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Dietary intake of inorganic nitrate in vegetarians and omnivores and its impact on blood pressure, resting metabolic rate and the oral microbiome

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Abstract

Vegetarian diets are commonly associated with lower blood pressure levels. This has been related to greater consumption of inorganic nitrate, since vegetables are the main source of this anion. Dietary nitrate is reduced to nitrite by commensal bacteria in the mouth, which in turn leads to increased circulatory nitrite availability. Nitrite can form nitric oxide by several pathways promoting a reduction in the vascular tone and lower blood pressure. This study tested whether vegetarians have higher concentrations of nitrite in saliva and plasma, and lower blood pressure and resting metabolic rate (RMR), due to higher intakes of nitrate, compared to omnivores. Following a non-randomized, cross-over and single-blinded design we measured dietary nitrate intake, blood pressure and RMR in young and healthy vegetarians (n= 22) and omnivores (n= 19) with similar characteristics after using placebo or antibacterial mouthwash for a week to inhibit oral bacteria. Additionally, we analyzed salivary and plasma nitrate and nitrite concentrations, as well as the oral nitrate-reduction rate and oral microbiome in both groups. Dietary nitrate intake in vegetarians (97 ± 79 mg/day) was not statistically different ($P > 0.05$) to omnivores (78 ± 47 mg/day). Salivary and plasma nitrate and nitrite concentrations were similar after placebo mouthwash in both groups ($P > 0.05$). The oral nitrate-reducing capacity, abundance of oral bacterial species, blood pressure and RMR were also similar between vegetarians and omnivores ($P > 0.05$). Antibacterial mouthwash significantly decreased abundance of oral nitrate-reducing bacterial species in vegetarians (−16.9 %; $P < 0.001$) and omnivores (−17.4 %; $P < 0.001$), which in turn led to a significant reduction of the oral nitrate-reducing capacity in vegetarians (-78%; $P < 0.001$) and omnivores (-85%; $P < 0.001$). However, this did not lead to a significant increase in blood pressure and RMR in either groups ($P > 0.05$). These findings suggest that vegetarian diets may not alter nitrate and nitrite homeostasis, or the oral microbiome, compared to an omnivore diet. Additionally, inhibition of oral nitrite synthesis for a week with antibacterial mouthwash did not cause a significant raise in blood pressure and RMR in healthy, young individuals independent of diet.

Introduction

Inorganic nitrate has emerged over the last decade as potentially beneficial to cardiovascular health (1, 2). Green leafy vegetables, such as rocket, spinach, kale and certain types of lettuce, and also beetroot are the main source of this dietary compound (2). Sodium and potassium nitrate are also commonly used as food additives in cured and processed meats, but contribute less than 5% of overall nitrate intake (3). The nitrate content of foods has been a concern because of a potential link to the formation of carcinogenic *N*-nitrosamines (4). This evidence is based on early studies in rodents and the methodological limitations of these studies have been highlighted by Bryan et al. (5). In humans, there is a lack of evidence for the association between dietary nitrate intake and cancer (5), but the European Food Safety Authority (EFSA) has maintained an Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/kg body mass/day (3).

New evidence suggests that consumption of inorganic nitrate, mainly in form of supplements at doses that can exceed the ADI (> 500 mg/day), induces a blood pressure lowering effect (6-8). This seems to be modulated by the activity of oral bacteria. Briefly, nitrate is rapidly absorbed in the upper gastrointestinal tract with 20-25% of circulatory nitrate actively taken up by the salivary glands and about 75% being excreted in the urine (9). In the oral cavity, facultative bacteria reduce salivary nitrate to nitrite by the action of nitrate reductases (10). Once nitrite is swallowed, it spontaneously decomposes to nitric oxide (NO) in the acidic stomach, however a small proportion is directly absorbed into the bloodstream (11). Circulatory nitrite is reduced to NO in different tissues and organs enhancing the bioavailability of this important vasodilator (12). Inhibition of oral bacteria using antibacterial mouthwash has been shown to disrupt the oral nitrate-nitrite pathway and to markedly reduce plasma nitrite levels (13, 14). Importantly, this has also been related to a significant increase in blood pressure both in healthy individuals (15) and those with hypertension (16).

On the other hand, increased nitrate availability through a short period (3 days) of pharmacological supplementation (sodium nitrate) has been found to decrease resting metabolic rate (RMR) in healthy volunteers (17). A limitation of this and previous studies on blood pressure using dietary nitrate supplements is that they were acute and short-term interventions (< 4 weeks). A recent study by Blekkenhorst et al (18) showed that increased intake of nitrate-rich vegetables (~150 mg) for four weeks did not lower blood pressure in pre-hypertensive individuals. In contrast to this, an epidemiological study from the same group suggested that older individuals consuming more nitrate (~115 mg) had lower risk of atherosclerotic vascular disease than those individuals with lower nitrate intake (19). However, low nitrate intake may be also associated with reduced consumption of vegetables, which in turn, can impair availability of other dietary compounds such as polyphenols which are well-known for reducing cardiovascular risk (20). Thus, the current evidence about the effect of dietary nitrate on blood pressure and RMR at long term is inconsistent. From this viewpoint, vegetarians are an interesting population to investigate for several reasons. Firstly, it has been assumed that they consume large quantities of nitrate since vegetables provide over 80% of dietary nitrate (21). Secondly, this may induce greater bioavailability of nitrate and nitrite (22). Thirdly, vegetarian diets have been commonly associated with lower blood pressure (23), which has been suggested to be related to greater nitrate consumption (9, 24, 25). However, no previous study has investigated all these questions together.

Thus, the main aims of this study were to estimate dietary nitrate intake in vegetarians compared to omnivores, to determine salivary and plasma concentrations of nitrate and nitrite, as well as the activity and diversity of oral bacteria in both groups. Secondly, this study aimed to measure blood pressure and RMR before and after inhibiting oral bacteria with antibacterial mouthwash. We hypothesized that vegetarians would consume greater amounts of nitrate than omnivores leading to higher concentrations of nitrite in saliva and plasma, and lower blood pressure and RMR. Inhibition of oral bacteria would raise blood pressure and RMR in both groups, but this response would be more accentuated in vegetarians than omnivores, as their vascular and metabolic response may be more dependent on dietary nitrate.

Methods

Participants

Healthy vegetarians (vegans and lacto-ovo vegetarians) and healthy omnivores aged between 18 and 45 years were recruited by poster and e-mail advertisements in the University of Plymouth. The sample size of this study was estimated to detect differences of 3 mmHg in systolic blood pressure after using antibacterial mouthwash. Thus, twenty-two individuals in each group were required to have an 85% power at the 5% significance level. Prior to enrolment, individuals were screened using a questionnaire and excluded if they were smokers, taking any medications or recreational drugs that might have affected the study outcomes, or had pre-existing medical conditions such as hypertension, diabetes or dental conditions (gingivitis). Additionally, individuals using mouthwash or tongue scrapes were excluded from this study. Participants provided written consent to participate in this study. The study was approved by the Ethics Committee of the Faculty of Health & Human Sciences (University of Plymouth) and was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving human subjects. This study was also registered on http://www.clinicaltrials.gov (NCT03871777).

Main protocol

The study used a single blinded, non-randomized, cross over design. Participants visited the laboratory on three different occasions. At the first visit, they were informed about the main aims of the study and instructed by a researcher to complete a seven-day food and physical activity record. They received 14 tubes of 10 mL placebo mouthwash (ultrapure unflavoured water) with which they rinsed their mouth for one minute, twice a day for 7 days. They were also given a small tube of the same toothpaste to standardise it throughout the study.

Participants returned to the laboratory after one week (second visit). At least 24 hours prior to their visit, they were sent written instructions to avoid drinks containing caffeine, such as tea or coffee, before the test and to refrain from strenuous exercise. They arrived at the lab between 8 and 9 am having fasted overnight. Body mass and stature were measured using a mechanical bathroom scale (Salter, Tonbridge, United Kingdom) and stadiometer (Seca, Birmingham, UK), respectively. Then, participants rested in a supine position for 30 min in order to measure RMR. Following this measurement, participants stayed supine whilst blood pressure was measured. After completing these measurements, a venepuncture was performed on the antecubital vein to obtain a blood sample (~ 12 mL) to analyse plasma nitrate and nitrite, blood glucose and blood lipids. Then, a non-stimulated salivary sample (3 mL) was taken into a sterile tube in order to analyse nitrate, nitrite, pH, lactate, glucose and composition and diversity of the oral bacteria. Finally, the oral nitrate-reducing capacity was measured. At the end of the visit, the participant was given breakfast and the food and activity diaries from the previous seven days were collected. A dietician checked the seven-day food diaries in order to confirm the foods and portion sizes consumed, preparation methods, recipes and any brand names. The food and activity diaries were then photocopied and returned to the participant who was requested to replicate the previous week's food intake and activity levels as closely as possible. The participant was given a further one-week supply of antibacterial mouthwash containing 0.2% chlorhexidine (Corsodyl, GlaxoSmithKline, UK), encouraged to use it as per the previous mouthwash (one minute, twice a day) and requested to return to the laboratory in 7 days to repeat all measurements in the same order.

Analyses

Resting Metabolic Rate

Oxygen uptake (VO_2), carbon dioxide production (VCO_2) and the Respiratory Exchange Ratio (RER) were measured continuously for 30 minutes using a ventilated hood connected to a respiratory analyser (Jaeger® Oxycon Pro, CareFusion, Germany), which was calibrated before each test using a reference gas (15.8% O2, 4.9% CO2). Data from the first 20 minutes was discarded and RMR was calculated as the average measurements of the final 10 minutes of the test by using the following equation (26):

$$RMR\ (kcal/day) = [3.941\ X\ (VO_2/1000) + 1.106\ (VCO_2/1000)] \times 1440$$

Blood Pressure

Systolic, diastolic and mean arterial blood pressure was measured following British Hypertension Guidelines (British Hypertension Society, 2014) (27). Three successive supine readings were taken (four if variation in systolic or diastolic blood pressure of > 4 mmHg was found) using an oscillometric device (Connex ProBP 3400 Digital Blood Pressure Device, Welch Allyn UK Ltd.) with a one minute rest between readings. The second and third readings were averaged to determine mean clinic blood pressure.

Plasma and salivary nitrate and nitrite

Whole blood was collected into lithium-heparin tubes (BD Vacutainer®, Becton Dickinson, Plymouth, UK) and rapidly centrifuged at 4,000 rpm and 4 ^{0}C for 10 min. The plasma was then separated, frozen at –80^{o}C until further analyses of nitrate and nitrite. Both anions were measured in plasma and saliva using ozone-based chemiluminescence as previously described (28).

Blood glucose, lactate and lipids

Blood markers were analyzed to assess differences between vegetarians and omnivores to control for diabetes and dyslipidaemia. Whole blood glucose and lactate was measured using a biochemistry analyser (YSI 2300 Stat Plus, YSI Life Sciences, USA). For blood lipids, 5 mL of blood was collected into a serum separator tube (serum separator tubes, BD Vacutainer®, Becton Dickinson, Plymouth, UK). Total cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoproteins (LDL) were analysed with enzymatic methods using the Roche 702 spectrophotometric module of a Cobas 8000 analyser (Roche Diagnostics Ltd, UK)

Salivary pH

Salivary pH was measured using a single electrode digital pH meter (Lutron Electronic Enterprise Co Ltd., Model PH-208, Taiwan) that was calibrated following the manufacturer's instructions.

Oral-nitrate reducing capacity

Participants were instructed to hold 10 ml of water containing sodium nitrate (80 µmol) in their mouth for 5 minutes. The mouth rinse was collected into a Falcon sterile tube and centrifuged (4,500 rpm, 4^{o}C) for 10 minutes. The supernatant was collected and stored at –80^{o}C before measurement of absolute nitrite concentration as indicted above.

Bacterial analysis

Saliva samples were immediately frozen at –80^{o}C in a single sterile tube. Before the analysis, the sample was centrifuged for 10 min at 14,000 rpm, 70 mg of the pellet was isolated and incubated in 50 mg/mL of lysozyme for 30 min at 37 ^{o}C to break the gram positive bacteria. Salivary DNA was extracted using a QIAamp® DNeasy Blood & Tissue Kit (Qiagen, Crawley, UK). PCR amplification of the 16S rRNA V1-2 region was carried out using universal 16S primers 27 F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 338 R (5′-GCW GCC WCC CGT AGG

WGT-3’). PCR’s contained 1 µL (10ng) of DNA template, 25 pmol/µL of each primer, 25 µL of MyTaq™ (Bioline, London, UK) and 22 µL of molecular grade water in a TC-512 thermal cycler (Techne, Staffordshire, UK). Initial denaturation was at 94°C (7 min), followed by 10 cycles at 94°C (30 s), n a touchdown of -1°C per cycle from 68 −57 °C (30 s) and 72 °C (30 s). A further 14 cycles were performed at 94°C (30 s), 56 °C (30 s), 72 °C (30 s), and a final extension 72°C (10 min). Single band PCR products were purified using 1.8 × of Agencourt® AMPure® XP paramagnetic beads (Beckman Coulter, High Wycombe), and quantified with Qubit 2.0 Fluorometer (Invitrogen, CA, USA Sequencing was performed on an Ion Torrent Personal Genome Machine (LifeTechnologies™) using a 318™ v2 chip (LifeTechnologies™) at the Systems Biology Centre in Plymouth University (UK), according to the manufacturer’s instructions for 400bp sequencing. Samples were demultiplexed and filtered by the Torrent server, removing adapter sequences and low quality reads, then exported as fastq files.

Bioinformatic analyses were performed on the fastq files using Cutadapt (v1.18) to remove primers and trim sequences (29). Trimming was performed to a maximum length of 160bp based on the initial quality scores. Following this, chimeras were removed in Qiime (version 1.8) using Chimera Slayer (30). Sequences were clustered *de novo* and binned into operational taxonomic units (OTUs) based on 99% identity. Taxonomy was assigned using the RDP classifier trained to the Greengenes database (31). After quality filtering and removal of singleton reads from the dataset 11074283 sequences remained with an average of 143822 sequences per sample. Alpha diversity parameters were calculated in Qiime2 (version 2018.6; https://qiime2.org/) using a sampling depth of 14,700 reads.

Dietary and physical activity records

Macro- and micronutrient intake of seven-day food diaries were analysed using a nutritional analysis software programme (Microdiet, Downlee Systems, Chapel-en-le-Frith, UK). In addition, a standard protocol was adapted to ensure consistency of coding from food diaries (32). Total polyphenol (determined by folin assay) was obtained using an on-line database, Phenol-Explorer (33). Nitrate content of vegetables was mainly obtained from the European Food Safety Authority (34) and additional data for spinach and lettuce from the Food Standards Agency (35). Nitrate and total polyphenol figures were uploaded to the Microdiet database prior to analysis. Overall, 37 values were imported for nitrate for vegetables.

Exercise diaries were used to assess the total time of physical activities such as running, going to the gym, swimming, cycling etc.

Statistical analyses

Data are presented as mean ± SD. Normal distribution of the sample was assessed using Shapiro-Wilk test. Differences in physical and nutritional parameters between groups were analyzed using unpaired *t*-tests (data normally distributed) or Mann-Whitney U test (data non-normally distributed). Then, a two-way repeated measures ANOVA was performed to assess the main and interactions effects between groups (vegetarian and omnivores) and treatments (placebo and mouthwash). Sphericity was assessed with Mauchly's test and the Greenhouse-Geisser correction was used if sphericity assumption was not met. When the ANOVA revealed a significant interaction ($P < 0.05$), individual comparisons were performed. Paired *t*-tests were used to compare differences between treatments in the same group and unpaired *t*-tests to compare differences between vegetarians and omnivores. OUTs assigned to the main bacterial salivary phyla and genus level were analyzed using linear discriminant analysis (LDA) effect size (LEfSe) method (36). Relationships between the oral nitrate-reducing capacity (absolute nitrite levels), OUTs and salivary and plasma concentrations of nitrate and nitrite were analysed using a Wilcoxon Signed Ranks Test.

Results

Baseline data

The study was conducted from May 2016 to August 2017. Twenty-two vegetarians and nineteen omnivores completed the study. Both groups were matched by age, gender, BMI, blood pressure and physical activity levels (Table 1).

-Table 1-

Dietary intake

Results of the analyses of the seven-day dietary records are shown in Table 2. Dietary fibre (non-starch polysaccharide) intake was higher in vegetarians compared to omnivores (P = 0.037). On the other hand, protein intake was lower in vegetarians compared to omnivores

(P = 0.033). Energy, carbohydrate, saturated fat and total fat consumption did not statistically differ between vegetarians and omnivores ($P > 0.05$). The average consumption of nitrate was 24% higher in vegetarians than in omnivores, but these differences were not statistically different (P = 0.14). A large variability in inorganic nitrate intake was found in vegetarians (range: 13-294 mg/day) compared to omnivores (range: 6-160 mg/day).

-Table 2-

Plasma and salivary concentration of nitrate and nitrite

Figure 1 shows plasma and salivary nitrate and nitrite concentrations in vegetarians and omnivores after treatment with placebo and antibacterial mouthwash. Similar concentrations ($P > 0.05$) of plasma nitrate and nitrite were found in both groups following a week of placebo (Figure 1A). After treatment with antibacterial mouthwash, plasma and salivary concentrations of nitrite were significantly lower in vegetarians and omnivores (Figure 1B, 1D). On the other hand, salivary nitrate increased by 31% (P = 0.014) in vegetarians after using antibacterial mouthwash, but this was more attenuated (6%; $P > 0.05$) and not significant in omnivores (Figure 1C).

-Figure 1-

After placebo, plasma nitrate concentrations in vegetarians correlated strongly with salivary nitrate (r_s= 0.62; P = 0.003) and salivary nitrite (r_s= 0.70; P = < 0.001). Plasma nitrite in vegetarians was also positively associated with salivary nitrate (r_s= 0.47; P = 0.032), but not with salivary nitrite (r_s= 0.41; P = 0.056). In omnivores, no significant correlations were found between plasma nitrate and nitrite and salivary concentrations of both anions (plasma nitrate-salivary nitrate: r_s= 0.43, P = 0.067; plasma nitrate-salivary nitrite: r_s= 0.33, P = 0.18; plasma nitrite-salivary nitrate: r_s< 0.1, P = 0.76; plasma nitrite-salivary nitrite: r_s= 0.18, P = 0.47). On the other hand, salivary nitrate and nitrite concentrations were strongly associated in vegetarians (r_s= 0.66; P = 0.001) and omnivores (r_s= 0.60; P = 0.007) after placebo.

Oral nitrate-reducing capacity and salivary concentration of lactate, glucose and pH.

Both groups showed similar oral nitrate-reducing capacity after using placebo (Figure 2). Antibacterial mouthwash significantly ($P < 0.001$) reduced the oral nitrate-reducing capacity in vegetarians and omnivores (Figure 2A). Interestingly, this was accompanied by a significant decrease in salivary pH (Figure 2B) and increase in lactate (Figure 2C) and glucose (Figure 2D) concentrations in both groups (Figure 2).

-Figure 2-

Blood glucose and lipids

Blood glucose and cholesterol did not differ between vegetarians and omnivores after using placebo (Table 3). Triglycerides were significantly lower in omnivores compared to vegetarians after placebo (P = 0.005). The use of antibacterial mouthwash did not have a significant impact on glucose concentration in both groups. Regarding lipids, antibacterial mouthwash induced a significant increase of triglycerides in omnivores (P = 0.029), but this response was not observed in the vegetarian group. In vegetarians, an increase in plasma concentration of low-density lipoproteins (LDL) was observed after using antibacterial mouthwash (P = 0.02) (Table 3).

-Table 3-

Blood pressure

Systolic, diastolic and mean arterial blood pressure results are presented in Figure 3. No differences were found under baseline conditions between both groups ($P > 0.05$). After using antibacterial mouthwash, systolic blood pressure increased, but not significantly in both groups (vegetarians: 1.2 ± 4.7 mmHg, P = 0.26; omnivores: 1.0 ± 3.7 mmHg, P = 0.31). Given the lack of differences between both groups under baseline conditions (placebo), we also analyzed the effect of antibacterial mouthwash on blood pressure taking all the participants together. Systolic blood pressure was again slightly higher after using antibacterial mouthwash (1.1 ± 4.3 mmHg), but differences were not statistically significant (P = 0.103). No changes were observed in diastolic blood pressure and mean arterial blood pressure either ($P > 0.05$).

-Figure 3-

Resting metabolic rate (RMR)

Respiratory values and RMR are shown in Table 4. The RMR was similar between vegetarians and omnivores after using placebo mouthwash ($P > 0.05$) and no changes were observed in any group after using antibacterial mouthwash ($P > 0.05$).

-Table 4-

Oral microbiome

The oral microbiome of 15 omnivores and 21 vegetarians was successfully analysed. The total amount of phyla and OTUs assigned to the main nitrate-reducing genera and species were similar between both groups after placebo (Figure 4). After placebo, numbers of OTUs assigned to Actinobacteria were positively and significantly correlated with the oral nitrate-reducing capacity in omnivores (r_s= 0.52; P = 0.046), while in vegetarians only Firmicutes correlated significantly with the oral nitrate-reducing capacity (r_s= 0.46; P = 0.037).

Antibacterial mouthwash caused a significant reduction of OTUs assigned to Bacteroidetes ($P < 0.001$) and an increase of Proteobacteria (P = 0.005) in omnivores. The content of other minor salivary phyla was also significantly reduced (P = 0.002) (Figure 4). All these changes led to a significant reduction of bacterial alpha diversity as shown by Shannon's index (Figure 4C). Changes caused by antibacterial mouthwash were more attenuated in vegetarians compared to omnivores. While OTUs assigned to Bacteroidetes ($P < 0.001$) and other minor salivary phyla were significantly reduced (P = 0.001), the increase in Proteobacteria did not reach statistical significance in vegetarians (P = 0.054). However, this group showed a reduction in Shannon's index as well (Figure 4C). A negative and significant correlation (r_s= -0.59; P = 0.022) was found between OTUs assigned to Proteobacteria and the oral nitrate-reducing capacity in omnivores, but not in vegetarians after using antibacterial mouthwash (r_s= -0.21; P = 0.37).

Antibacterial mouthwash caused a significant drop in genera containing bacteria implicated in nitrate reduction in vegetarians (−16.9 %; $P < 0.001$) and omnivores (−17.4 %; $P < 0.001$) with *Prevotella and Actinomyces* most affected (Table 4). On the other hand, *Rothia* increased significantly in vegetarians (P = 0.046), but not in omnivores ($P > 0.05$). *Prevotella* and *Leptotrichia* correlated strongly with the oral nitrate-reducing capacity after using antibacterial mouthwash in omnivores (r_s= 0.75; P = 0.001). In vegetarians, negative and significant correlations were found between *Fusobacterium* (r_s= -0.51; P = 0.019), *Neisseria* (r_s= -0.50; P = 0.021), *Porphyromonas* (r_s= -0.49; P = 0.024) and the oral nitrate reducing capacity after the same treatment.

Antibacterial mouthwash lowered the number of OTUs assigned to of nitrate-reducing species such as *Prevotella malaninogenica* (P < 0.001) and *Rothia dentocaricosa* (P < 0.001) in both groups. On the other hand, OTUs assigned to *Rothia mucilaginosa* (P < 0.03) increased after using antibacterial mouthwash. Omnivores also showed an increase of *Veilonella parvula* (P = 0.04), but this was not found in vegetarians (P = 0.14).

-Figure 4-

-Table 5-

Discussion

This study did not confirm that dietary nitrate intake was greater in vegetarians than omnivores. These findings were strengthened by showing similar concentrations of nitrate and nitrite in saliva and plasma in both groups. Additionally, a vegetarian diet did not lead to higher activity or abundance of oral nitrate-reducing bacteria compared to an omnivore diet. After using antibacterial mouthwash, blood pressure and RMR did not significantly increase in either group despite oral nitrate-reducing bacteria and nitrite bioavailability were significantly reduced in both groups.

These findings are contrary to two previous Polish studies showing higher consumption of dietary nitrate in a group of vegetarians (37), and also greater concentrations of salivary nitrate and nitrite, respectively (22). This discrepancy may be attributed at least to two different factors. Firstly, our vegetarians could have consumed smaller amounts of nitrate-rich vegetables compared to the vegetarians in the study by Mitek et al (37). To achieve the nitrate intake reported by Mitek et al (37) at least 75 g of rocket, 300 g of fresh spinach or 150 g of lettuce would need to be consumed daily. Methodological differences between studies could also explain some of the differences as Mitek et al (37) used a food frequency questionnaire which apparently was not validated for nitrate. We used a 7-day dietary record which has been reported to be a more robust approach to assess dietary intake (38). An older study by Trackzyk et al (22) also reported higher salivary nitrite concentrations in vegetarians compared to omnivores, but this was not confirmed in our study. Dietary nitrate intake in vegetarians and omnivores was not reported by Trackzyk et al (22) so it may be suggested that differences they reported on salivary nitrite between vegetarians and omnivores could be due to low consumption of vegetables in omnivores.

Another relevant finding of the current study was that dietary nitrate intake in all the participants, except two vegetarians, was below the ADI (39). This is relevant since studies investigating the effect of dietary nitrate supplements on vascular health usually provide amounts above the ADI suggesting that this amount is achievable while following a diet rich in vegetables (2). However, this study was not able to confirm this in vegetarians. It is also important to highlight that there is a lack of studies in humans looking at health and safety when nitrate intake exceeds the ADI, thus, it seems appropriate to raise a word of caution in this regard. Studies promoting the consumption of nitrate-rich vegetables have shown controversial results. While short interventions (1 week) have found a lowering blood pressure effect in healthy individuals (40), this has not been confirmed in longer trials (4 weeks) in hypertensive individuals (18). On the other hand, a recent observational study found greater consumption of nitrate-rich vegetables (~115 mg/day) was associated with lower risk of cardiovascular disease in older people (19). Despite vegetarians in this study were near to these values of nitrate intake, we could not confirm that it reduced the risk of cardiovascular disease as blood pressure levels were similar between both groups before and after inhibiting oral bacteria.

On the other hand, this study did not show the expected lower blood pressure levels in vegetarians compared to omnivores under baseline conditions as other epidemiological studies reported (23, 41, 42). Additionally, we did not see differences in blood glucose, but blood triglycerides were higher in vegetarians than omnivores after placebo. This is contrary to what we expected, and it may be related to genetic factors rather than lifestyle as familial hypertriglyceridemia affects one in every 250 people in the UK (43). Despite controlling for blood glucose and lipids to exclude individuals with metabolic alterations, it is possible that lipid disorders could occur in vegetarians but be partially masked by their dietary pattern. Additionally, older studies reporting low values of blood lipids in vegetarians compared to omnivores did not control for some key variables such as physical activity levels (44-46). The current study controlled for BMI, gender, age, physical activity and energy intake. Thus, it seems that the assumption that vegetarian diets automatically lead to lower blood pressure compared to healthy omnivore diets must be challenged, at least, in healthy young individuals performing similar levels of exercise.

Systolic blood pressure increased slightly in vegetarians (1.2 mmHg) and omnivores (1 mmHg) after using antibacterial mouthwash, but this was not statistically significant. Kapil et al (15) and Bondonno et al (16) reported greater increases (2.3 mmHg) after using the same type of antibacterial mouthwash for seven and three days, respectively. This occurred despite similar reductions in the oral nitrate-reduction rate (> 78%), salivary nitrite (> 50%) and plasma nitrite (> 19%) concentrations (15, 16). In agreement with our findings, a more recent study by Sundqvist et al (47) did not find a change on blood pressure after giving antibacterial mouthwash for three days in a group of healthy females. These authors suggested that this could be due to an upregulation of the L-Arginine/NO Synthase pathway in order to compensate the inhibition of the oral nitrate-nitrite pathway since they did not observe a decrease in plasma nitrite after providing antibacterial mouthwash (47). This should be investigated more in depth in future studies regarding also the diversity and activity of the oral microbiome to analyse individual responses.

The RMR in our study was also unaffected by the inhibition of the oral nitrate-nitrite pathway, which concurs with a recent study in healthy females (47). Using a different methodological approach, Larsen et al (17) found a significant decrease of RMR in healthy volunteers after providing a supplement of inorganic nitrate. These findings suggest that inorganic nitrate from diet and oral nitrite synthesis do not seem to control oxygen demands and energy expenditure under resting conditions.

This study did not find differences in the salivary microbiota between the groups under baseline conditions (placebo), which is in agreement with a previous study reporting similar composition of the oral microbiota in vegetarians and omnivores (48). In contrast to this, another recent study by Hansen et al (49) reported differences at genera and species level between vegans and omnivores. They showed greater abundance of some nitrate-reducing species such as *Neisseria subflava*, *Rothia mucilagionasa* and *Haemophilus parainfluenzae* in vegans, while *Prevotella malaninogenica* was more abundant in omnivores. These changes were also associated with dietary fatty acid consumption (49). However, we also analysed fat consumption and all the above oral species, and no differences were found between vegetarians and omnivores. This discrepancy may be due to different feeding regimes as we also combined vegans and lacto-ovo vegetarians in the present study or different methodological approaches such as sample size or genetic analysis of the 16S rRNA (V4 vs V1-V2).

On the other hand, this is the first study showing that antibacterial mouthwash not only reduced the activity, but also the abundance of oral nitrate-reducing species especially in omnivores. At phylum level, omnivores showed significant reductions in Bacteroidetes and other minor phyla groups, and a significant increase of Proteobacteria. Similar changes were found in vegetarians in Bacteroidetes and minor phyla groups, but abundance of Proteobacteria did not change significantly after using antibacterial mouthwash. In addition, Actinobacteria was significantly higher in vegetarians compared to omnivores after using antibacterial mouthwash. Although more knowledge is needed about the potential effect of these changes in health, previous studies have found that lower abundance of Actinobacteria was associated with greater risk of oral cancer (50). From this viewpoint, vegetarian diets may be an interesting approach to reduce changes in the oral ecosystem induced by oral treatments such as mouthwashes and antibiotics that may potentially trigger other co-morbidities.

At genus level, *Prevotella* was the most affected by the use of antibacterial mouthwash. Interestingly, it has been shown that several members of these genera may significantly contribute to nitrate reduction in the oral cavity (51). *Prevotella* belong to the major phyla Bacteroidetes, which in turn, was reduced in both groups after antibacterial mouthwash treatment leading to a significant imbalance between Firmicutes and Bacteroidetes. The lack of colonization of these bacteria could lead to a state of nitric oxide insufficiency (51). On the other hand, an excess of nitrogen species may also alter nitric oxide homeostasis and the oral microbiome as shown by a recent study by Vanhatalo et al (52). Large doses of inorganic nitrate (12 mmol/day) in form of beetroot juice caused a significant increase of oral phyla Proteobacteria. We found a similar pattern in omnivores after using antibacterial mouthwash, and this was strongly associated with lower oral-nitrate reducing capacity. It is unknown whether a similar association occurs when abundance of oral Proteobacteria raises due to dietary nitrate supplementation at large doses. Future research must elucidate this question as studies using dietary nitrate supplements are using large quantities of this anion that may substantially affect the oral ecosystem and NO homeostasis at long term.

Antibacterial mouthwash caused a significant increase in salivary lactate and glucose, and a reduction of pH. In contrast, previous studies showed a rapid increase of salivary pH after using different types of mouthwashes, but this was only found acutely (53, 54). Recent studies from our laboratory have confirmed this oral acidic response of antibacterial mouthwash (unpublished data). Whether these changes are the main cause or just a consequence of modifications observed in the oral microbiome remain to be elucidated, but higher acidity of the oral cavity is a major risk of periodontal disease (55). Furthermore, periodontitis has been associated with lipoprotein alterations and higher risk of cardiovascular disease (56). Importantly, we found a significant increase in triglycerides and LDL in vegetarians and omnivores, respectively, after using antibacterial mouthwash. Thus, this is the first study showing that antibacterial mouthwash not only has a detrimental effect on nitrate-reducing activity of oral bacteria, it may also have a detrimental effect on lipid metabolism.

This study has some limitations. Firstly, the treatment was not randomized due to the lack of available data indicating the time needed for the full recovery of the oral microbiome after one-week use of antibacterial mouthwash. The protocol mirrored a previous study by Kapil et al (15) in order to compare the results with previous evidence. Secondly, the sample size calculation of this study aimed to detect differences of at least 3 mmHg in systolic blood pressure so this could limit us to report statistical significance when smaller differences occurred, and they might be clinically relevant (57). Additionally, other analyses could also be underpowered. For instance, consumption of nitrate by vegetarians was 24% higher than in omnivores, but it was not statistically different, potentially due to a low sample size. However, to recruit vegetarians and omnivores of similar characteristics and meeting the inclusion criteria was challenging. Nonetheless, the sample size of this study was larger than the majority of studies in this area of research (15, 16, 47, 58). Estimation of dietary nitrate through dietary records has also some limitations as the nitrate content of vegetables can substantially vary depending on soil conditions, season, cooking methods, etc. It would be also interesting to analyze nitrate and nitrite urine excretion to provide deeper knowledge of the metabolism of both anions in vegetarians and omnivores. We analyzed the oral microbiome from saliva samples, which represent the totality of bacterial communities in the oral cavity, but it must be noted that bacteria in saliva may include those shed from biofilms which may be less metabolically active than those found in the tongue (59, 60). However, we decided to analyse bacteria in saliva because we could compare it with other salivary markers such as pH, lactate and glucose. Finally, this study was performed in young and healthy individuals, and the physiological effect of vegetarian diets may differ in other populations such as patients with cardiovascular disease or aged individuals under cardiovascular risk.

In conclusion, we showed that dietary nitrate consumption was not statistically different between vegetarians and omnivores. This was confirmed by similar salivary and plasma concentrations of nitrate and nitrite and oral microbiome characteristics in both groups. The inhibition of the oral nitrate-nitrite pathway by antibacterial mouthwash led to a similar response in omnivores and vegetarians in decreasing salivary and plasma nitrite, but did not induce any change in blood pressure or RMR. However, further attention should be given to the changes caused by antibacterial mouthwash on the diversity of the oral microbiome regarding oral health and other associated conditions.

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Figure 1: Plasma (A, B) and salivary (C, D) concentrations of nitrate and nitrite in vegetarians and omnivores after using placebo mouthwash or antibacterial mouthwash.

Figure 2: Oral nitrate-reducing capacity (ONRC) (A), salivary pH (B), salivary lactate (C) and salivary glucose (D) in vegetarians and omnivores after using placebo mouthwash or antibacterial mouthwash.

Figure 3: Systolic (A), diastolic (B) and mean arterial blood pressure (C) in vegetarians and omnivores after using placebo mouthwash or antibacterial mouthwash.

Figure 4: Relative abundance of the main bacterial phyla (A), ratio between the relative abundance of Firmicutes and Bacteroidetes (B) and the Shannon Diversity Index values (C) in vegetarians and omnivores in vegetarians and omnivores after using placebo mouthwash or antibacterial mouthwash (* $P < 0.05$ between placebo and mouthwash; # $P < 0.05$ between vegetarian and omnivore groups).

Table 1: Main characteristic of participants

	Vegetarians	Omnivores
Age (years)	26 ± 6	26 ± 7
Gender (F:M)	16:6	11:8
BMI	22.9 ± 3.8	22.1 ± 2.9
Physical Activity (min/wk)	315 ± 221	336 ± 216
Heart Rate (b/min)	59 ± 8	59 ± 11
Resting Metabolic Rate (kcal/day)	1175 ± 208	1202 ± 267

Table 2: Daily dietary intake of vegetarians and omnivores (mean ± SD)

	Vegetarians	**Omnivores**
Energy (Kcal)	1,827 ± 526	2,021 ± 560
Protein (g)	61 ± 19	91 ± 36[#]
Fat (g)	71 ± 27	78 ± 24
Saturated fat (g)	21 ± 10	28 ± 11
Carbohydrate (g)	234 ± 71	246 ± 76
Polyphenols (g)	182 ± 124	178 ± 116
Non-starch polysaccharide (fibre) (g)	21.6 ± 7.7	17.3 ± 4.9*
Nitrate (mg)	97 ± 79	78 ± 47
Nitrate (mmol)	1.5 ± 1.2	1.2 ± 0.8

([#] $P < 0.05$ between vegetarian and omnivore groups)

Table 3: Blood glucose and lipids in vegetarians and omnivores after using placebo mouthwash or antibacterial mouthwash

	Vegetarians		**Omnivores**	
	Placebo	**Mouthwash**	**Placebo**	**Mouthwash**
Glucose (mmol/L)	4.28 ± 0.31	4.26 ± 0.31	4.44 ± 0.30	4.36 ± 0.31
Cholesterol (mmol/L)	4.17 ± 1.21	4.01 ± 1.09	4.26 ± 0.64	4.25 ± 0.68
Triglycerides (mmol/L)	0.87 ± 0.30	0.83 ± 0.28	0.65 ± 0.18[#]	0.74 ± 0.22*
HDL (mmol/L)	1.52 ± 0.50	1.52 ± 0.46	1.66 ± 0.43	1.65 ± 0.42
LDL (mmol/L)	2.24 ± 0.71	2.10 ± 0.68*	2.32 ± 0.50	2.28 ± 0.51
Chol:HDL	2.80 ± 0.77	2.69 ± 0.74	2.69 ± 0.55	2.69 ± 0.59

HDL: high density lipoproteins; LDL: low density lipoproteins; Chol:HDL: ratio between total cholesterol and high density lipoproteins. (* $P < 0.05$ between placebo and mouthwash; [#] $P < 0.05$ between vegetarian and omnivore groups).

Table 4: Oxygen uptake (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER) and resting metabolic rate (RMR) in vegetarians and omnivores after using placebo mouthwash or antibacterial mouthwash.

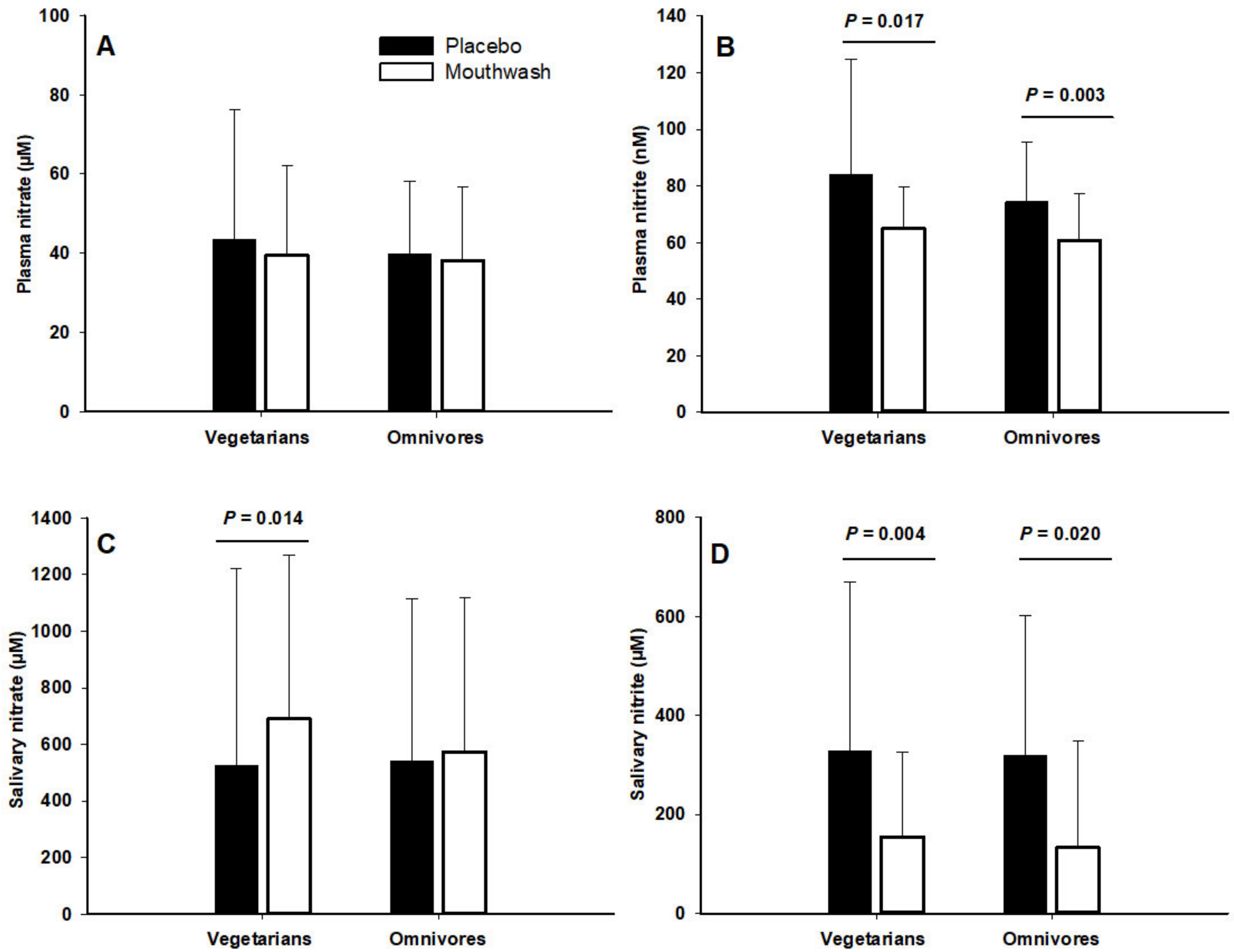
	Vegetarians		**Omnivores**	
	Placebo	**Mouthwash**	**Placebo**	**Mouthwash**
VO2 (mL/min)	211 ± 39	211 ± 38	212 ± 47	212 ± 48
VCO2 (mL/min)	148 ± 29	150 ± 31	145 ± 32	148 ± 36
RER	0.70 ± 0.07	0.70 ± 0.07	0.69 ± 0.07	0.70 ± 0.06
RMR (kcal/day)	1175 ± 208	1185 ± 212	1202 ± 267	1200 ± 272

Table 5: Relative abundance of genera and species (%) which have previously been implicated in nitrate reduction in vegetarians (*n* = 21) and omnivores (n = 15) after using placebo mouthwash or antibacterial mouthwash.

	Vegetarians		**Omnivores**	
OTU (%)	**Placebo**	**Mouthwash**	**Placebo**	**Mouthwash**
GENERA				
Prevotella	27.7 ± 6.5	9.6 ± 6.0*	26.1 ± 9.5	8.6 ± 11.1*
Veiolonella	12.3 ± 3.7	12.2 ± 5.8	12.7 ± 5.0	13.2 ± 7.1
Actinomyces	9.5 ± 4.9	5.7 ± 5.1*	8.5 ± 5.2	3.9 ± 2.9*
Neisseria	5.6 ± 6.1	12.2 ± 10.2	4.9 ± 5.3	12.2 ± 10.3
Rothia	4.0 ± 2.8	7.7 ± 6.8*	4.6 ± 3.5	4.9 ± 7.0
Porphyromonas	3.3 ± 2.8	2.7 ± 2.7	3.3 ± 3.0	4.0 ± 5.3
Fusobacterium	1.7 ± 3.7	0.7 ± 0.7*	1.6 ± 1.0	0.9 ± 0.9*
Leptotrichia	1.0 ± 0.8	1.6 ± 2.0	1.2 ± 0.8	1.3 ± 1.7
SPECIES				
Prevotella malaninogenica	18.1 ± 7.0	6.2 ± 7.0*	16.7 ± 9.0	5.2 ± 9.9*
Veilonella dispar	10.2 ± 3.9	10.1 ± 5.4	10.7 ± 3.8	10.5 ± 5.3
Neisseria subflava	5.0 ± 5.7	10.6 ± 9.8	4.7 ± 5.1	10.1 ± 9.4
Rothia mucilaginosa	3.8 ± 2.8	6.8 ± 6.5*	3.8 ± 3.1	5.6 ± 6.9*
Veilonella parvula	0.6 ± 0.2	0.5 ± 0.3	0.6 ± 0.2	0.8 ± 0.5*
Haemophilus parainfluenzae	0.5 ± 0.4	0.6 ± 0.6	0.3 ± 0.2	0.8 ± 0.8
Rothia dentocaricosa	0.09 ± 0.07	0.04 ± 0.10*	0.09 ± 0.07	0.02 ± 0.03*

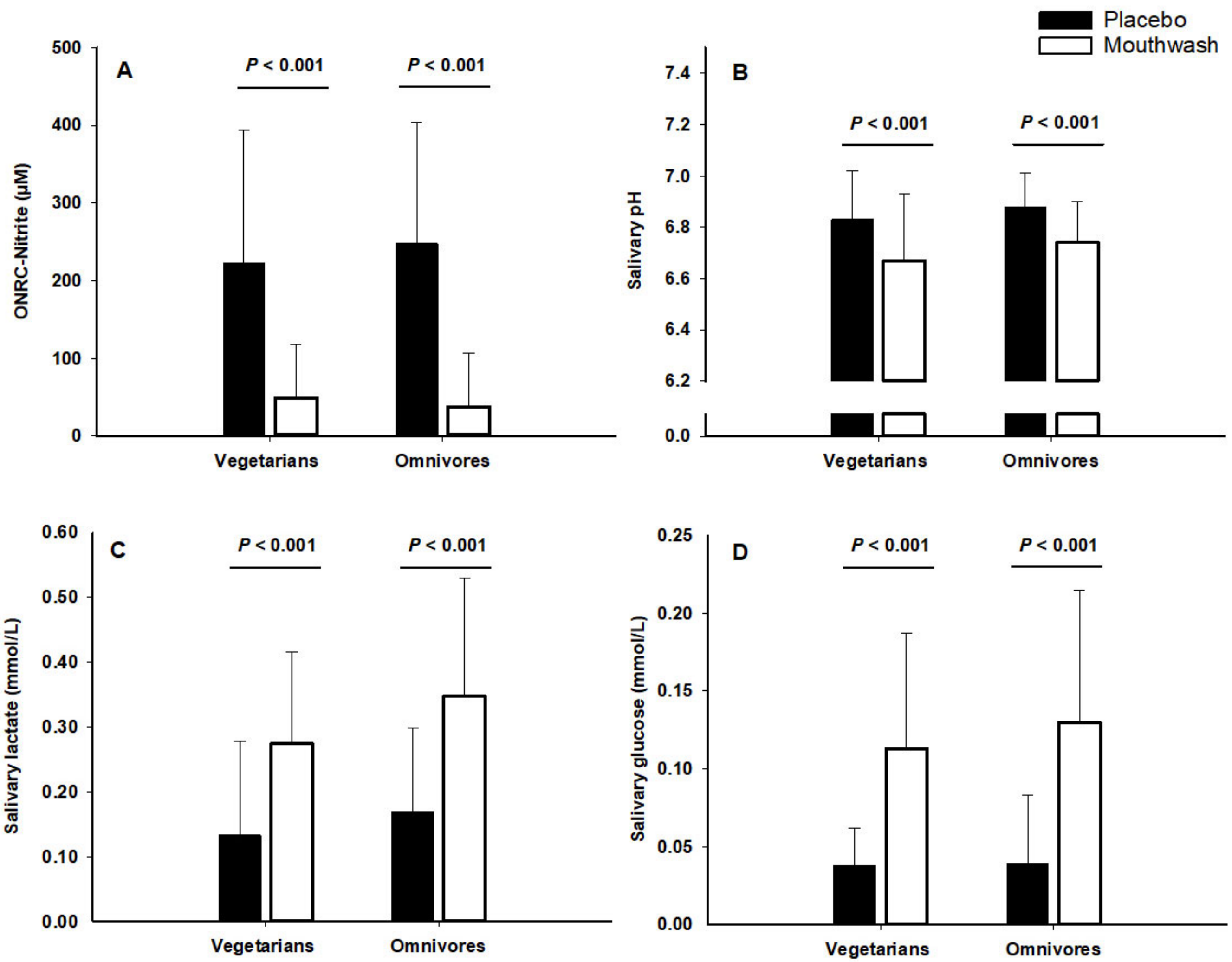
(* $P < 0.05$ between placebo and mouthwash)

Figure 1



A
Placebo
Mouthwash
Plasma nitrate (µM)
100
80
60
40
20
0
Vegetarians
Omnivores
B
P = 0.017
P = 0.003
Plasma nitrite (nM)
140
120
100
80
60
40
20
0
Vegetarians
Omnivores
C
P = 0.014
Salivary nitrate (µM)
1400
1200
1000
800
600
400
200
0
Vegetarians
Omnivores
D
P = 0.004
P = 0.020
Salivary nitrite (µM)
800
600
400
200
0
Vegetarians
Omnivores

Figure 2



Placebo
Mouthwash
A
P < 0.001
P < 0.001
ONRC-Nitrite (μM)
500
400
300
200
100
0
Vegetarians
Omnivores
B
Salivary pH
7.4
7.2
7.0
6.8
6.6
6.4
6.2
0.0
C
Salivary lactate (mmol/L)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
D
Salivary glucose (mmol/L)
0.25
0.20
0.15
0.10
0.05
0.00

Figure 3

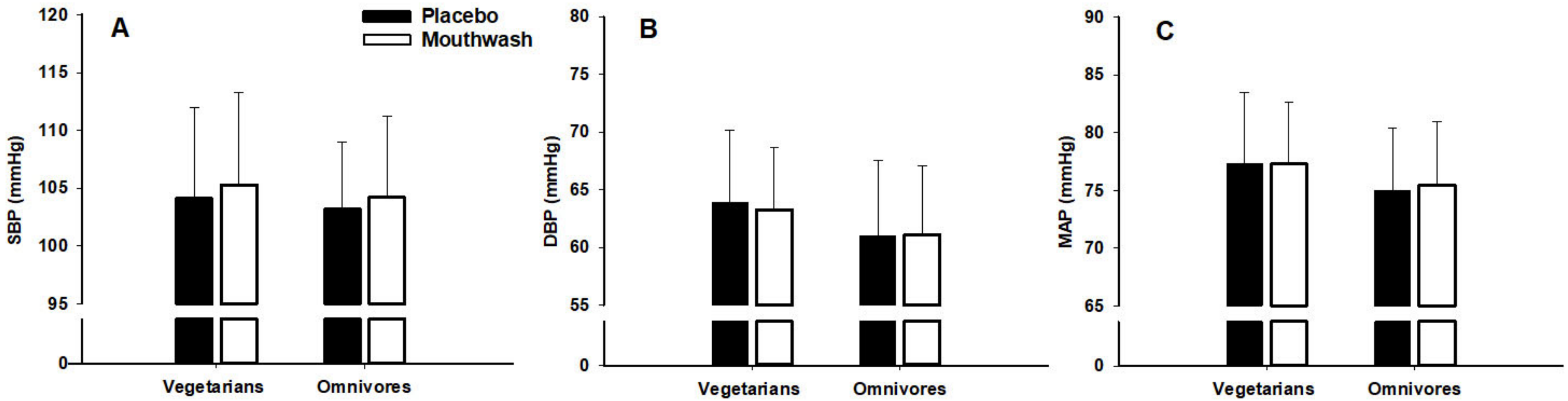
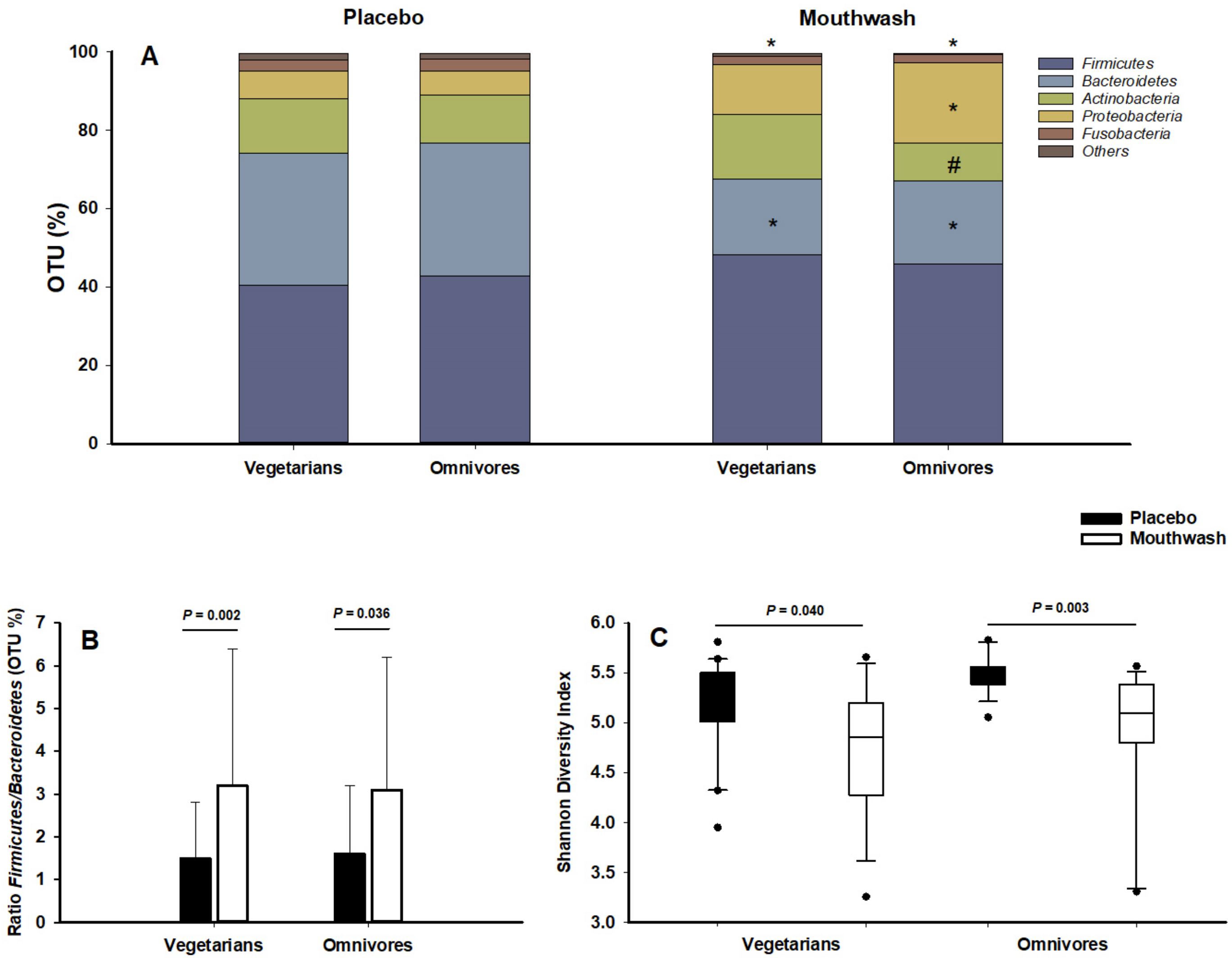

A
Placebo
Mouthwash
SBP (mmHg)
120
115
110
105
100
95
0
Vegetarians
Omnivores
B
DBP (mmHg)
80
75
70
65
60
55
0
Vegetarians
Omnivores
C
MAP (mmHg)
90
85
80
75
70
65
0
Vegetarians
Omnivores

Figure 4


Placebo
Mouthwash
100
80
60
40
20
0
OTU (%)
A
*
*
*
#
*
*
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Fusobacteria
Others
Vegetarians
Omnivores
Vegetarians
Omnivores
Placebo
Mouthwash
Ratio Firmicutes/Bacteroidetes (OTU %)
7
6
5
4
3
2
1
0
B
P = 0.002
P = 0.036
Vegetarians
Omnivores
Shannon Diversity Index
6.0
5.5
5.0
4.5
4.0
3.5
3.0
C
P = 0.040
P = 0.003
Vegetarians
Omnivores