prevotella dentalis,* $r = -0.88$, FDR<0.0001; and *Prevotella intermedia,* $r = -0.72$, FDR = 0.001), and $MM8_O$ (*Treponema PMZ 838,* r = 0.62, FDR<0.001); *T. marseille Q4132,* $r = 0.80$, FDR = 0.04). $MM6_O$ was the only module where significant correlations with plasma Δ[NO_2^-] coincided with sensitivity to nitrate (*i.e.*, significant change in OTUs detected by LEfSe). There were no significant Δ correlations in the young group between changes in oral microbiome and •NO biomarkers.

3. Discussion

The present study is the most comprehensive investigation to date examining the influence of dietary nitrate-induced changes in the oral microbiome on cardiovascular outcomes in humans. The principal original findings of this study were that the oral microbiomes of healthy young and older adults responded differently to 2 weeks of nitrate supplementation, and that in older adults the elevation of plasma nitrite concentration was associated with a decrease in the oral microbiome module dominated by *Prevotella* and *Veillonella*. The older group had

Table 3
Physiological variables (mean ± standard deviation) measured pre and post placebo (PL), nitrate (BR) and antibacterial mouthwash (MW) conditions in the older group. *P*-values are given for time-by-condition interaction effect for each variable. * = different from pre within same condition, a = different from same time point in PL, b = different from same time point in BR, c = different from same time point in MW. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PWV, pulse wave velocity; WSR, wall shear stress; aucttp, area-under-curve for time-to-peak dilation.

	Placebo		Nitrate		Mouthwash		*P*-value
	Pre	Post	Pre	Post	Pre	Post	
Body mass (kg)	67.8 ± 11.1	67.8 ± 11.0	67.8 ± 11.0	68.0 ± 10.8	67.6 ± 11.1	67.5 ± 10.8	0.12
Saliva flow rate (g/min)	0.41 ± 0.22	0.42 ± 0.24	0.39 ± 0.22	0.44 ± 0.22	0.45 ± 0.29	0.45 ± 0.25	0.29
Plasma [NO_3^-] (μM)	52 ± 20	87 ± 67 *bc	61 ± 29	1353 ± 416 *ac	51 ± 20	54 ± 21 ab	**<0.001**
Plasma [NO_2^-] (nM)	131 ± 40	185 ± 146 b	139 ± 58	1164 ± 1232 *ac	145 ± 59	141 ± 49 b	**<0.001**
Saliva [NO_3^-] (μM)	1402 ± 1664	1777 ± 1523 b	1379 ± 859	18030 ± 6561 *ac	1155 ± 797	1919 ± 1231 *b	**<0.001**
Saliva [NO_2^-] (nM)	179 ± 97	355 ± 460 *b	214 ± 153	2203 ± 1535 *ac	191 ± 145	287 ± 214 *b	**<0.001**
Heart rate (bpm)	65 ± 10	65 ± 7c	63 ± 7	68 ± 9 *c	62 ± 6	62 ± 6 ab	**0.002**
Brachial SBP (mmHg)	133 ± 11	130 ± 13	134 ± 10	127 ± 12 *c	133 ± 12	134 ± 13 b	**0.001**
Brachial DBP (mmHg)	71 ± 6	70 ± 7 *	71 ± 6	69 ± 6 *c	71 ± 6	71 ± 6 b	**0.030**
Brachial MAP (mmHg)	93 ± 7	91 ± 8 *c	93 ± 7	89 ± 8 *c	93 ± 8	93 ± 8 ab	**0.003**
Brachial pulse pressure (mmHg)	61 ± 9	60 ± 9	62 ± 9	57 ± 9 *c	62 ± 9	63 ± 10 b	**0.013**
Central SBP (mmHg)	127 ± 11	124 ± 12 *	127 ± 10	120 ± 12 *c	127 ± 13	128 ± 13 b	**0.003**
Central DBP (mmHg)	73 ± 6	71 ± 7 *	72 ± 6	70 ± 7 *	71 ± 7	72 ± 6	**0.010**
Central MAP (mmHg)	94 ± 7	92 ± 8 *	94 ± 7	90 ± 8 *c	93 ± 8	95 ± 7 b	**0.002**
Central pulse pressure (mmHg)	54 ± 9	53 ± 9	55 ± 9	50 ± 10	55 ± 9	56 ± 10	0.10
Carotid-radial PWV (cm/s)	8.7 ± 0.9	8.6 ± 0.8	8.4 ± 0.7	8.4 ± 0.9	8.6 ± 0.9	8.7 ± 0.9	0.60
Carotid-femoral PWV (cm/s)	9.1 ± 1.4	9.0 ± 1.3	9.1 ± 1.4	8.9 ± 1.3	9.3 ± 1.7	9.5 ± 1.6	0.33
FMD variables							
WSR baseline (1/s)	50 ± 23	45 ± 21	50 ± 30	45 ± 20	47 ± 37	35 ± 16	0.39
WSR peak (1/s)	519 ± 145	500 ± 157	505 ± 148	482 ± 170	500 ± 123	469 ± 104	0.91
Δ WSR peak-baseline (1/s)	469 ± 141	455 ± 150	455 ± 135	437 ± 157	452 ± 111	435 ± 102	0.98
WSR aucttp (au)	10851 ± 3498	10266 ± 4008	10704 ± 3457	10162 ± 4440	10631 ± 3632	9627 ± 2819	0.81
Diameter baseline (mm)	3.5 ± 0.7	3.5 ± 0.6	3.5 ± 0.7	3.6 ± 0.7	3.5 ± 0.6	3.5 ± 0.7	0.40
Diameter peak (mm)	3.6 ± 0.7	3.6 ± 0.6	3.6 ± 0.7	3.7 ± 0.7	3.6 ± 0.7	3.6 ± 0.7	0.47
Δ Diameter (mm)	0.11 ± 0.08	0.11 ± 0.06	0.11 ± 0.07	0.10 ± 0.07	0.12 ± 0.06	0.09 ± 0.07	0.33
Δ Diameter (%)	3.3 ± 2.2	3.3 ± 1.9	3.2 ± 2.2	3.1 ± 2.4	3.4 ± 1.6	2.5 ± 1.9	0.16
Time to peak diameter (s)	47 ± 18	49 ± 24	53 ± 25	49 ± 20	47 ± 24	45 ± 17	0.37

higher baseline blood pressure compared to young controls and showed clinically relevant ~7 mmHg reductions in systolic blood pressure. There were, however, no blood pressure effects following the high-nitrate diet in healthy young adults, whose baseline blood pressure was in the normal range for their age. The overall compositions of oral microbiomes were unaffected by 2-week placebo and antiseptic mouthwash interventions in both young and older adults. However, twice-daily use of antiseptic mouthwash over 2 weeks decreased oral microbiome diversity and reduced vascular reactivity (as indicated by FMD) compared to 2 weeks of high-nitrate diet in healthy young adults. Collectively, these findings suggest that ageing modulates responsiveness to dietary nitrate and mouthwash interventions, and that the oral microbiome and the nitrate-nitrite-•NO pathway mediates the greater effects of dietary nitrate (in terms of •NO biomarkers and blood pressure) in healthy older people compared to young controls ([13]; present study).

The microbiome composition of healthy older adults did not differ from young controls at baseline prior to placebo, high-nitrate, or mouthwash conditions according to non-metric multidimensional scaling analysis. Dietary nitrate supplementation significantly altered the oral microbiomes in both young and older groups, but there were marked differences in the responses between the age groups. Similar to previous studies in older people [14,21], there were increases within genera *Neisseria*, *Haemophilus* and *Rothia*, and decreases in *Gemella*, *Megasphaera*, *Prevotella*, *Tannerella*, and *Veillonella* in the older group in response to dietary nitrate supplementation. The young group also

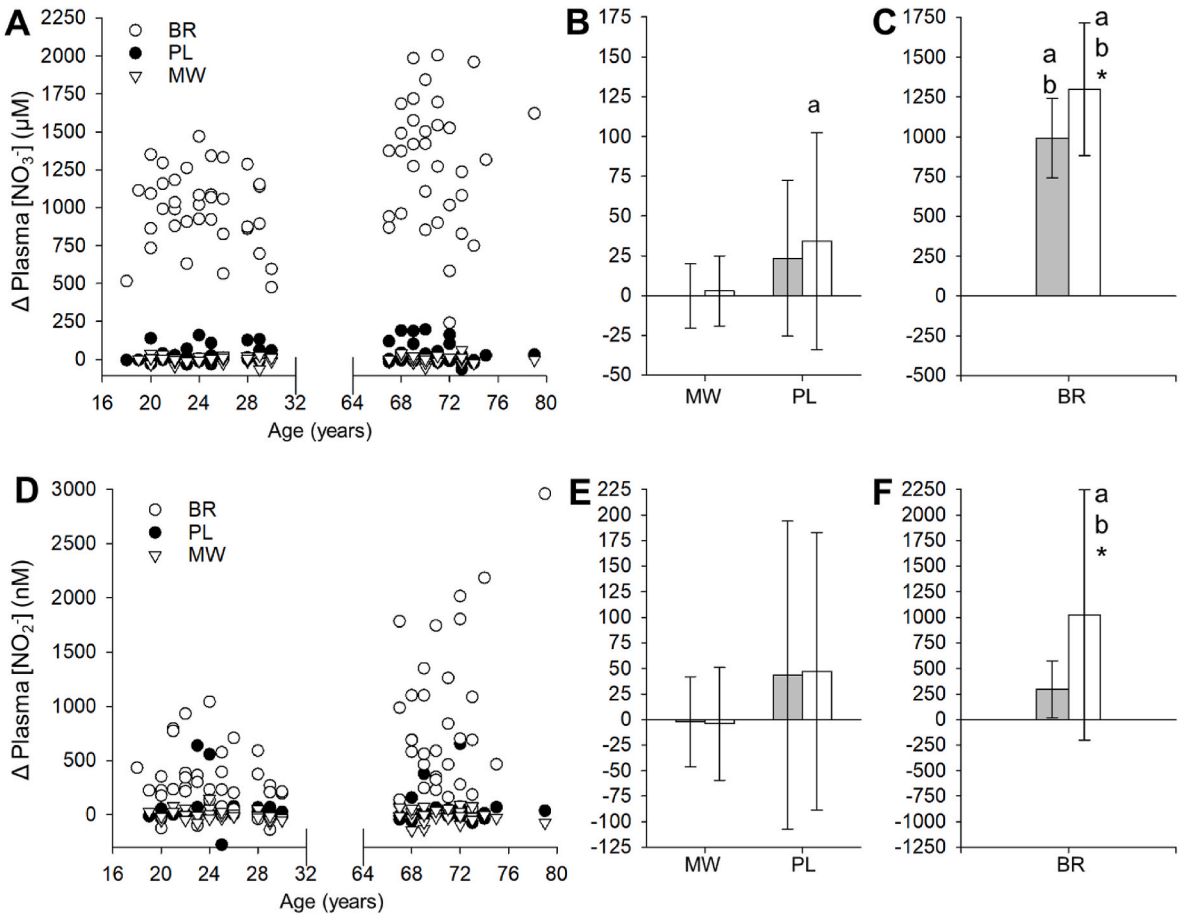

Fig. 4. Changes (Δ) in plasma nitrate concentration ([NO_3^-], panels A, B and C), and plasma nitrite concentration ([NO_2^-], panels D, E and F) during 2 weeks of placebo (PL), nitrate (BR) and mouthwash (MW) interventions. Notice the 10-fold difference in the y-axis scale between panels B and C, and panels E and F. * = within-condition difference between young (grey bars) and older (white bars); a = different from MW within the age group; b = different from PL within age group. Error bars indicate standard deviations.

showed an increase in *Neisseria* and decreases in *Megasphaera* and *Gemella*, but following the high-nitrate diet the older group had a greater relative abundance of *Neisseria* than the young (26 *vs* 18 %). These discrete changes in the oral microbiomes of young and older adults occurred alongside a greater increase in the mean plasma nitrite concentration in the older group compared to the young group, and a significant decrease in blood pressure in the older group which was absent in the young group. In the older group, the increase in plasma nitrite concentration correlated with the decrease in blood pressure and with decreases in oral bacteria *Crassaminicella* sp. *SY095, Prevotella dentalis, Prevotella intermedia* and *Selenomonas* sp. oral taxon 126. Of note, *Prevotella dentalis*, *Prevotella intermedia* and *Selenomonas.* oral taxon 126 are known to encode ammonia forming nitrite reductases of the dissimilatory nitrate reduction to ammonia (DNRA) pathway (based on search performed on https://www.uniprot.org/). This DNRA pathway 'short circuits' the nitrate-nitrite-•NO cycle by removing •NO precursors from the circulation such that DNRA bacteria are considered detrimental for the maintenance of systemic •NO homeostasis [22,23]. *Prevotella*, *Selenomonas* and *Megasphaera* genera clustered together in $MM6_O$ which diminished following nitrate supplementation, and species within these three genera have been identified as risk factors for hypertension during a 10-year follow-up in women over 60 years of age [24]. In contrast, the *Neisseria* species that increased in abundance particularly in the older group in the present study (*Neisseria bacilliformis, Neisseria elongata, Neisseria flavescens, Neisseria mucosa*) have been shown to contain genes for the denitrification pathway (NirK, NorB or NosZ; based on search performed on https://www.uniprot.org/), which restores •NO precursors to the nitrate-nitrite-•NO cycle thus helping maintain systemic •NO availability. *Neisseria* was consistently among the top five most abundant genera across all conditions, while *Capnocytophaga and Rothia* occurred in smaller relative abundances (<5 %), even after nitrate supplementation. The net effect of nitrate supplementation, therefore, was a shift in the metabolism of the oral ecosystem towards greater nitrite production which manifested as elevated systemic nitrite concentration. The decrease in $MM6_O$ module of oral bacteria in the older group (which contained several bacteria encoding the ammonia-producing DNRA nitrate reduction pathway) drove the increase in circulating levels of the NO precursor, nitrite, which in turn correlated with the reduction in blood pressure. The greater abundance of the *Neisseria* genus in older compared to young participants following BR ingestion in this study is thus consistent with the greater elevation in plasma nitrite concentration and greater reduction in blood pressure in older compared to young groups.

We used WGCNA adapted for 'weighted microbiome co-occurrence network' to explore oral microbiome interactions with physiological variables. A strength of this method is that it enables characterisation of the oral ecosystem of synergistic and antagonistic relationships among bacteria, which in turn may be related to physiological outcomes measured following dietary interventions. We previously showed a consensus correlation across PL and BR endpoints (two layers) between a low brachial MAP and a high abundance of a microbiome module dominated by *Streptococcus* and *Rothia* [14]. In the present study, we took a more stringent approach of seeking consensus between four layers of correlation networks and found a preserved correlation

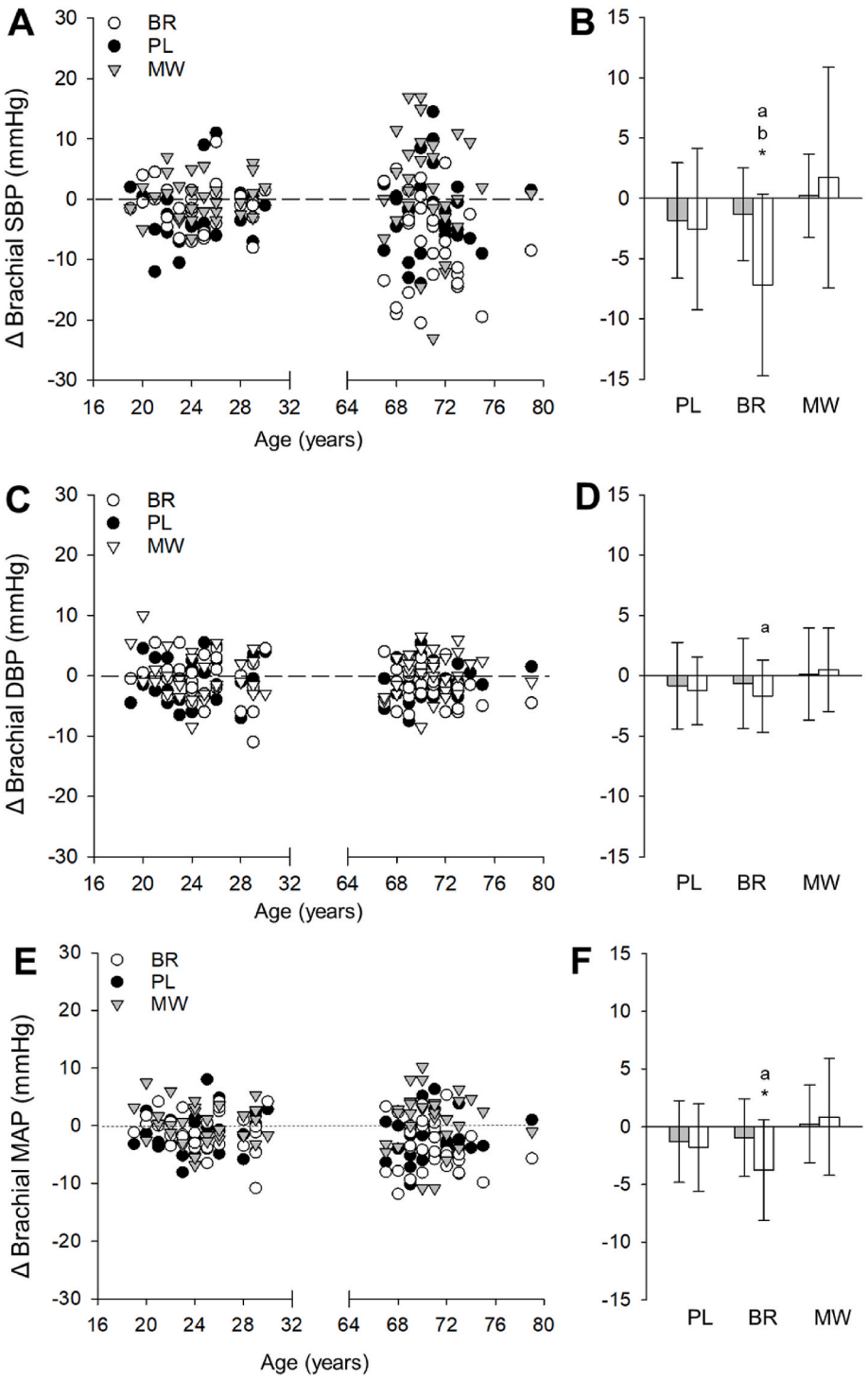

Fig. 5. Changes (Δ) in supine brachial systolic (SBP, panels A and B), diastolic (DBP, panels C and D) and mean arterial blood pressure (MAP, panels E and F) during 2 weeks of placebo (PL), nitrate (BR) and mouthwash (MW) interventions. * = within-condition difference between young (grey bars) and older (white bars); a = different from MW within age group; b = different from PL within age group. Error bars indicate standard deviations.

between central MAP and $MM2_O$, indicating that a high cMAP was associated with a high abundance of bacteria belonging in $MM2_O$. This module contained *Rothia* and *Streptococcus* but also a broader range of other OTUs than seen in our previous study [14] including *Actinomyces, Capnocytophaga, Gemella, Rothia, Streptococcus* and *Tannerella* species. The number, size and makeup of microbiome modules based on correlations between OTUs varies between study cohorts and interventions such that precise replication of consensus correlations is unlikely. The causality of potential relationships between blood pressure indices and oral microbiome modules should be explored using metagenomic shotgun sequencing to enable comprehensive identification of DNRA and denitrification metabolic pathways.

We found that a twice-a-day, two-week antiseptic mouthwash treatment significantly decreased the FMD response compared to a nitrate supplemented condition (but not placebo) in healthy young adults, while there were no significant differences between conditions in FMD in the older adults. Mouthwash use did not alter the overall microbiome compositions in young or older groups, but regular use of antiseptic

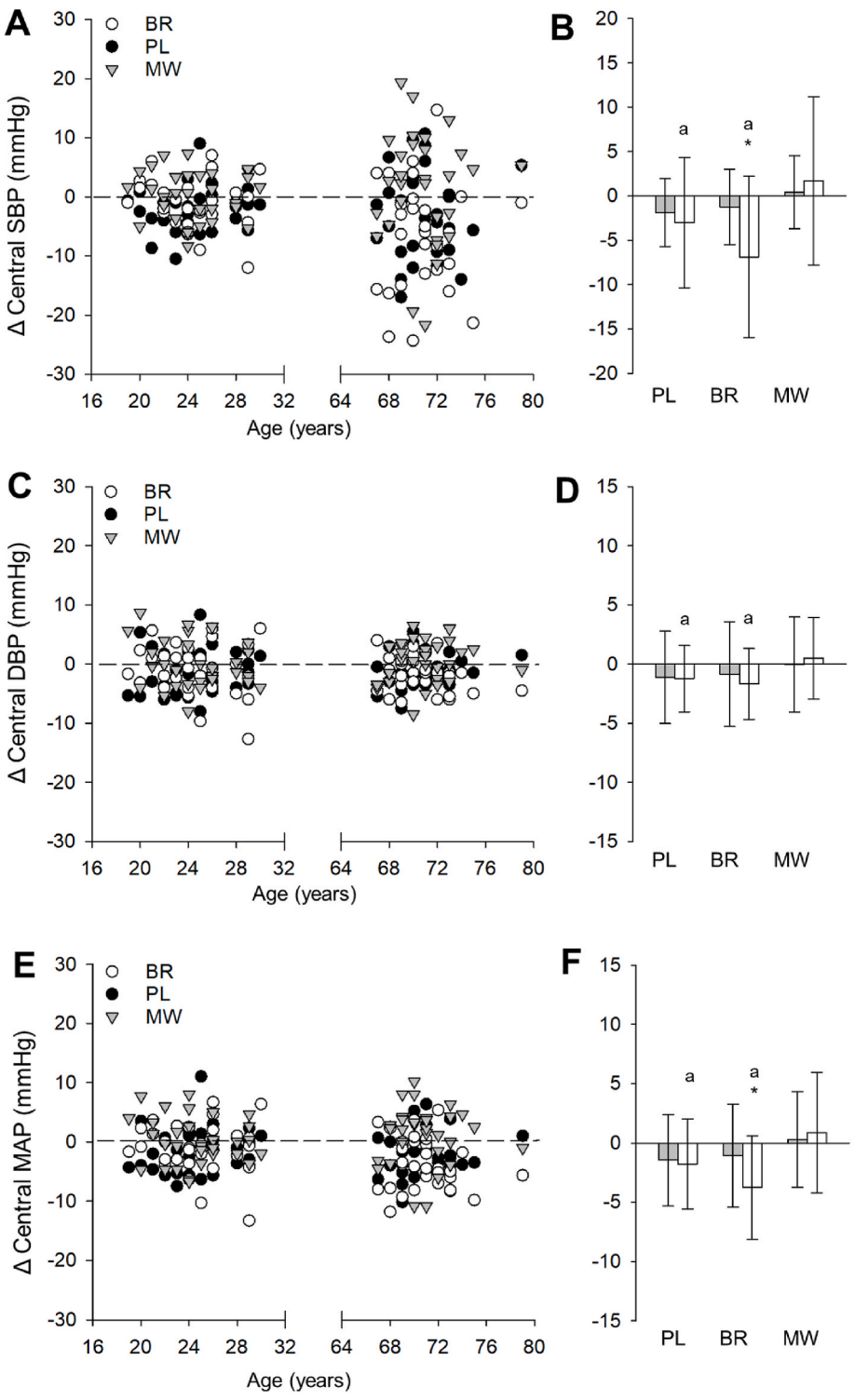

Fig. 6. Changes (Δ) in supine central systolic (SBP, panels A and B), diastolic (DBP, panels C and D) and mean arterial blood pressure (MAP, panels E and F) during 2 weeks of placebo (PL), nitrate (BR) and mouthwash (MW) interventions. * = within-condition difference between young (grey bars) and older (white bars); a = different from MW within the age group. Error bars indicate standard deviations.

mouthwash may decrease the thickness of oral biofilms and thus decrease the total number of nitrate-consuming and/or nitrite-producing bacteria in the oral cavity. However, we did not observe decreases in plasma or saliva nitrate or nitrite concentrations following the mouthwash condition. Chlorhexidine-containing mouthwash has been shown to decrease the oral microbiome diversity, cause changes in relative abundances of bacteria [25,26], and to attenuate the plasma nitrite increase following acute bolus nitrate ingestion [27]. Seven days of chlorhexidine mouthwash use resulted in a 90 % decrease in salivary nitrite concentration and increased blood pressure [11]. The Listerine® antiseptic mouthwash used in the present study does not contain chlorhexidine, with active ingredients including eucalyptol, menthol, methyl salicylate, and thymol dissolved in 25 % ethanol. Although we did not see significant changes in mean plasma or saliva nitrite

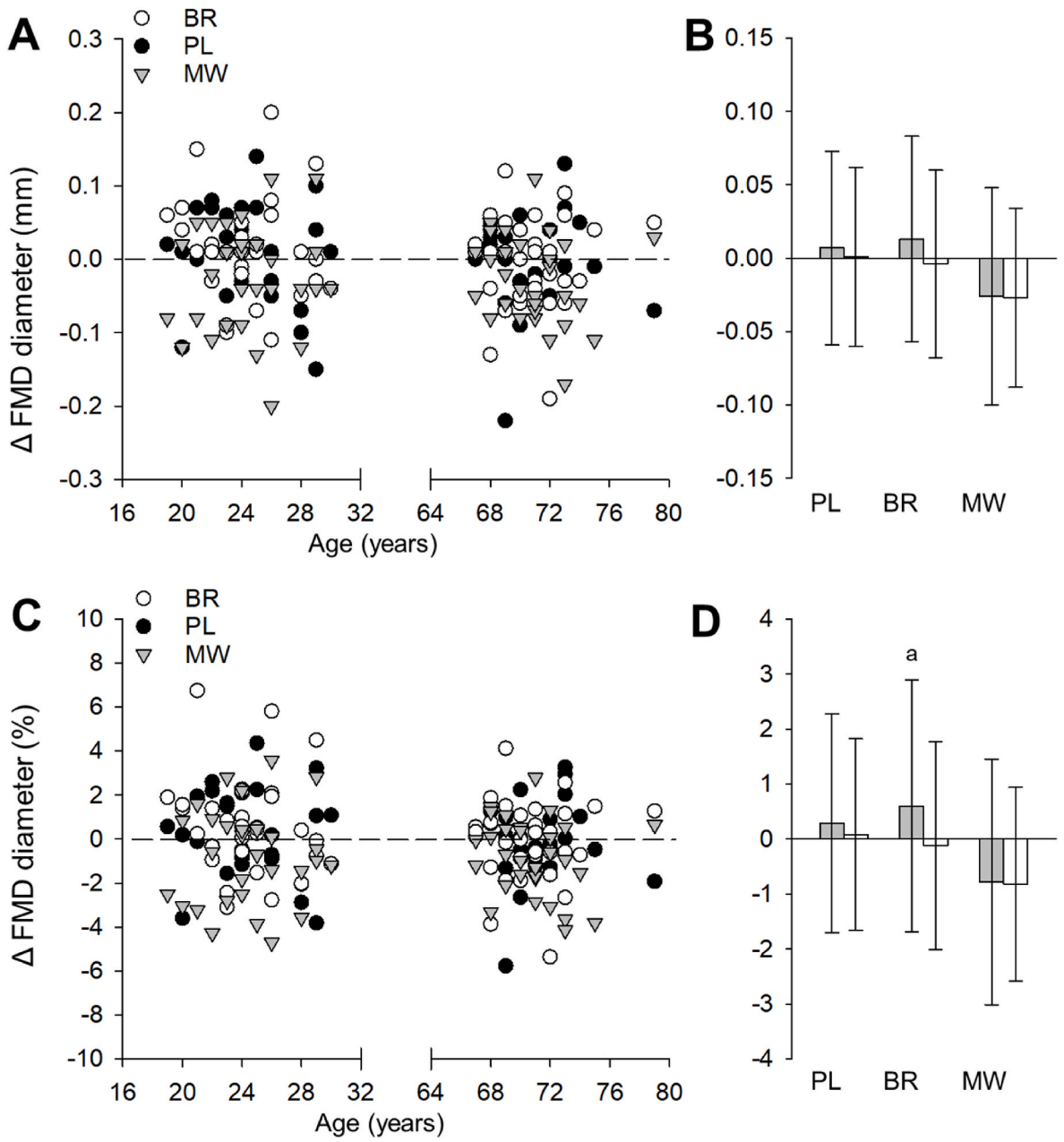

Fig. 7. Changes (Δ) in brachial artery diameter in response to flow mediated dilatation (FMD) in mm (panels A and B) and as a %Δ (panels C and D) during 2 weeks of placebo (PL), nitrate (BR) and mouthwash (MW) interventions. a = different from MW within the age group. Error bars indicate standard deviations.

concentrations or relative abundances of oral bacteria following mouthwash use in this study, there was a decrease in oral microbiome alpha diversity (Shannon index) in the young group, and the differences in plasma nitrite and nitrate concentrations between the positive and negative controls (high-nitrate and mouthwash conditions) correlated with peak brachial artery diameter and FMD, respectively, in the young and older participants. The present results extend previous evidence [11,12,28] in suggesting that oral hygiene practices in healthy adults may have long-term cardiovascular health implications due to disruption of bacterial nitrate reduction in the oral cavity. These results also highlight scope for development of pre- and probiotic interventions to support the rehabilitation of a healthy oral microbiome, and to attenuate disruption of $^{\bullet}$NO homeostasis and adverse vascular responses, during recovery from oral diseases and surgery where bactericidal mouthwash forms a part of prescribed care regime.

While there were clinically relevant decreases in brachial and central blood pressure in nitrate supplemented compared to mouthwash and placebo conditions in the older group, we found no significant differences in the young adults in the change in blood pressure from pre to post between the three conditions (Figs. 5 and 6). The absence of a blood pressure lowering effect of nitrate in the young group is consistent with some studies [13,29] but is different from other reports showing reductions in blood pressure in normotensive adults [10,30–32]. The inverse relationship between baseline blood pressure and the reduction in blood pressure following nitrate supplementation shown in this study, and in previous studies [10,11], may reflect healthy NOS function in normotensive young populations, such that the relatively low baseline blood pressure of the young group (~117/72 mmHg) precluded substantive blood pressure reduction following the BR intervention.

The greater effect on blood pressure in older than young adults in the present study contrasts with some previous research suggesting that an ageing-associated loss of vascular responsiveness may blunt the

response to dietary nitrate supplementation [33]. The older participants in our study were in good overall health with a baseline blood pressure of 126/80 mmHg and their baseline •NO biomarker concentrations were not different from those measured in young adults. There were also no alterations in FMD or PWV variables in the older group after nitrate supplementation which may reflect good baseline cardiovascular health status of these participants with little scope for improvement through elevated NO bioavailability. Our results support the notion that healthy ageing is associated with not only the maintenance of •NO bioavailability and bioactivity but perhaps also a superior ability to utilise and benefit from supplemental nitrate compared to young adults. It should be noted that while there was no significant difference in the sex-ratio between the age groups, the older group consisted of 67 % females and the young group of 49 % females. The structure and function of the oral microbiome have been found to be largely resilient to hormonal fluctuations during the menstrual cycle [34], such that not controlling for menstrual phase in younger women in this study was unlikely to constitute a substantial confounder on oral microbiome outcomes in this study. Hormonal fluctuations during the female lifespan, including pregnancy and menopause, is an important area for future dietary nitrate research to enable development of better personalised nutrition guidance for women. Other factors that may have contributed to differences between age groups include physical activity and diet which were not controlled in the present study beyond advising participants to maintain their habitual activity and dietary patterns throughout the trial and self-reporting adherence to supplementation regimen. Further research is needed to explore the causes of the variability in responses to dietary nitrate supplementation beyond age, including potential sex-specific differences, the influence of hormonal fluctuations across female lifespan, as well as lifestyle factors such as habitual diet, physical activity and smoking history.

We found that the nitrate-depleted beetroot juice placebo caused an elevated mean plasma nitrate concentration in young and older participants (Tables 2 and 3) and had a beneficial effect on blood pressure in the older group (Table 3). This is in contrast with a previous trial showing no significant change in blood pressure after chronic placebo beetroot juice supplementation, although within the same trial acute placebo beetroot juice ingestion reduced diastolic blood pressure [21]. The placebo *BeetIt Sport* shot (James White Drinks Ltd.) is produced by the manufacturer using a chromatography column containing a nitrate-specific anion-exchange resin (Purolite A520e) which selectively removes nitrate from the juice [35]. During the present study, the placebo juice nitrate concentration was monitored by random sampling from batches delivered and confirmed as <0.03 mmol/70 ml shot each time. Beetroot juice naturally contains phytochemicals including betacyanins, polyphenols and flavonoids [36] that may promote nitrite reduction, conserve •NO, or increase NOS activity [37]. A meta-analysis of 22 beetroot juice supplementation trials showed a significant 3.6 mmHg reduction in SBP across all studies, but the effect size was smaller for those studies that had used the placebo juice product instead of a pure control [38]. The strengths of our study included a 'pre vs post' design with a negative control (MW), a high dose of nitrate, and a large sample size. These factors revealed subtle effects of the placebo that were smaller than in the high-nitrate condition, and different from the mouthwash condition, that may have been masked in some previous studies which solely compared PL vs BR endpoints in the absence of baseline measurements.

In conclusion, dietary nitrate represents an acceptable and cost-effective dietary intervention to enhance cardiovascular health in older age via modification of the oral microbiome. This study showed that the elevation in plasma nitrite concentration following dietary nitrate supplementation in older adults was associated with discrete changes in oral microbiome, namely, a decrease in co-occurring module of bacteria dominated by *Prevotella*. Greater blood pressure benefits from supplemental dietary nitrate in healthy older people compared to young were mediated primarily by the suppression of potentially pathobiotic bacteria of the $MM6_O$ module which are known to contain genes that encode nitrate reduction to ammonia in the oral cavity. The finding that dietary nitrate diminished the pro-inflammatory $MM6_O$ module of oral bacteria dominated by *Prevotella* and *Veillonella* in older people has important clinical implications for conditions underpinned by chronic low-level inflammation, including cognitive impairment, recovery from radio therapy, rheumatoid arthritis, COVID-19, low immunity, frailty and pneumonia.

4. Materials and methods

Ethical approval

This study (ClinicalTrials.gov ID: NCT03467061) received ethical approval from the host institution's Ethics Committee (170712/B/03; University of Exeter, UK) and conformed to the World Medical Association (WMA) Declaration of Helsinki.

4.1. Participants

Eligible participants were healthy adults aged 18–30 y (young group) and 65–80 y (older group). Exclusion criteria included individuals receiving medication for pulmonary, cardiovascular, or metabolic conditions; ulcerative colitis or renal disease; having an active oral disease or dentures; smoking; having resting BP > 140/90 mmHg; having used antibiotics within 3 months; lacking willingness or capacity to give informed consent. One-hundred-and-four individuals were screened for eligibility and 42 young, and 36 older adults completed the trial (Supplemental Fig. S1 and S2). The baseline characteristics of study participants are presented in Table 1.

Participants were instructed to arrive at the Diabetes and Vascular Research Centre (NIHR Exeter Clinical Research Facility) in a rested and hydrated state, following an overnight fast and having avoided strenuous exercise, and alcohol and caffeine intake in the 24 h preceding each laboratory visit. Participants were otherwise asked to follow their habitual diet and physical activity pattern throughout the study and to refrain from using mouthwash (MW) until instructed to do so. Adherence to these instructions was monitored through self-reporting during each laboratory visit. All laboratory visits were scheduled at approximately the same time of day (±2 h) for each participant.

4.2. Trial design

Participants were first assigned in a double-blind, randomised, crossover design to receive 14 days of dietary supplementation with concentrated NO_3^- -rich beetroot juice (BR; 2 × 70 ml $\bullet d^{-1}$, each containing ~595 mg NO_3^-, BeetIt Sport, James White Drinks, Ipswich, UK) and NO_3^- -depleted beetroot juice (PL; 2 × 70 ml $\cdot d^{-1}$, beetroot juice containing ~1 mg NO_3^-, James White Drinks, Ipswich, UK). Participants were randomised to BR and PL in a 1:1 ratio using minimisation to ensure balance on age (18–30, 65–80 years) and sex (male, female; N.B., balancing on sex was not possible in the older group where females were predominant). The randomization sequence was computer generated and assigned in a strict sequence at the point of randomization. The dietary supplements and mouthwash were kept in a secure location and only supplied to participants when they commenced each 2-week intervention. The investigators maintained accurate records of the dispensing of supplements in accordance with the trial randomization.

Participants were instructed to consume one of the 70 ml beverages in the morning and another in the evening for 13 days, and on day 14 of each supplementation period, to consume 2 × 70 ml–2 h prior to attending the laboratory. Following completion of PL and BR conditions, participants were assigned to receive mouthwash (MW; Original Listerine® Antiseptic Mouthwash; Johnson & Johnson Consumer Inc., New Jersey, USA) for 14 days during which they rinsed the oral cavity with MW for 30 s every morning and every evening for 13 days, and on day

14, used the MW for the final time ~ 2 h prior to attending the laboratory. The MW condition was always the third arm of the intervention to avoid any carry-over effects, since the wash-out period required for the oral microbiome to return to baseline after cessation of mouthwash use was not known. Active ingredients in Listerine ® antiseptic mouthwash include eucalyptol (0.092 %), menthol (0.042 %), methyl salicylate (0.060 %), and thymol (0.064 %) dissolved in ethanol (25 %). Each treatment period was separated by a 14-day washout to ensure the systemic elimination of nitric oxide metabolites and allow compositional changes in oral bacteria to return to baseline. Adherence to intervention was monitored during laboratory visits and participants were asked to return the empty bottles to the laboratory upon completion of each supplementation period. Participants visited the laboratory before and after each 14-day treatment period for the measurement of primary and secondary outcomes to enable detection of possible carry over effects and between-group baseline-to-treatment comparisons. In total, participants visited the laboratory on six occasions to provide saliva, blood and tongue swab samples, and undergo macrovascular assessment. Participants completed a saliva flow rate questionnaire at baseline [39]. Plasma and saliva [NO_3^-] and [NO_2^-] were assessed using ozone-based chemiluminescence.

4.3. DNA extraction and 16S illumina sequencing

Genomic DNA (gDNA) from the tongue swab samples was extracted using the method of Goode et al. [20]. Briefly, DNA stabilisation buffer and cell lysis solution was added to the swab samples, mixed, and incubated at room temperature for 30 min. RNAse solution was added to the samples and these were incubated on ice for the removal of RNA. Proteinase K solution and protein precipitation solution were then added to remove proteins and lipids. For isolation of gDNA, glycogen solution and isopropanol were added and the mixture was centrifuged (3000 g) at 4 °C for 30 min. The gDNA was purified using an ethanol wash and rehydrated with Tris-EDTA. DNA concentration was quantified using Qubit high-sensitivity fluorescence detection (Qubit 3.0, ThermoFisher Scientific, Waltham, MA). Library preparation employed a NEXTflex 16S V1 - V3 Amplicon-Seq Kit (Bioo Scientific, Austin, TX). The 16S V1-V3 rDNA was selectively amplified using universal primers. 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. Paired-end 300 metagenomic next generation sequence analysis was performed on the MiSeq Illumina platform (Illumina, San Diego, CA) using v3 MiSeq reagents. Nucleotide sequence data in FASTQ format was trimmed and processed by the Kraken 2 Taxonomic Sequence Classification System [40]. Variations in the V1-V3 rDNA regions enabled taxonomic identification.

4.4. Weighted microbiome co-occurrence network

WGCNA was adapted to group highly correlated OTUs and to correlate the groups with physiological responses to interventions (WGCNA R version 6.3). Data were filtered by removing counts of less than five in >50 % of the samples and transformed in accordance with methods described by Langfelder & Horvath [41]. An analysis of the network topology was performed using a signed consensus network for PL, BR and MW where the soft thresholding value was at least 0.8. In module identification, spurious associations were minimised by transforming the adjacency matrix to a topological overlap matrix. A hierarchical clustering tree was used for OTU clustering and module identification (Supplemental Fig. 3). Module eigengenes for PL, BR and MW were correlated with the physiological traits. A consensus network was used to determine the concordance and differences between the networks [41]. Linear discriminant analysis (LDA) of effect size (LEfSe) was used to identify key biomarkers within in each module and compare the statistically different features in PL, BR and MW. The 'all-against-all' strategy for multiclass analysis was used with a logarithmic LDA score threshold of 2 [42].

4.5. Brachial and central artery blood pressure

Blood pressure of the brachial artery was measured in the supine position to enable the participant to remain at rest in the same position throughout the subsequent assessments of pulse wave velocity (PWV) and brachial flow-mediated dilatation (FMD). As described previously [43], central artery blood pressure (BP) was estimated from the applanation tonometry of the radial artery using a SphygmoCor system (AtCor Medical Pty Ltd, West Ryde, Australia). Briefly, participants lay supine on an examination bed and right radial artery pressure waveforms were recorded for 10 s with a high-fidelity micromanometer (SPT-304, Millar Instrument, Houston, TX) attached to the SphygmoCor system. Dedicated software (SphygmoCor version 8.2) processed acquired waveforms to calculate an ensemble-averaged radial pressure waveform with a calibration of brachial systolic and diastolic BP (as per the manufacturer's suggestion) using an automated oscillometric device (M5-I, Omron, Japan). A corresponding central artery BP waveform was then derived using a previously validated transfer function [44,45]. Three separate waveforms were acquired for each participant and the average of these acquisitions was used for statistical analysis. Following central blood pressure parameters were obtained for data analysis: 1) central systolic pressure (CSBP), 2) central diastolic pressure (CDBP) and 3) central pulse pressure (CPP).

4.6. Carotid-femoral and carotid-radial pulse wave velocity

Carotid-femoral pulse wave velocity (cfPWV) was assessed by sequentially recording the ECG-gated carotid and femoral pulses using a SphygmoCor system (version 8.2, AtCor Medical Pty Ltd, West Ryde, Australia) as described previously [46]. The time difference between the R-wave of the ECG and foot of the carotid and femoral pulse waves was calculated using the intersecting tangent algorithm. The path-length for cfPWV was obtained by measuring the straight distance between the carotid and femoral artery measurement sites and multiplying the measured distance by 0.8 [47].

Carotid-radial pulse wave velocity (crPWV) was also assessed by sequentially recording the ECG-gated carotid and radial pulses using the same SphygmoCor system. The time difference between the R-wave of the ECG and foot of the carotid and radial pulse waves were calculated using the intersecting tangent algorithm. The path-length for crPWV was obtained by measuring the distance between the suprasternal notch and radial artery measurement site, with the distance between the carotid artery measurement site and suprasternal notch being subtracted.

4.7. Brachial artery flow-mediated dilatation

Brachial artery FMD was assessed in accordance with established guidelines [48] and as previously described elsewhere [49,50]. Briefly, participants lay supine on an examination bed with the right arm fixed in position and immobilised using a positioning pillow on a metal table. A small blood pressure cuff was placed around the proximal part of the forearm. B-mode ultrasound images and multi-gate Doppler velocity data from the brachial artery were obtained using the Ultrasound Advanced Open Platform ULA-OP; Microelectronics Systems Design Laboratory, University of Florence, Italy [51] as previously described [49,50]. Baseline brachial artery and blood velocity were recorded for 60 s, and once obtained, the forearm blood flow was occluded for 5 min by rapidly inflating the forearm cuff to 250 mmHg (AI6, Hokanson, Bellevue, WA). At 5 min, the cuff was rapidly deflated to induce reactive hyperaemia. Recording of brachial artery image and blood velocity was re-started 30 s before cuff-deflation and continued until 5 min after cuff-deflation.

A custom-designed signal elaboration system was used to extract

wall shear rate (WSR) and diameter parameters as previously described [49,50]. The following WSR parameters were obtained for data analysis: 1) WSR at baseline (WSRbase), 2) WSR at peak hyperaemia (WSRpeak), 3) area under the WSR curve until time to peak dilatation (WSRaucttp). In addition, the following diameter parameters were obtained for data analysis: 1) diameter at baseline (DIAMbase), 2) diameter at peak hyperaemia (DIAMpeak), 3) absolute diameter increase from baseline (DIAMΔ), 4) percentage diameter increase from baseline (DIAMΔ%), and 5) time to peak diameter (DIAMTp).

4.8. Statistical analyses

Between-treatment differences in baseline-to-treatment (delta data) were analysed using one-way ANOVAs (IBM SPSS Statistics, Ver 27, Armonk, NY, USA). Significant effects were followed up using Bonferroni-adjusted *t*-tests. Non-metric multidimensional scaling (NMDS) was used to assess the level of similarity in microbiomes between treatments using non-parametric relationships and were analysed using ADONIS (Vegan R Software). Differences between treatments in bacteria that made up >0.01 % of total bacteria were assessed using Benjamini-Hochberg-adjusted paired t-tests (R statistical software; 42). The Shannon-Wiener diversity index (H') was used to explore differences in diversity (Vegan R Software). Statistical significance was defined as $P \leq 0.05$.

CRediT authorship contribution statement

Anni Vanhatalo: Validation, Data curation, Writing – review & editing, Supervision, Conceptualization, Writing – original draft, Funding acquisition, Visualization, Formal analysis. **Joanna E. L'Heureux:** Writing – review & editing, Data curation, Visualization, Methodology, Formal analysis. **Matthew I. Black:** Validation, Methodology, Investigation, Writing – review & editing, Data curation. **Jamie R. Blackwell:** Resources, Methodology, Writing – review & editing, Investigation, Validation. **Kuni Aizawa:** Writing – review & editing, Formal analysis, Validation, Data curation, Methodology, Investigation. **Christopher Thompson:** Writing – review & editing, Investigation. **David W. Williams:** Funding acquisition, Writing – review & editing, Conceptualization, Methodology, Investigation. **Mark van der Giezen:** Conceptualization, Writing – review & editing, Methodology, Funding acquisition. **Paul G. Winyard:** Conceptualization, Writing – review & editing, Methodology, Funding acquisition. **Andrew M. Jones:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of conflicts

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2025.07.002.

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