# THE IMPACT OF DIETARY INORGANIC NITRATE ON BLOOD PRESSURE AND OTHER CARDIOVASCULAR DISEASE RISK MARKERS

By

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## DECLARATION

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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August 2021.

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## PUBLISHED PAPERS FROM THESIS

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**Published conference abstract**

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**Alzahrani H.S., Jackson K.G., Hobbs D.A. and Lovegrove J.A. (2018).** The Role of Oral Bacteria in the Bioconversion of Nitrate to Nitrite. *Food Antioxidants and functional Ingredients – Shelf Life Extension, Nutrition and Health, London* - March 2018.

**Alzahrani H.S., Jackson K.G., Hobbs D.A. and Lovegrove J.A. (2021).** The Impact of Different Sources of Dietary Nitrate on Blood Pressure and Other Risk Factors for Cardiovascular Diseases in a Representative UK Population. *American Society of Nutrition, USA* – June 2021.

## GENERAL ABSTRACT

Epidemiological studies have demonstrated an inverse association between high vegetable consumption and lower blood pressure (BP), an important modifiable cardiovascular disease (CVD) risk marker. The high nitrate content of root (e.g. beetroot) and green leafy vegetables is a key dietary component related to the beneficial effects on vascular function and BP. However, the role of commensal oral bacteria which convert dietary nitrate to nitrite and potent vasodilator nitric oxide, in BP regulation and maintenance of vascular tone is unclear. A literature review (Chapter 2) was performed to determine the association between the oral microbiome and real time measures of vascular function. Elimination of oral bacteria with antiseptic mouthwash was reported to reduce conversion of inorganic and supplemental nitrate to nitrite and be associated (in some studies) with an increase in BP. This review highlighted the need for further research to focus on the effects of dietary nitrate on oral bacteria composition and CVD risk markers, and to determine underlying mechanisms.

The relationship between dietary nitrate consumption from different sources (vegetable, processed meat and water) with BP and other CVD risk factors was investigated in the UK NDNS cross-sectional cohort (Chapter 3). This data analysis included 3407 adults aged 19-64 years old. A comprehensive database was developed to accurately estimate the nitrate and nitrite levels in vegetables, processed meats and drinking water. Across increasing quartiles of dietary nitrate intake from vegetables and drinking water, BP and glycated haemoglobin were significantly lower in Q3 (95-130 mg nitrate/day) than Q1 (3-65 mg nitrate/day). In addition, glucose and high sensitivity C-reactive protein concentrations were lower in Q4 compared to Q1. In contrast, there were no differences in CVD risk markers across quartiles of nitrate or nitrite from processed meats or dietary nitrite from vegetables and drinking water.

Analysis of urinary nitrate and nitrite concentrations in n=1340 NDNS participants revealed a significant but weak correlation with total dietary nitrate intake (r=0.121, p<0001). In contrast to the dietary data, there were no significant difference in BP across increasing quartiles of urinary nitrate concentration which suggests that urinary nitrate may not be an ideal biomarker of intake (Chapter 4).

To address the role of the oral microbiome in contributing to the beneficial effects of dietary nitrate on BP and vascular function, two human studies were performed. In REBOC1 (Chapter 5), a three-arm sequential pilot study in n=20 healthy adults, the salivary nitrite concentration was found to be significantly higher after rinsing the mouth with beetroot juice (1.09±0.34 μM) compared with the use of an antibacterial mouthwash (to eliminate oral bacteria) prior to mouth rinsing with beetroot juice (0.021±0.01 μM; $p < 0.001$). Within the oral cavity, the posterior tongue was identified as the main site of nitrate reduction, with beetroot juice promoting an increase in the abundance of *Neisseria*. In a follow-up study incorporating a double-blind randomised cross-over design, the chronic effects of dietary nitrate intake on BP and other CVD risk markers were related to the changes in oral and gut microbiota compositions (Chapter 6). Consumption of nitrate-rich beetroot juice (3.7 mg/kg body weight) for 8 weeks reduced systolic and diastolic BP by 4.1 and 2.8 mmHg, respectively and increased endothelial independent microvascular reactivity by 4% compared with a control beetroot juice (n=19 healthy participants). These findings were associated with an increase in the nitrate-reducing capacity and abundance of *Neisseria* in the oral cavity and reduction in *Clostridium* in the gut microbiota after chronic dietary nitrate intake. Future research should address the impact of inorganic nitrate as a potential dietary strategy to improve cardiovascular health, particularly in those individuals with raised BP, and to determine the utility of urinary nitrate as a biomarker of intake.

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## ABBREVIATIONS

**Ach:** Acetylcholine

**ADI:** Acceptable daily intake

**ANCOVA:** Analysis of covariance

**AOAC:** Association of official analytical chemists

**AUC:** Area under the curve

**BH4:** Tetrahydrobiopterin

**BMI:** Body mass index

**BP:** Blood pressure

**BW:** Body weight

**CFU:** Colony forming unit

**cGMP:** Cyclic guanosine monophosphate

**CHD:** Coronary heart disease

**CRP:** C-reactive protein

**CVD:** Cardiovascular diseases

**DASH:** Dietary approach to stop hypertension

**DBP:** Diastolic blood pressure

**eNOS:** Endothelial nitric oxide synthase

**FMD:** Flow mediated dilation

**HbA1c:** Glycated haemoglobin

**HDL-C:** high-density lipoprotein cholesterol

**IAUC:** Incremental AUC

**LDI:** Laser doppler imaging

**LDL-C:** Low-density lipoprotein cholesterol

**MAP:** Mean arterial pressure

**NDNS:** National diet and nutrition survey

**NEFA:** Non-esterified fatty acids

**NO:** Nitric oxide

**$NO_2$:** Nitrite

**$NO_3$:** Nitrate

**NOS:** Nitric oxide synthase

**OTU:** Operational taxonomic unit

**PBS:** phosphate-buffered saline

**PP:** Pulse pressure

**PWA:** Pulse Wave Analysis

**RCT:** Randomised control trial

**SBP:** Systolic blood pressure

**SCFA:** Short-chain fatty acids

**SNP:** Sodium nitroprusside

**TAG:** Triacylglycerol

**Total-C (T-C):** Total cholesterol

# CHAPTER 1: GENERAL INTRODUCTION

Cardiovascular diseases (CVDs) are one of the leading causes of death globally (1). High blood pressure (BP) or hypertension (140/90 mm Hg) is a modifiable risk, responsible for 13% of global deaths each year (2). Evidence shows healthy dietary patterns such as the Mediterranean diet and Dietary Approaches to Stop Hypertension (DASH) diet are associated with lower BP and reduced risk of CVDs (3), which has been suggested to be due in part to the higher abundance of vegetables in these diet patterns, some of which are rich in nitrate and nitrite(4)(5). An increasing number of studies have shown that inorganic dietary nitrate and its reduction to nitrite and further to NO, has BP lowering effects and lead to improvements in endothelial function(6). Although increases in dietary nitrate from dietary intervention studies have been promising(7, 8), determination of population intakes are extremely limited due to the lack of detailed data on dietary databases (difficulty was in obtaining accurate values for the nitrate content of vegetables and water due to seasonal variability) and suitable biomarkers of intake. To date, urinary nitrate has been proposed as a possible biomarker for dietary nitrate exposure. However, few studies have used this biomarker to assess intake and relate this to BP and other CVD risk markers. Furthermore, the lack of studies performed in UK populations warrants a need for research to be conducted.

## Dietary nitrate, blood pressure and vascular function

The physiological generation of NO has an important role in maintaining vascular homeostasis. The basic NO-cyclic guanylate monophosphate (cGMP)-protein kinase G signalling pathway causes vascular relaxation when the endothelium NO diffuses into the underlying vascular smooth muscle cells. NO also has an impact on thrombosis prevention by reducing platelet adhesion, activation and agonist-induced

secretion(6). That has been shown to promote platelet disintegration and prevent the binding of fibrinogen(9). Endothelium-derived NO not only affects the vasculature but also inhibits leukocyte adherence and injury-induced intimal proliferation by reducing smooth muscle cell proliferation and migration(10). When NO's bioavailability is reduced, however, these positive effects are lost, and endothelial dysfunction takes over. Endothelial dysfunction, as defined by decreased production or activity of NO may be one of the earliest signs of atherosclerosis and is associated with the development of several cardiovascular disorders such as hypertension(11). Therefore, increasing and or improving NO bioavailability in the vasculature are likely to have considerable implications for the development of cardiovascular disorders. There is increasing evidence to suggest that dietary nitrate may improve NO bioavailability, increase vasodilation, inhibit platelet aggregation and thus improve cardiovascular health(12).

The L-arginine–NOS pathway can produce (about 1.7 mmol/d) of NO to sustain an individual’s health(13). Some conditions such as diabetes mellitus and ageing have been found to affect the bioactivity of endogenously generated NO by increasing degradation of NO(14). However, diet can provide an alternative source of NO, and an increasing body of evidence indicates that dietary nitrate intakes contribute substantially to NO availability in the human body via the nitrate-nitrite-NO pathway. Nitrate is widely available in the diet, with vegetable sources include green leafy vegetables and beetroot accounting for approximately 60%–80% of total daily consumption(15). Inorganic nitrate can raise plasma nitrate concentrations for up to 5–6 hours after ingestion. Although approximately 75% of this nitrate is excreted at the kidneys, 25% is extracted by the salivary glands. As part of the nitrate-nitrite-NO pathway, salivary nitrate is reduced to nitrite via bacteria in the oral cavity. This nitrite-

rich saliva is swallowed and further reduced in the acidic stomach to nitrogen oxides, including NO(16).

Currently, the acceptable daily limit for nitrate is 3.7 mg/kg of body weight (BW)/day, which is equivalent to approximately 222 mg/day for a 60-kg adult. However, it is worth noting that cardioprotective dietary patterns rich in vegetables, such as the Mediterranean and Dietary Strategies to Prevent Hypertension (DASH) diets, are also naturally high in dietary nitrate, providing approximately 174–1222 mg of nitrate per day(17), a level of intake that far exceeds the intake on an average Western diet, which provides approximately 75 mg/day(15). There is an increasing need within the scientific community for a comprehensive investigation into the role of inorganic nitrate concerning a variety of CVD risk factors and related outcomes to further consolidate the impact of dietary nitrate as a potential novel food solution to this major health problem.

It is possible that nitrate, via the nitrate-nitrite-NO pathway, is responsible for some of the blood pressure-lowering effects of green leafy vegetables and beetroot juice consumption. The positive effects of nitrate consumption on blood pressure, arterial stiffness, endothelial function, platelet function, and cerebral blood flow have all been studied in humans(18). There is currently strong evidence from human intervention trials that short-term dietary nitrate intake lowers blood pressure in healthy populations. Also, most chronic studies have used relatively high doses of nitrate. A study conducted in 20 healthy volunteers were consuming lower (1mg/kg) and higher (10mg/kg) nitrate intake reported that 1 mg nitrate /kg led to significant increase in FMD after 1 h of nitrate consumption(19). However, the effects of long-term consumption of 3.7 mg of nitrate/kg of body weight on blood pressure and cardiovascular reactivity in healthy people, on the other hand, are unknown.

A systematic review with meta-analysis by Siervo et al (20) identified that acute intakes of inorganic nitrate significantly lowers resting in SBP but not DBP. This review included intervention studies in which inorganic salts (NaNO3, KNO3) and Beetroot Juice were used. Then another systematic review with meta-analysis by Jackson et al(6) included high dietary nitrate studies (that provide an example of how nitrate is consumed in the general population) as well as beetroot juice and inorganic salt. This review investigated all major risk factors for the development of atherosclerosis and CVD such as BP, arterial stiffness, platelet aggregation, and endothelial function measured by Flow-mediated dilation (FMD). This study reported a significant reduction in SBP which were consistent with Siervo et al. However, a statistically significant reduction in resting DBP was also detected in contrast to Siervo findings. Findings from the Jackson analysis provided promising evidence to support the role of inorganic nitrate for reducing CVD given that a 5-mmHg reduction in SBP and 2-mmHg reduction in DBP has been estimated to reduce the prevalence of hypertension by 20% and is believed to result in 14% fewer deaths from stroke, 9% fewer deaths from coronary heart disease, and 7% fewer deaths overall(21). This meta-analysis found that inorganic nitrate consumption (130–259 mg) of Beetroot Juice was found to be a highly effective method for reducing resting BP and could represent a promising basis for making more specific dietary recommendations for hypertension prevention(6).

The reduced BP may be linked to the high variability of nitrate contents seen in green leafy vegetables, which can alter considerably depending on cultivation, cooking, and processing practices. By comparison, a prospective cohort study (n=1544) by Golzarland et al(22) identified a 37% reduction in 3-year risk of hypertension in adults with a higher intake of nitrate-containing vegetables. These results indicate a promising long-term effect of high dietary nitrate. Subgroup analysis revealed

inorganic nitrate consumption led to greater reductions in resting BP for healthy participants compared with patient groups (22). It would be interesting to know whether these larger periodic reductions in resting BP could be clinically relevant and reduce CVD morbidity or mortality in the long term.

FMD is the measurement of the percentage changes in brachial artery diameter in response to shear stress stimulus that produces a nitric oxide-dependent response. Noninvasive ultrasound FMD of the brachial artery is now widely quoted in the human trail It was found that for every 1% increase in FMD there was a 10% reduced risk of CVD(23). The results from the human studies indicate that inorganic nitrate consumption can significantly improve FMD response (23, 24, 25). Also, indicate that inorganic nitrate consumption led to significantly reduced arterial stiffness and PWV (23, 24, 26). Furthermore, inorganic nitrate consumption significantly reduced platelet aggregation by 18.9% in response to platelet agonists(6). It is important to mention, Although the review findings represent the effect of short-term nitrate intakes on CVD risk factors, it would be reasonable to hypothesize that increased long-term consumption of dietary nitrate would lead to a reduced risk of atherosclerosis and CVD.

Oral bacteria have a significant role in the conversion of 85% of dietary nitrate to nitrite in the mouth leading to the formation of nitric oxide. Nitrate-reducing facultative anaerobic bacteria such as *Neisseria, Actinomyces, Veillonella and Rothia* have a symbiotic relationship with the host, in which they use nitrate from the host for their own respiration, resulting in production of nitrite(27). This relationship is particularly important for nitrite bioavailability since humans are unable to complete this process independent of the nitrate-reducing bacteria. Nitrate-reducing bacteria has been reported to represent approximately 20% of the oral bacteria in some people, and their

presence is correlated with increased oral nitrate-reducing capacity resulting in salivary and plasma nitrite concentrations(28). This might contribute to the improvements in endothelial function and blood pressure. However, further work is required to determine the impact of higher dietary nitrate intakes on the location and composition of the oral nitrate reducing bacteria and its possible role on blood pressure and vascular function.

### 1.1. AIMS, OBJECTIVES AND HYPOTHESIS OF THE THESIS

The overarching aim of my thesis was to investigate the role of the oral microbiome and dietary nitrate on BP and other CVD risk markers. This thesis includes two human dietary intervention studies to investigate the impact of the oral microbiome and high dietary nitrate intakes on vascular reactivity and blood pressure. In addition, a cross-sectional analysis was performed using dietary data, and urine samples from the National Diet and Nutrition Survey (NDNS). A dietary database of the food and drink nitrate and nitrite compositions was developed, as well as measurement of urinary nitrate and nitrite to determine the utility of this as a biomarker of nitrate intake.

**CHAPTER 2:**

**Objective:** To critically review the evidence on the potential role of dietary nitrate and the oral microbiome on vascular function including BP and vascular tone. Furthermore, to evaluate the subject determinants that are responsible for the inter-individual differences in oral bacterial composition.

**CHAPTER 3:**

**Objective:** To investigate the relationships between daily dietary nitrate consumption on BP and other CVD risk factors in a representative UK population (NDNS). Furthermore, to compare the impact of nitrate from different dietary sources (vegetables, cured and processed meats and drinking water) on CVD risk markers.

**Hypothesis:** Higher consumption of nitrates from vegetables and drinking water are associated with lower BP and beneficial CVD risk markers compare with nitrate intakes from cured and processed meats.

**CHAPTER 4:**

**Objective:** To examine the efficacy of 24-hour urinary nitrate concentrations as a marker of dietary nitrate exposure and to determine the relationship with BP and other CVD risk factors.

**Hypothesis:** Urinary nitrate and nitrite represent a suitable biomarker to estimate dietary nitrate intake.

**CHAPTER 5:**

**Objective:** To investigate the influence of beetroot juice ingestion and mouthwash on the abundance and genus of nitrate-reducing bacteria. Furthermore, to identify the major sites and specific nitrate-reducing bacterial species within the oral cavity.

**Hypothesis:** The mouth rinse with beetroot juice will increase nitrate-reducing bacteria in the oral cavity and antibacterial mouthwash will reduce the number of nitrate-reducing bacteria in the oral cavity of healthy volunteers.

**CHAPTER 6:**

**Objective:** To investigate the chronic effects of beetroot juice consumption on BP, microvascular reactivity, blood lipids, markers of insulin control and inflammation in 28 healthy people.

**Hypothesis:** The 8-wk supplementation of beetroot juice containing 3.7mg/body weight/d of nitrate will result in a decrease in BP, improvements in microvascular function and CVD risk markers compared with matched control.

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# CHAPTER 2: LITERATURE REVIEW

## Chapter 2

The following review has been published in Nutrition Research Review Journal.

## Contribution towards the paper

The authors responsibilities were as follows: H.S.A., D.A.H, K.G.J. and J.A.L. contributed to the conception of the literature search strategy. H.S.A. undertook the literature review. D.A.H., K.G.J. and J.A.L. provided feedback and guidance on previous drafts of the review and J.A.L. was responsible for final content. The authors have no conflicts of interest to declare.

**The Role of Dietary Nitrate and the Oral Microbiome on Blood Pressure and Vascular tone**

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**Running title:** Dietary nitrate, oral bacteria and vascular tone.

**Keywords:** Nitrate, nitrite, nitric oxide, oral microbiome, blood pressure, mouthwash.

## 2.1. ABSTRACT

There is increasing evidence for the health benefits of dietary nitrates including lowering blood pressure and enhancing cardiovascular health. Although commensal oral bacteria play an important role in converting dietary nitrate to nitrite, very little is known about the potential role of these bacteria in blood pressure regulation and maintenance of vascular tone. The main purpose of this review is to present the current evidence on the involvement of the oral microbiome in mediating the beneficial effects of dietary nitrate on vascular function and to identify sources of inter and intra-individual differences in bacterial composition. A systematic approach was used to identify the relevant articles published on PubMed and Web of Science in English from January 1950 until September 2019 examining the effects of dietary nitrate on oral microbiome composition and association with blood pressure and vascular tone. To date, only a limited number of studies have been conducted, with n=9 in humans and n=3 in animals focusing mainly on blood pressure. In general, elimination of oral bacteria with use of a chlorhexidine based antiseptic mouthwash reduced the conversion of nitrate to nitrite and was accompanied in some studies by an increase in blood pressure in normotensive subjects. In conclusion, our findings suggest that oral bacteria may play an important role in mediating the beneficial effects of nitrate-rich foods on blood pressure. Further human intervention studies assessing the potential effects of dietary nitrate on oral bacteria composition and relationship to real time measures of vascular function are needed, particularly in individuals with hypertension and those at risk of developing cardiovascular diseases.

## 2.2. INTRODUCTION

Cardiovascular diseases (CVDs), including coronary heart disease and stroke, are one of the leading causes of death globally. In 2017, the World Health Organization (WHO) reported that 18 million people had died from CVDs worldwide which represents 31% of deaths[1]. Abnormally raised blood pressure, defined as greater than 140 (systolic)/90 (diastolic) mmHg, is an independent risk factor for CVDs and this silent killer is associated with a three-fold higher risk of having a stroke or developing heart failure[2,3]. High blood pressure affects more than 1 in 4 adults in England, around 12.5 million people. However, the prevalence of hypertension appears to differ between sexes, with 31% reported amongst men and 26% amongst women[4]. Dysfunction of the endothelium, which controls vascular tone and strongly associated with hypertension, is now recognised as an early, but potentially reversible, step in the development of CVDs[5].

The control of vascular function is known to be influenced by dietary factors, with nitrate-rich vegetables considered an important modulator[6,7,8]. This has been demonstrated in many observational and cohort studies which have shown consumption of nitrate and nitrite-rich foods to significantly improve cardiovascular health[9] such as lowering blood pressure[10] in both healthy[11] and hypertensive individuals[12], reducing endothelial dysfunction[13,14] [15,16,17] and inflammation[18], protection from ischemia reperfusion injury[19], and improved exercise performance in patients with heart failure[20]. A prospective cohort study has also concluded that an increased adherence to a diet high in nitrate is accompanied by a significant reduction in the risk of suffering both cardiovascular complications and death due to any cause[21]. Clinically, nitrate supplementation or use of nitrate as a medication to increase the bioavailability of nitrite and nitric oxide (NO) can reduce blood pressure[22]. The interest

in using dietary nitrates as a treatment for lowering blood pressure is growing but mechanisms underlying the effects are unclear which limits their current application as a dietary treatment for hypertension[22]. Furthermore, there is some evidence to suggest that high dietary nitrate intakes are associated with negative effects on health, which has led to the development of the Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/kg body weight/day and for nitrite of 0.07 mg/kg body weight/day[23]. The ADI for nitrate is based on the risk of methaemoglobinaemia commonly known as blue baby syndrome, which can occur following high nitrate intake in some babies, and can be fatal [24]. In addition, some epidemiological studies have reported an association between dietary nitrite intake and colorectal cancer. However, the weight of evidence only supports a significant relationship between cancer and red and processed meat[25], with little known about vegetables and drinking water. The nitrate and nitrite within processed meat may be a contributing factor in the association with cancer, although this needs further confirmation.

Humans are naturally colonised by an array of microorganisms, such as commensal or symbiotic communities, whose metabolic activity is important for host physiology and health. Commensal oral bacteria and those residing in the gastrointestinal (GI) tract play an important role in converting dietary nitrate to nitrite and the potent vasodilator NO[26,27,28,29,30,31]. Up to 85% of ingested nitrate is reduced to nitrite by the nitrate-reducing bacteria in the oral cavity[32] raising the salivary nitrite concentration to 1000 times that of plasma[28]. A cohort study conducted in 281 volunteers found that the high abundance of nitrate reducing bacteria was associated with blood pressure in normotensive individuals, although this association was not found in those with hypertension[33]. To date, very little is known about the role of these oral bacteria in the control of vascular function, and the variation in composition that exists between

individuals. The aim of this review is to present the current evidence on the potential role of dietary nitrate and the oral microbiome on vascular function including blood pressure and vascular tone. Important determinants of the number and composition of the oral bacteria will also be described. However, the impact of dietary nitrate interventions on vascular function only will not be specifically addressed in this instance due to the large number of review articles which already exist in this research area[13,14 15,16,17]. Before presentation of the methodology and results of the literature review, we provide a general overview of dietary nitrate sources, the pathways for the conversion of dietary nitrate and nitrite to NO, location and type of nitrate-reducing bacteria in the oral cavity and their potential role in regulating vascular tone.

## 2.3. NITRATE, NITRITE AND NITRIC OXIDE SOURCES AND NITRIC OXIDE PATHWAY

NO, the most effective form of nitrate, was first recognised in 1998 as an important signalling molecule in the cardiovascular system[34]. NO plays a significant role in virtually all organs in the body, and higher circulating concentrations are associated with a lower CVD risk[35]. In additional to the dietary (exogenous) sources of nitrate and nitrite which leads to the production of nitrite, and subsequently NO, via the oral bacteria, the body can also derive NO endogenously (figure1). The endogenous pathway can occur in a number of different tissues in the body using three forms of NO synthase (NOS) enzyme, neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS). eNOS was initially discovered in endothelial cells and is important in modulating vascular tone and upholding endothelial integrity. However, eNOS can also be expressed in various tissues and requires the presence of oxygen, calcium and calmodulin to be activated[36]. Within the endothelium, L-arginine undergoes a 5-electron oxygen dependent oxidation to produce NO and L-citrulline, catalysed by the

synthase enzymes. Five cofactors required by the NOS enzymes are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and heme iron[28]. Once produced in the endothelial cell, NO rapidly diffuses to the underlying smooth muscle layer where it mediates blood vessel vasodilation. Any NO remaining in the circulation is rapidly converted to nitrate by oxyhaemoglobin or superoxide before it enters the enterosalivary pathway. Therefore, the NO produced has a relatively short half-life in the order of seconds to minutes[37].

## 2.4. NITRATE METABOLISM, ENTEROSALIVARY CIRCULATION AND GASTROINTESTINAL TRACT

High levels of inorganic nitrate are found in vegetables (such as beetroot and spinach) as well as drinking water, and these dietary sources accounts for 80% of the daily intake. In contrast, the intake of dietary nitrite is very low, being approximately 100 times lower[38] than that of nitrate [39]. Although the process of re-circulation of nitrates in the body has been known since 1970s, the importance of the oral nitrate-reducing bacteria in the enterosalivary circulation has only recently been recognised[27] (Figure 2). The key role these bacteria play in nitrate reduction was supported by a previous human study in which a significant correlation was found between high abundance of oral nitrate-reducing bacteria and nitrite level in saliva[40]. Nitrate secretion from the salivary glands leads to a 10 fold rise in salivary nitrate levels[41] and this nitrate enriched saliva appears to be a supportive environment for the growth of the oral bacteria particularly the nitrate-reducing bacteria on the tongue[42]. These bacteria are mostly facultative anaerobes which use nitrate as an alternative electron acceptor for their respiration[43]. A symbiotic relationship therefore exists between the oral commensal bacteria in which they receive nitrate from the host for their own respiration

and in return produce nitrites required by the host[42]. This relationship is particularly important for nitrite bioavailability since humans are unable to complete this process independent of the nitrate-reducing bacteria, with 80% of nitrates swallowed and present in the stomach produced by the oral commensals[44]. Once in the stomach, contact with the gastric acidity leads to the protonation of nitrites to form nitrous acid ($HNO_2$), which then decomposes into not only NO but also several other nitrogen oxides[45] which have localised benefits on maintaining the gastric mucosa layer[46] and enhancing mucosal blood flow[45] which increases the thickness of the mucosal layer[47]. This process is referred to as non-enzymatic conversion which does not require bacteria. However, the presence of *Helicobacter pylori* can contribute to a more acidic environment within the stomach and increase non-enzymatic conversion[48]. Residual nitrates and nitrites are then absorbed in the small intestine with the half-life of circulating nitrate in the blood stream of around 5-6 hours[49]. In contrast, plasma nitrite concentrations start to increase within 15 minutes of nitrate ingestion and reach a peak level in 2 hours[50]. A large portion, approximately 70-75% of the plasma nitrate, is excreted in the urine whereas the remaining 25% is stored in the salivary gland and then recycled in the enterosalivary pathway[51].

The role of the nitrate-reducing bacteria can persist past the oral cavity as most of these bacteria move into the stomach with both swallowed food and saliva. Limited studies have investigated the existence of these bacteria in the stomach and have confirmed that the gastric acidity is not a germ-free environment[52]. Although the gastric pH is below 5, some bacteria species can tolerate the stomach acidity, with a culture based study reporting *Clostridium* spp, *Veillonella* spp and *Lactobacillus* spp as the most predominant gastric species[53], with *Veillonella* spp identified as the most abundant nitrate reducing bacteria[43]. There are many factors that can influence gastric

acidity such as inflammation and long-term use of proton pump inhibitors. The pH level has been found to have a positive impact on nitrate and nitrite concentration in the gastric juice. In a study conducted in 99 patents with dyspepsia, results showed that when the pH level of gastric mucosal surface increased there was a comparable increase in both nitrate and nitrite concentrations. Findings from another study conducted in participants with achlorhydria , in which gastric pH ranged from 6-8, reported three genera of nitrate reducing bacteria: *Streptococci* and *Neisseriae* to be responsible for the nitrite accumulation in the gastric secretions[54].

The small intestine and colon contain many different species of bacteria including both facultative and obligate anaerobes which are involved in the bioconversion of nitrite to NO, although they are not necessarily the same as the nitrate reducing bacteria found in the oral cavity[55]. A study conducted in germ-free and normal rats has shown that NO can be produced by the bacteria resident in the small intestine of normal rats, but not in germ free rats[56]. Furthermore, two studies have identified *Lactobacilli, Bifidobacteria*[56]*, Escherichia coli and Shigella* as the predominant nitrate reducing bacteria in the large intestine[57]. However, an *in-vitro* study which used pure strains of gut bacteria incubated in agar media with nitrate then nitrite, found that in the presence of nitrite, both *Bifidobacterial* and *Lactobacilli* generated large amounts of NO, up to 5000 parts per billion (ppb), but only approximately 35 ppb of nitrate[58]*.* Interestingly, Sobko et al reported that the NO formed was being utilised by *Escherichia coli* and *Staphylococcus aureus* [46]. These authors speculated that these gut bacteria may consume NO in order to help adapt to their environment in this *in vitro* experiment. Therefore, it appears that the presence of NO and other nitrate metabolites in the large intestine may be dependent on the relevant abundance of these bacteria species and their production and utilisation of NO [59]. Localised effects of the NO could include

altering blood flow which could potentially increase the uptake of nitrate and nitrite in the proximal small intestine where the majority are absorbed[44]. However, the NO level in the GI tract could also be influenced by other factors such as pH level, inflammation, oxygen tension and the level of dietary nitrate intake of an individual. Further studies are need to determine the direct effects of nitrate and nitrite on gut bacteria composition and nitrate metabolism.

## 2.5. BACTERIAL NITRATE REDUCTION IN THE ORAL CAVITY, COMPOSITION AND LOCATION

A continuous flow of saliva, specialized mucosal surfaces and teeth in the human oral cavity provide a unique microbial habitat for bacteria. Most of these bacteria are found on the dorsum (surface) of the tongue and around the teeth where a wash of 1 ml of saliva can contain up to $10^7 – 10^8$ microorganisms[44]. However, only 700 species have currently been identified[44]. The majority of these bacteria shelter in the gingival crevices between teeth which represents a conducive anaerobic environment. Here, the gingival crevicular fluid bathes the bacteria within a nutritionally rich medium supporting their proliferation[60]. In contrast, the smoother surfaces of teeth have much lower levels of bacteria due to the forces that act on these areas during eating and drinking. However, the nitrate-reducing bacteria are found predominately on the rear dorsum of tongue, with a higher proportion of gram-negative bacteria found within the papillae of the tongue compared to the surface. Some studies have identified the genus and species of these bacteria that can produce nitrate reductases and nitrite reductases that aid in the production of nitric oxides. These include: *Veillonella atypical Veillonella dispar, Actinomyces eslumdii, A. odontolyticus, Staphylococcus epidermids, Neisseria flarescens, Haemophilus, Porphyromonas, Rothia mucilaginosa, Rothia dentocarisa, Prevotella and Leptotrichia*[42,43]. The two major

groups of oral nitrate-reducing bacteria are the strict anaerobes such as *Veillonella atypica* and *Veillonella dispar* and the facultative anaerobes such as *Actinomyces odontolyticus* and *Rothia mucilaginosa*[42]. Facultative anaerobes are mostly prevalent on the surface of the tongue, with a study stratifying participants according to oral nitrate reduction capacity observing a higher abundance of *Streptococcus*, *Granulicatella, Prevotella, Neisseria, and Haemophilus* on the posterior surface of the tongue compared to *Actinomyces*[43]. Interestingly, although lower in prevalence, *Actinomyces* have been reported to be more efficient reducers of dietary nitrates under anaerobic conditions.

## 2.6. MECHANISMS BY WHICH BACTERIA MAY CONVERT NITRATE TO NITRITE

The three mechanisms through which nitrates are converted to nitrites and other components by bacteria are denitrification, assimilation and dissimilation. The first process, denitrification, occurs in the oral cavity under aerobic conditions[61] and is also called the respiratory nitrate reduction process. During microbial respiration, oxygen is replaced by nitrogen oxides as terminal electron acceptors and ultimately reduces nitrate to nitrous oxide or free nitrogen[62]. Most of the bacteria which have genes for respiratory nitrate reductases (*nirS* and *nirK*) prefer aerobic conditions[63] such as *Rothia spp* and *Neisseriae spp*. However, some denitrification species of bacteria also reside in anaerobic conditions[44] such as *Veillonella*. The specialised surface of the tongue dorsum therefore represents a microaerophilic environment which allows denitrification to occur under both aerobic and anaerobic conditions. In the oral cavity, nitrite ($NO_2$) is initially formed from salivary nitrate ($NO_3$) by some oral bacteria such as *Actinomyces*[43] that are considered to possess the nitrate reductase enzyme (*nar*) and further converts nitrite to NO through either enzymatic (*nir*) or non-enzymatic

denitrification. The latter process is a well-established step in the gastric environment of the stomach. NO is then converted to nitrous oxide ($N_2O$) by nitric oxide reductase (*nor*) and finally to nitrogen ($N_2$) by nitrous oxide reductase (*nos*). The nitrogen oxides and enzymes that participate in the process of denitrification are as follows:

$$NO_3 \xrightarrow{nar} NO_2 \xrightarrow{nir} NO \xrightarrow{nor} N_2O \xrightarrow{nos} N_2$$

In the second pathway known as dissimilation, nitrate is reduced to ammonia ($NH_4+$) by periplasmic nitrate reductase (*nap*), with the intermediate product being nitrite[64]. This two-step process is strictly anaerobic and occurs in the human gut by the facultative anaerobes[55].

$$NO_3 \xrightarrow{nap} NO_2 \xrightarrow{nrf} NH_4+$$

Assimilation, which occurs predominantly in plants, water and soil[65], is the third pathway. Similar to denitrification, the conversion of nitrate to ammonia occurs but during this pathway, the enzyme cytoplasmic nitrate reductase (*nas*) is used[65]. In this biosynthetic anabolic pathway, nitrite is further reduced to ammonia, which can then undergo ammonium assimilation by incorporating the amino acid glutamine[44]. The assimilation and dissimilation processes are therefore important in the utilization of nitrates**.** Nitrifying bacteria (including *Nitrobacter, Nitrococcus* and *Nitrosomonas*)[66] are responsible for the dissimilation and ammonification of nitrates and oxidises ammonium salts and nitrites to nitrates in a process called nitrification. It has been hypothesised that this process might happen in the gut, but to date, this has not been described[67]**.**

In humans, nitrate reduction seems to occur either directly, such as in assimilatory nitrate reduction, or during a series of reactions during respiratory nitrate reduction.

Notably, the latter process needs more than one enzyme for further reduction which is mediated by the bacterial communities[44]. This suggests that nitrate reducing capacity of nitrate-reducing bacteria is related to the bacterial species, cellular location of enzymes and environmental conditions such as oxygen level. Therefore, dissimulation would occur more in the gut and denitrification in the oral cavity[67]. Although the role of oral bacteria in mediating the beneficial effect of nitrate on vascular function is poorly understood, this review aims to address this knowledge gap by focussing on studies that used antibacterial mouthwash and toothpaste to determine the importance of the presence of oral microbiome on blood pressure and vascular tone.

### 2.7. METHOD*S*

A systematic approach was used to identify the relevant human and animal studies which investigated the role of dietary nitrate and the oral microbiome on blood pressure. PubMed and Web of Science were used for the literature search which included all relevant articles published in English from January 1950 until September 2019. There were three stages in the selection process. The combinations of the key terms used in the search strategy were as follows: (“Nitrate” OR “Nitrite” OR “Nitric Oxide”) AND (“Oral Bacteria” OR “Oral Microbiom” OR “Nitrate-Reducing Bacteria”) AND (“Blood Pressure” OR “Hypertension” OR “Cardiovascular” OR “Vascular Function”) AND (“Mouth Wash” OR “Antiseptic” OR “Antibacterial”). The titles and abstracts of the identified papers were screened by one member of the review team (HA) who identified potentially relevant papers. This review was restricted to animal studies and human studies which used antibacterial mouthwash or toothpaste to determine the effects on oral nitrate reduction on blood pressure and vascular tone. Only published peer-reviewed literature was considered and ‘grey’ literature such as dissertations, conference proceedings, reports, letters to editors and other non-peer-

reviewed research, was excluded. After duplicates were removed, the abstract and full papers were screened for eligibility. In addition, a hand-search of the bibliographies of the articles found from the electronic database searches was also conducted. An overview of the literature search is shown in Figure 3.

The quality of the included human RCTs and animal studies were assessed for the risk of bias using the Cochrane risk of bias tool[68] for human studies and SYRCLE's tool[69] for animal studies.

## 2.8. RESULTS AND DISCUSSION

The systematic search identified 160 publications. Of these, 11 relevant publications were included, with 9 describing studies conducted in humans and 3 in animals. The risk of bias assessment summaries for each study are presented in Supplementary Tables 1 and 2, respectively. Animal studies will be discussed before studies including human participants. This will be followed by discussion of the non-modifiable and modifiable factors affecting intra-individual variability in number and composition of oral bacteria, with potential mechanisms of action.

### 2.8.1. Animal studies

Of the 14 animal studies which have investigated the effect of nitrate on blood pressure, only 3 studies have determined whether oral bacteria are important in mediating the improvements in blood pressure and endothelial function (Table 2). Formation of bioactive NO takes place within the gastric environment of the stomach as a result of the enterosalivary circulation of nitrate, as well as systemically in the blood vessels. In 2009, Petersson and his colleagues[70] reported daily mouthwash treatment for 7 days in rats to attenuate both the gastroprotection provided by NO and the diastolic blood pressure lowering effect of sodium nitrate. A similar pattern was also evident for the mean arterial pressure in the rats treated with mouthwash and

nitrate, but the lack of an effect in the rats treated with mouthwash and nitrite suggested that oral bacteria play an important role in the metabolism of nitrate to NO and mediated vasodilation. Furthermore, these rats also had reduced oral bacteria suggesting that nitrite could bypass the reduction step by the oral bacteria and was being reduced in the circulation or within endothelial cells to NO, or via effects on the formation of the intermediate nitrosothiols[70]. However, dietary nitrite intake is generally lower than that of nitrate, and the half-life in plasma shorter (seconds versus hours) which suggests that even if nitrite directly stimulates NO signalling, the quantity and kinetics of nitrite versus nitrate indicates that the critical aspect of this mechanism is the reduction of nitrate. Therefore, the role that dietary nitrite plays in blood pressure lowering may be more limited relative to nitrate.

In agreement, Hyde et al[29] also reported a significant reduction in diastolic blood pressure and increase in plasma nitrite concentrations following the addition of sodium nitrate to drinking water in male Wistar rats. However, in this study, mouthwash treatment was unable to diminish the blood pressure lowering effects of the nitrate supplementation. The authors speculated that the direct application of the chlorhexidine-based mouthwash (Vedco, St. Joseph, MO) to the tongue surface using a swab might not have enabled sufficient time for the mouthwash to exert its full extent on the bacteria relative to mouthspray[29]. A novel aspect of this longer-term supplementation study was the focus on the changes in the microbiota composition on the rat tongue in response to the treatments. Compared with baseline, there was a greater relative abundance of nitrate reducing bacteria (*Haemophilus spp* and *Steptococcus spp*) after 6 days of sodium nitrate consumption, and of these *Haemophilus parainfluenzae* has also been identified as 1 of 14 species contributing to nitrate reduction in the oral cavity of healthy adults. Co-supplementation of

mouthwash with nitrate was found to increase the diversity of the oral bacteria present relative to nitrate intake only, with increases found in the low abundance taxa such as Enterobacteriaceae, *Corynebacterium*, and *Morganella*. Therefore, the use of mouthwash appeared to disturb the oral microbiome by reducing the abundance of the normally dominant taxa but not completely to impact nitrate reduction. These findings suggest that the lower abundance taxa which were evident after mouthwash treatment may be functionally important in the bioactivation of dietary nitrate. However, the authors did caution against translating these findings on the oral bacteria composition to humans since the oral human microbiome has been shown to be more diverse and of a differing composition compared with the rat[29].

The impact of mouthwash on chronic changes in blood pressure in response to nitrate or nitrite supplementation was further examined by Pinheiro et al[71] in both control and hypertensive rats. After 4 weeks, significant reductions in mean arterial pressure and systolic blood pressure were evident in both the nitrate and nitrite groups, with concordant increases found in circulating plasma nitrate and nitrite levels. Interestingly, co-supplementation with mouthwash attenuated the rise in plasma nitrite levels by 25-30% in both groups but was only found to blunt the blood pressure lowering effect of nitrate, with little impact found on blood pressure in the mouthwash and nitrite group. In agreement with Petersson et al[70], these findings suggested that anti-hypertensive effects of nitrite were potentially occurring via non-enzymatic reactions within the gastric environment after swallowing this ion independently of the enterosalivary pathway and potentially via non-enzymatic reactions within the gastric environment after swallowing this anion. Analysis of the endogenously produced vasodilatory compound S-nitrosothiol and levels of vascular nitrosylation revealed mouthwash to reduce nitrosylation responses to nitrate only, leading the authors to

speculate that S-nitrosylation was an important mediator of the blood pressure lowering effects of both nitrate and nitrite[70,71]. Studies have also reported that the foods consumed with dietary nitrites, such as conjugated fatty acids, are also a target of nitrating species in the stomach leading to the formation of nitro-fatty acids (such as nitro-conjugated linoleic acid). These electrophiles have been shown to have anti-hypertensive effects independent of S-nitrosothiols suggesting that they may also play a role in mediating the effects of nitrate and nitrite on blood pressure [72]. Antiseptic mouthwash was proposed to attenuate the beneficial effects of dietary nitrate intake on blood pressure by reducing the amount of nitrite formation by the oral bacteria and therefore reaching the stomach, inhibiting gastric formation of S-nitrosothiols. However, the positive benefits on blood pressure of raised S-nitrosothiols was only found in the antihypertensive rats, supporting previous observations in both animals and humans that raised blood pressures often show a greater sensitivity to the anti-hypertensive effects of medication and/or dietary modification.

Studies performed in animals may provide useful insights into the mechanisms underlying the effects of oral bacteria in the bioactivation of nitrate. However, findings in rats and mice need to be interpreted with caution due to differences in physiology and dependence on nitrate as a source of NO between organisms. In contrast to humans, rats and mice do not recirculate nitrate in saliva[73] and so salivary nitrate concentrations never exceed those levels found in plasma[74] and they also have other nitrate reducing mechanisms that may work in tandem with nitrate reduction by the oral bacteria to control nitrite and NO level[73].

### 2.8.2. Human Studies

The publications describing the human studies were divided into those which examined 1) the association between oral bacteria with nitrate/nitrite levels and/or blood pressure (n=5; Table 2) and 2) the combined effects of nitrate ingestion and oral

bacteria on nitrate/nitrite levels and/or blood pressure (n=4; Table 3). The role of the oral bacteria in mediating systemic nitrite production after nitrate intake has been primarily investigated with the use of an antiseptic mouthwash to remove the bacteria prior to the measurement of the outcomes of interest. The type of mouthwash has been shown to be important, with the strong antibacterial chlorhexidine-based mouthwash (Corsodyl) found to be more effective at reducing *Veillonella dispar* (nitrate reducing bacteria) in the oral cavity than Listerine (mixture of essential oils), Isodine and Cepacol (antibacterial) in healthy adults[75]. In support of these findings, gargling with 10 ml of chlorohexidine mouthwash (Corsodyl) twice for 1 min was also found to reduce the bacterial count of nitrate reducing bacteria by approximately 80% and virtually abolish the oral nitrate reducing capacity compared with no mouthwash in healthy subjects[27]. Although nitrate accumulated in saliva after ingestion of sodium nitrate in both studies, a significant reduction in the conversion of salivary nitrate to nitrite after mouthwash was associated with 30% lower plasma nitrate concentrations at 3 h post-ingestion, compared with no prior use of mouthwash. In contrast, a randomised cross-over study found an antibacterial toothpaste to have no effect on salivary or plasma nitrate concentrations in 16 women after consuming 400 mg of nitrate before brushing their teeth with antibacterial toothpaste (0.3% triclosan) or toothpaste containing no antibacterial agent[76]. The lack of an effect observed with the antibacterial toothpaste may reflect either the lower prevalence of the nitrate reducing bacteria on the surface of the teeth, relative to the tongue, or the less efficient removal of the bacteria sheltering within the gingival crevices between the teeth compared with mouthwash.

Four studies have determined the impact of mouthwash on changes in oral nitrate reducing capacity and blood pressure (Table 2). Compared with no mouthwash, Kapil

et al[41] reported that using 0.2% chlorhexidine twice daily for 7 days significantly increased systolic and diastolic blood pressure measured using 3 different techniques (clinic, ambulatory and home measurements) by approximately 3 and 2 mmHg respectively in 19 healthy normotensive subjects. Interestingly, the effects of mouthwash treatment on blood pressure was evident after only a single use of the chlorhexidine mouthwash and was maintained for the following 6 days. The rise in blood pressure was significantly correlated with the significant reduction in plasma nitrite levels, with only a trend for a relationship with the salivary nitrite, highlighting the potential importance of the oral nitrate-reducing bacteria in blood pressure modulation. In 15 subjects treated with anti-hypertensive medication, the attenuation found in oral nitrate reducing capacity after daily use of chlorhexidine mouthwash for 3 days was associated with an increase in systolic blood pressure of 2.3 mmHg, but only a trend for a decrease in plasma nitrite concentrations compared with the control (tap water)[77]. The lack of a significant effect on the plasma nitrite response relative to Kapil et al[41] was thought to be due to the study visit being performed 12 h after prior use of the mouthwash treatment or related to the age or medication use of the hypertensive participants. In order to determine the mechanism underlying the effects of dietary nitrate intake on blood pressure, plasma cGMP, a mediator of NO-dependant smooth muscle relaxation in the endothelium and a good marker of NO production, can be measured. Although increases in plasma nitrite and cGMP after dietary nitrate intake have been previously associated with blood pressure lowering, no effects were evident on cGMP concentrations after 3 days of using mouthwash. This may be related to the lack of a nitrate challenge on the study visit (which provides an important source of NO under hypoxic conditions) but could also suggest that dietary nitrate may impact on vascular tone via direct effects on smooth muscle function.

In contrast to these two studies, Tribble et al [78]reported use of chlorhexidine mouthwash twice daily for 7 days to be associated with a highly variable effect on clinic systolic blood pressure (an increase of at least 5 mmHg found in n=9 subjects whereas a decrease was observed in n=4) in an orally healthy cohort. Post-hoc data analysis revealed the inclusion of tongue cleaning as part of the daily dental hygiene routine to play a significant role in the responses observed both on blood pressure and the diversity of the oral bacteria at baseline and during the study. Specifically, regular tongue cleaning was associated with a greater ability to reduce nitrite to NO whereas the lack of tongue cleaning resulted in an oral microbiome composition which favoured conversion of nitrite to ammonia and not NO. The authors speculated the use of chlorhexidine mouthwash was having a chemo-stimulatory effect on the oral bacteria, with the temporary loss of bacterial numbers proposed to stimulate a rapid population recovery and increase in bacterial nitrate reductase activity. However, these effects may also reflect a protective upregulation of the nitrate, nitrite and NO regulating mechanisms in the microbiota suddenly detached from their biofilms during tongue cleaning and warrants further investigation.

In a cross-over study, treatment with chlorhexidine (0.2%) for 3 days was shown to have no effect on clinic or 24 h ambulatory blood pressure in 17 young females compared with a placebo mouthwash [79]. Although a reduction in salivary nitrite and oral nitrate reducing capacity was found after the antibacterial mouthwash, comparable changes were not evident in either the plasma or urine samples collected. The lack of effects observed relative to other studies may reflect the short intervention time with the mouthwash treatments or inclusion of female participants only. Based on a previous study conducted by the same research group in athletes, they speculated that cross-talk may exist between the enterosalivary nitrate-nitrite-NO pathway and

eNOS, with a greater intake of dietary nitrate associated with a lower eNOS activity. However, whether a reduction in nitrate-nitrite-NO with antibacterial mouthwash leads to an upregulation in eNOS is yet to be established.

In the studies presented in Table 3, measures of blood pressure have been related to salivary and plasma nitrate/nitrite levels following nitrate intake and use of mouthwash. In agreement with previous findings, Woessner et al [30] found antibacterial mouthwashes to attenuate postprandial salivary and plasma nitrite concentrations following dietary nitrate intake (concentrated beetroot juice) compared with the weaker antiseptic mouthwash and control. Although changes in clinic systolic blood pressure 0-3 h after the treatments were not related to plasma/salivary nitrite or nitrate levels, systolic blood pressure at 4 h was 2-5 mmHg higher after Chlorhexidine and Cepacol mouthwashes compared with control and Listerine mouthwash. These findings potentially suggest an important role of the nitrate-nitrite-NO enterosalivary pathway but should be interpreted with caution due to the small sample size, inclusion of male subjects only and the short duration of the study visit relative to the expected peak in plasma nitrite concentrations (approximately 3 h). Furthermore, these findings may have been influenced by the large inter-individual variability observed in blood pressure responses following the mouthwash treatments.

In the study of McDonagh and co-workers [80], consumption of 2 x 70 ml shots of concentrated beetroot juice and daily use of strong or weak antibacterial mouthwash for 6 days were found to have limited effects on baseline blood pressure and salivary and plasma nitrate/nitrite levels compared with the control (water). However, differences were evident 2-4 h after drinking the beetroot juice, with the rise in plasma nitrite found to be attenuated after use of the strong and weak mouthwash for 6 days. These changes were associated with a reduced oral nitrate reducing capacity after the

strong mouthwash, with lower nitrite levels compared with both the weak and placebo mouthwashes. Although changes in resting measures of blood pressure (supine and seated) and pulse wave analysis (arterial stiffness) after the juice were not influenced by the strength of the mouthwash used, differences were evident in blood pressure during low-intensity activity on the treadmill. In particular, there was a greater increase in systolic blood pressure and mean arterial pressure after rinsing with the strong (Chlorhexidine) compared with the control (water) mouthwash. The lack of effect on arterial stiffness even in the presence of lower salivary and plasma nitrite levels after the strong mouthwash indicates that either the availability of NO was not altered sufficiently over the 4 h acute test period in these young active participants or that their higher physical active level may have masked any effects of the mouthwash on the vascular function measures. However, this is one of the only studies to incorporate a measure of blood vessel elasticity to determine the role of oral bacteria in mediating the beneficial effects of beetroot juice on vascular function, and so further studies are needed in which to compare these findings and determine the underlying mechanisms. As highlighted in the human studies, oral bacteria composition appears to vary between individuals, with both non-modifiable (e.g. age, sex, genetics and tongue physiology) and modifiable (e.g. diet, health conditions, life style and dental hygiene routine) factors considered to impact on the abundance and prevalence of nitrate reducing bacteria in the oral cavity. These factors are important to consider during interpretation of the study findings and for informing the design of future studies exploring the role of oral nitrate reducing bacteria on the regulation of vascular function. The following section summarises the main factors identified from the human studies.

### 2.8.3. Intra-individual variability in number and composition of oral bacteria

- ***Non-modifiable factors***

Geographical location and culture have all been suggested to impact on oral bacteria composition. Findings from a study including participants from Northern and Southern Europe, reported a higher abundance of *Rothia* and unclassified *Gemellaceae* in Finnish populations compare to Spanish while *Lactococcus, Fusobacterium* and *Porphyromonas* genus were significantly higher in Spanish compare to Finnish groups[81]. Comparing findings of this study with another study which investigated the differences in oral bacteria between people living in Africa, Alaska and Germany showed that oral bacteria composition is highly variable between countries[82]. These differences may represent the sex and age distributions of these different populations, genetic make-up and habitual food preferences[83,82].

Moreover, the dorsal surface of the tongue plays a major role in nitrate reduction and represents a highly papillated surface area. The papillary structure of the human tongue is unique in nature and supports a higher bacterial density than the mucosal surface, accumulating oral debris and anaerobic bacteria on the rear of tongue[42]. There are three kinds of papillae on the tongue: fungiform, circumvallate and foliate papillae. The fungiform papillae have a mushroom shape and are found predominately on the dorsal surface of the tongue covering up to two-thirds of the surface. Their shape supports a higher bacterial density[84]. However, the shape and number of papillae varies between individuals which has been related to differences in oral bacteria composition. Studies have shown that a number of factors can affect the papillary number on the tongue including ageing (with lower number of papillae observed in those individuals over 60 years), genetic make-up, ethnicity[81], demographics and environment[84].

Within the oral cavity, the presence of teeth increases the bacterial density compared

to those with permanent tooth loss since the gingival crevices between teeth represent a greater surface area and environment for bacterial growth[85]. Other important factors considered to impact on the variety of nitrate reduction bacteria present in the oral cavity are ageing and sex. However, in a recent human study conducted in n=9 participants < 22 years and n=9 > 70 years, a similar salivary microbiome at baseline and after placebo beetroot juice was found in both groups. Comparable changes in bacterial composition (increases in *Rothia* and *Neisseria*) were also evident in both age groups in response to consuming 140 ml of beetroot juice (≈ 12 mmol nitrate) daily for 10 days[86] suggesting that age was not an important modulator of the oral bacteria composition in this study. Few studies have determined differences in oral bacteria composition between men and women. In order to address this knowledge gap, Kapil and colleagues[87] examined the impact of sex on nitrate reducing bacteria abundance in 13 male and 13 females age 18-45 years. Oral bacteria samples were collected before and after nitrate supplementation and all samples were analyzed by 16S rRNA sequencing. Significant sex dependent effects on oral nitrate reducing bacteria composition were not found in this study. However, sub-group analysis indicated females to have a non-significant tendency for a higher activity of nitrate reducing bacteria than men[87,74] but these findings need to be confirmed in a suitably powered study.

- ***Modifiable factors***

Several modifiable factors have been reported to influence and change the oral nitrate reducing bacteria composition, with dietary nitrate intake considered to be one of the most important factors[27,88]. In a recent cross-over study conducted in 18 volunteers assigned to receive a nitrate supplement or a placebo for 10 days, an increase in the abundance of some nitrate reducing bacteria, particularly *Rothia and Neisseria* was

linked with the ability of an individual to reduce the nitrate supplement. However, changes were not observed with the *Prevotella and Veillonella* species[86]. Interestingly, these results corroborate findings from another study which reported the reduction in *Prevotella* and *Veillonella* species in the oral cavity of elderly adults following dietary nitrate intake to be associated with a lower mortality risk in this population[88]. Furthermore, the increased prevalence of *Rothia* and *Neisseria* species relative to the *Prevotella* and *Veillonella* species was linked to higher NO bioavailability in both saliva and plasma[86]. These findings imply that the oral bacteria community is responsive to changes in the level of dietary nitrate intake[89]. However, the authors also reported that individuals with a higher abundance of *Campylobacter concisus* and *Prevotella melaninogenica* in their oral cavity at baseline may not be as responsive to dietary nitrate intake than those with a lower proportion of these bacteria[86]. This might reflect the fact that both *Campylobacter. concisus* and *Prevotella. melaninogenica* are predominately nitrite, but not nitrate, reducers in the oral cavity. Therefore, dietary nitrate availability may affect the growth and composition of particular groups of oral bacteria which can be related to improved cardiovascular health[89]. Of particular note, drinking beetroot juice rich in dietary nitrate can increase the oral cavity pH from 7.0 to 7.5 which is close to the optimal pH of 8 required for nitrate reductase activity[90]. Therefore, the effect of pH is also important in terms of the proliferation and inhibition of different populations within the oral bacterial community[86].

In a similar fashion, some health conditions have also been reported to influence the oral bacterial composition, with a lower density of nitrate reducing bacteria and a different bacterial composition found in people with raised blood pressure (hypertensives) than normotensive subjects[31]. A recent novel study has provided further evidence on the relationship between differences in oral bacteria composition

with hypertension in postmenopausal women (n=446). This study analysed oral bacterial samples by using 16S RNA sequencing and found that the abundance of *Prevotella oral species 317* and *Streptococcus oralis* were significantly lower in women with elevated blood pressure compared with those with normal blood pressure[91]. Furthermore, the differences in the oral bacteria communities between groups also seemed to be associated with the severity and progression of the hypertension [33]. Conversely, a higher abundance of nitrate reducing bacteria were observed in individuals who suffer from migraines (a vascular driven process associated with changes in NO). Interestingly, the dominant nitrate reducing bacteria in these individuals were *Pseudomonas* and *Streptococcus* which are not common in subjects who did not suffer with migraines. Oligotyping (the technique for differentiation between closely related microbial taxa)[92] was performed for both genera to investigate the strain-level differences across the bacterial population. *Pseudomonas* decompose to 2 oligotypes (different strains of the same species) and has differential abundance patterns with significantly higher abundance in oligotype 2 in those suffering from migraines compared with non-sufferers[93]. These results suggest that the type of these oral bacteria may be more prevalent in people with migraines. However, more work is needed to find the link and the mechanism to explain how these bacteria adapt genetically to their host environment.

Therefore, there may be an optimum number and composition of nitrate reducing bacteria which has beneficial effects, and a greater level may have a negative impact on conditions associated with blood vessel dilation such as migraine. However, it should be acknowledged that nitrate reduction and metabolism cannot be attributed to single bacterial species as they are unlikely to express all of the enzymes required to decompose nitrate simultaneously. More likely, these individual nitrate reducing

bacteria are considered to work in synergy with other members of the microbial community. This has been demonstrated by Hyde et al, [43] who found that mixed colonies of high and low nitrate reducers showed a greater capacity for nitrate reduction than mixes of either multiple high reducers or individual nitrate reducing bacteria. This highlights the complexity of the oral microbiome and the impact on dietary nitrate metabolism.

Cardiometabolic diseases including obesity, the metabolic syndrome and type II diabetes are major contributors to global CVD disease burden. Whilst some studies have reported plasma nitrate/nitrite levels to be negatively associated with waist circumference [94], obesity [95] and blood pressure, others have observed positive associations between plasma nitrite and BMI, fasting blood glucose [96], systolic blood pressure and the fasting lipid profile. In support of these findings, Akram et al (2018)[97], found plasma nitrite levels to be higher in individuals with both obesity and the metabolic syndrome followed by those with obesity alone, with the lowest levels in those with normal weight. Whether high plasma nitrite levels play a role in the worsening of the cardiometabolic risk markers is a public health issue since higher dietary nitrate intakes may also cause higher levels of plasma NOx (sum of nitrate and nitrite levels). Furthermore, these data are associations, do not indicate whether cardiometabolic risk markers change in response to varying nitrate/nitrite intakes and do not prove cause and effect. Interestingly, a review of the evidence suggests the contrary, with dietary nitrate supplementation found to reverse or improve some of the features of the metabolic syndrome and be protective against the development of CVD[98]. Although these beneficial effects may be related to improvements in NO metabolic pathways and glucose control, we cannot discount that favourable changes in the gut microbiota in response to dietary nitrate intake may also represent an

important mechanism since dysbiosis (a term to describe microbial imbalance) is a common feature of the cardiometabolic diseases. However, very few studies have determined the impact of dietary nitrate supplementation on the gut microbiota in humans, with a very short-term study with nitrate-rich fruit and vegetable juice suggesting a reduction in the Firmicutes to Bacteroides ratio after 3 days which was related to higher plasma nitrate/nitrite levels [99]. Furthermore, a one-year intervention with the Mediterranean diet, rich in vegetables, was associated with increased abundance of specific taxa that were inversely associated with inflammatory markers [100]. More studies are needed to address this research gap which also include analysis of the oral microbiome to determine whether increases in the abundance of nitrate reducing bacteria are related to improvements in cardiovascular health.

Oral hygiene habits, including daily use of an antibacterial mouthwash or tongue scraper have been found to not only reduce acute bacterial infection, but also numbers of bacteria present[30]. On the other hand, poor oral hygiene contributes to dysbiosis by accumulating a plaque biofilm which contains large number of microbes including nitrate reducing bacteria [101]. This can cause dental infections and gingivitis by increasing pathogenic bacteria such as (*porphyromonas gingivalis)*[102]. Studies have shown that patients with periodontal disease to have higher levels of salivary nitrite which may be partly derived from the reduction of nitrates by the oral bacteria. Since nitrite has been shown to have an antimicrobial effect against gastrointestinal and oral pathogens, it has been speculated that the salivary glands may respond to the periodontal infection by enhancing the secretion of nitrate and production of nitrite by the nitrate reducing bacteria as a host defence mechanism[103]. This is thought to reduce the prevalence of the acidogenic bacteria which contribute to the development of dental caries. In agreement, Doel et al [42] has reported a significant reduction in

dental carries in study participants with high salivary nitrate concentration. Epidemiological studies have reported an association between periodontal disease with CVD. Although the cause and effect relationship has not been proven, studies have suggested that inflammation caused by the oral infection may contribute to the development and progression of the atherosclerotic plaque. Interestingly, periodontal pathogens have been identified in the atherosclerotic plaque suggesting a direct role in CVD. However, to date, periodontal disease has not been considered to be a CVD risk marker[104]. Lifestyle habits such as smoking can also influence oral bacteria composition[74]. In a study conducted in 9 non-smokers aged 20-45 y and n=5 healthy active smokers (>20 cigarettes per week) aged 30-60 years, nitrate reduction activity was found to be over 80% lower in smokers compared to non-smokers[74]. However, the low numbers of individuals within each group may have influenced the results observed.

As previously mentioned, dietary nitrates have been shown to interact with other food components such as lipids[72], with similar reports for polyphenols[105], alcohol[106] and proteins [25]. In particular, foods and beverages rich in polyphenols including apple, tea and orange juice have been shown to lead to a 3 fold increase in NO production in the stomach[107] and reduce endogenous N-nitrosamine formation. Along with polyphenols, the content of ethanol in red wine can also interact with nitrite forming ethyl nitrite which works as a nitrosation agent and may mediate NO effects. These interactions with other dietary components may therefore play a role in modulating the circulating NO levels and bioavailability of the nitrate and nitrite contained within foods.

In summary, a systematic approach was used to identify the studies that have determined the impact of oral bacteria on blood pressure in response to nitrate intake, from dietary sources or supplements. However, only a very limited number of human

(n=2) and animal (n=3) studies have addressed this research question, with the remaining studies examining the importance of the oral bacteria on the nitrate reducing capacity on circulating nitrite concentrations and blood pressure. Based on our observations from these studies, there is accumulating evidence to suggest that absence of nitrate-reducing oral bacteria was associated with increasing blood pressure even when accompanied by a high nitrate intake. However, some of the studies failed to see any effects, which may be due to type of mouthwash used in the human studies or the method of application of the mouthwash in the animal study[29]. Sex, hypertension, and tongue cleaning were all found to be important potential determinants of the variability in the responses between participants. Of these, the dental hygiene practice of tongue cleaning, which is recommended by the American Dental Association, appeared to promote oral microbiota diversity and be associated with a greater ability to recover the tongue microbiome after mouthwash use. Potential mechanisms to explain the blood pressure lowering effects of dietary nitrates included increases in plasma nitrite, S-nitrosothiols, nitro-fatty acids and vascular nitrosylation and cross-talk between the enterosalivary nitrate-nitrite-NO pathways and eNOS activity in the endothelial cells. However, the limited number of studies performed make it difficult to draw any firm conclusions from this literature review.

## 2.9. CONCLUSIONS

With the increasing prevalence of non-communicable diseases there is an urgent need for further studies to investigate the role of the oral bacteria on cardiovascular health in response to dietary nitrate intake, and to determine the underlying mechanisms. With vascular function now recognised as an important prognostic marker for future CVD risk, studies incorporating real time measures of vascular reactivity and tone are required. Furthermore, the use of rigorous methods to determine changes in the

abundance and composition of the oral bacteria in response to intake of dietary nitrate would help to identify important nitrate-reducing bacteria related to changes in vascular function and determine whether these bacterial groups are also evident in the gut microbiome, a proposed modulator of chronic disease risk. Diets containing nitrate-rich foods may contain other bioactive components which could also contribute to CVD risk reduction, including fibres, vitamins, minerals and flavonoids. Such diets may offer a number of advantages over nitrate/nitrite supplemental use, not only due to the availability of other bioactive components, but also because of reports of vascular adaptation and risk of marked acute hypotension after supplemental nitrate use, not found with nitrate-rich diets[108]. With hypertension a major risk factor for CVD, more studies are needed to determine whether diets higher in nitrate-rich foods can be recommended for blood pressure lowering and disease prevention in healthy individuals and those at greater CVD risk.

## 2.10. ACKNOWLEDGEMENTS AND AUTHOR CONTRIBUTIONS

The authors responsibilities were as follows: H.S.A., D.A.H, K.G.J. and J.A.L. contributed to the conception of the literature search strategy. H.S.A. undertook the literature review. D.A.H., K.G.J. and J.A.L. provided feedback and guidance on previous drafts of the review and J.A.L. was responsible for final content. The authors have no conflicts of interest to declare.

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# TABLES

**Table1: Commonly reported nitrate reducing bacteria species found in the oral cavity**

| Bacteria species | Condition | Change in abundance in response to nitrate intake | Location in the oral cavity |
|---|---|---|---|
| *Veillonella dispar*[42,43] | Anaerobic | ↑ | Tongue |
| *Actinomyces odontolyticus*[42,43] | Facultative anaerobic | ↑ | Tongue |
| *Prevotella salivae*[42,43] | Anaerobic | ↑ | Tongue |
| *Rothia mucilaginosa*[42,14] | Aerobic | ↑↑ | Tongue |
| *Neisseria flavescens*[43,14] | Aerobic | ↑↑ | Tongue |

**Table 2: Animal studies investigating the importance of oral nitrate reducing bacteria on blood pressure in response to nitrate intake**

| Reference | Animals | Study design and duration | Intervention | Measurement | Outcome measures |
|---|---|---|---|---|---|
| Petersson 2009[70] | n= 4-7 Male Sprague Dawley rats each group (190-360 g, B and K, Sollentwia, Sverge). | Parallel groups with 7 day treatment periods:<br>1) No treatment (control).<br>2) $NaNO_3$ only<br>3) Mouthwash<br>4) Mouthwash + $NaNO_3$ or $NaNO_2$ | Water supplemented with 10 mM $NaNO_3$ or 1 mM $NaNO_2$<br><br>Mouthwash groups: Chlorhexidine mouthwash spray (0.3 ml), 2X daily. | Plasma | $\Delta$ $NO_2$ $\downarrow$ after mouthwash + $NaNO_3$ vs control $p<0.05$. |
| | | | | HR<br><br>SBP | NS<br><br>NS |
| | | | | DBP | $\downarrow$after $NaNO_3$. DBP lowering absent in mouthwash treated rats |
| | | | | MAP<br><br><br><br>Oral bacteria | $\downarrow$ after $NaNO_3$ and mouthwash + $NaNO_2$ vs mouthwash only. MAP lowering absent in mouthwash + $NaNO_3$ rats<br><br>$\downarrow$ viable bacteria on tongue after mouthwash |
| Hyde 2014[29] | n= 8 Male Wistar rats 7 weeks old | 19 day sequential intervention:<br>0-5 control (water)<br>6-12 $NaNO_3$,<br>13-19 $NaNO_3$ + mouthwash<br><br>Blood collected at day 1, 5, 6, 12, 13 & 19. BP (telemetry) and tongue swab every day | $NaNO_3$ (1 g/L) in drinking water<br><br>Mouthwash regime: 0.3 ml of chlorohexidine applied 2X daily to tongue dorsal surface (days 13-19) | SBP | NS |
| | | | | DBP | $\downarrow$ after $NaNO_3$ and mouthwash + $NaNO_3$ vs control |
| | | | | Plasma NOx | NS |

| | | | | | |
|---|---|---|---|---|---|
| Pinheiro 2016[71] | n = 10, Male Wistar rats each group (190-210 g)<br><br>2 kidney, 1 clip (2K1C) hypertensive group.<br><br>Sham operated control group | 6 weeks – 2 weeks baseline followed by 4 weeks treatment<br><br>Experiment 1<br>Vehicle<br>$NaNO_2$<br>Mouthwash<br>Mouthwash + $NaNO_2$<br><br>Experiment 2<br>Vehicle<br>$NaNO_3$<br>Mouthwash<br>Mouthwash + $NaNO_3$<br><br>6 h after last treatment, blood and tongue swab collected. | 15 mg $NaNO_2$/kg or 140 mg $NaNO_3$/kg (gavage)<br><br>Mouthwash groups: Daily mouth clean with Chlorhexidine (0.12%) soaked swab. | Plasma | $\Delta$ $NO_2$ $\downarrow$ 25–30% after mouthwash **vs** $NaNO_2$ and $NaNO_3$ groups ($P < 0.05$)<br>$\Delta$ $NO_3$ $\downarrow$ 45% after mouthwash vs $NaNO_2$ group ($P < 0.05$) |
| | | | | BP | $\downarrow$ SBP (40 mmHg) and MAP with $NaNO_2$ and $NaNO_3$ ($P= 0.01$).<br>Mouthwash blunted MAP and SBP lowering effect of $NaNO_3$ ($p <0.05$) but not $NaNO_2$ |
| | | | | Oral bacteria | $\downarrow$CFU 50-70% with mouthwash |

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, NS: Not Significant, $NO_2$: Nitrite Concentration, SBP: Systolic Blood Pressure, $NO_3$: Nitrite Concentration, CFU: Colony Forming Unit (number of viable bacteria).

**Table 3: Human studies determining the effects of oral bacteria on salivary and plasma nitrite concentrations, and/or blood pressure in response to nitrate intake**.

| Reference | Subject characteristics | Study design and duration | Nitrate dose | Type of mouthwash | Measurement | Significant outcomes |
|---|---|---|---|---|---|---|
| **ACUTE STUDIES** | | | | | | |
| Mitsui et al., 2017 [75] | n=12 (6M/6F)<br>Normotensive,<br>Age 19-44 y<br>Non-smoking, | Acute, RCT, CO<br>4 visits 10 h in duration with 1 wk washout.<br><br>Saliva and oral bacteria collected 0, 1 and 10 h. | 100 g lettuce (110 mg $NO_3$) with breakfast. Lunch at 5 h. | 1. Water (control)<br>2. Listerine (antiseptic)<br>3. Isodine (povidone-iodine, 0.35%)<br>4. Chlorhexidine 0.0025%<br>Treatment for 3 min prior to nitrate ingestion | Saliva<br><br>Oral bacteria | Relative to baseline:<br>↑ $NO_3$ and $NO_2$ after each treatment ($P < 0.05$)<br><br>↓ nitrate reducing bacterium *V. Dispar* at 1 and 5 h after Chlorhexidine |
| Govoni et al 2008[27] | n=7<br>Normotensive<br>Age 24-51y<br>BMI 23 kg/$m^2$<br>Non-smoking | Acute, RCT, CO<br>2 visits of 3 h in duration.<br><br>Blood and saliva samples collected before and for 3 h after nitrate intake.<br>Oral bacteria collected in n=4 after mouthwash only. | 10 mg/kg $NaNO_3$ in 100 ml water | Mouthwash vs no mouthwash<br><br>Corsodyl (Chlorhexidine) gargled twice for 1 min, 15 min before nitrate ingestion. | Saliva<br><br>Plasma<br><br>Oral bacteria | ↑$NO_3$ on both visits<br>↓ $NO_2$ vs no mouthwash<br><br>$NO_3$ ↓ 29 nM and $NO_2$ ↓ 250 nM at 3 h vs no mouthwash<br><br>↓ bacteria count and (80%) and nitrate reducing capacity after mouthwash. |

| | | | | | | |
|---|---|---|---|---|---|---|
| Woessner et al 2016[30] | n=12 (M)<br>Normotensive<br>$\overline{x}$ age 36 y and BMI 24 kg/m$^2$<br><br>Non-smoking | Acute, RCT, CO<br>4 visits, 4 h in duration with 1 wk washout.<br><br>BP, blood and saliva collected before and for 4 h after juice consumption | 140 ml of concentrated beetroot juice (8.4 mmol nitrate) | 1) Water (control)<br>2) Listerine (antiseptic)<br>3) Cepacol (antibacterial)<br>4) Chlorhexidine (0.12%)<br><br>Treatment 15 min after beetroot juice for 60s. | SBP | ↓ Listerine and control vs Cepacol and Chlorhexidine (P ≤ 0.05) |
| | | | | | DBP | NS |
| | | | | | Saliva | ↑ $NO_3$ all treatments<br>↑ $NO_2$ control vs all mouthwashes and ↓ $NO_2$ Chlorhexidine and Cepacol vs antiseptic (P ≤0.05) |
| | | | | | Plasma | ↑ $NO_3$ all treatments<br>↓ $NO_2$ Chlorhexidine vs all treatments and Cepacol vs control (P ≤0.05) |
| Bondonno et al 2012[76] | N=16 F<br>Normotensive<br>$\overline{x}$ age 52±11y (F)<br>Non-smokers, | Acute, RCT, CO<br>5 visits of 3 h in duration.<br>1 wk washout.<br>Blood and saliva samples collected before and for 3 h after nitrate intake | 0, 100, 200, 400 mg NaNO3 in water | 1) Antibacterial toothpaste (0.3% triclosan)<br><br>2) Toothpaste without antimicrobial agent (control) | Saliva<br><br>Plasma | ↑ $NO_3$ all treatments<br><br>↑ $NO_3$ all treatments |

| **ACUTE WITHIN CHRONIC** | | | | | | |
|---|---|---|---|---|---|---|
| McDonagh et al 2016 [80] | n=12 (6M/6F) Normotensive $\overline{x}$ age 22±2y (F) and 24±2 y (M). Non-smokers, | Acute within chronic, RCT, double blind<br>6 visits over 8 weeks<br>Each treatment 6 days, with acute visits (4 h) on days 0 and 6.<br><br>Acute visits: Rinse with mouthwash 15 min before ingesting 2 x 70 ml beetroot juice. Measurements at 0, 2 and 4 h. BP and PWA measured at rest and during 10 min of treadmill walking. Saliva and plasma samples collected. | 70 ml of beetroot juice (6.2mmol nitrate) twice a day | 1) Strong - Corsodyl (Chlorhexidine)<br>2) Weak - Vademecum med (non-chlorhexidine-containing antibacterial mouthwash)<br>3) Deionised water (con)<br>3X daily 15 mins before beetroot juice and meals, for 6 days | SBP<br><br><br><br><br><br><br>DBP<br><br><br>MAP<br><br><br><br><br>HR<br><br><br><br>PWA | Relative to baseline (0 h):<br>Resting - NS<br>After 10 min exercise, ↑ 3 mmHg after strong mouthwash vs control (P = 0.07) 4 h after beetroot juice<br>Resting and during exercise – NS<br>Resting - NS<br>After 10 min exercise, ↑ after strong mouthwash vs control (P<0.05) at 4 h.<br>During exercise ↑ after strong vs control and weak (P<0.05).<br>NS |
| | | | | | Plasma<br><br><br><br><br><br>Saliva | ↑$NO_3$ all treatments<br>Δ NO2 ↓ after strong vs other treatments at 2 and 4 h, and weak vs control (P<0.05) at 2 h<br>Δ NO3 ↑ and Δ NO2 ↓ after strong vs weak and control (P<0.05) at 4 h. |

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, RCT: Randomized Controlled Trial, NS: Not Significant, PWA: Pulse Wave Analysis, SBP: Systolic Blood Pressure, CO: Cross Over.

**Table 4: Chronic human studies investigating the involvement of oral bacteria in the blood pressure lowering effect of nitrate**.

| Reference | Subject characteristics | Study design and duration | Oral nitrate reducing capacity | Mouthwash regime | Measurement | Significant outcome between treatment |
|---|---|---|---|---|---|---|
| Tribble et al., 2019 [78] | n=26 (16F/10M)<br>Normotensive<br>Age 22-71 y | Sequential<br>4 visits over 14 days: days 1 (baseline), 7 (post mouth wash), 10 (recovery) and 14 (recovery)<br><br>Clinic BP and oral bacteria at each visit. n=6 oral nitrate reducing capacity for 8 h after 30 s mouthwash | Mouth rinse with 1 mM $NaNO_3$ for 2 min | Chlorhexidine (0.12%)<br>2 x daily for 30 sec | SBP<br><br>DBP<br><br>Oral bacteria<br><br>Oral nitrate reducing capacity | In response to mouthwash, ↑ 5mmHg (n=9) and ↓ (n=4) NS<br><br>↓ Species diversity and abundance with mouthwash for 7 days.<br>↑ bacterial metabolic activity at day 14.<br>↓ $NO_3$:$NO_2$ ratio for 6-8 h after mouthwash. |
| Sunqvist et al 2016 [79] | n=17 (F)<br>Normotensive<br>$\overline{x}$ age 23 y<br>BMI = 22 kg/m$^2$<br>Non-smoking. | RCT, CO, double blind<br>Each treatment 3 days with a 28 day washout<br>4 visits (days 3 and 4 of each treatment)<br><br>24 h ABP and urine sample.<br>Clinic BP, saliva and plasma samples and oral nitrate reducing capacity | Mouth rinse with 10 mM $NaNO_3$ for 5 min | Chlorhexidine (0.2%) or placebo mouthwash<br>3 x daily after meals for 60s. | BP<br><br>Saliva<br><br>Plasma<br><br>Urine<br><br>Oral nitrate reducing capacity | No difference in ABP or clinic BP<br><br>↑ $NO_3$ and ↓ $NO_2$ after mouthwash (P ≤ 0.01)<br><br>No change in $NO_3$ and $NO_2$ with mouthwash vs placebo<br>excretion of $NO_3$ with mouthwash vs placebo<br>↓$NO_2$ after mouthwash (8 µM) vs placebo (234 µM)(P<0.001) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Bondonno et al 2015 [77] | n=15 (8M/7F)<br>Hypertensives taking medication<br>BP 120-159/100 mmHg.<br>Age 53-69 y and BMI 20-35 kg/m$^2$.<br>Non-smokers | RCT, CO<br>Each treatment 3 days with a 10-12 day washout.<br><br>Visits at day 0 and 3 of each treatment.<br><br>Saliva sample, oral nitrate reducing capacity and plasma sample. BP measured at home. | Ratio of $NO_2$ and $NO_3$ measured in saliva. | Chlorhexidine or tap water (control)<br>2x daily with 20 ml for 30 sec after brushing teeth | SBP | ↑ 2.3 mmHg after mouthwash vs water (P= 0.01) |
| | | | | | DBP | NS |
| | | | | | Saliva | ↑ NO3 and ↓NO2 after mouthwash vs control (P= 0.001) |
| | | | | | Plasma | ↓ NO2 after mouthwash vs control (P= 0.09). $NO_3$ - NS |
| | | | | | Oral nitrate reducing capacity | ↓ nitrate reductase ratio after mouthwash |
| Kapil et al 2013 [41] | n=19,<br>Normotensive,<br>Age 18-45y, BMI 18-40 kg/m$^2$,<br>Non-smokers,<br><br>No self-reported use of mouthwash or antibiotic | Sequential<br>2 visits (0 and 14 days).<br><br>At each visit, clinic BP, blood, urine and saliva samples and oral nitrate reduction capacity.<br><br>Fitted with ABP unit for 24 h and BP measured at home. | Mouth rinse after holding 3 doses of $KNO_3$ (0, 0.8 and 80 µmol) in the mouth for 5 min. | Chlorhexidine (0.2%)<br>2x daily days 8-14 only. | Clinic SBP | Relative to baseline, use of mouthwash<br>↑ 3.5mmHg (P = 0.003) |
| | | | | | Clinic DBP | ↑ 2.2mmHg (P = 0.038) |
| | | | | | A-SBP | ↑2.4 mmHg (P= 0.017) |
| | | | | | A-DBP | ↑2.2 mmHg (P= 0.014) |
| | | | | | Home SBP | ↑2.9 mmHg (P< 0.001) |
| | | | | | Home DBP | ↑2.0 mmHg (P< 0.001) |
| | | | | | HR | NS |
| | | | | | Saliva | ↑$NO_3$ and ↓$NO_2$ 90% (P< 0.001) |
| | | | | | Plasma | ↑$NO_3$ and ↓$NO_2$ 25% (P= 0.001) |
| | | | | | Urine | ↑$NO_3$ and ↓$NO_2$<br>At baseline, $NO_2$ in mouth rinse dose dependent (0<0.8<80 µmol $KNO_3$) |
| | | | | | Oral nitrate reducing capacity | After mouthwash, ↓ 90% $NO_2$ in mouth rinse for 0.8 and 80 µmol $KNO_3$. |

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, RCT: Randomized Controlled Trial, NS: Not Significant, PWA: Pulse Wave Analysis, SBP: Systolic Blood Pressure, CO: Cross Over, ABP: Arterial Blood Pressure

# FIGURES

## FIGURE LEGENDS

**Figure 1:** Diagram of the endogenous generation of nitric oxide (NO) by NO synthase (NOS) (right panel highlighted in pink), and exogenous generation of NO from the diet (left panel highlighted in blue)[28]. In biological fluids, NO is oxidized to nitrite ($NO_2$) and nitrate ($NO_3$) (dashed arrows).

**Figure 2:** Overview of the nitrate enterosalivary circulation and nitrate metabolism in humans.

Ingested inorganic nitrate is converted to nitrite in the oral cavity by nitrate reducing bacteria with reduction to NO and nitrogen oxides occurring within the acidic environment of the stomach. Remining nitrate and other nitrate components are then rapidly absorbed into the bloodstream via the small intestine. A large proportion of nitrate is then excreted by the kidneys into the urine, with up to 25% being recycled by the salivary glands and then concentrated in saliva.

**Figure 3:** Flow of information through the different phases of the literature review

**FIGURE 1**

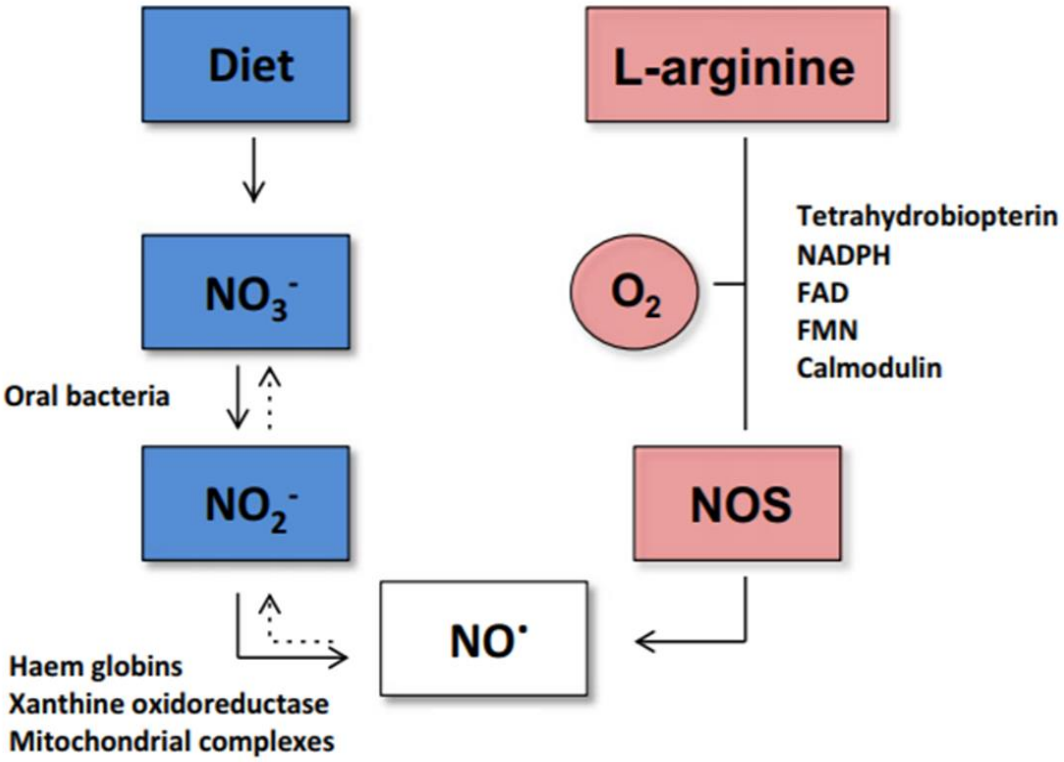

Diet
L-arginine
$NO_3^-$
$O_2$
Tetrahydrobiopterin
NADPH
FAD
FMN
Calmodulin
Oral bacteria
$NO_2^-$
NOS
NO·
Haem globins
Xanthine oxidoreductase
Mitochondrial complexes

**FIGURE 2**

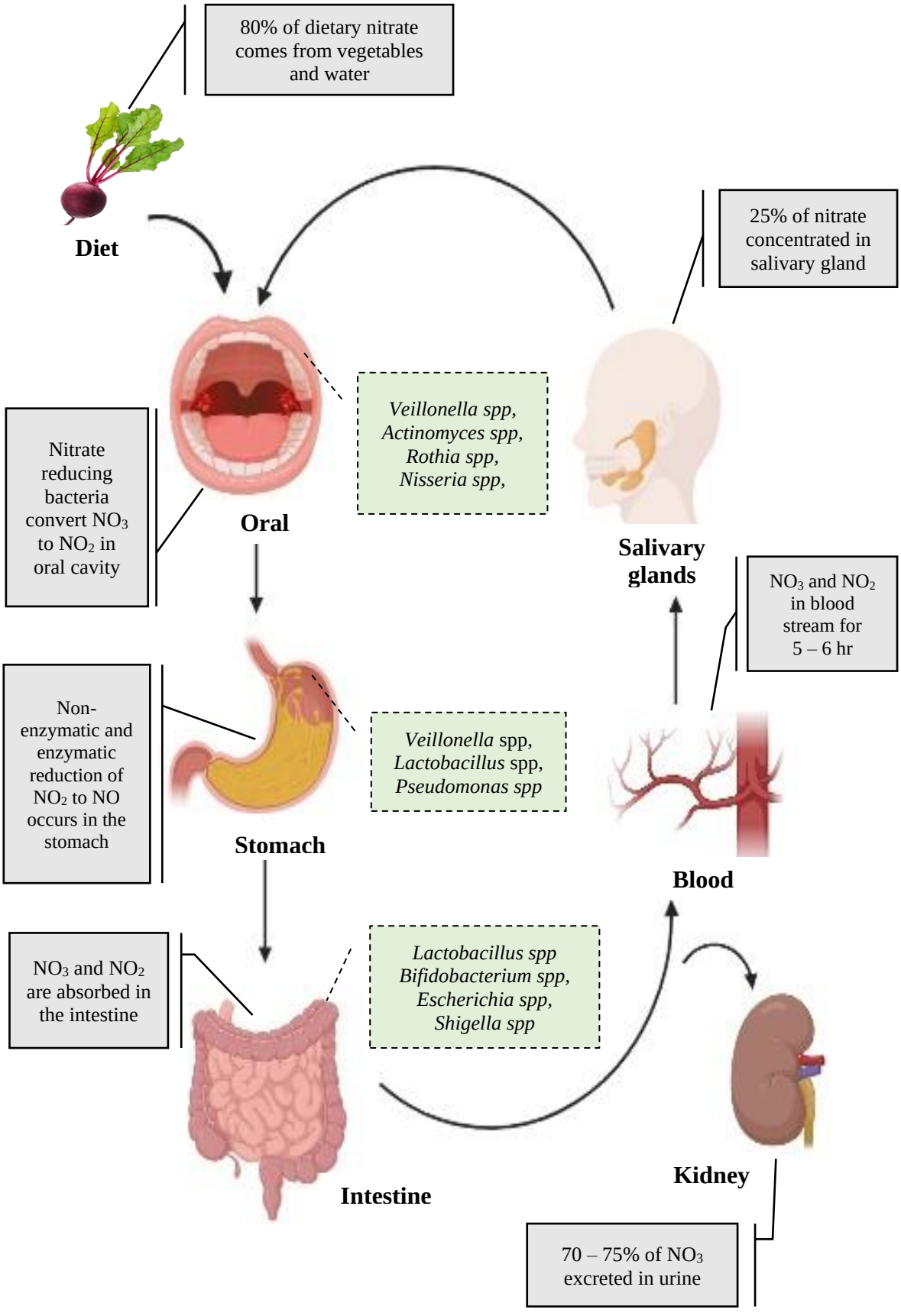
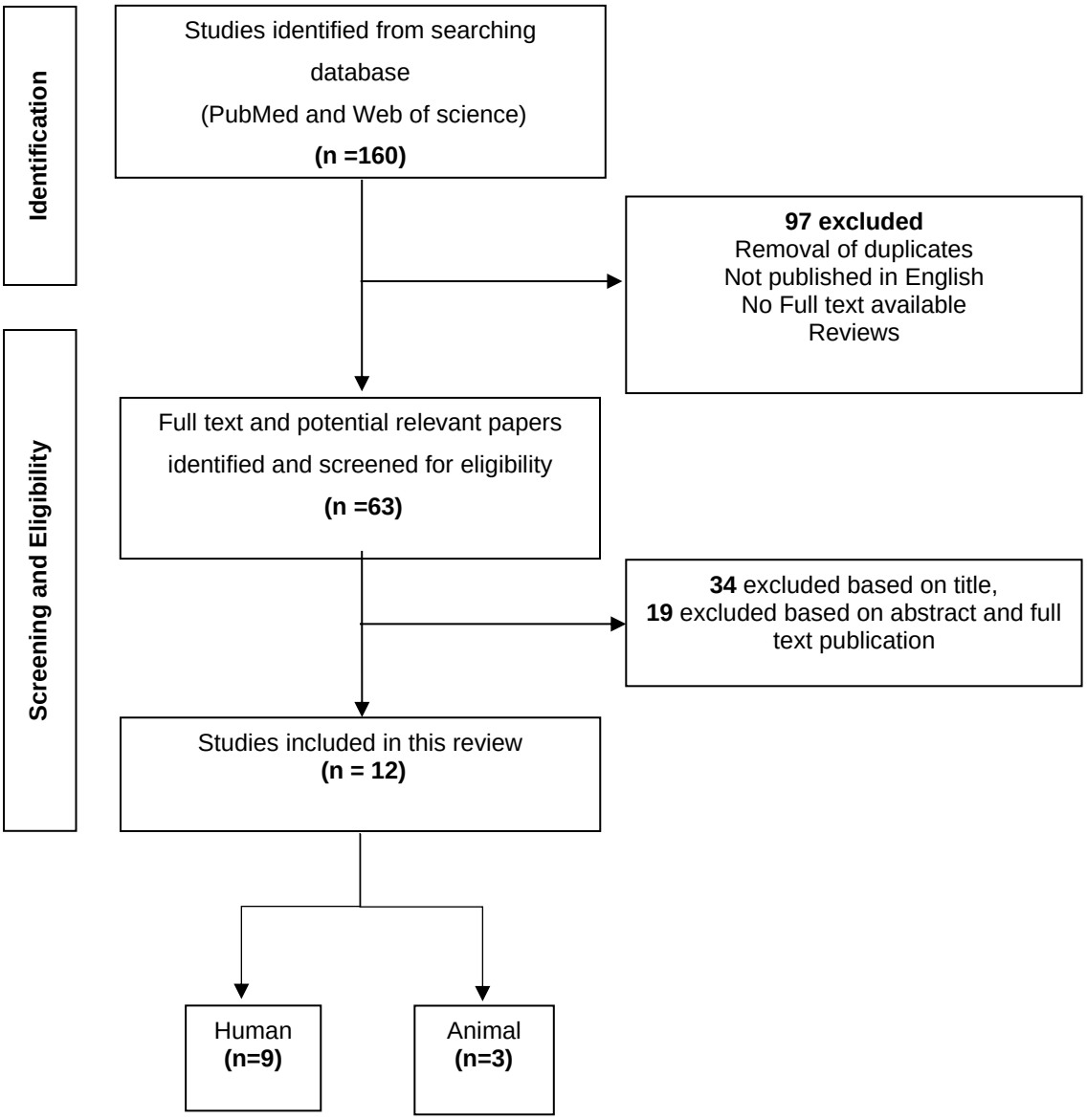

80% of dietary nitrate comes from vegetables and water
Diet
25% of nitrate concentrated in salivary gland
Veillonella spp,
Actinomyces spp,
Rothia spp,
Nisseria spp,
Nitrate reducing bacteria convert $NO_3$ to $NO_2$ in oral cavity
Oral
Salivary glands
$NO_3$ and $NO_2$ in blood stream for 5 – 6 hr
Non-enzymatic and enzymatic reduction of $NO_2$ to NO occurs in the stomach
Veillonella spp,
Lactobacillus spp,
Pseudomonas spp
Stomach
Blood
$NO_3$ and $NO_2$ are absorbed in the intestine
Lactobacillus spp
Bifidobacterium spp,
Escherichia spp,
Shigella spp
Intestine
Kidney
70 – 75% of $NO_3$ excreted in urine

**FIGURE 3**


Identification
Studies identified from searching database (PubMed and Web of science) (n =160)
97 excluded
Removal of duplicates
Not published in English
No Full text available
Reviews
Screening and Eligibility
Full text and potential relevant papers identified and screened for eligibility (n =63)
34 excluded based on title,
19 excluded based on abstract and full text publication
Studies included in this review (n = 12)
Human (n=9)
Animal (n=3)

# CHAPTER 3: THE ASSOCIATION BETWEEN DIETARY SOURCES OF NITRATE, BLOOD PRESSURE AND OTHER CARDIOVASCULAR DISEASE RISK FACTORS IN A REPRESENTATIVE UK POPULATION

**Chapter 3**

The following chapter will be submitted for publication in Journal of Nutrition.

**Contribution towards the paper**

I was involved with creating and establishing a comprehensive database of nitrate and nitrite content in food as well as drinking water, I carried out the data and statistical analysis and I was responsible for writing the first draft of this paper.

**The association between dietary sources of nitrate, blood pressure and other cardiovascular disease risk factors in a representative UK population**

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**Running title:** Sources of dietary nitrate and blood pressure

**Keywords:** Blood pressure; Cardiovascular disease risk factor ; Drinking water; Inorganic nitrate ; Processed meat ; Vegetables.

## 3.1. ABSTRACT

Dietary inorganic nitrate has been shown to lower blood pressure (BP) and improve endothelial function. Our objective was to determine the relationships between nitrate and nitrite from different dietary sources and BP and other cardiovascular disease risk markers in a representative UK population. Data from the cross-sectional National Diet and Nutrition Survey years 1-8, including 3,407 adults aged 19-64 years was used. Initially, a comprehensive database of nitrate and nitrite concentrations in drinking water, vegetables, processed meats and composite dishes was developed to estimate dietary nitrate and nitrite intakes of the study participants. The population was then stratified into quartiles of increasing daily nitrate or nitrite intakes from vegetables (including drinking water) and processed meats to determine the relationships with biomarkers of CVD risk (BP, lipid profile, C-reactive protein (CRP), anthropometric measures and glycaemic control). Across increasing quartiles of dietary nitrate intake from vegetables, systolic BP (SBP), diastolic BP, waist circumference, waist to hip ratio and glycated haemoglobin were significantly lower in Q3 (95-130mg/d) than Q1 (3-65mg/d) ($P \leq 0.038$) and participants in Q4 (>131mg/d) had significantly lower pulse pressure, glucose, CRP and total cholesterol concentrations compared to Q1. Similar beneficial associations on SBP and lipid profiles were also evident for dietary nitrite intake from vegetables ($P \leq 0.05$). In contrast, there were no difference in CVD markers across quartiles of nitrate from processed meats, with higher SBP in Q4 (1.8-3 mg/d) vs Q1 (0.1- 0.8 mg/d) for dietary nitrite intake from processed and cured meats ($p=0.010$). These data suggest the source of dietary nitrate and nitrite may play an important role in determining the relationship with BP and other CVD risk markers.

## 3.2. INTRODUCTION

Cardiovascular diseases (CVD) are the greatest cause of death worldwide (1) with elevated blood pressure (BP) an independent risk factor for its development (2). CVD risk factors can be mitigated, in part, by addressing BP management, modifying the diet (3) and adopting a healthier lifestyle (4). In particular, diets high in fruits and vegetables are associated with lower BP and lower risk of heart disease and stroke (5). This is supported by a significant BP reduction associated with compliance with the Dietary Approaches to Stop Hypertension (DASH) diet (6), which is high in vegetables, amongst other components.

Dietary nitrates are found in root (e.g. such as beetroot) and green leafy vegetables. High levels of consumption is becoming increasingly recognised as a potential dietary strategy to reduce BP and enhance cardiovascular health in both healthy (7) and hypertensive (8) individuals. Although vegetables are the main source of dietary nitrates, contributing 70-80% to the total nitrate consumed (9), water, and to a lesser extent cured and processed meats, also contribute to their intake (10). However, dietary nitrate and nitrite intake estimation is challenging, and often inaccurate, due to a general lack of available data on nitrate and nitrite content in commonly used dietary analysis software databases. Furthermore, it has been recently hypothesised that dietary nitrate from vegetables produces greater bioactive effects, which are thought to enhance the potency and bioactivity of dietary nitrate due to other components such polyphenols that consumed with nitrate(11). In contrast, nitrites are added to cured and processed meats as a preservative and to enhance taste, with high intakes associated with an increased risk of non-communicable diseases such as cancer(12). It is thought that nitrates and nitrites from processed meats produce N-nitroso compounds, which are potentially carcinogenic. The International Agency for

Research on Cancer (IARC)2015, reviewed over 800 research publications and concluded that eating 50g of processed meats per day was associated with a 18% higher risk of colorectal cancer, suggesting that processed meats can be carcinogenic(13). However, the impact of nitrate from different foods on BP and other CVD risk factors remains unclear.

Studies of short duration have reported that dietary nitrates lower BP in small sample sizes representative of young, healthy individuals(7). However, there is a paucity of data on long-term habitual intake of dietary nitrate on BP and CVD outcomes in a larger population. Furthermore, no studies have investigated the relationships between the daily consumption of dietary nitrate from different sources (vegetables, proceeded meat and drinking water) on CVD risk factors in the UK population. To fill this knowledge gap, a retrospective data analysis was performed to accurately estimate dietary nitrate consumption from various sources and investigate the relationship with BP and other CVD risk factors in UK participants (aged 19-64 years) from the National Diet and Nutrition Survey (NDNS). We hypothesise that higher consumption of nitrates from vegetables and drinking water are associated with lower BP and beneficial effects on CVD risk markers compared with nitrate intakes from cured and processed meats.

## 3.3. METHOD

### 3.3.1. Study design

The dataset used for this study was obtained from the NDNS years 1-8 (2008-2016) (14). The NDNS is a continuous cross-sectional observational survey that has been ongoing in the UK since 2008. Currently, it is conducted by NatCen Social Research in conjunction with the MRC Epidemiology Unit at the University of Cambridge. The NDNS aims to gather information on food consumption, nutrient intake, and nutritional

status within a representative sample of the UK population. The NDNS design has been previously described in detail and published elsewhere (14). Briefly, individuals completed a four-day food diary and attended an interview to discuss habitual dietary intake and gather information on, for example, socio-economic class. At a follow-up visit, volunteers had a blood sample collected, gave a 24-hour urine samples and completed a range of anthropometric and BP measurements, including weight, BMI, waist and hip circumferences, systolic (SBP) and diastolic BP (DBP), Pulse rate and pulse pressure, calculated by subtracting the mean DBP from the mean SBP. Blood samples were analysed for CVD risk biomarkers, including lipids (triacylglycerol [TAG], total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C] and high-density lipoprotein cholesterol [HDL-C]), C-reactive protein (CRP), glucose and glycated haemoglobin (HbA1c). The NDNS was conducted according to the guidelines laid down in the Declaration of Helsinki, and ethical approval for all procedures was granted by Local Research Ethics Committees covering all areas in the survey. All participants gave informed consent. The ethical approval was gained from NREC for the retrospective analysis in the urine samples and registered at clinicalTrials.gov.

In this retrospective observational study, diet diaries from 4,745 adults aged 19-64 years were analysed by the NDNS researchers using the NDNS Nutrient Databank; however, the dietary analysis software did not include data on the nitrate content of vegetables and other nitrate-rich foods. The researchers in the current study developed a comprehensive database of the nitrate and nitrite content of vegetables, processed meats and water, and the diet diaries were re-analysed manually using this database.

### 3.3.2. Development of the dietary nitrate and nitrite database

A comprehensive database of the nitrate and nitrite content of vegetables, water and processed meats, was established. This was based on published databases of nitrate

and nitrite content(15). The researchers, D.A.H. and H.B.M. manually searched the NDNS food database to remove food items that did not contain nitrate or nitrite and identified existing databases of food nitrate and nitrite concentrations. For this analysis, the nitrate concentrations of vegetables were taken from a database created as part of a meta-analysis of 255 publications assessing nitrate content in 180 vegetables and 22 herbs from worldwide samples published in 2017 (16). The nitrate content of each vegetable was converted to mg of nitrate or nitrite per gram. Nitrite values for cured and processed meats were taken from papers (with or without meta-analysis) reporting the nitrite content of cured and processed meats (17,18,15,10). To improve accuracy, the values from these three papers were then averaged to create a final representative value for these nitrate and nitrite concentrations.

The recipes for standard dishes (composite foods) were sourced from McCance and Widdowson's *The Composition of Foods Collection* (19) or McCance and Widdowson's supplementary textbooks, namely *Meat and Meat Dishes* (20) and *Vegetables and Vegetable Dishes* (21). For a more accurate estimation of dietary nitrate, the loss of nitrate content during processing and cooking was calculated. The average loss was set at 47.5% which represented the percentage loss of nitrate and nitrite from boiling given in the EFSA report 2008 (22), unless a specific and referenced value was taken from the literature, especially for processed meats (10). The nitrate content of each vegetable and processed meat was converted to mg of nitrate or nitrite per gram. The dietary nitrate content was calculated by multiplying the food items in grams by the nitrate content (mg) per gram including vegetables and meat within composite dishes.

To estimate the nitrate intake from drinking water of the NDNS participants, all of the UK water authorities were contacted to request information on the nitrate and nitrite

content of the drinking water over the time period of the NDNS survey (supplementary Table1). For water authorities that did not provide this information, the nitrate and nitrite concentrations were compiled from the Drinking Water Inspectorate (DWI) Annual Reports for 2008-2016. These data were collated and sent to the offices of NatCen Social Research, where a staff member linked the water authority data (based on the participants' postcodes and the date the diet diaries were completed) to the anonymised subject ID code. To protect the identities of NDNS participants living in the smaller water-authority areas, their data was either combined with a larger adjoining water-authority area or excluded completely from the dataset. Total nitrate and nitrite intakes from the local tap water were then calculated by multiplying each participant's total water intake in litres (for drinking and making coffee and tea) from the diet diary with the nitrate and nitrite concentration obtained from the water authorities in mg/L. Finally, the individual participants total nitrate and nitrite consumption was calculated by summing the nitrate and nitrite from vegetables, water and processed meats consumed in the 4-day dietary collection period and expressed as total nitrate or nitrite per day. The total dietary nitrate and nitrite intake data were then matched to the subject ID codes by NatCen employees, which allowed the relationship between these and anthropometric and CVD biomarkers to be determined.

### 3.3.3. Statistical analysis

Statistical analysis was completed using IBM SPSS version 24 software. Weighting factors were applied to ensure the data collected from different years of NDNS survey were comparable and could be pooled for the statistical analysis. Data were checked for normality using Q-Q plots and transformed to log10 where necessary. Data were stratified according to quartiles of daily nitrate and nitrite intake from all sources, representing diets with the lowest (Q1) and highest (Q4) intakes. Daily nitrate and

nitrite intakes were calculated separately based on dietary sources (i.e. vegetables including drinking water, and processed meats) before being stratified according to quartiles, representing diets with the lowest (Q1) and highest intakes (Q4). An analysis of covariance (ANCOVA) was then used to determine differences between the CVD risk factors, including BP, anthropometric measurements, blood lipids, glucose and CRP across increasing quartiles of intake of nitrate and nitrite from each dietary source. Age, sex, dietary energy intake (MJ) and BMI were used as covariates. A Bonferroni post-hoc test was used to detect differences between quartiles of nitrate and nitrite intake when a significant difference was identified by ANCOVA. For the ANCOVA analysis, the estimated marginal means ± SEM are presented in the tables. Statistical significance was accepted when $p < 0.05$.

## 3.4. RESULTS

This retrospective observational study investigated dietary nitrate and nitrite intake based on food diary records for 4,745 adults aged 19-64 years old in years 1-8 of the NDNS survey. Dietary data was sourced from n=2,697 from years 1-4, n=964 from years 5-6 and n=1081 from years 7-8. After removing participants with incomplete anthropometric, BP or blood biomarker outcome measures, 3,408 subjects were included in this analysis, of which 1,404 were men and 2,002 were women. These subjects had a mean BMI of 26.3±7.1 kg/m$^2$, and their mean age was 43±12 years.

### 3.4.1. Dietary nitrate intake

Stratification of data according to quartiles of total dietary nitrate, revealed SBP to be on average 2 mmHg lower in Q3 (150-192 mg/d nitrate) vs Q1 (<113 mg/d nitrate; p=0.041) and pulse rate to be slower in Q3 (67 bpm) and Q4 (69 bpm) vs Q1 (71 bpm; p<0.001). Waist circumference and waist/hip ratio were also lower in Q3 and Q4 vs Q1(p=0.027), with no differences in the blood CVD biomarkers between quartiles

(Table 1).

Similar findings were observed across increasing intakes of nitrate from vegetables and drinking water (Table 2). Participants in Q3 (95-130 mg/d nitrate) had lower SBP (-2 mmHg) and DBP (-3 mmHg) vs Q1 (<65 mg/d nitrate) (p=0.041 and p=0.011, respectively). Pulse rate was also significantly slower in Q4 (>130 mg/d nitrate) vs Q1 (p=0.008). Additionally, waist circumference, waist/hip ratio and HbA1c were lower in Q3 vs Q1 (p≤0.017, respectively). T-C, glucose and CRP concentrations were found to be lower in Q4 vs Q1 (p≤0.046), with no differences in other biomarkers between quartile groups.

For nitrate intake from processed meats, differences were not found in any of the measures of BP (SBP, DBP or pulse pressure) across quartile groups. However, pulse rate was lower in Q3 (68 bpm) vs Q1 (71 bpm, p=0.001). Hip circumference was higher in Q4 (>77 mg/d nitrate) vs Q2(33-52 mg/d nitrate) (p=0.016). Accordingly, the waist/hip ratio was significantly different between quartiles with a lower ratio in Q4 than Q2 (p=0.003). No significant differences were observed in any of the blood biomarkers.

### 3.4.2. Dietary nitrite

Across quartiles of increasing total nitrite intake, SBP was 3 mmHg higher in Q3 (2-3 mg/d nitrite) and Q4 (3-15mg/d nitrite) vs Q1 (<1 mg/d nitrite; p<0.0001), with no significant changes observed in DBP, pulse pressure or pulse rate. Waist and waist/hip ratios were lower in Q3 than Q1 (p=0.015 and p=0.027, respectively). For the CVD biomarkers, total and LDL-cholesterol were lower in Q4 than Q3 (p≤0.038). HDL-C was higher in Q3 vs Q1 (p=0.023) and CRP was significantly lower among all quartiles compared to Q1 (p<0.0001) (Table 3).

Nitrite intake was also separated based on sources (vegetable and drinking water, or processed meats). In general, nitrites from vegetables and water were associated with more beneficial CVD risk marker profiles than nitrites from processed meat (Table 4).

Participants in Q4 (1.5-9.2 mg/d nitrite) had lower SBP (-2mm Hg) than Q1 (>0.3 mg/d nitrite, p=0.007). Nitrite intake from vegetable and water was associated with lower waist circumference in Q3 vs Q1 (p=0.005) and lower waist/hip ratio in both Q2 and Q3 vs Q1 (p=0.001). Moreover, HDL-C was higher in in Q3 than Q1 (p=0.020) and there was a lower TC: HDL-C ratio and HbA1c in Q4 vs Q1 (p≤0.028). CRP concentrations were lower in Q2-Q4 compared to Q1 (p<0.001).

Higher nitrite intakes from processed meats were associated with a significantly greater SBP, +3 mmHg in Q4 (1.8-3 mg/d nitrite) vs Q1 (0.1-0.8 mg/d nitrite) (p=0.010). Furthermore, hip circumference was higher in quartile Q4 vs Q2 (p=0.008). No differences were observed in any of the other biomarkers.

### 3.5. DISCUSSION

This study aimed to evaluate the associations between total dietary nitrate and nitrite intake with different dietary sources and markers of CVD risk. Initially a comprehensive database of the nitrate and nitrite content of foods and water was developed and used to assess the daily dietary intake of these bioactives in vegetables, drinking water, and processed meats. Using data from the NDNS it was found that dietary nitrate consumption of 150-192 mg/d from all sources (vegetables, water and processed meats) was associated with the lowest SBP and DBP which reached significance when compared to 114-149 mg/d. However, when the different sources of dietary nitrate were examined, it was shown that only vegetable and water sources (95-130 mg/d nitrate) were associated with lower SBP and DBP and also longer-term glycaemic control (HbA1c). Furthermore, intakes of greater than 131 mg/d of nitrate from vegetables and water were associated with lower plasma total cholesterol and glucose concentrations, independent risk marker for CVD and type 2 diabetes, respectively. Favourable effects on CVD risk markers were not observed with intake

of dietary nitrate from processed meats. These data give further support for the benefits of dietary nitrate from vegetables (and water) on these CVD risk markers, and suggests that other components within processed meats (such haem iron and sodium) may have attenuated these beneficial effects. The current study also identified a differential impact of dietary nitrite from vegetables and water compared with processed meat sources. The highest intake of vegetable and water-derived nitrite (1-1.7mg/d) was associated with lowest SBP and more beneficial lipid profile (lower TC: HDL-C ratio), markers of glycaemia (lower HbA1c) and inflammation (lower CRP) compared to the lowest intake. This is in contrast to the consumption of the highest compared with the lowest dietary nitrite from processed meat (>3.1 mg/d vs 0.1-0.8 mg/d) that was found to be associated with a higher SBP. No studies have investigated the impact of nitrite intake on BP and CVD risk factors. However, these data support the previously reported benefits of vegetable consumption on CVD risk and detrimental impact of processed meats(17).

To the best of our knowledge, no previous studies have investigated the impact of daily dietary nitrate intake from different sources on BP and CVD risk factors in a healthy UK population. However, a limited number of studies have determined the impact of dietary nitrates on CVD mortality. A cohort study of 1226 elderly women aged 70-85 years found that vegetable-nitrate intake of more than 70 mg/d was associated with a 21% lower risk of atherosclerotic vascular disease mortality. Compared to a nitrate intake of from vegetables of less than 39.5 mg/d, this relationship appeared to plateau at intakes of 63.5 mg/d and above, with no additional benefit observed in participants with higher intakes of 93.8 mg/d(23). These findings agreed with another study conducted among 2,229 men and women aged over 49 years with a follow-up period of 14 years. A significant reduction of CVD mortality,

coronary heart disease and stroke hazard ratios were reported among those participants with vegetable nitrate intake of more than 99.7-137.8 mg/day (24). However, only intakes of vegetable nitrate of more than 138 mg/day was significantly associated with lower hazard ratios for non-CVD and all-cause mortality. Interestingly, this study also showed that non-vegetable nitrate was not associated with a lower hazard ratio for CVD mortality (24). However, similar to the previous study, a dose-dependent relationship between dietary nitrate intake and CVD mortality was not evident, with lower hazard ratios only found in those individuals consuming between 99.7-137.8 mg and not >138 mg of nitrate/day from vegetables (24). This finding is in agreement with our data that showed a nitrate intake of 131 mg/day or above was not associated with lower BP, suggesting a threshold effect may exist for the beneficial effects of nitrate on vascular function. In contrast to the studies demonstrating a beneficial effect of vegetable nitrate on mortality, an observational study conducted among 536,969 participants (male and female, aged 50-71 years) with 16-year follow-up found that processed and unprocessed red meats were associated with a higher hazard ratio of all-cause mortality. This study linked components of processed red meats such as nitrate, nitrite and haem iron to nine causes of death, with nitrate associated with 50% of overall mortality; of which approximately three quarters were attributed to CVD, while haem iron was strongly associated with deaths from cancer (25). In support of these findings, we observed a lack of beneficial effects of nitrate intake from processed meats CVD risk markers including BP, lipid profile and glycaemia. Another study reported that vegetarians have a 24% lower risk of death due to ischemic heart disease and a 20% lower risk of death from any cause compared with occasional meat eaters, which could, in part, reflect greater consumption of vegetable rather than meat-based nitrate (26).

Obesity is a modifiable risk factor for developing CVD, with greater fat accumulation around the waist associated with dyslipidaemia and insulin resistance(29). In the current study, waist circumference and waist to hip ratio was lower in Q3 of vegetable and water nitrate intake compared to Q1. Our findings are in agreement with those of a cohort study which reported a higher urinary nitrate concentration (used as a biomarker of nitrate intake) to be inversely associated with abdominal obesity (27). In contrast, those individuals consuming the highest intake of nitrate from processed meats had a greater hip circumference and lower waist to hip ratio than those in Q2, with no differences found in waist circumference. A study found that waist and hip circumference was positively associated with cardiovascular risk factors with no protective effect of a large hip circumference against CVD(30). However, another study reported that hip and waist circumferences need to be considered both together in the clinical setting to best identify those at increased risk of CVD deaths. A strong relationship was shown between waist circumference and risk of CVD death for all hip circumference categories, with smaller waist circumference associated with lower risk of death(31). Although higher intakes of vegetable nitrate were related to a lower waist circumference, further studies are needed to determine whether the lower waist circumference was related to dietary nitrate or reflective of a healthier lifestyle.

The mechanism that nitrate can lower blood pressure and enhancing vascular function due to NO role in controlling vascular tone, smooth muscle cell proliferation and growth, platelet activity and aggregation, leukocyte trafficking, expression of adhesion molecules and inflammation (32). Dietary nitrate is converted to nitrite and then NO. NO rapidly diffuses across vascular smooth muscle cell membranes, binding to and activating guanylyl cyclase to produce cyclic GMP. GMP leads to the activation of myosin phosphatase, which in turn leads to smooth muscle cell relaxation and

vasodilation (34). In addition to regulating vascular tone, NO can facilitate many other important functions, such as preventing the development of atherosclerosis by upholding vascular integrity with its anti-inflammatory (35), anti-aggregatory (36) and antioxidative properties (37).

Consumption of dietary nitrate with other food such as alcoholic drinks and unsaturated fatty acids may react with each other to form a variety of bioactive compounds. Alcoholic drinks interact with nitrite in the acidic stomach, leads to the production of ethyl nitrite (40). Ethyl nitrite is a potent smooth muscle relaxant and may have a vasodilatory role in the cardiovascular system (41). Furthermore, the reaction of NO with unsaturated fatty acids such as linoleic acid can produce nitroalkenes which may support multiple-cell signaling events such as vasodilation and reduced inflammation (39).

The nitrate–nitrite–NO pathway may affect endothelial nitric oxide synthase enzyme (eNOS) function via stimulation of AMP-activated protein kinase (AMPK). Nitrites and nitrates can activate this important regulator of cellular energy homeostasis (42), which in turn may activate eNOS by stimulating enzyme phosphorylation (43). As AMPK is central to regulating glucose metabolism, its activation by nitrate and nitrite may also explain the beneficiary effects of these anions observed in experimental models of metabolic syndrome and type 2 diabetes. This may support the findings of our study as glucose and HbA1 were lower in high nitrate quartile.

### 3.5.1. Strengths and limitations of the study

One strength of this study was the development of the comprehensive database of nitrate and nitrite from different dietary sources, including the main dietary contributors: vegetables, drinking water and meat. This database enabled more accurate assessment of nitrate and nitrite intakes from these different dietary sources and to examine the relationships with established CVD risk markers. To the best of our knowledge, this study is the first to include data from all water authority areas in the UK. Additionally, calculating nitrates in cooked food, considering the nitrate loss due to cooking methods, gave a more accurate estimation of dietary nitrate intake. The use of data from the NDNS ensured that the population studied was representative of the typical UK population.

The main limitation of this study was its cross-sectional nature, which did not prove cause or effect. Due to some incomplete datasets, the total number of participants was reduced to 3,408 men and women, which although lower than the total survey population, was none the less of reasonable size. Participants were generally healthy, so data may not be representative of those with CVD or other co-morbidities. Although we controlled for confounding factors in the statistical analysis other factors could have affected BP and blood biomarkers, including other dietary components, physical activity level, oral hygiene habits and smoking.

## 3.6. CONCLUSION

Currently, available evidence indicates a role of high vegetable intake in lowering CVD morbidity and mortality, with inorganic nitrate considered to be an important dietary mediator. This study showed that higher habitual nitrate and nitrite intake from vegetables and water was associated with lower BP and other CVD risk markers. However, nitrate from processed meat was not associated with these beneficial

outcomes, and processed meat-derived nitrite was associated with higher BP. These findings support previous evidence that vegetables rich in nitrates could play a role in BP control and CVD risk management, but that consumption of high amounts of processed meat should be discouraged. There is an urgent need to determine the long-term effects of sustained inorganic vegetable nitrate and nitrite consumption on the development of CVD and its consequences.


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### 3.9. CONFLICT OF INTREST

The authors have no conflicts of interest to declare.

### 3.10. AUTHOR CONTRIBUTIONS

The author's responsibilities were as follows: J.A.L, KGJ, DAH and HAS designed the research; H.S.A. conducted the research and analysed the data and drafted the paper K.G.J. and J.A.L. provided feedback and guidance on previous drafts of this paper and J.A.L. was responsible for final content.

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# TABLES

Table 1: Blood pressure, anthropometric measures and other CVD risk factors in NDNS subjects according to quartiles of total dietary nitrate.

| | Q1, (19-113 mg/d) | Q2, (114-149mg/d) | Q3, (150-192mg/d) | Q4, (193-572mg/d) | P value |
|---|---|---|---|---|---|
| **Systolic BP, mmHg** | 122± 0.58[ab] | 123± 0.53[b] | 121± 0.51[a] | 123± 0.53[ab] | 0.041 |
| **Diastolic BP, mmHg** | 73±0.55 [ab] | 74±0.53 [b] | 72±0.52 [a] | 73±0.53 [ab] | 0.044 |
| **Pulse Pressure, mmHg** | 37±0.85 | 39±0.79 | 39±0.76 | 39±0.78 | 0.141 |
| **Pulse rate, bpm** | 71±0.46[b] | 70±0.43[ab] | 67±0.41[a] | 69±0.42[a] | <0.001 |
| **Waist circumference, cm** | 91.4± 0.23[b] | 90.8± 0.21[ab] | 90.2±0.20[a] | 90.4±0.21 | 0.001 |
| **Hip circumference, cm** | 104.6±0.16 | 104.9±0.17 | 104.7±0.15 | 104.9±0.16 | 0.485 |
| **Waist:hip ratio** | 0.87±0.02[b] | 0.86±0.02[ab] | 0.86±0.02[a] | 0.86±0.02[a] | 0.002 |
| **Total-C, mmol/l** | 5.09±0.04 | 4.97±0.04 | 4.97±0.04 | 5.01±0.04 | 0.248 |
| **TAG, mmol/l** | 1.35±0.03 | 1.24±0.03 | 1.26±0.03 | 1.23±0.03 | 0.082 |
| **HDL-C, mmol/l** | 1.45±0.01 | 1.41±0.01 | 1.44±0.01 | 1.43±0.01 | 0.239 |
| **LDL-C, mmol/l** | 3.06±0.04 | 3.05±0.03 | 2.95±0.03 | 3.05±0.3 | 0.360 |
| **Total: HDL-C ratio** | 3.74±0.05 | 3.77 ±0.04 | 3.65±0.04 | 3.73±0.04 | 0.334 |
| **Glucose, mmol/l** | 5.05±0.03 | 5.16±0.03 | 5.10±0.03 | 5.11±0.03 | 0.120 |
| **CRP, mg/l** | 2.19±0.01 | 1.98±0.01 | 1.99±0.01 | 2.05±0.01 | 0.089 |
| **HbA1c %** | 5.45±0.02 | 5.48±0.02 | 5.44±0.02 | 5.43±0.02 | 0.389 |

ANCOVA was used for this analysis, values are estimated marginal means ± SEM. Values shown for quartiles of total dietry nitrate consumpton are min and max grams consumed per day. Age, gender, energy intake and BMI were used as covariates and weighting factors were used in the analysis. Different superscript letters indicate significant difference.

**ABBREVIATIONS**: **BP**: Blood Pressure, **CRP**: C-reactive Protein, **HbA1c**: Glycated haemoglobin, **HDL-C**: High density lipoprotein cholesterol, **LDL-C**: Low density lipoprotein cholesterol, **TAG**: Triacylglycerol, **Total-C**: Total cholestrol.

Table 2: Blood pressure, anthropometric measures and other CVD risk factors in NDNS subjects according to quartiles of dietary nitrate from vegetables (including drinking water) and processed meats.

| | Vegetables and Water Nitrate | | | | | Processed meats Nitrate | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Q1 (3- 65 mg/d)** | **Q2 (66-94 mg/d)** | **Q3 (95-130 mg/d)** | **Q4 (131-450 mg/d)** | **P value** | **Q1 (3-32 mg/d)** | **Q2 (33-52 mg/d)** | **Q3 (53-76 mg/d)** | **Q4 (77-372 mg/d)** | **P value** |
| **Systolic BP, mmHg** | 123±0.56$^{b}$ | 122±0.52$^{ab}$ | 121±0.53$^{a}$ | 122±0.50$^{ab}$ | 0.041 | 124±0.66 | 123±0.61 | 123±0.62 | 124±0.66 | 0.515 |
| **Diastolic BP, mmHg** | 74±0.54$^{b}$ | 72±0.49$^{ab}$ | 71±0.52$^{a}$ | 73±0.50$^{ab}$ | 0.011 | 74±0.49 | 74±0.45 | 73±0.46 | 73±0.49 | 0.430 |
| **Pulse Pressure, mmHg** | 39±0.83 | 38±0.77 | 37±0.78 | 39±0.75 | 0.47 | 36±0.98 | 39±0.93 | 40±0.96 | 39±0.99 | 0.077 |
| **Pulse rate, bpm** | 71±0.45$^{b}$ | 69±0.42$^{ab}$ | 69±0.42$^{ab}$ | 68±0.41$^{a}$ | 0.008 | 71±0.51$^{b}$ | 70±0.48$^{ab}$ | 68±0.50$^{a}$ | 69±0.52$^{ab}$ | 0.001 |
| **Waist circumference, cm** | 91±0.23$^{b}$ | 90±0.21$^{ab}$ | 89±0.21$^{a}$ | 90±0.20$^{ab}$ | 0.001 | 91±0.26 | 91.±0.25 | 91±0.26 | 91±0.27 | 0.291 |
| **Hip circumference, cm** | 105 ±0.17 | 104±0.16 | 105.±0.16 | 105 ±0.15 | 0.16 | 105±0.20$^{ab}$ | 105±0.19$^{b}$ | 105±0.19$^{ab}$ | 106±0.205$^{a}$ | 0.016 |
| **Waist:hip ratio** | 0.87±0.02$^{b}$ | 0.86±0.02$^{ab}$ | 0.85±0.02$^{a}$ | 0.86±0.02$^{ab}$ | 0.002 | 0.86±0.02$^{ab}$ | 0.87±0.02$^{b}$ | 0.86±0.02$^{a}$ | 0.86±0.03$^{a}$ | 0.010 |
| **Total-C (mmol/L)** | 5.30±0.08$^{b}$ | 5.24±0.07$^{ab}$ | 5.26±0.08$^{ab}$ | 5.00±0.07$^{a}$ | 0.046 | 5.12±0.03 | 5.13±0.03 | 5.16±0.03 | 5.13±0.03 | 0.520 |
| **TAG (mmol/L)** | 1.08±0.01 | 1.21±0.09 | 1.09±0.09 | 1.07±0.09 | 0.65 | 1.14±0.08 | 1.13±0.08 | 1.12±0.6 | 1.16±0.7 | 0.63 |
| **HDL-C (mmol/L)** | 1.45±0.17 | 1.42±0.16 | 1.44±0.4 | 1.42±0.16 | 0.68 | 1.42±0.01 | 1.45±0.01 | 1.44±0.01 | 1.47±0.01 | 0.126 |
| **LDL-C (mmol/L)** | 3.16±0.07 | 3.11±0.07 | 3.14±0.07 | 2.98±0.06 | 0.22 | 3.13±0.03 | 3.10±0.03 | 3.15±0.03 | 3.16±0.03 | 0.20 |
| **Total: HDL-C ratio** | 3.76±1.3 | 3.79 ±1.2 | 3.77±1.1 | 3.72±1.2 | 0.616 | 3.82±0.04 | 3.77 ±0.04 | 3.81±0.04 | 3.65±0.04 | 0.14 |
| **Glucose (mmol/L)** | 5.19±0.03$^{b}$ | 5.11±0.03$^{ab}$ | 5.06±0.06$^{ab}$ | 5.04±1.2$^{a}$ | 0.027 | 5.16±0.002 | 5.21±0.002 | 5.19±0.002 | 5.16±0.002 | 0.46 |
| **CRP (mg/L)** | 2.19±0.14$^{b}$ | 2.08±0.13$^{a}$ | 2.04±0.14$^{a}$ | 2.02±0.13$^{a}$ | 0.001 | 2.16±0.01 | 2.14±0.01 | 2.17±0.01 | 2.25±0.01 | 0.151 |
| **Hb A1c (%)** | 5.50±0.02$^{b}$ | 5.44±0.02$^{ab}$ | 5.40±0.02$^{a}$ | 5.45±0.6$^{ab}$ | 0.017 | 5.52±0.001 | 5.51±0.001 | 5.45±0.001 | 5.48±0.001 | 0.127 |

ANCOVA was used for this analysis, values are estimated marginal means ± SEM. Values shown for quartiles of nitrate consumpton from each dietary source are min and max grams consumed per day. Age, gender, energy intake and BMI were used as covariates and weighting factors were used in the analysis. Different superscript letters indicate significant difference.

**ABBREVIATIONS**: **BP**: Blood Pressure, **CRP**: C reactive Protein, **Hb A1c**: Glycated haemoglobin, **HDL-C**: High density lipoprotein, **TAG**: Triacylglycerol, **Total-C**: Total Cholestrol, **LDL-C**: Low density lipoprotein.

Table 3: Blood pressure, anthropometric measures and other CVD risk factors in NDNS subjects according to quartiles of total dietary nitrite intake.

| | Q1 (0.1-0.9 mg/d) | Q2 (1-1.8 mg/d) | Q3 (1.9-3.1 mg/d) | Q4 (3.2-15.9 mg/d) | P value |
|---|---|---|---|---|---|
| **Systolic BP, mmHg** | 121±0.62[b] | 122±0.62[ab] | 124±0.65[a] | 124±0.69[a] | <0.0001 |
| **Diastolic BP, mmHg** | 73±0.36 | 73±0.39 | 74±0.38 | 73±0.40 | 0.174 |
| **Pulse Pressure, mmHg** | 38±0.74 | 38±0.79 | 37±0.76 | 40±0.73 | 0.062 |
| **Pulse rate, bpm** | 69±0.50 | 69±0.60 | 68±0.67 | 67±0.76 | 0.413 |
| **Hip circumference, cm** | 104.4 ±0.18 | 104.6 ±0.19 | 104.4±0.19 | 104.7±0.2 | 0.635 |
| **Waist circumference, cm** | 91.0 ±0.24[b] | 90.6 ±0.25[ab] | 89.9±0.25[a] | 90.5±0.27[ab] | 0.015 |
| **Waist: hip ratio** | 0.87±0.02 | 0.86±0.02 | 0.85±0.02[a] | 0.85±0.02[a] | 0.027 |
| **Total-C, mmol/l** | 4.99±0.01[ab] | 5.01±0.01[ab] | 5.10±0.01[b] | 4.92±0.01[a] | 0.023 |
| **TAG, mmol/l** | 1.06±0.03 | 1.08±0.03 | 1.11±0.03 | 1.05±0.05 | 0.298 |
| **HDL-C, mmol/l** | 1.40±0.01[b] | 1.41 ±0.01[ab] | 1.46±0.01[a] | 1.45±0.01[ab] | 0.023 |
| **LDL-C, mmol/l** | 3.03±0.03[ab] | 3.05±0.03[ab] | 3.09±0.002[b] | 2.94±0.04[a] | 0.038 |
| **Total: HDL-C ratio** | 3.82±0.04[b] | 3.73±0.04[ab] | 3.74±0.04[ab] | 3.58±0.05[a] | 0.008 |
| **Glucose, mmol/l** | 5.53±0.002 | 5.45±0.001 | 5.548±0.002 | 5.45±0.002 | 0.079 |
| **CRP, mg/l** | 2.33± 0.01[b] | 1.97± 0.01[a] | 2.03± 0.01[a] | 1.81± 0.01[a] | <0.0001 |
| **HbA1c, %** | 5.48±0.002 | 5.44±0.002 | 5.43±0.002 | 5.42±0.002 | 0.193 |

ANCOVA was used for this analysis, values are estimated marginal means ± SEM. Values shown for quartiles of total dietary nitrite consumpton are min and max grams consumed per day. Age, gender, energy intake and BMI were used as covariates and weighting factors were used in the analysis. Different superscript letters indicate significant difference.

**ABBREVIATIONS: BP**: Blood Pressure, **CRP**: C reactive Protein, **Hb A1c**: Glycated haemoglobin, **HDL-C**: High density lipoprotein, **TAG**: Triacylglycerol, **Total-C**: Total Cholestrol, **LDL-C**: Low density lipoprotein.

Table 4: Blood pressure, anthropometric measures and other CVD risk factors in NDNS subjects according to quartiles of dietary nitrite intake from vegetables (including drinking water) and processed meats.

| | Vegetables and Water Nitrite | | | | | Processed meat Nitrite | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Q1 (0.1- 0.3 mg/d)** | **Q2 (0.4- 0.7 mg/d)** | **Q3 (0.8- 1.4 mg/d)** | **Q4 (1.5- 9.2 mg/d)** | **P value** | **Q1 (0.1- 0.8 mg/d)** | **Q2 (1- 1.7 mg/d)** | **Q3 (1.8- 3 mg/d)** | **Q4 (3.1- 15.9 mg/d)** | **P value** |
| **Systolic BP, mmHg** | 124±0.48$^{b}$ | 123±0.46$^{ab}$ | 123±0.45$^{ab}$ | 122±0.45$^{a}$ | 0.007 | 120±0.70$^{a}$ | 121±0.93$^{ab}$ | 121±0.81$^{ab}$ | 123±0.33$^{b}$ | 0.010 |
| **Diastolic BP, mmHg** | 72±0.42 | 73±0.50 | 72±0.56 | 72±0.64 | 0.57 | 73±0.51 | 73±0.68 | 73±0.59 | 74±0.22 | 0.13 |
| **Pulse pressure, mmHg** | 38±0.75 | 38±0.77 | 37±0.77 | 38±0.77 | 0.22 | 37±1.7 | 38±1.5 | 38±1.3 | 39±1.2 | 0.89 |
| **Pulse rate, bpm** | 70±0.53 | 69±0.62 | 69±0.70 | 68±0.78 | 0.37 | 69±0.57 | 71±0.75 | 70±0.65 | 69±0.26 | 0.08 |
| **Waist circumference, cm** | 91±0.19$^{b}$ | 91±0.19$^{ab}$ | 90±0.19$^{a}$ | 91±0.19$^{ab}$ | 0.005 | 92.2±0.19 | 92±0.19 | 92±0.19 | 92±0.19 | 0.507 |
| **Hip circumference, cm** | 105±0.14 | 105±0.14 | 105±0.14 | 105±0.15 | 0.27 | 105±0.14$^{b}$ | 105±0.14$^{ab}$ | 105±0.14$^{ab}$ | 106±0.15$^{a}$ | 0.008 |
| **Waist:hip ratio** | 0.87±0.02$^{b}$ | 0.86±0.02$^{a}$ | 0.86±0.02$^{a}$ | 0.86±0.02$^{ab}$ | 0.001 | 0.87±0.02 | 0.87±0.02 | 0.87±0.02 | 0.87±0.02 | 0.725 |
| **Total-C (mmol/L)** | 5.00 ±0.03 | 5.04±0.03 | 5.06±0.03 | 4.93±0.03 | 0.229 | 4.89±0.05 | 5.08±0.07 | 5.09±0.06 | 5.05±0.03 | 0.059 |
| **TAG (mmol/L)** | 1.06±0.08 | 1.09±0.08 | 1.09±0.08 | 1.05±0.08 | 0.501 | 1.08±0.08 | 1.14±0.08 | 1.12±0.08 | 1.10±0.08 | 0.244 |
| **HDL-C (mmol/L)** | 1.40±0.01$^{b}$ | 1.42±0.01$^{ab}$ | 1.47±0.01$^{a}$ | 1.46±0.01$^{ab}$ | 0.020 | 1.42±0.01 | 1.43±0.01 | 1.46±0.01 | 1.45±0.01 | 0.06 |
| **LDL-C (mmol/L)** | 3.03±0.03 | 3.07±0.03 | 3.06±0.03 | 2.95±0.03 | 0.148 | 2.9±0.04 | 3.03±0.06 | 3.04±0.05 | 3.05±0.03 | 0.082 |
| **Total:HDL-C Ratio** | 3.87±0.04$^{b}$ | 3.71 ±0.04$^{ab}$ | 3.60±0.04$^{ab}$ | 3.59±0.04$^{a}$ | 0.017 | 3.81±0.04 | 3.76 ±0.04 | 3.75±0.04 | 3.70±0.04 | 0.058 |
| **Glucose (mmol/L)** | 5.18±0.002 | 5.14±0.002 | 5.11±0.002 | 5.17±0.002 | 0.267 | 5.18±0.002 | 5.14±0.002 | 5.15±0.002 | 517±0.002 | 0.389 |
| **CRP (mg/L)** | 2.40±0.01$^{b}$ | 2.14±0.02$^{a}$ | 2.12±0.02$^{a}$ | 2.00±0.01$^{a}$ | <0.001 | 2.20±0.01 | 2.14±0.02 | 2.12±0.02 | 2.15±0.01 | 0.823 |
| **Hb A1c (%)** | 5.55±0.002$^{b}$ | 5.48±0.001$^{ab}$ | 5.54±0.002$^{ab}$ | 5.45±0.002$^{a}$ | 0.028 | 5.50±0.002 | 5.48±0.001 | 5.47±0.002 | 5.44±0.002 | 0.094 |

ANCOVA was used for this analysis, values are estimated marginal means ± SEM. Values shown for quartiles of nitrite consumpton from each dietary source are min and max grams consumed per day. Age, gender, energy intake and BMI were used as covariates and weighting factors were used in the analysis. Different superscript letters indicate significant difference.

**ABBREVIATIONS: BP**: Blood Pressure, **CRP**: C-reactive Protein, **Hb A1c**: Glycated haemoglobin, **HDL-C**: High density lipoprotein, **TAG**: Triacylglycerol, **Total-C**: Total Cholestrol, **LDL-C**: Low density lipoprotein.

## SUPPLEMENTARY INFORMATION

Table 1: The UK Water Authorities list.

| *No.* | *Water Company Names* | *Location* |
|---|---|---|
| 1 | Albion Water | Herts |
| 2 | Independent Water Networks | Cardiff |
| 3 | SSE Water Ltd | Reading |
| 4 | Peel Water Networks | The Trafford Centre |
| 5 | Veolia Water | London |
| 6 | Northern Ireland water | Northern Ireland |
| 7 | Affinity Water | Essex |
| 8 | Affinity Water | Kent |
| 9 | Anglian Water Services Ltd | Huntingdon |
| 10 | Bristol Water plc | Bristol |
| 11 | Cholderton & District Water Company Ltd | Wiltshire |
| 12 | United Utilities Water plc | Warrington |
| 14 | Northumbrian Water Ltd | Durham |
| 15 | Portsmouth Water plc | Hants |
| 16 | Sembcorp Bournemouth Water Ltd | Bournemouth |
| 17 | Dee Valley Water plc | North Wales |
| 18 | Northumbrian Water Ltd | Durham |
| 19 | Dwr Cymru Welsh Water | Welsh |
| 20 | Sembcorp Bournemouth Water Ltd | Bournemouth |
| 21 | Severn Trent Water Ltd | Coventry |
| 22 | South East Water Ltd | Kent |
| 23 | Southern Water Services Ltd | Sussex |
| 24 | South Staffordshire Water plc | Cambridge |
| 25 | South West Water Ltd | Exeter |
| 26 | Sutton & East Surrey Water plc | Surrey |
| 27 | Thames Water Utilities Ltd | Reading |
| 28 | Wessex Water Services Ltd | Bath |
| 29 | Yorkshire Water Services Ltd | Bradford |
| 30 | Severn Trent Water Ltd | Coventry |

# CHAPTER 4: UTILITY OF URINARY NITRATE AS A BIOMARKER OF DIETARY NITRATE INTAKE AND RELATIONSHIP WITH CARDIOVASCULAR DISEASE RISK MARKERS IN A REPRESENTATIVE UK POPULATION

**Chapter 4**

The following chapter will be submitted for publication in the British Journal of Nutrition.

**Contribution towards the paper**

I was involved with the design of laboratory study, and I performed the urinary nitrate and nitrite analysis. I carried out the data and statistical analysis and I was responsible for writing this paper.

**Utility of urinary nitrate and nitrite as a biomarker of dietary nitrate intake and relationship with cardiovascular disease risk markers in a representative UK population**

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**Keywords:** Blood Pressure; CVD biomarkers; Dietary Nitrate; Urinary Nitrate; Urinary Nitrite.

## 4.1. ABSTRACT

Inorganic dietary nitrate intake has been associated with beneficial health outcomes, including lower blood pressure (BP). However, it is difficult to accurately estimate dietary nitrate intake due to a lack of data on nitrate and nitrite content of food and drinking water on dietary analysis software. Therefore, this study aimed to determine if urinary nitrate and nitrite were related to dietary nitrate intake and biomarkers of cardiovascular disease (CVD) risk in a representative UK population. This cross-sectional study determined 24-hour urinary nitrate excretion of 1340 National Diet and Nutrition Survey (NDNS) participants and investigated the relationship between daily urinary nitrate and nitrite excretion with BP and other CVD health biomarkers. Urinary nitrate and nitrite concentrations were stratified into quartiles prior to ANCOVA analysis. Our data analysis showed no significant differences in BP between the urinary nitrate or nitrite quartiles. However, Total-C was lower in Q4 vs Q1($p= 0.035$). For urinary nitrite only, HDL-C was higher in Q4 vs Q1, and waist circumference ($p = 0.041$) and waist-hip ratio were lower in Q4 vs Q2 ($p = 0.0320$). CRP was higher ($p = 0.036$) and HbA1c lower in Q4 vs Q2 ($p = 0.021$). Furthermore, Spearman's correlation revealed urinary nitrate and nitrite concentrations to be weakly, but significantly, associated with total dietary nitrate intake ($r_s = 0.121$, $p < 0001$). These data suggest that urinary nitrate and nitrite were not ideal biomarkers of dietary nitrate intake and were not associated with BP or other measured markers of health. Further studies are needed to confirm these findings.

## 4.2. INTRODUCTION

Diets high in vegetables are associated with lower cardiovascular disease (CVD) risk, the leading cause of death worldwide(1). This has been attributed to a number of bioactive components, including the inorganic nitrate content(2). Many studies have reported that inorganic nitrate can lower blood pressure (BP) in both hypertensive(3) and healthy(4) groups and improve endothelial function(5). Dietary nitrate is mostly found in vegetables (green leafy and root vegetables), processed meats and drinking water(6). However, there are limited comprehensive databases of nitrate and nitrite content of foods and drinks, and to date, no dietary analysis software contains information on these bioactive compounds. This lack of dietary data significantly compromises the investigation of dietary nitrates and nitrite on health promotion and disease prevention. Identification of a suitable biomarker of intake would be of great utility in this endeavor and allow objective estimation of population dietary nitrate and nitrite exposure.

Dietary and salivary nitrate is converted into nitrite in the oral cavity by nitrate-reducing bacteria(10) and further reduced in the stomach to NO. Dietary nitrate is absorbed almost entirely owing to its bioavailability in the stomach and the small intestine, and about 75% is excreted in the urine, while the remaining, up to 25%, is recycled by the salivary glands(11). Nitrate can be endogenously produced from nitrite or, nitric oxide, in the circulation(7). A study that measured endogenously produced nitrate found that the human body can produce approximately 1 mmol of nitrate per day(8). However, the endogenous production of nitrate has been found to be influenced by health-related conditions, with lower levels of production evident in patients with hypertension(9). This highlights that the production of endogenous nitrate varies between subjects, and urinary nitrate may contain both endogenously produced nitrate

and dietary nitrate. Therefore, urinary nitrate may be a suitable biomarker for total nitrate exposure since up to 75% of both exogenous and endogenous nitrate is excreted in urine after 24 hours. Furthermore, urine is relatively easy to collect and is non-invasive.

With hypertension now affecting one in four UK adults (13), there is considerable interest in inorganic nitrate as a potential dietary strategy for BP lowering. However, mechanisms underlying the beneficial effects on CVD risk are lacking. This highlights the need for a biomarker of inorganic nitrate intake to assess the relationship with BP, CVD risk markers and mortality. To date, few studies have used urinary nitrate as a biomarker of dietary nitrate intake to assess the relationship with BP, CVD-risk biomarkers and mortality. Studies performed in two populations, the Italian Chianti cohort study (12) and the US National Health and Nutrition Examination Survey (NHANES)(13), have shown a significant correlation between increasing urinary nitrate and lower BP in elderly people(12) and a lower prevalence of hypertension(13), although few studies have been performed in the UK.

To address this knowledge gap, urine samples from the National Diet and Nutrition Survey (NDNS), conducted in a representative sample of the UK population, were analysed for nitrate and nitrite. The daily dietary nitrate and nitrite intake was also estimated using a previously developed database of nitrate and nitrite content of foods and drinking water (14). The association between diets containing high and low intakes of nitrate and nitrite were compared with and 24-hour urinary nitrate and nitrite concentrations to determine if the measurement of nitrate and its metabolites in urine represents a suitable biomarker for dietary nitrate intake. We also examined the association between 24-hour urinary nitrate and nitrite excretion, a marker of both endogenous and dietary nitrate exposure, with blood pressure and other CVD risk

factors.

## 4.3. METHOD

### 4.3.1. Study design

In this cross-sectional observation study 24-hour urine samples from a total of 2442 men and women aged 19–64 years were obtained during years 1 to 5 of the NDNS, conducted between 2008 and 2013, the detailed methodology of which has been previously published(15). Briefly, individuals interested in participating in the survey were asked to complete a 4-day food diary and attend an interview to discuss habitual dietary intake and to measure characteristics such as socioeconomic class. At a follow-up visit, volunteers gave a blood sample and were provided with detailed instructions for the 24-hour urine collection, their eligibility was checked, written consent taken and equipment delivered to the participants. The nurse also arranged a date for the 24-hour urine sample collection and for the second visit which was completed on the day after the urine collection. During the second visit, the nurse recorded the weight of the urine sample, collected four aliquots and completed a range of anthropometric measurements, including weight, BMI, waist and hip circumferences, blood pressure (systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse). The waist to hip ratio was calculated and pulse pressure was determined by subtracting the mean DBP from the mean SBP. Blood samples were analysed for CVD risk biomarkers, including triacylglycerol, total cholesterol, low- and high-density lipoprotein cholesterol, C-reactive protein (CRP), glucose and glycated haemoglobin (HbA1c). The NDNS participants consented for their bio-banked urine samples to be used for nutrition and health-related research, and ethical approval for the analysis of the urine samples for nitrate and nitrite was obtained from the NHS Ethics Committee (18/NS/0085 IRAS Project ID: 238212) and the University

of Reading ethics committee (STUDY Number – 11/18).

Total dietary nitrate intakes were estimated using a comprehensive database created by the researcher and previously described in detail(14) . In brief, the nitrate and nitrite concentrations of vegetables and processed meat were taken from a published database. The nitrate and nitrite content of each vegetable and meat was converted to mg of nitrate or nitrite per gram. Composite foods and recipes with consideration the loss of nitrate content during processing and cooking was calculated as well as nitrate and nitrite from water.

### 4.3.2. Urinary nitrate and nitrite analyses

Urinary nitrate and nitrite concentrations were measured using HPLC-NO. Briefly, the samples were diluted 1:1 with mobile phase (carrier solution, (Eicom,Germany) diluted to 1L with 0.1L methanol and 0.9L water) to ensure the sample concentrations were within the linear range of the standard curve (12.5–2000 µM). All diluted samples were prepared in 96-well plates before analysis. For quality assurance, six control samples of known nitrate and nitrite concentrations and five blank samples were used to determine the accuracy and reproducibility of the analyses. The plate was rejected if the coefficient of variation (CV%) was ±15%(16). Nitrate and nitrite concentrations were corrected for the dilution factor and then multiplied by the 24-hour-urine volume to represent the total urinary nitrate and nitrite excretion in µM/day.

### 4.3.3. Sample size and statistical analyses

The sample size was based on multiple linear regression analysis, which was performed to determine the relationships between the estimated dietary nitrate intake determined by the urine and food-diary analysis and blood pressure (SBP, DBP and pulse pressure), other CVD risk markers (including BMI, fasting lipid profile, glucose and CRP). A conservative approach and an anticipated effect size (r2) of 0.02 were

chosen, with a two-sided significance level of 0.01 and a power of 90%. With a large number of predictors (estimated as up to 50), the required sample size was 2409.

The statistical analyses were completed using IBM SPSS, Version 24. The data normality was checked using Q-Q plots, and log10 transformation where required. As the data were derived from different NDNS years, weighting factors were applied to ensure that data were comparable from the different years of the NDNS survey . Urinary nitrate and nitrite data were stratified into quartiles representing the lowest to the highest excretion concentrations. An analysis of covariance was used to detect statistically significant differences between the level of urinary nitrate and nitrite and risk factors for CVD, including BP, anthropometric measurements, blood lipids, glucose and CRP. Age, sex, total energy intake (MJ) and BMI were included as covariates in the analysis. Bonferroni post-hoc tests were used to detect differences between the quartiles of nitrate and nitrite intake. Statistical significance was accepted when $p \leq 0.05$. Data were presented as estimated marginal mean ± SEM. Finally, Spearman's correlation analysis was performed to compare the urinary nitrate and nitrite concentrations with the estimated dietary nitrate and nitrite intake data.

## 4.4. RESULTS

### 4.4.1. Urinary nitrate

A total of 2442 urine samples were analysed for nitrate and nitrite. Of these, 800 were excluded due to poor analytical performance (> 15% CV) and a further 350 were excluded due to incomplete data, such as BP and other anthropometric measurements. Therefore, 1350 participants, comprising 571 men and 769 women, were included in these analyses. The participants had a mean age of 43 years (SD 12), BMI of 26 kg/m$^2$ (SD 7), SBP of 94 mmHg (SD 55) and DBP of 56 mmHg (SD 33). The data were stratified into quartiles according to increasing urinary nitrate with

concentration: Q1: 3–376 µM/day, Q2: 377–599 µM/day, Q3: 600–877 µM/day and Q4: 878–7133 µM/day. There were no significant differences in SBP and DBP across quartiles of increasing urinary nitrate concentrations (Table 1). Total cholesterol and LDL-C were significantly higher in Q4 (5.21 ± 0.06 mmol/L) compared to Q1 (4.89 ± 0.09 mmol/L) ($p = 0.025$).

The urinary nitrite quartiles were Q1: 0–0.40 µM/day, Q2: 0.41–0.99 µM/day, Q3: 1–1.99 µM/day and Q4: 2-46 µM/day. There were no significant differences in SBP or DBP between urinary nitrite quartiles (Table 2). However, participants with the highest urinary nitrite concentration (Q4) had a significantly lower waist circumference and waist:hip ratio compared with Q2 ($p = 0.032$ and $p = 0.041$, respectively). Lower CRP and HbA1c concentrations were also evident in Q4 vs Q2 ($p = 0.036$ and $p = 0.021$, respectively). No other relationships were observed with the anthropometric and CVD risk markers. Among urinary nitrate quartiles, total dietary nitrate intake was higher in Q4 than all other quartiles, nitrate intake from vegetable and water was higher in Q4 vs Q1 with no significant change evident for nitrate from cured and processed meats. Furthermore, in urinary nitrite quartiles total dietary nitrate intake was higher in Q4 than Q1 and Q2 ($p<0.0001$) with no changes in nitrate intake of vegetable and water. However, meat nitrate intake was significantly higher in Q4 vs Q2. Nitrite intake was higher in Q2 vs Q1 in urinary nitrate quartiles.

### 4.4.2. Relationship between urine nitrate and nitrite with dietary nitrate

The total urinary nitrate and nitrite were combined and found to be weakly but significantly associated with dietary nitrate ($r_s = 0.117$, $p < 0001$). However, urinary nitrate ($r_s = 0.121$, $p < 0001$) but nitrite, ($r_s = 0.008$, $p = 0.77$) was found to explain this significant relationship. Furthermore, nitrate from vegetable and water was very weakly associated with urinary nitrate ($r_s=0.072$, $p = 0.009$) but was not significantly associated with nitrate from meat ($r_s=0.045$, $p = 0.102$).

## 4.5. DISCUSSION

As far as the authors are aware, this is the first study conducted in a representative UK population to assess the utility of urinary nitrate and nitrite as biomarkers of dietary nitrate and nitrite intake, and to determine the association with BP and other CVD risk markers. After stratifying the urinary nitrate and nitrite concentrations into quartiles, no associations with BP were observed, with beneficial effects evident on the fasting lipid profile in those individuals with the highest versus the lowest urinary concentrations.

Accurate estimation of dietary nitrate and nitrite intake is challenging as there are few databases of their content in foods and drinks (including tap water), and currently, no dietary analysis software available which includes these data. Therefore, biomarkers of intake, such as urinary nitrate and nitrite, could represent an objective measure of intake, that does not rely on the recording of dietary intake or accuracy of the nitrate and nitrite content of foods and drinks. It is estimated that around 70% of dietary nitrate is excreted in urine over 24 hours, and less than 1% is excreted in faeces(17). For these reasons urinary nitrate concentrations have been proposed as potential biomarker of chronic dietary nitrate intake. However, their utility as a reliable measure of dietary exposure has not been adequately assessed. In an attempt to determine the rate of excretion of dietary nitrate, one study investigated the long-term impact of dietary nitrate intake on salivary, plasma and urine concentrations in a total of 19 men and women who consumed 400 mg/day of inorganic nitrate from spinach and green leafy vegetables for 7 days (18). Plasma nitrate was measured at baseline (day 0) on completion of the nitrate ingestion period (day 7) then at 2, 7 and 14 days after returning to a habitual diet, whereas urinary nitrate was determined at baseline (day 0), day 7 and 14 days following completion of high nitrate consumption. It was reported that salivary and plasma nitrate concentrations returned to baseline concentrations

within 2 days of stopping the high nitrate diet, and urinary nitrate concentrations were 7-fold lower 14 days following consumption of the high nitrate diet compared with directly after the diet (day 7). This suggests that nitrate excreted in urine more accurately reflects the previous 24-hour consumption and represent only a short-term marker of intake (18). For our analysis data was not available on the timing of the completion of the diet diary and collection of the urine sample, which may have influenced the strength of the associations observed.

Data from the NDNS cohort analysis illustrated that the relationship between urinary nitrate and dietary nitrate (determined by 4 day estimated diet diaries) was significant, yet weak. Moreover, urinary nitrite was not associated with dietary nitrite intake. Only one study has found a moderate positive correlation between urinary nitrate and vegetable intake (19). However, nitrate intake from other sources such as meat and water were not included in this study which may have impacted on the association reported. This was not matching our findings as nitrate from vegetables and water was very weakly associated with urinary nitrate ($r_s$=0.072, p = 0.009). Furthermore, urinary nitrate and nitrite excretion have been found to be influenced by a number of different factors, such as health conditions and medications. For example, urinary nitrate was found to be lower in patients with hypertension and individuals with diabetes mellitus(9) and greater in those with low parathyroid hormone concentrations(20). In addition, some medications such as Methylprednisolone, has been found to increase urinary nitrate(23, 24) . Furthermore, sex, race and smoking also influences urinary nitrate concentrations with white Americans having significantly higher urinary nitrate than black Americans(22). smoker men were found to have statistically significantly lower urinary nitrate than women (22), while urinary nitrate in non-smokers was found to be significantly higher in men than women (18). In addition, the amount of caffeine

consumed or vitamin C was reported to have a positive association with levels of urinary nitrate (22, 23). The mechanistic links between these diet, chronic conditions and lifestyle factors with urinary nitrate are unclear and could have a differential impact on the excretion or appearance of nitrate in the urine, which requires further investigation. Taken together, this weak association between dietary nitrate and urinary nitrate and factors found to impact urinary nitrate suggests that urinary nitrate may be not an ideal biomarker of nitrate intake if measured after 24 hours of nitrate consumption and if subjects have other health conditions. Therefore, there is an urgent need for further studies to confirm this finding.

In contrast to our findings, three previous studies found a significant association between urinary nitrate and lower BP and the prevalence of congestive heart failure(12, 13 , 24). The first study was conducted in a total of 919 men and women aged between 21 and 95 from the Italian National Institute of Research and Care on Aging. The study found that urinary nitrate excretion of more than 1 mM was associated with lower SBP and DBP by 4–7 and 2–3 mmHg, respectively(12). Importantly the excretion of 1 mM of nitrate was above that recorded for all participants included in the NDNS cohort (<7.1 µM/day) and therefore could reflect the difference in nitrate excretion and exposure in the different populations. Furthermore, the participants in this Italian study were older (85% of the participants > 64 years old) than the participants in our study (aged 19–64 years). The Italian study also used a colorimetric assay incorporating a Griess reagent, with vanadium which was used to determine the total urinary nitrate. This method has several limitations, including low sensitivity and selectivity compared to the HPLC-NO used in our study. Moreover, the Italian study was unable to conclude whether dietary nitrate or endogenous synthesis made the greatest contribution to urinary nitrate excretion in elderly people, which

make it difficult to conclude if urinary nitrate was a suitable biomarker of inorganic dietary nitrate. The US NHANES study conducted in 17,618 adults with a median age of 45 years found that high urinary nitrate excretion (>726 µM/L) was associated with a 32% lower prevalence of hypertension(13). Moreover, another study conducted in 14,894 participants from the same NHANES population found that high urinary nitrate (>26.1 mg nitrate/g creatinine) was significantly associated with lower rates of congestive heart failure(24). Although both these studies were highly powered, they measured nitrate concentrations using spot urine samples and presented the urinary concentration in µM/L or mg nitrate/g creatinine which makes it difficult to compare with other studies (such as ours) that report total daily nitrate excretion. Furthermore, adjustment of spot urine nitrate concentrations for creatinine may not be appropriate, as creatinine excretion can vary considerably between individuals according to factors such as age, health and kidney function, which may impact on the final calculated value (25).

Our results showed that urinary nitrite was associated with other CVD risk factors such as total cholesterol, HDL-C in Q4 than Q1, CRP and HbA1c in Q4 than Q2. The mechanism underlying this association is unknown, and no studies have separately measured urinary nitrite or linked the level of intake to BP and other CVD risk factors. However, higher urinary nitrite may occur due to urinary tract infection (26) which may represent an important confounding factor in the association we found between urinary nitrite and other CVD risk factors. Further work is needed to investigate the association between urinary nitrite and CVD risk markers and to determine underlying mechanisms of action.

The main strength of this study was the use of complete 24-hour urine samples for the analysis. Furthermore, urinary nitrate and nitrite were determined by HPLC-NO, which

is considered a more sensitive measure (27) compared with other assays(28). The study also included a relatively large representative UK population. However, a study limitation is the varying time periods between the dietary analysis and urine collection, the observational and cross-sectional nature of the study which prevented identification of cause and effect. Therefore, further studies are needed to confirm these findings.

## 4.6. CONCLUSION

Biomarkers are increasingly used to assess nutritional intake due to their objectivity and lack of reliance on dietary recording or nutrient analysis. There is increasing interest in the possible use of urinary nitrate as a biomarker of dietary nitrate intake, although, to date, this has not been adequately assessed. This study indicated that urinary nitrate was weakly associated with dietary nitrate intake, which suggests that they may have some use as a biomarker of intake. However, its utility as a suitable biomarker was difficult to assess, due to the lack of coordination of dietary assessment and urine collection. Further studies, which include 24-hour urine samples collected the day following dietary nitrate assessment, are required to determine whether urinary nitrate and nitrite could be used as effective dietary biomarkers. In addition, RCT which include different quantities of nitrate-rich foods over varying periods, coordinated with the urine sample collection would further elucidate the relationship between these measures.

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### 4.9. CONFLICT OF INTREST

The authors have no conflicts of interest to declare.

### 4.10. AUTHOR CONTRIBUTIONS

The author's responsibilities were as follows: J.A.L, KGJ, DAH and HAS designed the research; H.S.A. conducted the urine samples analysis, statistical analysis and drafted the paper K.G.J. and J.A.L. provided feedback and guidance on previous drafts of this paper and J.A.L. was responsible for final content.

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# TABLES

Table 1: BP, anthropometric measurment and other CVD risk factors according to quartiles of urinary nitrate and nitrite.

| | Urinary Nitrate (n=1350) | | | | | Urinary Nitrite (n=1350) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Q1 (3- 376 µM/d) | Q2 (377-599 µM/d) | Q3 (600-877 µM/d) | Q4 (878-7133 µM/d) | P value | Q1 (0- 0.40 µM/d) | Q2 (0.41-0.99µM/d) | Q3 (1-1.99 µM/d) | Q4 (2-46 µM/d) | P value |
| **Systolic BP, mmHg** | 124±1.16 | 124±1.07 | 124±0.92 | 122±0.89 | 0.38 | 123±1.01 | 121±1.05 | 124±0.92 | 124±0.99 | 0.25 |
| **Diastolic BP, mmHg** | 74±0.88 | 74±0.82 | 74±0.70 | 73±0.68 | 0.57 | 74±0.76 | 73±0.81 | 74±0.70 | 74±0.76 | 0.57 |
| **Pulse Pressure, mmHg** | 40±1.97 | 38±1.82 | 38±1.57 | 40±1.53 | 0.07 | 40±1.71 | 40±1.80 | 38±1.58 | 41±1.70 | 0.58 |
| **Pulse rate, bpm** | 74±1.03$^{a}$ | 69±0.92$^{b}$ | 70±0.81$^{b}$ | 70±0.78$^{b}$ | 0.001 | 71±0.88 | 71±0.93 | 69±0.83 | 70±0.86 | 0.32 |
| **Waist circumference, cm** | 91.3±0.52 | 90.5±0.47 | 90.3±0.41 | 90.7±0.40 | 0.47 | 91.0±0.44$^{ab}$ | 91.4±0.46$^{b}$ | 90.8±0.41$^{ab}$ | 89.6±0.44$^{a}$ | 0.03 |
| **Hip circumference, cm** | 105.1±0.39 | 105.1±0.35 | 104.4±0.31 | 105.1±0.30 | 0.51 | 105.2±0.33 | 104.7±0.35 | 105.3±0.31 | 104.6±0.33 | 0.31 |
| **Waist to hip ratio** | 0.86±0.05 | 0.85±0.04 | 0.86±0.04 | 0.86±0.04 | 0.91 | 0.86±0.05$^{ab}$ | 0.87±0.04$^{b}$ | 0.86±0.04$^{ab}$ | 0.85±0.04$^{a}$ | 0.04 |
| **Total-C (mmol/L)** | 4.89±0.09$^{b}$ | 4.99±0.08$^{ab}$ | 4.99±0.07$^{ab}$ | 5.21±0.06$^{a}$ | 0.02 | 4.91±0.07$^{b}$ | 4.98±0.08$^{ab}$ | 5.05±0.07$^{ab}$ | 5.21±0.06$^{a}$ | 0.035 |
| **TAG (mmol/L)** | 1.15±0.02 | 1.11±0.01 | 1.12±0.01 | 1.12±0.01 | 0.97 | 1.13±0.01 | 1.16±0.01 | 1.06±0.01 | 1.17±0.01 | 0.32 |
| **HDL-C (mmol/L)** | 1.40±0.01 | 1.43±0.01 | 1.40±0.01 | 1.49±0.01 | 0.21 | 1.37±0.02$^{b}$ | 1.41±0.02$^{ab}$ | 1.43±0.02$^{ab}$ | 1.49±0.02$^{a}$ | 0.02 |
| **LDL-C (mmol/L)** | 2.92±0.05 | 3.02±0.05 | 3.04±0.05 | 3.19±0.05 | 0.051 | 2.98±0.06 | 3.00±0.07 | 3.10±0.06 | 3.19±0.06 | 0.21 |
| **Total: HDL-C Ratio** | 3.63±0.10 | 3.6 ±0.08 | 3.79±0.08 | 3.71±0.07 | 0.66 | 3.72±0.08 | 3.78 ±0.09 | 3.66±0.07 | 3.68±0.08 | 0.74 |
| **Glucose (mmol/L)** | 5.18±0.04 | 5.12±0.04 | 5.03±0.02 | 5.05±0.02 | 0.20 | 5.04±0.04 | 5.15±0.05 | 5.04±0.04 | 5.12±0.04 | 0.20 |
| **CRP (mg/L)** | 2.30±0.02 | 2.01±0.02 | 2.03±0.02 | 1.99±0.02 | 0.29 | 1.95±0.02$^{ab}$ | 2.36±0.02$^{b}$ | 1.93±0.02$^{ab}$ | 2.07±0.02$^{a}$ | 0.03 |
| **HbA1c (%)** | 5.49±0.03 | 5.40±0.03 | 5.50±0.03 | 5.43±0.03 | 0.08 | 5.47±0.03$^{ab}$ | 5.54±0.03$^{b}$ | 5.43±0.02$^{ab}$ | 5.40±0.03$^{a}$ | 0.02 |
| **Dietary nitrate intake(mg/d)** | 150±3.65$^{b}$ | 156±3.54$^{b}$ | 152±3.15$^{b}$ | 170±2.97$^{a}$ | <0.0001 | 153±3.31$^{b}$ | 147±3.43$^{b}$ | 164±3.21$^{ab}$ | 167±3.2$^{a}$ | <0.0001 |
| **Veg+water nitrate intake (mg/d)** | 74±2.49$^{b}$ | 76±2.49$^{ab}$ | 77±2.49$^{ab}$ | 83±2.51$^{a}$ | 0.021 | 77±2.44 | 75±2.44 | 78±2.44 | 78±2.44 | 0.81 |
| **Meat nitrate intake (mg/d)** | 54±1.70 | 53±1.70 | 55±1.70 | 57±1.70 | 0.276 | 53±1.69$^{ab}$ | 50±1.69$^{b}$ | 56±1.69$^{ab}$ | 60±1.69$^{a}$ | 0.001 |
| **Dietary nitrite intake(mg/d)** | 1.85±0.08$^{a}$ | 2.19±0.08$^{b}$ | 2.11±0.08$^{ab}$ | 2.11±0.08$^{ab}$ | 0.040 | 1.99±0.08 | 2.02±0.08 | 2.11±0.08 | 2.14±0.08 | 0.54 |

ANCOVA was used for this analysis, values are estimated marginal means ± SEM, covariates have been used in the statistical analysis. Values shown for quartiles of total urinary nitrate are min and max µM per day. Different superscript letters indicate significant difference.
**BP**: Blood Pressure, **CRP**: C reactive Protein, **Hb A1c**: Glycated haemoglobin **HDL-C**: High-density lipoprotein cholesterol, **LDL-C**: Low-density lipoprotein cholesterol, **TAG**: Triacylglycerol, **Total-C**: Total cholesterol. **(Range of sample in each quartile**: **Q1**=132, **Q2**=152, **Q3**=182, and **Q4**=171**).**

# CHAPTER 5: IDENTIFICATION OF THE TYPE AND LOCATION OF ORAL NITRATE-REDUCING BACTERIA IN RESPONSE TO INORGANIC NITRATE CONSUMPTION

**Chapter 5**

The following chapter will be submitted for publication as a short communication in Molecular Nutrition and Food Research.

**Contribution towards the paper**

I was involved with the design of REBOC1 study, subject recruitment and coordination of the study days. I was responsible for conducting the study and performing the laboratory analysis. I conducted the data and statistical analysis and wrote the manuscript.

**Identification of the type and location of oral nitrate-reducing bacteria in response to inorganic nitrate consumption**

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**Running title:** Dietary nitrate and nitrate reducing bacteria in the oral cavity

**Key words:** Nitrate; Nitrite, Beetroot juice; Oral microbiome; Mouthwash; *Neisseria*

## 5.1. ABSTRACT

Higher intakes of dietary inorganic nitrate have been reported to have health benefits including improvements in vascular function. Oral nitrate-reducing bacteria have been proposed to play an important role in the conversion of dietary nitrate to nitrite but few studies have determined their composition or location in the mouth. This study aimed to identify the major sites of nitrate-reducing bacteria in the human oral cavity and investigate whether dietary inorganic nitrate intake and mouthwash use influence oral nitrate reduction. A three-arm sequential study involving 20 healthy adults (10 M and 10 F, mean age 31 ± 9 y and BMI 23.8 ± 3.1 kg/$m^2$) was conducted. On separate occasions, the participants were assigned to treatments in the following order: rinsing the mouth for 5 min with 10 ml of (I) beetroot juice, (II) low-nitrate mineral water (control) or (III) beetroot juice after the use of an antibacterial mouthwash. Samples of saliva were collected for the measurement of nitrate and nitrite concentrations, and nitrate reduction was measured in different sites in the oral cavity using filter paper. Nitrate-reducing bacteria were isolated using a cotton swab before and after each treatment and identified by using 16S rDNA sequencing.

The salivary nitrite concentration was increased after rinsing the mouth with beetroot juice (5.18 ± 1.41 μM) but decreased after prior use of antibacterial mouthwash (0.03 ± 0.01 μM) ($p<0.001$). A total of 109 nitrate-reducing bacteria were identified from seven sites in the oral cavity, with the majority isolated from the rear tongue under aerobic conditions after rinsing the mouth with beetroot juice. Identification of bacterial genera showed an increase in the abundance of *Neisseria* after beetroot juice, and these bacteria were abolished after prior use of mouthwash. This study revealed nitrate-reducing bacteria to play an important role in converting dietary nitrate (beetroot juice) to nitrite in the mouth, with the rear tongue identified as an important location of these bacteria in the oral cavity.

## 5.2. INTRODUCTION

Evidence has shown that healthy dietary patterns and lifestyles can help prevent CVDs, a leading cause of death globally. Diets high in nitrate-rich vegetables have been shown to lower blood pressure and improve endothelial function(1). The commensal oral bacteria are essential in the conversion of nitrate to nitrite and stimulate the formation of nitric oxide (NO) through denitrification(2). Within the oral microbiome, it has been reported that nitrate-reducing bacteria represent approximately 20% of the total bacteria(3), and their presence is correlated with increased nitrite concentrations in saliva(4) and plasma(5). This has been shown to influence human health, such as beneficial effects on vascular function(6) and risk markers for developing cardiometabolic disease(7). To determine the role of these bacteria in the conversion of nitrates to nitrites, studies have used antibacterial mouthwash to reduce the abundance of oral bacteria(5). Kapil et al reported that using 0.2% chlorhexidine twice daily for 7 days significantly increased systolic and diastolic blood pressure measured using 3 different techniques (clinic, ambulatory and home measurements) by approximately 3 and 2 mmHg respectively in 19 healthy normotensive subjects(8). Several non-modifiable risk factors have been found to influence the presence and composition of the bacteria in the oral cavity, such as age, genetic make-up and tongue physiology(9). However, the main modifiable factor is dietary nitrate intake(10), with inorganic nitrate found abundantly in some vegetables (such as beetroot and spinach) and drinking water(11).

Among the 700 bacterial species found in the oral microbiome, nitrate-reducing bacteria such as *Veillonella dispar, Actinomyces odontolyticus, Rothia mucilaginosa* (12) and *Neisseria*(13) have been identified. Only a few studies have focused on the role of the oral microbiome in mediating the effects of inorganic nitrate on blood

pressure. One study conducted in nine elderly adults (70–79 yrs; 6 F and 3 M) and nine young adults (18–22 yrs; 5 F and 4 M) showed an increase in the abundance of the nitrate-reducing bacteria *Rothia and Neisseria* after 10 days of drinking 170 ml of beetroot juice (~12.4 mmol nitrate) compared with a placebo. However, no increase in the abundance of *Prevotella and Veillonella* species were observed(14). Moreover, the increased prevalence of *Rothia and Neisseria* species relative to the *Prevotella and Veillonella* species was linked to higher nitrite concentrations in both saliva and plasma(14) which was related to the reduction in systolic and diastolic blood pressure in the older participants only. These findings support the hypothesis that the oral microbiome community is responsive to changes in the level of dietary nitrate intake.

Doel et al have reported that the posterior surface of the tongue was the main area responsible for the majority of nitrate reduction in the oral cavity(12). The effect of nitrate-rich foods on the composition of the human oral microbiome has not been fully elucidated. This pilot study aimed to investigate the influence of rinsing the mouth with beetroot juice and antibacterial mouthwash on the abundance and genera of nitrate-reducing bacteria, and to identify the major sites (rear, mid and front tongue, tooth surface, buccal surface, hard palate and sublingual) in the oral cavity in which the nitrate-reducing bacteria are found.

## 5.3. METHOD

### 5.3.1. Volunteers

The School of Chemistry, Food and Pharmacy Research Ethics Committee, University of Reading gave a favourable ethical opinion for conduct (STUDY Number-32/16) and the study was registered at clinicaltrials.gov (NCT03068962). Thirty healthy male and female volunteers were recruited from the University of Reading and the surrounding areas by email, displaying posters and advertising on social media. A medical and

lifestyle questionnaire was used to assess their eligibility for the study before being invited to attend a screening visit. Subjects were selected if they met the study inclusion criteria: non-smokers, aged 18–55 y, body mass index between 18 and 30 kg/m$^2$ and no recent history of periodontal disease or taking medication shown to influence oral bacteria, including antibiotics. Interested volunteers were invited to the Hugh Sinclair Unit of Human Nutrition at the University of Reading for a short screening visit where the study was explained in greater detail and all the volunteers gave their written informed consent before the study visit measurements were taken. These included weight, height and office blood pressure. Potentially eligible participants were invited to a further screening session during which a dentist checked for evidence of dental diseases (e.g. current dental cavities or periodontal infection) as periodontal disease can affect the composition of oral bacteria. Those with dental disease were unable to participate in the study.

### 5.3.2. Study design

Eligible participants were invited to take part in the three-arm sequential study. Volunteers attended the clinical unit within the Hugh Sinclair Unit of Human Nutrition for each of their study visits, which were separated by at least one week. Volunteers consumed a low-nitrate diet and drank only low-nitrate mineral water (Buxton) on the day before each study visit and refrained from strenuous exercise and drinking alcohol. They were then required to fast overnight and only drink low-nitrate mineral water during this time. On the morning of the study visit, they refrained from performing their usual dental care routine (brushing teeth, flossing or using mouthwash) before attending the clinical unit.

Participants were assigned three treatments in the following order: visit 1: rinse mouth with 10 ml of low-nitrate mineral water followed by holding 10 ml of beetroot juice (James White, Beet it, organic, ~6.2 mmol nitrate) in the mouth for 5 min; visit 2: rinse

mouth with 10 ml of low-nitrate mineral water followed by holding 10 ml of low-nitrate mineral water in the mouth for 5 min (control); visit 3: rinse mouth with a chlorhexidine mouthwash (0.2% chlorhexidine digluconate, GlaxoSmithKline) for 1 min before holding beetroot juice in the mouth for 5 min. After 5 min, the whole mouth rinse was collected into sterile ice-chilled Falcon tubes that were placed on ice and centrifuged immediately after collection for 10 min at 5000 rpm (3000 × g) at 4°C. Three aliquots of the supernatant were then stored at −80°C until analysis.

To assess the oral nitrate-reducing capacity, filter paper squares were first soaked in the relevant treatment according to the study visit and then placed in seven specific sites in a volunteer's mouth for 90 s. They were then removed and placed into labelled Eppendorf tubes containing 500 µl of 0.5 M sodium hydroxide. The Eppendorf tubes were then centrifuged immediately for 10 min at 8000 rpm (6000 × g), and the supernatants were stored at −80°C until it was analysed for nitrate and nitrite concentrations.

### 5.3.3. Isolation of nitrate-reducing bacteria

Samples of oral bacteria were collected before and after each of the treatments from the same seven sites in the oral cavity (rear, mid and front tongue, tooth surface, buccal surface, hard palate and sublingual) by sliding TePe brushes between two back molars and by swabbing a sterile cotton swab along the buccal cervical margin of the premolars and on the rear, mid and front of the tongue. Samples were placed into Eppendorf tubes containing 1 ml of sterile phosphate-buffered saline (PBS). The samples were then stored in a freezer at −80°C until bacterial DNA was extracted for 16S rDNA sequencing.

Initially, a novel method was developed to isolate nitrate-reducing bacteria from the oral bacterial samples before these bacteria were identified on agar plates using Griess reagent. In this method, 100 µl of PBS was placed into a 10 ml Hungate tube

containing 10 ml of anaerobic nitrate broth and a 20 ml Bojota bottle containing 10 ml 10 ml of aerobic nitrate broth, each with 0.5 % cysteine followed by incubation for 24 h under anaerobic and aerobic conditions, respectively. After this incubation, 100 µl of each sample was spread onto a Petri dish of nutrient agar and further diluted to a final concentration of $1 \times 10^{-4}$ of bacteria before spreading them onto a Petri dish. When growth was observed after 24-48h, a single bacterial colony was transferred onto a further Petri dish of nitrate agar and incubated for another 24 h to grow. If growth was observed on the nitrate agar, a single colony of this bacterium was picked up using an inoculation loop and placed directly onto another Petri dish containing nitrate agar to purify the sample (see Supplementary Figure 2). The pure samples then were stored separately in 50% glycerol at -80°C before extracting the bacterial DNA.

### 5.3.4. Identification of nitrate-reducing bacteria

The samples that were stored in 50% glycerol were then spread onto nutrient agar and incubated for 24 h. If growth occurred, a colony was isolated using an inoculation loop and placed again in 10 ml of nitrate broth to select for nitrate-reducing bacteria. A sample from the nitrate broth (100 µl) was then placed between a 10-ml layer of agar with potassium nitrate and a 10-ml layer of agar containing Griess reagent. A pink halo was indicative of a presence of bacteria that could reduce nitrate to nitrite (see Supplementary Figure 2). The positive bacteria for the Griess reagent were counted as nitrate-reducing bacteria.

Bacterial DNA was extracted from the identified nitrate-reducing positive bacteria isolated from 3 sites of the tongue only (as indicated that tongue was the major site of nitrate reduction) of the participants as well as baseline (before treatments) samples for the tongue by using the QIAamp DNA Mini Kit (Qiagen, Manchester, United Kingdom) according to the manufacturer's instructions. The extracted DNA was pipetted in a 96-well PCR plate and stored at −20°C before being sent to the company

for 16S sequencing analysis to identify the abundance of the bacterial genus. All DNA samples were analysed by using 16S amplicon sequencing at the Animal and Plant Health Agency (Surrey, United Kingdom). Sequencing was performed on an Illumina MiSeq with 2 × 300 base reads according to the manufacturer's instructions (Illumina Cambridge UK), and bioinformatic analysis was performed using QIIME v1.9.1.

### 5.3.5. Statistical analysis

The sample size for this pilot study was based on a previously published research investigating the number and location of oral microbiota in humans. Ten subjects was deemed sufficient to find a significant correlation between nitrate and oral bacteria composition in all the subjects in this previous study; however, this study group was heterogeneous and included both men and women(12). To determine the differences in oral microbiota between sexes, this pilot study was performed in 20 adult volunteers with equal numbers of men and women. IBM SPSS Statistics version 24 was used for all statistical analyses and the results are presented in the tables and text as mean ± SD. Data were checked for normality and log-transformed where necessary. General Linear Model (Repeated Measures) was used to determine the effects of treatments over the study period. The change in the abundance of bacteria genus over the study visits was analysed using non-parametric Kruskal-Wallis test as our data was stratified into a group according to nitrate-reducing abundances if it was low <10 or ≥ 10. Statistical significance was accepted when $p < 0.05$.

## 5.4. RESULTS

### 5.4.1. Baseline and descriptive data

A total of 20 volunteers (10 men and 10 women) completed this study. The characteristics of the study participants are shown in Table 1 for the whole group and then according to the men and women matched for age and BMI.

### 5.4.2. Identification of nitrate-reducing bacteria

From the 20 subjects, a total of 109 nitrate-reductase-positive colonies were counted and identified from seven sites around the oral cavity. A greater proportion of the colonies were isolated under aerobic conditions (n= 88) rather than anaerobic conditions (n= 21). More nitrate-reducing bacteria were isolated after the mouth rinse containing beetroot juice (n= 62) compared with the control mouth rinse (n= 37) and prior use of the antibacterial mouthwash before the beetroot juice mouth rinse (n= 10). The results obtained illustrate the effects of the treatments on the numbers of nitrate-reductase-positive bacteria (Supplementary Figure 3). Figure 1 shows the presence of bacteria in each volunteer and the inter-individual variability; five subjects had six to eight positive nitrate-reducing bacteria in their oral cavity, whereas 15 subjects had only one to four positive nitrate-reducing bacteria after rinsing their mouth with beetroot juice.

A greater number of the positive nitrate-reducing bacteria were isolated from the rear tongue (n = 14), mid tongue (n = 12) and front tongue (n=10) after rinsing the mouth with beetroot juice, whereas after rinsing with control, most of the nitrate-reducing bacteria were isolated from the mid tongue (n = 11) and front tongue (n = 9). The nitrate-reducing bacteria were almost absent from the rear tongue, mid tongue and front tongue after the third treatment (beetroot juice mouth rinse after the prior use of mouthwash) (Figure 2). When the data were stratified by sex, the results were similar, with no significant differences in numbers of positive nitrate-reducing bacteria found between the male and female participants after each treatment ($p$ = 0.112, $p$ = 0.062 and $p$ = 0.061, respectively).

The results presented in Table 2 show the numbers of the positive nitrate-reducing bacteria found in the tongue samples from all participants after each treatment. The numbers of *Nesseria* and *Staphylococcus* bacteria were significantly higher after the

beetroot juice treatment compared with baseline and the other treatments, whereas there was a significant decrease in the abundance of *Prevotella* after the beetroot juice treatment. The majority of the nitrate-reducing bacteria (90%) were significantly abolished after the use of the chlorhexidine medicated mouthwash. However, *Staphylococcus* persisted even after mouthwash use.

### 5.4.3. Salivary nitrate and nitrite concentrations

Our results presented in Table 2 indicate a significant difference in salivary nitrate concentration between treatments ($p < 0.001$). The control resulted in a significantly lower (68.8 ± 35.1 µM) salivary nitrate concentration than both the beetroot juice and beetroot juice with mouthwash treatments ($p < 0.001$), which showed similar salivary nitrate concentrations (452 ± 168 µM versus 453 ± 172 µM). The salivary nitrite concentration was significantly higher after the beetroot juice treatment (5.18 ± 1.41 µM) than after the control (0.49 ± 0.14µM) and beetroot juice plus mouthwash treatment (0.03 ± 0.01 µM) ($p < 0.001$). Sex had no significant impact on the salivary nitrate and nitrite concentrations after any of the treatments.

### 5.4.4. Oral nitrate-reducing capacity in different sites in the oral cavity

There was no significant difference in the nitrate concentrations measured on the different filter papers placed in several sites around the oral cavity after the beetroot juice, control or beetroot juice after prior use of mouthwash treatments ($p = 0.124$, $p = 0.99$ and $p = 0.241$, respectively) (Table 4).

There was a significant difference in nitrite concentration between sites after the beetroot juice treatment. The rear tongue showed a higher nitrate reduction (21.7 ± 7.0 µM; $p < 0.001$) than all other sites in the oral cavity. Moreover, the rear tongue showed a higher nitrite concentration (16.8 ± 7.2 µM; $p < 0.001$) than the other sites after the low-nitrate mineral water treatment. However, the nitrite concentration was very low in all of the sites around the oral cavity after prior use of mouthwash before

the beetroot juice mouth rinse with no significant difference in concentration between sites ($p$ = 0.346) (Table 4).

### 5.5. DISCUSSION

The nitrate-reducing bacteria that reside in the oral cavity play an important role in the conversion of dietary nitrate to nitrite and have been proposed to contribute to the systemic bioavailability of the potent vasodilator nitric oxide. In this pilot study, the prior use of mouthwash was found to abolish the numbers of all oral bacteria and reduce the salivary nitrite concentrations in response to beetroot juice. The tongue surface was identified as a location of greater abundance of nitrate reducing bacteria, with *Neisseria* and *Staphylococcus* bacteria found to be increased and *Prevotella* bacteria decreased, after the beetroot mouth rinse.

Measurements of nitrate and nitrite concentrations in different sites in the oral cavity revealed a higher oral nitrate-reducing capacity on the posterior surface of the tongue, which suggests that this is the main area responsible for the majority of the nitrate reduction. This supports the findings of Doel et al. who also identified the tongue surface as the primary contributor to nitrate reduction(12). In agreement with previous human(13) and animals(15) studies, we found a greater abundance of nitrate-reducing bacteria on the posterior surface of the tongue and that higher proportions of these bacteria were facultative anaerobes. Compared with other mucosal surfaces, the papillary structure of the human tongue supports a high bacterial density by providing a unique environment for both aerobic and anaerobic bacteria(16). The lack of differences between men and women observed in this study supports other studies(2),(17), although our study may not have been adequately powered to determine the impact of sex on the outcomes measured. A previous study reported that the oral microflora of men was more diverse than women(18), however it is difficult

to compare these studies directly as this pilot study, and those of Doel et al(12). and Kapil et al(2)., focused primarily on nitrate-reducing bacteria.

After the beetroot juice treatment, a higher abundance of nitrate-reducing bacteria was found to be associated with an increased salivary nitrite concentration, compared with the control which suggests that the inorganic nitrate may stimulate the growth rate of the nitrate-reducing bacteria within the oral cavity. The nitrate-reducing bacteria identified in the current study were *Nesseria* and *Staphylococcus*, which increased in abundance after the beetroot juice treatment with a concomitant decrease in the abundance of *Prevotella* and *Veillonella* bacteria. However, most of the nitrate-reducing bacteria were significantly abolished after the use of an antiseptic mouthwash. This finding agrees with that of Mitsui et al(19). who observed a decrease in the number of nitrate-reducing bacteria such as *Veillonella dispar* after using chlorhexidine for 3 min before nitrate ingestion in 12 healthy volunteers. Moreover, Govoni et al. found a reduction in the oral nitrate-reducing capacity after mouthwash use with an 80% reduction found in the oral bacterial count(20). These findings support our data that showed that some bacteria were still present after mouthwash use in three participants indicating that mouthwash did not abolish all the oral bacteria.

Analyses of the mouth rinse samples supported an increase in the oral nitrate reducing capacity after the beetroot juice compared with the control treatment, demonstrated by an increase in salivary nitrite concentrations. In agreement with our results, Govoni et al(20). reported that the circulating concentrations of nitrite increased after the ingestion of dietary nitrate due to the activity of the oral bacteria and that the removal of the nitrate-reducing bacteria by mouthwash use had a marked effect on systemic nitrite formation(8). Interestingly, the response of nitrite after the beetroot juice treatment was markedly attenuated after the medicated mouthwash treatment, which

can inactivate nitrate reductase activity by removing nitrate-reducing bacteria. However, Govoni et al did not relate these changes to blood pressure or any vascular function measurements(20).

One study reported that using 0.2% chlorhexidine twice daily for 7 days significantly increased systolic and diastolic blood pressure by approximately 3 and 2 mmHg respectively in 19 healthy subjects. The rise in blood pressure was significantly correlated with the concomitant reduction in plasma nitrite levels(8). Furthermore, in 15 subjects treated with anti-hypertensive medication, the attenuation found in oral nitrate-reducing capacity after daily use of chlorhexidine mouthwash for 3 days was associated with an increase in systolic blood pressure of 2.3 mmHg, but only a trend for a decrease in plasma nitrite concentrations compared with the control (tap water). These findings highlight the potential importance of the oral nitrate-reducing bacteria in blood pressure modulation(21).

The strength of this study was the use of 16S sequencing for bacterial genus identification. Also, an equal number of men and women, matched for age and BMI were included to compare the variation in responses between sexes. Furthermore, this study was the first study used a dietary nitrate source (beetroot juice) to simulate nitrate-reducing bacteria rather than nitrate salt which represented more physiological conditions.

The main limitation of this study was the use of an *in vitro* method for identification and isolate of the nitrate-reducing positive colonies which was based on the Griess assay. This may have resulted in the loss of up to 50% of bacterial growth and impact on the identification of the species observed since this reagent only reacts with nitrite, but not nitrate. Also, the study design was sequential which may have impacted on the findings observed.

In conclusion, beetroot juice high in nitrate increased the oral nitrate-reducing capacity, especially on the rear tongue and was associated with a greater abundance of *Neisseria*. The prior use of a medicated mouthwash confirmed the importance of nitrate-reducing bacteria in reducing dietary nitrate to nitrite. Further research should focus on the impact of dietary nitrate on specific nitrate-reducing bacterial species number and type and investigate their role in mediating the beneficial effects of nitrate-rich foods on vascular function.


## 5.6. ACKNOWLEDGEMENT

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## 5.8. CONFLICT OF INTREST

The authors have no conflicts of interest to declare.

## 5.9. AUTHOR CONTRIBUTIONS

H.S.A involved with the design of REBOC1 study, performing the study and conducting the statistical analysis and wrote the manuscript. Prof. Julie Lovegrove and Drs Kim Jackson, Ditte Hobbs and Gemma Walton contributed to the design of the REBOC1 study, provided guidance on the laboratory analysis, assisted with data interpretation, and provided feedback on each draft of this paper.

## TABLES

**Table 1: Baseline characteristics of the study participant.**

| Characteristic | Whole Group | Men (mean ±SD) | Women (mean ±SD) |
|---|---|---|---|
| **Number** | 20 | 10 | 10 |
| **Age (years)** | 31 ± 9 | 30.7 ± 9.5 | 32.6 ± 7.7 |
| **Body Mass Index (kg/m$^{2)}$** | 23.8 ± 3 | 24.0 ± 3.1 | 23.9 ± 2.8 |
| **Systolic blood pressure (mmHg)** | 116 ± 8 | 115± 7 | 109± 5 |
| **Diastolic blood pressure (mmHg)** | 67 ± 7 | 67± 8 | 67 ± 5 |
| **Pulse pressure (mmHg)** | 49 ± 9 | 49 ± 6 | 42 ± 6 |

**Table 2: Number nitrate-reducing bacteria identified after each treatment.**

| BACTERIA GENUS | PRESENT | | | | |
|---|---|---|---|---|---|
| | Baseline | beetroot | control | Beetroot + mouthwash | P value |
| k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella | 8* | 2 | 1 | 0 | 0.004 |
| k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g __Staphylococcus | 5 | 6 | 0 | 3 | 0.066 |
| k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g __Streptococcus | 6* | 3 | 1 | 0 | 0.024 |
| k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g __Veillonella | 3 | 1 | 0 | 0 | 0.101 |
| k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f __Oxalobacteraceae;g__Ralstonia | 3 | 4* | 0 | 0 | 0.048 |
| k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f __Neisseriaceae;g__Neisseria | 6 | 8* | 1 | 0 | 0.024 |
| k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f __Pasteurellaceae;g__Haemophilus | 2 | 0 | 0 | 0 | 0.108 |

Kruskal-Wallis test was used data presented as numbers. Statistical significance was accepted when $p < 0.05$.

**Table 3: Salivary nitrate and nitrite concentrations collected after each treatment:**

| | Beetroot juice | control | Beetroot juice +mouthwash | P-value |
|---|---|---|---|---|
| **Nitrate µM** | 452± 168 | 68.8± 35.1* | 453± 172 | p < 0.001 |
| **Nitrite µM** | 5.18± 1.41* | 0.49± 0.14 | 0.03± 0.01 | p < 0.001 |

Repeated measure ANOVA was used data presented as mean± SD. P<0.05, *nitrate level significantly lower than beetroot juice and beetroot juice+ mouthwash. *nitrite significantly higher than control and beetroot juice+ mouthwash treatments.

**Table 4: Oral nitrate reducing capacity determined by measuring nitrate (A) and nitrite (B) on filter paper placed in different sites within the oral cavity.**

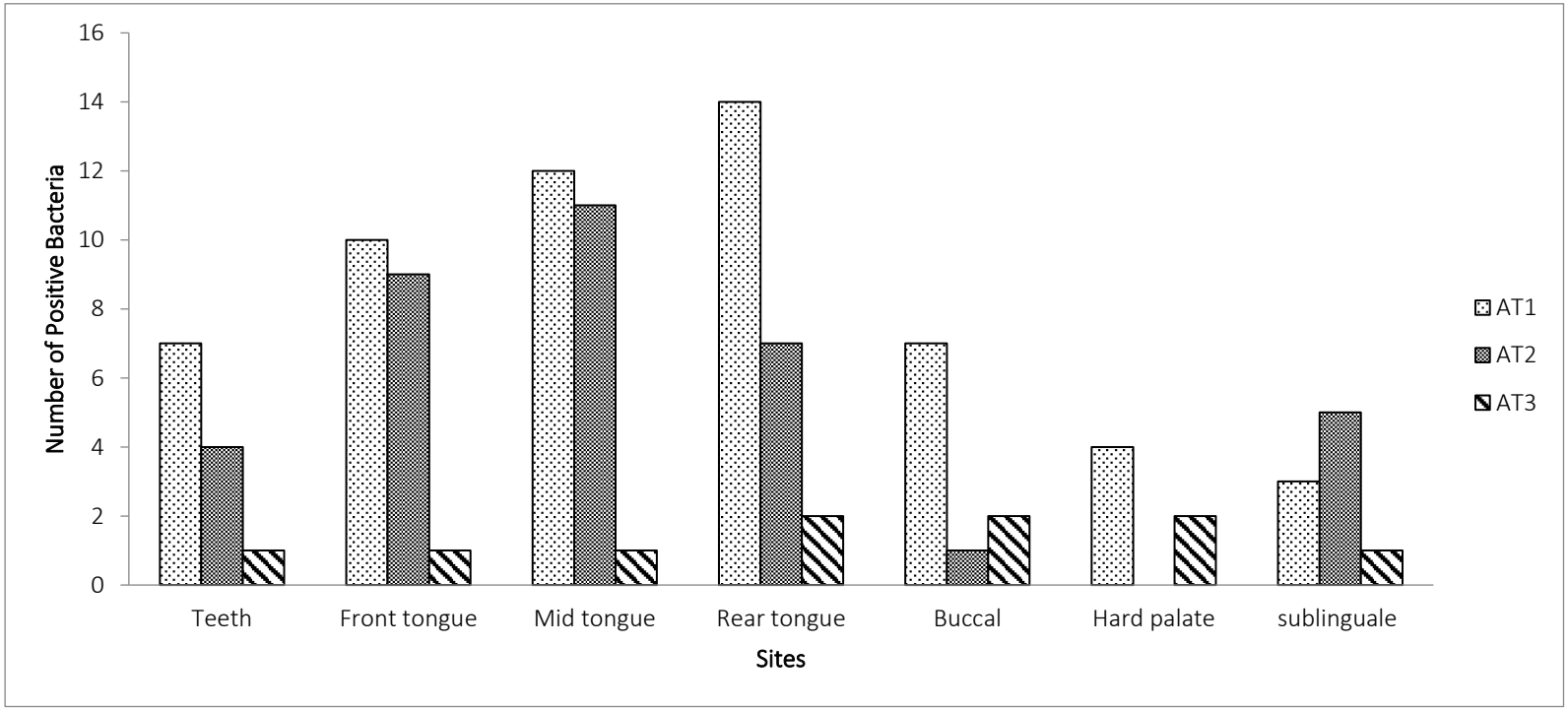
| A | nitrate, µM Beetroot juice | nitrate, µM control | nitrate, µM Beetroot juice +mouthwash | Treatment **P-value** |
|---|---|---|---|---|
| **Teeth** | 789 ± 145 | 584 ±164 | 690±. 138 | 0.090 |
| **Front tongue** | 745± 187 | 592 ±117 | 643± 182 | 0.152 |
| **Mid tongue** | 727± 177 | 566 ±113 | 649 ± 185 | 0.212 |
| **Rear tongue** | 713± 124 | 575±131 | 600 ± 175 | 0.051 |
| **Buccal** | 671± 143 | 577±137 | 650± 131 | 0.330 |
| **Sublingual** | 652± 167 | 582±145 | 6.2 ± 186 | 0.636 |
| **Hard pallet** | 653± 148 | 587 ±108 | 676 ±. 158 | 0.795 |
| Location **P-value** | 0.124 | 0.990 | 0.241 | |

| B | nitrite, µM Beetroot juice | nitrite, µM control | nitrite, µM Beetroot juice +mouthwash | P-value (Treatment) |
|---|---|---|---|---|
| **Teeth** | 8.50± 4.12 | 6.38± 3.95 | 0.52± 0.77 | 0.000 |
| **Front tongue** | 10.21± 5.95 | 7.87± 4.65 | 0.97± 1.82 | 0.000 |
| **Mid tongue** | 10.01± 8.74 | 6.77± 5.37 | 0.91± 0.97 | 0.001 |
| **Rear tongue** | 21.70± 6.98* | 16.76± 7.24* | 1.04± 1.25 | 0.000 |
| **Buccal** | 6.23± 2.02 | 2.96 ± 4.43 | 0.59± 0.62 | 0.000 |
| **Sublingual** | 5.81± 2.09 | 3.62± 4.96 | 0.79± 1.56 | 0.001 |
| **Hard pallet** | 7.41± 9.67 | 6.60± 2.46 | 0.56± 0.84 | 0.005 |
| Location **P-value** | 0.001 | 0.001 | 0.346 | |

Repeated measure ANOVA was used, data presented as mean± SD. Nitrate level in control was significantly lower than beetroot juice and beetroot juice+ mouthwash. Nitrite was significantly higher after beetroot juice than control and beetroot juice+ mouthwash. Statistical significance was accepted when $p < 0.05$.

## FIGURES

**Figure 1: The location of nitrate reducing bacteria in the oral cavity after each treatment.**




This figure presents the number of nitrate positive nitrate bacteria isolated from all participants based on location in the oral cavity. **AT1:** After Treatment 1 (beetroot juice). **AT2:** After Treatment 2 (control). **AT3:** After Treatment 3 (mouth wash and beetroot juice).

## 5.10. REFERENCES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## SUPPLEMENTARY INFORMATION

**Figure1: Overview of the new technique for the Isolation and identification of nitrate reducing bacteria from oral samples collected after each treatment**

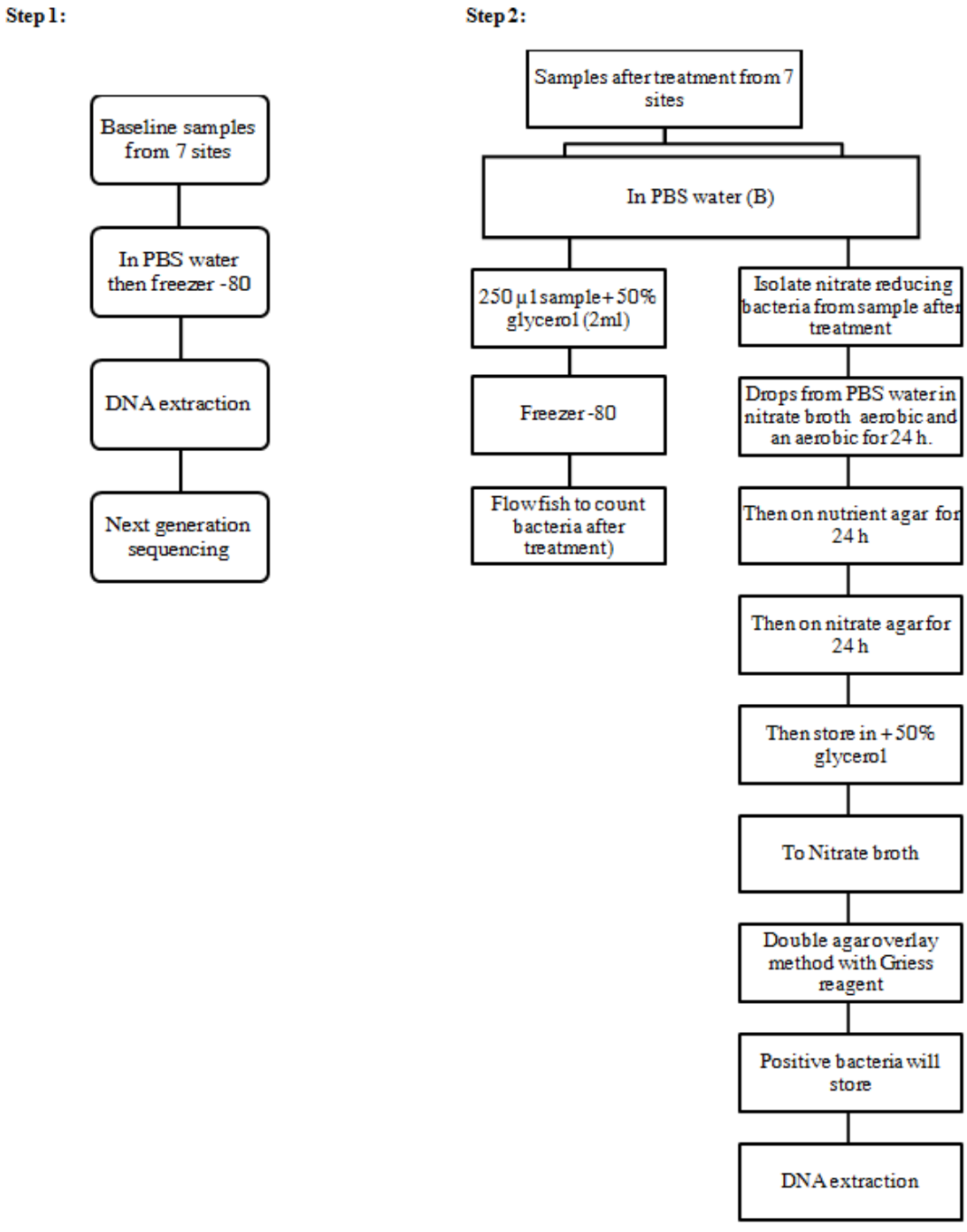


**Figure 2: The positive bacteria are identified by a pink hallo**

**(Indicated by the arrow)**

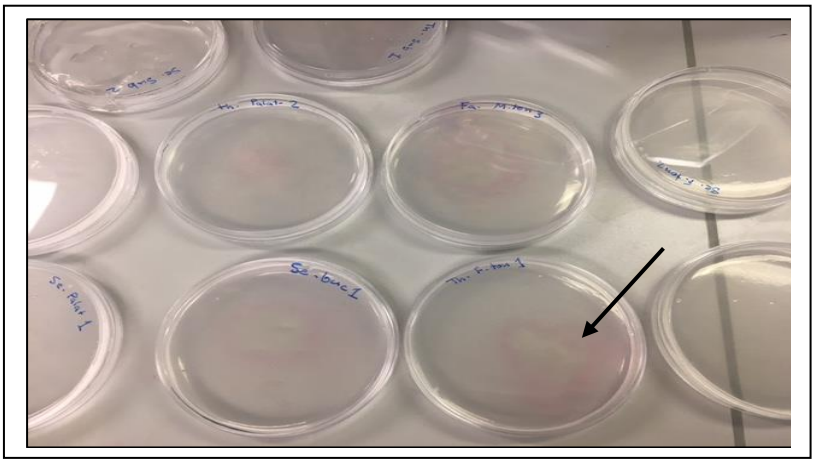
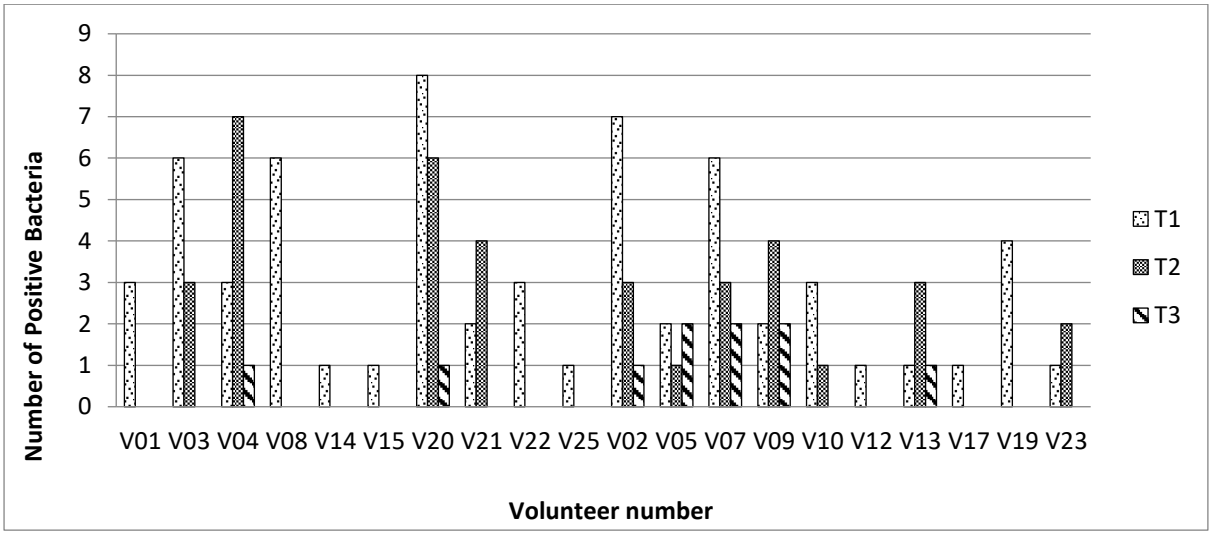


**Figure 3: Nitrate reducing bacteria identified in the oral cavity from each participant after each treatment.**




Treatment 1: beetroot juice. Treatment 2: low nitrate mineral water. Treatment 3: beetroot juice after prior used of mouthwash in each participant.

# CHAPTER 6: CHRONIC BEETROOT JUICE COMPOSITION HAS BENEFICIAL EFFECTS ON BLOOD PRESSURE, MICROVASCULAR FUNCTION, GUT AND ORAL BACTERIA COMPOSITION

**Chapter 6**

The following manuscript will be submitted for publication in The American Journal of Clinical Nutrition.

**Contribution towards the paper**

I was involved with the design of REBOC2 study, subject recruitment and coordination of the study days. I was the main researcher managing the running of the human study. I also performed the laboratory analysis and conducted the data and statistical analyses. I was responsible for writing the first draft of this paper.

**Chronic beetroot juice composition has beneficial effects on blood pressure, microvascular function, gut and oral bacteria compositions: Findings from the REBOC2 randomised crossover study**

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**Running title:** Dietary nitrate, blood pressure and vascular function

**Clinical trial registration:** Clinicaltrials.gov (NCT03784742)

## 6.1. ABSTRACT

**Background:** Oral nitrate-reducing bacteria have been proposed to contribute to the beneficial effects of high dietary nitrate intakes on blood pressure. However, very little is known about the changes in the oral and gut microbiome in response to chronic inorganic nitrate intake and relationship to measures of vascular function.

**Objective:** To compare the effect of daily consumption for 8 weeks of nitrate-rich beetroot juice (3.7 mg of nitrate/kg body weight) with a control beetroot juice on cardiovascular disease (CVD) risk markers, oral and gut microbiota compositions in healthy individuals.

**Design:** In this double-blind, randomised, controlled, cross-over study, 19 healthy adults consumed nitrate-rich and nitrate-free (control) beetroot juice for 8 weeks each, with a 4-week wash out period between treatments. Blood pressure and microvascular function were measured at the beginning and end of each intervention arm. Blood and saliva samples were taken for the analysis of CVD risk markers, nitrate and nitrite concentrations. Oral and stool samples were collected to determine microbiota composition.

**Results:** Compared with the control juice, beetroot juice consumption for 8 weeks significantly decreased systolic ($p=0.01$) and diastolic ($p=0.03$) blood pressure and increased the incremental area under the curve (IAUC) for the microvascular response to sodium nitroprusside (endothelium-independent vasodilation) ($p=0.002$). These findings were associated with significantly higher nitrate and nitrite concentrations in saliva ($p=0.002$), urine ($p<0.001$) and plasma ($p<0.001$) after the beetroot juice. Nitrate intake also impacted the oral and gut microbiome, with a significant increase in the abundance of *Neisseria* in the oral cavity by 4% ($p=0.014$) and a reduction in

*Clostridium* in the gut bacteria by 3% (p=0.002). There were no significant changes in any other CVD risk biomarkers.

**Conclusions:** The beneficial effects of the chronic ingestion of inorganic nitrate (3.7 mg of nitrate/kg body weight) on blood pressure and microvascular function were associated with a greater oral nitrate-reducing capacity and changes in the oral and gut microbiome compositions.

**Key words:** Beetroot juice, Laser Doppler imaging, Microvascular function, Blood pressure, Oral microbiome, Gut microbiome.

## 6.2. INTRODUCTION

Hypertension is an independent modifiable risk factor for cardiovascular diseases (CVDs), being responsible for 13% of deaths globally. Epidemiological evidence suggests that higher vegetable consumption is associated with reduced blood pressure and reduction in CVD risk by 80% (1), which is believed to be related to the high content of inorganic nitrate in green leafy and root vegetables (2). Dietary inorganic nitrate has been reported to have beneficial effect on vascular function (3, 4) by promoting the bioavailability of nitric oxide (NO) in the systemic circulation (5). Nitrate-reducing bacteria within the oral microbiome (6) exist in a symbiotic relationship with the host using nitrate and nitrite as the final electron acceptors in respiration and helping the host to convert nitrate to nitrite and then to NO (7). Inorganic nitrate consumption has been found to impact the composition of oral bacteria. For example, the supplementation of rich-nitrate Juice increased the nitrate-reducers *Neisseria* and *Rothia* in the saliva and decreased *Veillonella* and *Prevotella* bacteria(8). These changes were associated with greater plasma nitrite concentrations and a concomitant reduction in blood pressure by 2–6 mmHg (9). Therefore, it appears that the abundance and composition of nitrate-reducing bacteria in the oral cavity may be important in relation to cardiovascular health.

The potential of dietary nitrate to promote the oral and gut proliferation of nitrate-reducing bacteria was of interest in this study. However, the effect of dietary inorganic nitrate on bacteria has been found not only to influence the oral microbiome but also to affect the gut microbiome. A recent study reported that consumption of nitrate-rich fruit and vegetable juice for 3 days lead to a reduction in the *Firmicutes* to *Bacteroides* ratio. However, these changes may not be linked to nitrate intake directedly as the

nitrate was consumed with another food matrix, such as an apple (10). To date, very little is known about the composition of the oral and gut bacteria following chronic beetroot juice consumption and whether changes in oral nitrate-reducing capacity are related to changes in blood pressure and microvascular function.

The main aim of this study was to determine whether chronic intake of inorganic nitrate for 8 weeks can reduce blood pressure and improve microvascular reactivity. We chose to limit the dose of nitrate to 3.7 mg/kg body weight to determine whether the reported benefits on vascular function were also evident at the current acceptable daily intake (ADI). A further objective was to determine whether dietary nitrate influences the make-up of oral and gut bacteria in healthy people and whether relationships exist with changes in blood pressure and blood vessel elasticity

## 6.3. SUBJECTS AND METHODS

### 6.3.1. Study participants

The study was given a favourable ethical opinion for conduct by the University of Reading Research Ethics Committee (UREC 18/40) and was registered at Clinicaltrials.gov (NCT03784742). The study visits were conducted between November 2018 and March 2020. Healthy volunteers were recruited from the University of Reading and surrounding areas by email, poster displays and advertising on social media. Interested participants were provided with a participant information sheet before their eligibility was assessed using a health and lifestyle questionnaire. Subjects were selected if they met the following study criteria: non-smoker, no history of recent serious acute or chronic illness; not prescribed or taking antibiotics within 3 months of the start of the study or taking medication likely to affect blood pressure; no self-reported use of mouthwash or excess consumption of alcohol (>14 units/week).

Potentially eligible individuals were invited to a screening visit after a 12 h overnight fast at the Hugh Sinclair Unit of Human Nutrition, University of Reading. All subjects gave their written informed consent before the screening measurements were performed. These included office blood pressure and anthropometric measurements to ensure that participants were normotensive (blood pressure ≤120 (systolic)/80 (diastolic) mmHg) and BMI was between 18.5–39.9 kg/m$^2$. Two blood samples were then collected, one for the analysis of full blood count using a DxH520 haematology analyser (Beckman Coulter, USA) to assess anaemia (haemoglobin<115 g/l for women and <130 g/l for men) and the second for the analysis of fasting serum lipids, glucose and markers of liver and kidney function using the Randox Daytona+ clinical chemistry analyser (Randox Laboratories Ltd, UK) to ensure they were within the normal range. Subjects who met these initial inclusion criteria were invited to a further screening session during which a dentist checked for dental diseases (e.g. gingivitis or periodontal infection). The dental check was carried out to ensure that all participants had good dental health because periodontal disease can impact the composition of oral bacteria. Volunteers without periodontal disease were then invited to participate in the main study.

### 6.3.2. Study design

A chronic, double-blind, randomised, controlled, cross-over study design was conducted. Randomisation was carried out using MINM by the study researcher (H.S.A) to generate the sequence for the beetroot juice and control beetroot juice interventions. Subjects and researchers were blinded to the randomisation sequence. To minimize bias, the taste and packaging of the control nitrate-free and nitrate-rich beetroot juice were identical. Since the beetroot juice was concentrated (400 mg of nitrate in 70 ml) and to ensure that the ADI of nitrate (3.7/kg of body weight) was not

exceeded, the volume consumed daily by the participants was matched to their body weight.

Volunteers were randomly assigned to consume either (i) nitrate-rich beetroot juice (Beet It Sport, James White Drinks Limited, UK) or (ii) the same volume of control beetroot juice without nitrate (control, Beet It Sport, James White Drinks Limited, UK) daily for 8 weeks, with a 4-week washout period between the juice interventions. Study visits were performed at the beginning and end of each 8-week treatment arm (Figure 1). As drinking water is a major source of dietary nitrate, subjects were provided with a Brita water filter (Brita LP, USA) to reduce the nitrate level in their tap water for drinking and cooking during the study period. Volunteers were also provided with a toothbrush and low-nitrate toothpaste and were asked to maintain their usual toothbrushing regime and refrain from using any mouthwash or chewing gum that contained xylitol during this period. Finally, participants were asked to complete a 4-day food diary during each intervention period.

On the day before each study visit, volunteers were required to fast overnight for 12 h after consuming a standard low-nitrate evening meal provided by the researchers and to only drink low-nitrate mineral water (Buxton water) during this time. All subjects were instructed to refrain from strenuous exercise and alcohol consumption one day before each study visit. Furthermore, an anaerobic container and sachet were given to the volunteers to provide a fresh stool sample before attending their study visit.

On the morning of the study visit, the volunteers were asked not to brush their teeth and to collect a fresh stool sample in the anaerobic container (Fisher Scientific, Loughborough, UK) with a sachet (anaerobic atmosphere generation bag) to reduce the stool sample fermentation (Oxoid, Hampshire, UK) before arriving at the Hugh Sinclair Unit of Human Nutrition. Stool samples were collected to measure changes in

the gut microbiota. On arrival, the volunteers were asked to provide a spot urine sample to measure nitrate and nitrite excretion levels as well as creatinine before their body weight and body composition were measured using the Tanita scale (BC-418MA, Tanita, Ltd, UK). A saliva sample was then collected into a falcon tube and an oral bacteria sample was collected by swabbing the tongue with a sterile cotton swab (Thermo Fisher Scientific, USA). After resting in a supine position for 10 min in a temperature-controlled room (22.5 °C), blood pressure was measured followed by the assessment of microvascular reactivity on the same arm using Laser Doppler Imaging (LDI) with iontophoresis. A blood sample of 20 ml was then collected to measure CVD markers including the lipid profile, glucose, insulin, uric acid, non-esterified fatty acids (NEFA) and C-reactive protein (CRP), after which a light breakfast was provided.

### 6.3.3. Assessment of vascular function and blood pressure

The LDI technique assesses the response of cutaneous blood vessels to transdermal delivery of vasoactive agents by iontophoresis. Measurements were carried out using a Moor LDI (Moor Instruments Limited) as previously described (11),(12). Briefly, two chambers, an anodal and cathodal chamber, were set 1 cm apart on the alcohol-cleansed volar face of the forearm using double-sided adhesive rings. The chambers were filled with 2.5 ml of 1 % Ach (acetylcholine chloride) and 1 %SNP (sodium nitroprusside), respectively, prepared with 0.5 % sodium chloride (Sigma Aldrich, Poole, UK). Iontophoresis was then used to deliver Ach and SNP transdermally with electrical current via the anodal and cathodal chambers. Microvascular responses to Ach (endothelium-dependent vasodilation) and SNP (endothelium-independent vasodilation) were assessed over 21 scans to determine the area under the curve (AUC) and incremental area under the curve (IAUC) for flux versus time measured in arbitrary units. Blood pressure of the brachial artery was measured following 10 min

of lying down in a quiet room using Omron upper arm blood pressure monitor (Omron, HEM-72211-E8, Omron Healthcare Co.). A total of three measurements were taken and the mean of these measurements for systolic (SBP) and diastolic (DBP) blood pressure was recorded. Pulse pressure (PP) was then determined by subtracting the mean of DBP from that of SBP.

### 6.3.4. Food diary

Participants completed a 4-day weighed food diary during each treatment arm. The food diaries were analysed by using Nutritics software (v5.042, GB15 database), and the average daily intake over the 4 days was calculated. Since the Nutritics software did not include data for the nitrate content of juice, vegetable and meat-based dishes, the nitrate and nitrite intake were calculated manually by using dietary data from the NDNS dataset (1–8 y).

### 6.3.5. Oral bacterial and saliva samples

Stimulated saliva was collected into sterile ice-chilled falcon tubes, which were placed on ice and centrifuged immediately after collection for 10 min at 5000 rpm (3000 x g) at 4 $^{o}$C. Three aliquots of the supernatant were then stored at -80 $^{o}$C prior to analysis for nitrate and nitrite concentrations using HPLC-NO.

The sterile cotton swabs used to collect the oral bacteria samples during each of the study visits (weeks 0, 8, 12 and 20) were placed into Eppendorf tubes containing 1 ml of phosphate-buffered saline (PBS). The samples were then stored in a freezer at -80 °C until bacterial DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen, Manchester, UK) following the manufacturer's recommendations. The extracted DNA was pipetted in a 96-well PCR plate and stored at -20 °C before being sent to the Animal and Plant Health Agency (Surrey, United Kingdom) for 16S amplicon sequencing analysis to identify the abundant bacterial genus. The sequencing was

performed on an Illumina MiSeq with 2 × 300 base reads according to the manufacturer's instructions (Illumina, Cambridge, UK), and bioinformatic analysis was performed with QIIME v1.9.1.

### 6.3.6. Stool samples

The stool samples collected on the morning of each study visit were processed immediately. Briefly, after recording the weight, 20 g of stool was mixed with PBS to make a 10 % (w/w) faecal slurry. A portion (30 ml) was transferred into a 50-ml falcon tube with a glass bead and vortexed for 1 min before centrifuging at 1500 rpm (200 x g) for 2 min. A 1-ml portion of supernatant PBS faecal slurry was transferred to two Eppendorf tubes and spun in a microcentrifuge at 13,000 rpm for 10 min. Finally, the supernatant was transferred to two fresh Eppendorf tubes and then stored at -80 $^{o}$C. DNA was extracted from the faecal samples using the DNeasy Power Soil kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. All DNA samples were analysed using 16S amplicon sequencing at the Animal and Plant Health Agency (Surrey, UK). The sequencing was performed on an Illumina MiSeq with 2 × 300 base reads according to the manufacturer's instructions (Illumina, Cambridge, UK), and bioinformatic analysis was performed with QIIME v1.9.1.

### 6.3.7. Blood samples

For the measurement of nitrate and nitrite as a biomarker of dietary nitrate intake and vascular function, blood was collected into a lithium heparin tube and centrifuged for 4 min at 4500 rpm (2500 x g) and stored at 4 °C within 2 min of collection. A 200-µl sample of plasma was then aliquoted into an amber, low nitrite/nitrate Eppendorf tube and stored at -80 °C until analysis. Blood was also collected into a serum separator tube for the analysis of serum total cholesterol, triacylglycerol, HDL-C, glucose, NEFA, uric acid and CRP with a Randox Daytona+ clinical chemistry analyser. LDL-C was

estimated using the Friedewald formula, and insulin was measured by using an ELISA kit (Crystal Chem, USA). The analyses commenced after the intervention studies had been completed, and all samples for each subject were analysed within a single batch.

### 6.3.8. Urine samples

The volume of spot urine samples collected on each study visit were measured and recorded before an aliquot (30 ml) was centrifuged at 3000 rpm (1700 x g) for 10 min at 4 °C. The supernatant was then aliquoted and stored frozen at -80 °C until analysis. Nitrate and nitrite concentrations were measured in diluted urine samples prepared in 96 well plates using the HPLC-NO analyser (Eicom Corporation, Kyoto, Japan). A standard curve was included to ensure that measurements were within the linear range of the assay (12.5–2000 μM) and quality control and blank samples were used to ensure the accuracy of the technique. Creatinine was determined in all urine samples using a kit supplied by Randox using the Randox Daytona+ analyser. The determination of total urinary nitrate and nitrite were used as a biomarker of dietary nitrate intake and nitric oxide production in response to the intervention drinks.

### 6.3.9. Sample size

The sample size was based on the expected decrease in SBP (the primary outcome measure) by 10 mmHg between intervention juices, based on studies by Webb et al, (2008) (13). With a standard deviation of 12 mmHg, at the 5% significance level, and with a power of 80 %, 25 subjects were required. We recruited 28 subjects to account for a 10% drop-out rate.

### 6.3.10. Statistical analyses

IBM SPSS statistics version 25 was used for all statistical analyses, and data are presented in the tables and text as mean ± SEM. Data were checked for normality via the Shapiro-Wilk test and transformed where necessary. The change in the outcomes

measured in plasma, urine and saliva over each 8-week intervention were calculated (post- minus pre-treatment concentration) before paired-samples t-tests were used to assess differences between the beetroot and control juice treatments. The relationships between plasma nitrate, nitrite and vascular function measurements (BP and LDI) were assessed using Spearman's correlation coefficient (r). Statistical significance was accepted when $p<0.05$. The R statistical package version 4.1.1 was used to determine the alpha and beta diversity of the oral and gut microbiome. The change in the abundance in taxonomic levels before and after each treatment was calculated and analysed using paired samples t-tests. Spearman's correlation was used to assess whether these changes were associated with plasma and urinary nitrate and nitrite.

## 6.4. RESULTS

### 6.4.1. Subjects

Although 37 subjects were initially recruited in this study, eight subjects were excluded due to evidence of periodontal disease. During the study, two women withdrew after becoming pregnant, and one male developed gout (considered an adverse event), which excluded him from the study. In March 2020, the study was suspended due to lockdown restrictions associated with the COVID-19 pandemic; and during this time, seven participants withdrew from the study (Figure 2). In total, 19 volunteers (n=6 men and n=13 women) completed all aspects of the study and were included in the data analysis. The screening characteristics of these subjects are shown in Table 1.

It is worth mentioning that there were no differences in the outcome measures between visit 1 and visit 3 (the baseline visits for the beetroot juice and control juice

respectively) in blood pressure, LDI, anthropometric measurements, cardiovascular risk biomarker in serum, plasma, urine and saliva or food diaries).

### 6.4.2. Vascular function, anthropometric and CVD risk markers

- ***Blood pressure***

There was a differential effect of juice consumption on SBP and DBP, with a decrease observed relative to baseline after the nitrate-rich beetroot juice (SBP -4.1 mmHg and DBP -2.8 mmHg) and an increase observed following the control beetroot juice ($p=0.01$ and $p=0.03$, respectively) (Table 2). However, the change in PP in response to the beetroot juice and control juice interventions were not found to be significantly different ($p=0.21$).

- ***LDI measurements***

Although the AUC and IAUC responses to both the Ach (endothelium-dependent) and SNP (endothelium-independent) vasodilators were increased after nitrate-rich beetroot juice consumption (Table 2), a significant treatment effect was only evident for the change in IAUC for SNP-induced vasodilation ($P=0.01$). This was reflected in the differential response between the juice treatments, with an increase in IAUC observed after the nitrate-rich beetroot juice compared with the decrease in IAUC observed after the control beetroot juice.

- ***Anthropometric measurements and cardiovascular risk biomarker in serum, plasma, urine and saliva.***

There were no significant changes in serum total cholesterol, HDL-C, LDL-C, TAG, insulin, NEFA, CRP or uric acid following consumption of nitrate-rich beetroot juice compared with control juice (Table 3). However, there was a significant treatment effect observed for the change in nitrate and nitrite in saliva, plasma and urine, with increased concentrations evident after nitrate-rich beetroot juice compared with control beetroot juice consumption ($p\leq 0.018$). Also, after correcting for the creatinine

level, the change in urinary nitrate and nitrite concentration were significantly higher by 55% after nitrate-rich (nitrate p<0.001 and nitrite p=0.013) compared to control beetroot juice consumption. There were no significant changes in the anthropometric measurements following consumption of nitrate-rich beetroot juice or control beetroot juice (Table 2).

- ***Dietary analysis***

There was no significant difference in macronutrient, potassium and sodium intakes during each intervention arm (Table 4). As expected, the dietary nitrate level was significantly higher during nitrate-rich beetroot juice 338.2±17 mg/d compared with control beetroot juice 121.5±16 mg/d consumption whereas dietary nitrite intakes were similar during each treatment arm with p=0.04.

### 6.4.3. Changes in endothelium-independent vasodilation correlates with increases in plasma nitrate concentration

The changes in SNP-induced vasodilation (endothelium-independent) after nitrate-rich beetroot juice consumption were positively correlated with changes in plasma nitrate (r=0.445; p=0.05) but not plasma nitrite (p=0.759) concentrations.

### 6.4.4. Oral microbiome

- ***Effect of nitrate-rich beetroot juice and control beetroot juice on alpha and beta diversity***

There were no significant differences in baseline alpha (diversity within the samples) or beta diversity (the diversity between individuals) determined before the start of either beetroot juice treatment (V1 vs V3). The alpha diversity of oral microbiota showed no significant changes before and after control beetroot juice consumption (p= 0.239) or before and after nitrate-rich beetroot juice consumption (p=0.341). However, the change in alpha diversity over the 8-week intervention period was found to be

significantly different between the nitrate-rich beetroot juice and the control beetroot juice (p=0.034) (Figure 3).

A significant change(decrease) in the beta diversity of the oral microbiota was only evident after control beetroot juice consumption compared to baseline (p=0.034), with no change found for the nitrate-rich beetroot juice. The change in beta diversity after 8 weeks of intervention was significantly different between the nitrate-rich and control beetroot juice treatments with nitrate-rich beetroot juice was higher than control beetroot (p=0.0042) (Figure 4).

- ***Class-level changes in the taxonomic composition of oral samples***

The total sequence reads identified by the classification of operational taxonomic units (OTUs) for both the pre- and post-intervention samples were analysed, and the microbial composition of the oral samples were determined for all taxonomic levels. For the purposes of this manuscript, only the microbial composition at the class and genus levels will be presented. At the class level, there was a significant reduction (7%) in the abundance of Bacteroidia compare to 6% increase in control beetroot juice (p=0.05). Also, increase in the abundance of Betaproteobacteria (5%) after nitrate beetroot juice consumption compared to control beetroot juice with 5% reduction (p=0.031). The changes in Bacteroidia after nitrate-rich beetroot juice consumption were negatively associated with plasma nitrate (r=-0.595, p=0.009) but not nitrite (Figure 5).

- ***Genus-level changes in the taxonomic composition of oral samples***

At the genus level, a significant increase was observed in *Neisseria* abundance after nitrate-rich beetroot juice consumption (by 4%) relative to the baseline visit (week 0), whereas there was a 3% decrease after control beetroot juice consumption (p=0.014). A 7% reduction was observed in *Prevotella* after nitrate-rich beetroot juice

consumption (Figure 6). The increase in *Neisseria* abundance was positively associated with plasma nitrate after nitrate-rich beetroot juice consumption (r=0.562, p=0.015).

### 6.4.5. Gut microbiome analysis by 16S rDNA

- ***Alpha and beta diversity for stool genus***

There was a significant difference in both alpha diversity (p=0.006) and beta diversity (p=0.004) of the stool microbiome between baseline visits (V1 and V3) for the beetroot juice interventions. Relative to baseline, there was a significant impact of nitrate-rich beetroot juice consumption on the alpha which decreased diversity of the gut microbiome (p=0.005). However, the change in diversity after the nitrate-rich and control beetroot juice treatments were not statistically significant (p=0.103) (Figure 7). There was no change in the beta diversity of the gut microbiome after consumption of either beetroot juice for 8 weeks compared to baseline, or between the nitrate-rich and control beetroot juice interventions (p=0.908) (Figure 8).

- ***Class-level changes in the taxonomic composition of stool samples***

The relative abundance of stool bacteria at the class level showed a significant reduction in Clostridia after nitrate-rich beetroot juice consumption by 3% compared to control which increased by 5% (p=0.002). However, the changes in Clostridia observed were not associated with plasma nitrate (r=-0.434, p=0.072) concentrations measured after the nitrate-rich beetroot juice consumption (Figure 9).

- ***Genus-level changes in the taxonomic composition of stool samples***

At the genus level, there was a significant increase in the abundance of *Streptococcus* and *Granulicatella* and reduction in *Moryella* following nitrate-rich beetroot juice compared with control beetroot juice consumption (p=0.041, p=0.033 and p=0.046, respectively). Moreover, nitrate-rich beetroot juice consumption reduced the

abundance of *Fusobacterium* by 1.2% (p=0.016) and *Peptostreptococcus* by 1% (p=0.033) compared to baseline. The reductions in *Fusobacterium* and *Peptostreptococcus* were negatively associated with changes in plasma nitrate after nitrate-rich beetroot juice consumption (r=-0.558, p=0.016 and r=-0.456, p= 0.05, respectively). Changes in *Granulicatella* were positively associated and *Moryella* was negatively associated with the change in plasma nitrate concentrations (r=0.464, p=0.05 and r=-0.494, p= 0.03, respectively).

## 6.5. DISCUSSION

To the best of our knowledge, the present study was the first to investigate the effects of inorganic beetroot juice on BP and microvascular vasodilation over 8 weeks. Chronic consumption of dietary nitrate at the level of the ADI (3.7 mg of nitrate/kg body weight) was found to significantly reduce BP (SBP and DBP) and increase the IAUC for the endothelium-independent microvascular reactivity in 19 healthy volunteers compared with consumption of a control beetroot juice. These improvements observed in vascular function were associated with increases in the oral nitrate-reducing capacity and increase in plasma, saliva and urinary nitrate and nitrite concentrations, and changes in the oral and gut microbiota compositions after beetroot juice consumption.

Previous studies determining the impact of dietary and supplemental nitrate on vascular function have assessed reactivity of the brachial artery using the gold-standard technique flow-mediated dilation (FMD) (14),(15), (16). In a study performed in 21 volunteers aged over 60 years with mild hypertension, supplementation with sodium nitrate (150 µmol/kg body weight) for 4 weeks significantly improved FMD, with no effect evident with the control (14). Furthermore, a 4% improvement in FMD was

observed after consumption of beetroot juice (250 mL naturally nitrate-rich beetroot juice/day) for 6 weeks in hypercholesterolemic individuals compared with the control intervention (15). Further support for the beneficial impact of daily dietary nitrate intake on FMD was reported in 2015 by Kapil et al., who observed a 20% improvement in FMD responses following 4 weeks of beetroot juice supplementation (250 mL daily, as beetroot juice/day) in a hypertensive cohort (16). However, to date, very few studies have investigated the chronic effects of beetroot or inorganic nitrate consumption on microvascular vasodilation, a vascular bed which is important in the regulation of BP. In the current study, LDI was used to measures responses to cutaneous perfusion of the forearm with Ach and SNP (17). Ach measures the endothelium-dependent generation of nitric oxide, whereas SNP, a NO donor, measures the endothelium-independent generation of nitric oxide. We found a significant increase in the IAUC for the microvascular vasodilation in response to SNP after nitrate-rich beetroot juice compared to control beetroot juice consumption. Our findings are in agreement with a previous study carried out by our group (18) which reported a significant increase in endothelium-independent vasodilation (in response to SNP) after acute consumption of beetroot bread (containing 1.1 mmol of nitrate per 200 g) in healthy men compared to a control bread. The dose of nitrate used in this study was between 70-100 mg of nitrate which was 2 fold lower than the amount used in the current study. The findings from our study and others have shown that beetroot juice consumption appears to have beneficial effects on both macro- and micro-vascular function. Some studies have investigated different doses of nitrate impact on blood pressure and vascular function. Hobbs et al(19) found significant reductions in SBP ($P < 0.006$) and DBP ($P < 0.001$) for up to 24 hours after beetroot juice consumption of increasing doses (143 mg, 353 mg, and 707 mg). Peak reductions occurred at 2–3 hours after beetroot juice

intakes, and SBP (143 mg: 13.1 mmHg; 353 mg: 20.5 mmHg; 703 mg: 22.2 mmHg) and DBP (143 mg: 16.6 mmHg; 353 mg: 14.6 mmHg; 707 mg: 18.3 mmHg). SBP appeared to be reduced in a dose-dependent manner. This finding suggested that nitrate has beneficial impact on blood pressure and vascular function with low or high intake of nitrate.

With one in three adults in the UK estimated to have hypertension, there is increasing interest in dietary strategies which have favourable effects on BP. In the current study, consumption of nitrate-rich beetroot juice for 8 weeks was associated with a -4.1 mmHg and -2.8 mmHg reduction in SBP and DBP, respectively. Our findings are in agreement with previous studies including Kapil et al. (2015) which reported reductions in SBP and DBP of −7.7 mmHg and −2.4 mmHg, respectively (16) after 4 weeks of consumption of beetroot juice, and reduction in SBP of -4.1 mmHg by Ashworth et al. (2015) (20). The findings from our study did not reveal any significant changes in PP following beetroot juice consumption for 8 weeks compared with the control juice. The improvements in endothelium-independent vasodilation and the reduction in BP after beetroot juice consumption were associated with an increase in plasma nitrate concentrations. This is in agreement with our previous study, which investigated the acute effects of beetroot bread consumption on microvascular reactivity, indicating an endothelial-independent mechanism of action via the direct effects of nitrate reduction on nitrite and nitric oxide (21). This is thought to promote the activation of the nitric oxide-cyclic monophosphate-protein kinase G signalling pathway, thereby causing smooth muscle cell vasodilation (22). Also, greater biotransformation of SNP to NO in the presence of higher nitrate concentrations may directly affect the smooth muscle layer.

Serum lipid profile (including total cholesterol, LDL-C, HDL-C, TAG, NEFA), glucose, insulin, CRP and plasma uric acid were measured as markers of CVD risk. There were no changes in any of these CVD risk biomarker concentrations in this study, or in other chronic human studies intervening with nitrate-rich interventions (15). The insignificant reduction observed in plasma uric acid after chronic beetroot juice consumption may be related to the higher circulating plasma nitrite concentration. This increase in nitrite can reduce oxidative stress by decreasing the activity of nitrite reductase enzymes (such as xanthine oxidoreductase), which play an important role in converting xanthine to uric acid. This was supported by the findings of animal study that reported inorganic nitrate supplementation had anti-inflammatory effects. Reduced leukocyte and neutrophil numbers were observed in conjunction with increased plasma nitrate after inorganic nitrate supplementation which indicated anti-inflammatory effects of inorganic nitrate by reduced macrovascular content (23). Also, in a human study, a reduction in the number of CD11b, a molecule involved in the recruitment of leukocytes to the endothelium, was found 3 h following beetroot juice intake. Thus far, only a few studies have investigated the influence of dietary nitrate on the inflammatory and thrombotic profile in humans. We observed a tendency for a reduction in CRP in the current study after nitrate-rich beetroot juice but this did not reach statistical significance.

Our results showed no significant changes in anthropometric measurements between the beetroot or placebo consumption groups. Furthermore, no effect was observed for nutrient intake, apart from a higher dietary nitrate intake during nitrate-rich beetroot juice consumption. Our findings suggest that the reduction in SBP and DBP was due to the greater nitrate intake during the study period which was associated with higher NOx concentrations in plasma, saliva, and urine, as well as urinary nitrate after

correction for creatinine. Moreover, urinary nitrate was measured in this study as a biomarker of nitrate intake and the urinary nitrate was significantly higher after beetroot consumption compared to the control beetroot juice drink. However, urinary nitrate was not significantly associated with dietary nitrate intake or with changes in BP in this study. These findings are in agreement with our previous cross-sectional study which also indicated that urinary nitrate was not associated with BP or other CVD risk factors, and may not represent a suitable biomarker of dietary nitrate intake (24).

As far as the authors are aware, this was the first study to examine the composition of both oral and gut microbiota in response to dietary nitrate intake. The fermentation of non-digestible substrates like dietary fiber by gut microbiota can produce short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate(25). These SCFAs have been reported to have some beneficial effects on glucose as well as maintaining oxygen balance in the gut, preventing gut microbiota dysbiosis(26), reducing hepatic cholesterol metabolism and lipogenesis and some limited evidence on central appetite regulation(27). Although there is evidence that dysbiotic states correlate with cardio-metabolic disorders (26) including obesity, type 2 diabetes and CVD(28). However, the underlying mechanisms have not been elucidated. It is clear, however, that composition and functional capabilities of the human gut microbiota rapidly adapt to changes in dietary macronutrient intake which may reduce or prevent these diseases(28). Dietary inorganic nitrate has been shown to enhance the abundance of specific species of nitrate-reducing bacteria and influence the systemic plasma nitrite level. Our results indicated an increased relative abundance of bacteria genus Neisseria after nitrate-rich beetroot juice consumption compared to the 3% reduction in abundance after the control beetroot juice. These findings support those of Vanhatalo et al(8) in which an increase in *Neisseria* and a reduction in *Prevotella* were

observed after consumption of beetroot juice containing 12 mmol/d of nitrate for 10 days with no changes evident in species diversity between the placebo and beetroot juice treatments (8). The nitrate dose used in this study was very high and exceeded the ADI of 3.7 mg/body weight. High abundances of *Rothia* and *Neisseria* and low abundances of *Prevotella* and *Veillonella* were associated with high NO bioavailability as indicated by plasma concentrations of nitrate and nitrite, and reduced blood pressure in the old, but not young, participants(8). The reason for this difference was that the younger group had low baseline blood pressure of 112/63 compared to the older participants, and therefore more limited opportunity for reduction (8).

In the current study, inorganic nitrate not only changed the oral microbiome composition but also change the gut microbiome. The consumption of nitrate-rich beetroot juice reduced Clostridia by 3% and increased Bacteroide by 7%. To the best of our knowledge, following a comprehensive literature search, only one other human study has investigated the effects of beetroot or inorganic nitrate consumption on gut microbiota composition (10). In this short-term study, a significant decrease in Firmicutes and Proteobacteria and increase in Bacteroidetes and Cyanobacteria was evident in stool samples compared to baseline in twenty healthy adults after consuming only vegetable/fruit juices for 3 days. These changes were associated with higher plasma nitrite levels that reflect an increase of NO bioavailability, an important vasodilator which promotes cardiovascular health (10). An increase in Bacteroides in the gut has been reported to be associated with a decrease in markers of metabolic syndrome such BP(29), suggesting that the increase found in our study may have contributed to the beneficial effects of nitrate-rich beetroot juice on vascular function. Further studies are needed to investigate the impact of nitrate consumption on the gut microbiome and changes on systemic CVD risk factors.

A strength of the study is the use of a matched control beetroot juice and the robust double-blind randomised controlled crossover study design which helped to minimise bias. The main limitation was the loss of volunteers due to the lockdown restrictions associated with the COVID-19 pandemic, which resulted in study sample falling below the required sample size.

In conclusion, we observed a significant improvement in endothelium-independent vasodilation and a reduction in SBP and DBP after consumption of beetroot juice containing 3.7 mg of nitrate/kg of body weight for 8 weeks compared with a control nitrate-free beetroot juice. These findings were associated with an increase in oral nitrate-reducing capacity and increase in systemic nitrate concentrations. Moreover, the daily consumption of inorganic nitrate at the ADI level may represent a potential dietary intervention to improve the oral and gut microbiome composition and enhance vascular function. Further powered studies are needed to assess the long-term effects of consuming nitrate-rich foods such as beetroot on BP and cardiovascular health, particularly in groups at risk of CVDs.

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## 6.8. CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

## 6.9. AUTHOR CONTRIBUTIONS

The author's responsibilities were as follows: J.A.L, KGJ, DAH and HAS designed the research; H.S.A. conducted the research and analysed the data and drafted the paper K.G.J. analysed serum insulin; K.G.J. and J.A.L. provided feedback and guidance on all drafts of this paper and J.A.L. was responsible for final content.

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## TABLES

**Table1: Table1: Characteristics of the participants at screening[1]**

| | Whole Group (n=19) | Male (n=6) | Female (n=13) |
|---|---|---|---|
| **Age, y** | 37±12 | 37±14 | 37±11 |
| **BMI, kg/m$^2$** | 23.7±3.6 | 23.6±2.9 | 22.9±4.0 |
| **SBP, mm Hg** | 116±10 | 123±6 | 112 ±12 |
| **DBP, mm Hg** | 73±7 | 72±6 | 69±8 |
| **PP, mm Hg** | 40±18 | 46±16 | 38±17 |
| **Total cholesterol, mmol/L** | 4.80±1.0 | 4.90±0.96 | 4.81±1.05 |
| **TAG, mmol/L** | 0.84±0.5 | 0.96±0.6 | 0.80±0.3 |
| **Fasting glucose, mmol/L** | 4.90±0.6 | 4.99±0.43 | 5.20±0.7 |

[1]data presented as mean±SD. Abbreviations: **BMI:** body mass index; **DBP:** diastolic blood pressure; **PP:** pulse pressure; **SBP:** systolic blood pressure; **TAG:** triacylglycerol

**Table2: Vascular function measures at baseline and after chronic consumption of control and nitrate-rich beetroot juice[1].**

| | Control beetroot juice | | | Nitrate-rich beetroot juice | | | P |
|---|---|---|---|---|---|---|---|
| | **Baseline** | **Post** | Δ | **Baseline** | **Post** | Δ | |
| **SBP, mm Hg** | 116±3.2 | 119±4.2 | 2.1±2.3 | 118±2.9 | 114±3.4 | -4.1±2.0 | 0.01 |
| **DBP, mm Hg** | 68±2.0 | 70±2.5 | 2.4±1.8 | 70±2.2 | 67±2.2 | -2.8±1.4 | 0.03 |
| **PP, mm Hg** | 48±2.3 | 49 ±4.6 | 1.5±5.1 | 48±2.1 | 47±2.4 | -1.3±2.0 | 0.21 |
| **LDI-Ach, AUC** | 2854±1489 | 2566±1513 | -289±1750 | 2736±1550 | 3983±7191 | 1247±7247 | 0.33 |
| **LDI-Ach, IAUC** | 1324±720 | 1379±795 | 55±850 | 1393±1347 | 2679±4781 | 1286±4669 | 0.25 |
| **LDI-SNP, AUC** | 3589±2137 | 2275±1340 | -1314±2441 | 3245±2329 | 3433±5667 | 188±5937 | 0.31 |
| **LDI-SNP, IAUC** | 1913±1066 | 1283±797 | -630±1163 | 1080±873 | 1928±2373 | 848±2200 | 0.01 |
| **Weight, kg** | 63.4±2.5 | 63.4±2.4 | -0.02±0.2 | 63.9±2.5 | 63.7±2.4 | -0.2±0.3 | 0.6 |
| **BMI, kg/m$^2$** | 23.2±0.9 | 23.3±0.9 | 0.1±0.1 | 23.4±1.0 | 23.4±1.0 | 0.0±0.1 | 0.83 |
| **Waist circumference, cm** | 82.1±2.4 | 81.4±2.0 | -0.8±1.0 | 82.6±2.3 | 79.7±2.3 | -2.9±0.9 | 0.06 |
| **Hip circumference, cm** | 101.4±2.3 | 101.5±2.0 | 0.1±1.2 | 102.4±2.0 | 101.8±2.0 | -0.6±0.8 | 0.59 |
| **Body fat, %** | 24.5±2.2 | 25.2±2.1 | 0.7±0.4 | 25.4±2.2 | 25.3±2.1 | -0.1±0.8 | 0.29 |
| **Fat Mass, kg** | 16.2±1.815 | 16.4±1.7 | 0.2±0.3 | 16.6±1.9 | 15.8±1.9 | -0.8±0.6 | 0.29 |
| **Fat free mass, kg** | 47.6±2.0 | 47.2±2.0 | -0.4±0.2 | 47.2±1.8 | 47.3±1.9 | 0.1±0.6 | 0.29 |

[1]Values are mean ± SEM, n=19. Difference between the change in outcome measures following the control and beetroot juice interventions: P<0.05 (by paired t- test).
**Abbreviations: BMI:** Body Mass Index; **DBP:** diastolic blood pressure; **PP:** Pulse pressure; **SBP:** systolic blood pressure; **LDI- Ach, AUC:** laser Doppler iontophoresis-acetylcholine (area under curve); **LDI- Ach, IAUC:** laser Doppler iontophoresis-acetylcholine (incremental area under curve) **LDI-SNP, AUC:** laser Doppler iontophoresis-sodium nitroprusside (area under curve); **LDI-SNP, IAUC**: laser Doppler iontophoresis-acetylcholine (incremental area under curve).

**Table 3: Cardiovascular disease risk biomarkers in serum, plasma, urine and saliva at baseline and following the control and nitrate-rich beetroot juice interventions.**

| | | Control beetroot juice | | | Nitrate-rich beetroot juice | | | p¶ |
|---|---|---|---|---|---|---|---|---|
| | | Baseline | Post | Δ | Baseline | Post | Δ | |
| Serum | Total Cholesterol (mmol/l) | 4.69±0.21 | 4.73±1.92 | 0.04±0.1 | 4.73±1.97 | 4.68±1.93 | -0.05±0.1 | 0.2 |
| | TAG, (mmol/l) | 0.76±0.07 | 0.72±0.04 | -0.04±0.07 | 0.83±0.07 | 0.78±0.06 | -0.04±0.06 | 0.99 |
| | HDL-C (mmol/l) | 1.60±0.09 | 1.62±0.07 | 0.02±0.07 | 1.58±0.08 | 1.59±0.07 | 0.01±0.04 | 0.85 |
| | LDL-C (mmol/l) | 2.71±0.10 | 2.79±0.13 | 0.09±0.08 | 2.82±0.15 | 2.74±0.14 | -0.09±0.07 | 0.15 |
| | Glucose (mmol/l) | 4.79±0.09 | 4.89±0.08 | 0.10±0.07 | 4.85±0.1 | 4.75±0.09 | -0.09±0.08 | 0.08 |
| | CRP (mg/l) | 0.63±0.17 | 0.86±0.2 | 0.23±0.1 | 1.10±0.41 | 0.77±0.18 | -0.34±0.4 | 0.25 |
| | Uric acid (µmol/l) | 308±20 | 316±20 | 8.79±7.2 | 311±23 | 306±22 | -4.43±8.1 | 0.29 |
| | NEFA, µmol/L | 0.41+0.05 | 0.39±0.05 | -0.02±0.08 | 0.39±0.05 | 0.34±0.04 | -0.05±0.05 | 0.78 |
| | Insulin (pmol/l) | 21.4±2.9 | 21.3±3.2 | -0.14±3.4 | 20.0±2.8 | 15.6±2.2 | -3.82±1.9 | 0.2 |
| Plasma | Nitrate, µmol/L | 25.2±1.96 | 31.5±2.4 | 6.3±2.5 | 26.7±2.21 | 110.3±11.9 | 83.5±11.4 | <0.001 |
| | Nitrite, µmol/L | 0.22±0.02 | 0.31±0.03 | 0.08±0.04 | 0.27±0.03 | 0.97±0.09 | 0.705±0.1 | <0.001 |
| Urine | Creatinine (mmol/l) | 9.01±0.99 | 9.37±1.2 | 0.36±0.9 | 8.25±1.4 | 8.57±1.1 | 0.32±1.2 | 0.98 |
| | Nitrate, µmol/L | 1106±166 | 1204±140 | 97.2±90 | 1307 ±241 | 2807±260 | 1500 ±324 | <0.001 |
| | Nitrite, µmol/L | 1.30±0.18 | 1.62±0.25 | 0.31±0.28 | 1.40±0.19 | 3.50±0.48 | 2.10±0.46 | 0.005 |
| | Nitrate (µmol/L/mmol/L creatinine) | 130±15.9 | 149 ±25.17 | 18.34±24.95 | 171 ±24.36 | 381±56.8 | 210±53.4 | <0.001 |
| | Nitrite (µmol/L/mmol/L creatinine) | 0.19±0.04 | 0.20±0.03 | 0.012±0.04 | 0.21±0.03 | 0.51±0.10 | 0.30±0.07 | 0.013 |
| Saliva | Nitrate, µmol/L | 422 ±62 | 513±99 | 90.6±100 | 468±74 | 2127±496 | 1659 ±446 | 0.002 |
| | Nitrite, µmol/L | 300 ±48 | 323±49. | 22.71±41 | 296±46 | 503±78 | 207 ±72 | 0.018 |

Values are means ± SEM. n = 19, Difference between the change in outcome measures following the control and beetroot juice interventions: P<0.05 (by paired t- test). **Abbreviations**: **CRP:** C-reactive protein; **TAG:** triacylglycerol; **HDL-C:** high-density lipoprotein; **LDL-C:** low-density lipoprotein; **NEFA:** non-esterified fatty acids.

**Table 4: Nutrient intake during the control and nitrate-rich beetroot juice intervention arms.**

| | Control beetroot juice | Nitrate-rich beetroot juice | P |
|---|---|---|---|
| **Total energy MJ/d** | 7.1±0.5 | 6.9±0.5 | 0.77 |
| **Total fat % of TE** | 38.5±1.8 | 38.4±1.3 | 0.90 |
| **SFA, % of TE** | 12.7±0.5 | 13.1±0.7 | 0.67 |
| **MUFA, % of TE** | 10.5±0.9 | 11.2±0.9 | 0.35 |
| **PUFA, % of TE** | 4.6±0.3 | 5.1±0.4 | 0.26 |
| **Protein, % of TE** | 19.2±1.2 | 18.3±1.4 | 0.33 |
| **Carbohydrates, % of TE** | 41.7±1.7 | 42.4±1.7 | 0.67 |
| **Dietary fiber,** AOAC | 22.4±1.7 | 20.3±1.6 | 0.13 |
| **Free sugar, g/d** | 10.3±1.9 | 12.84±1.9 | 0.21 |
| **Sodium, g/d** | 1.8±0.2 | 1.8±0.1 | 0.74 |
| **Potassium, g/d** | 2.3±0.2 | 2.1±0.2 | 0.45 |
| **Nitrate, mg/d** | 121.5±16 | 338.2±17 | 0.04 |
| **Nitrite, mg/d** | 1.24±0.2 | 1.21±0.2 | 0.85 |

Values are means ± SEM. n = 19, Difference between placebo and beetroot juice analysed by paired t-test). **Abbreviations**: **AOAC:** Association of Official Analytical Chemists; **TE:** Total energy; **SFA:** saturated Fatty Acid; **MUFA:** monounsaturated fatty acid; **PUFA:** polyunsaturated fatty acid

## FIGURES

**Figure 1: Participants randomly assigned to beetroot juice interventions for 8 weeks in a crossover design study.**

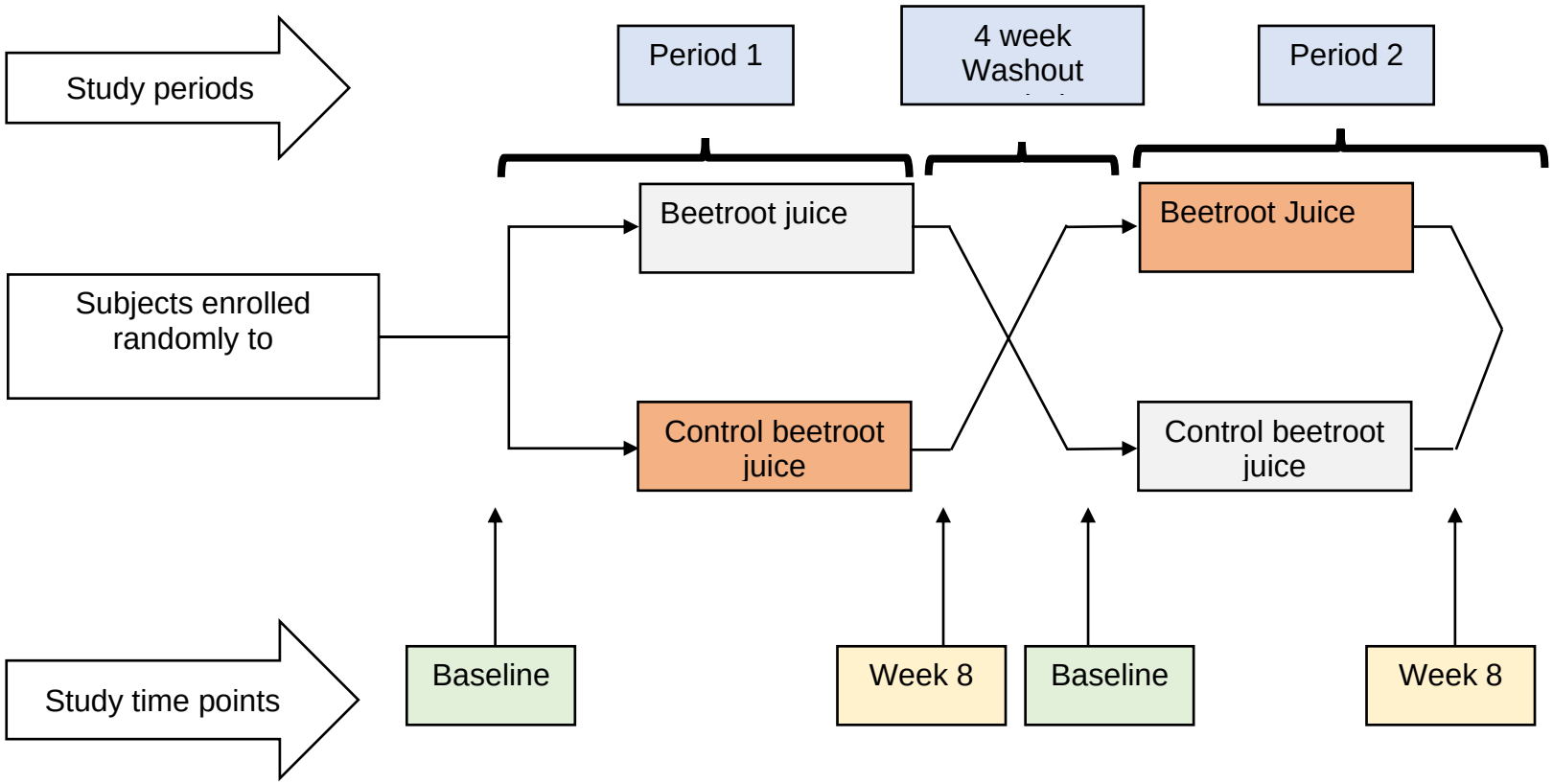


**Figure 2: CONSORT flowchart of study.**

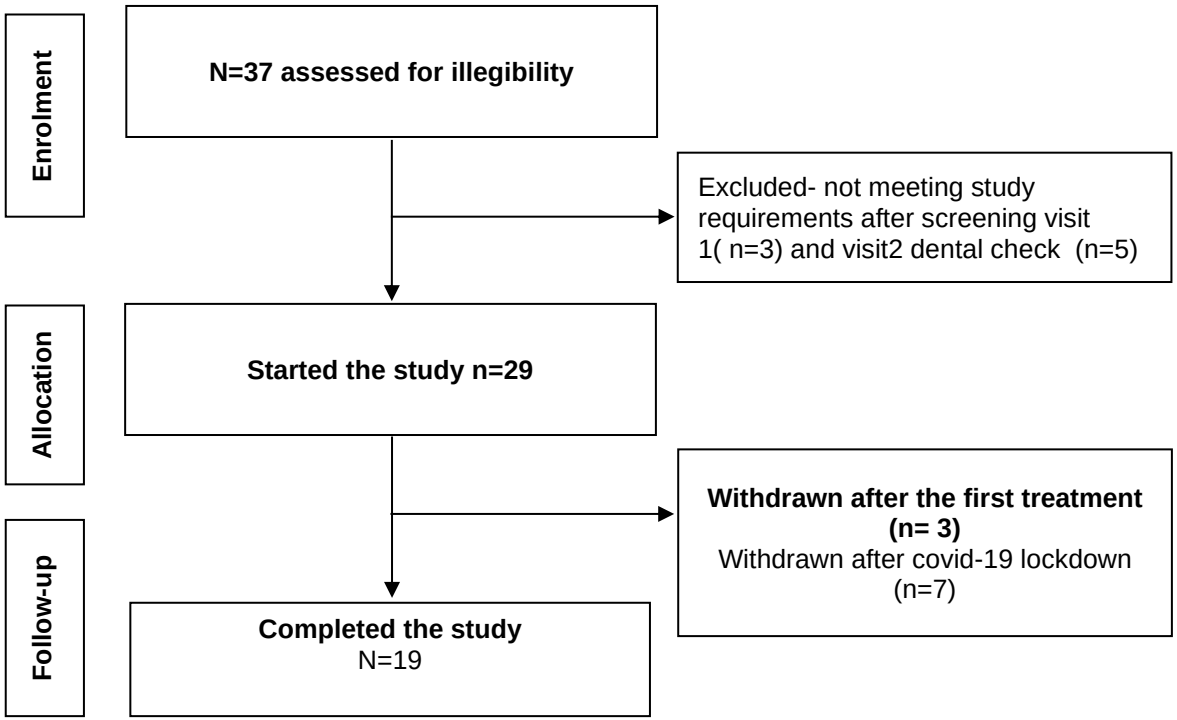


**Figure 3: Alpha diversity (changes within the samples) by study visit for the oral microbiome.** Shannon diversity index is plotted for participants before and after control juice and beetroot juice. The line inside the box represents the mean, the whiskers represent the smallest and largest values within the range. * indicate significant difference.

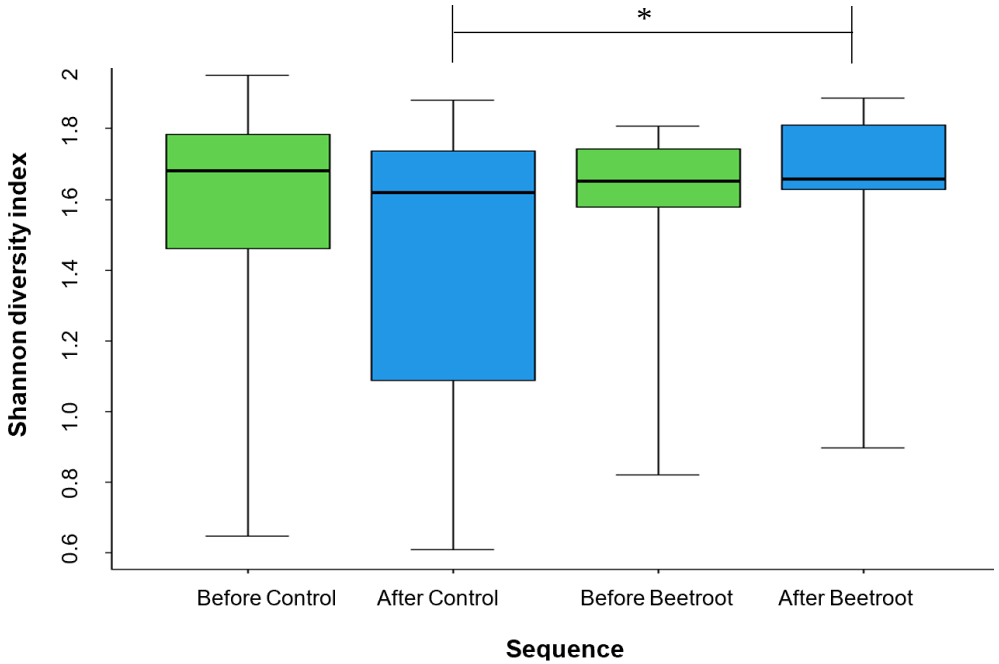


**Figure 4: Beta diversity (changes between the samples) by study visit for oral microbiome before and after the control and beetroot juice interventions.** The line inside the box represents the mean, the whiskers represent the smallest and largest values within the range. * indicate significant difference.

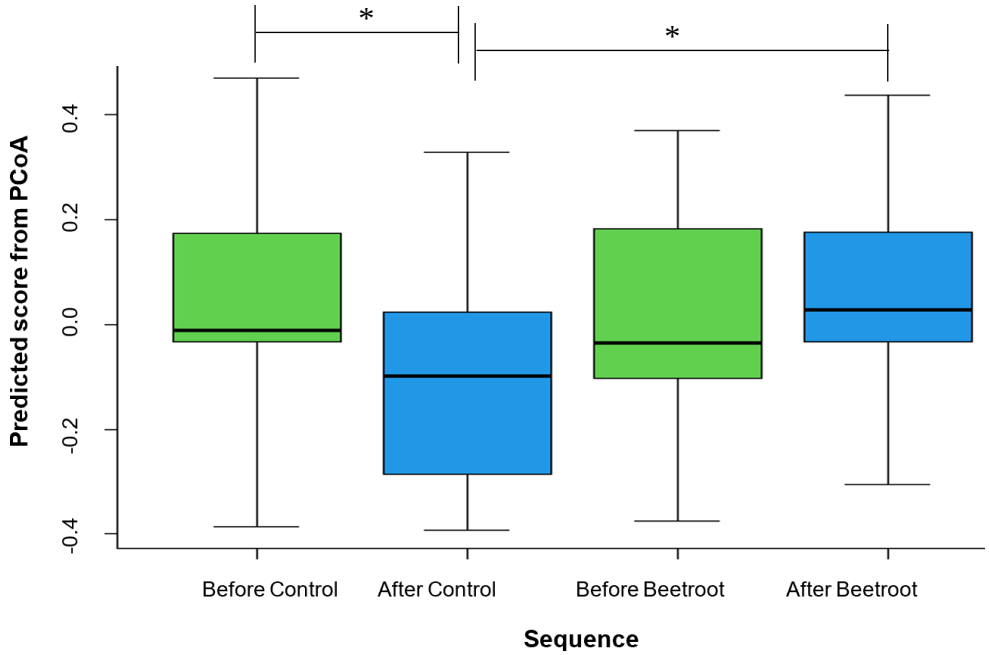


**Figure 5: Changes in relative abundance (%) distributions of microbial class in oral bacteria samples collected at baseline and after 8 weeks of consuming each intervention drink. Each column represents before and after the control and nitrate-rich beetroot juice treatments and the colours represent bacteria class.**

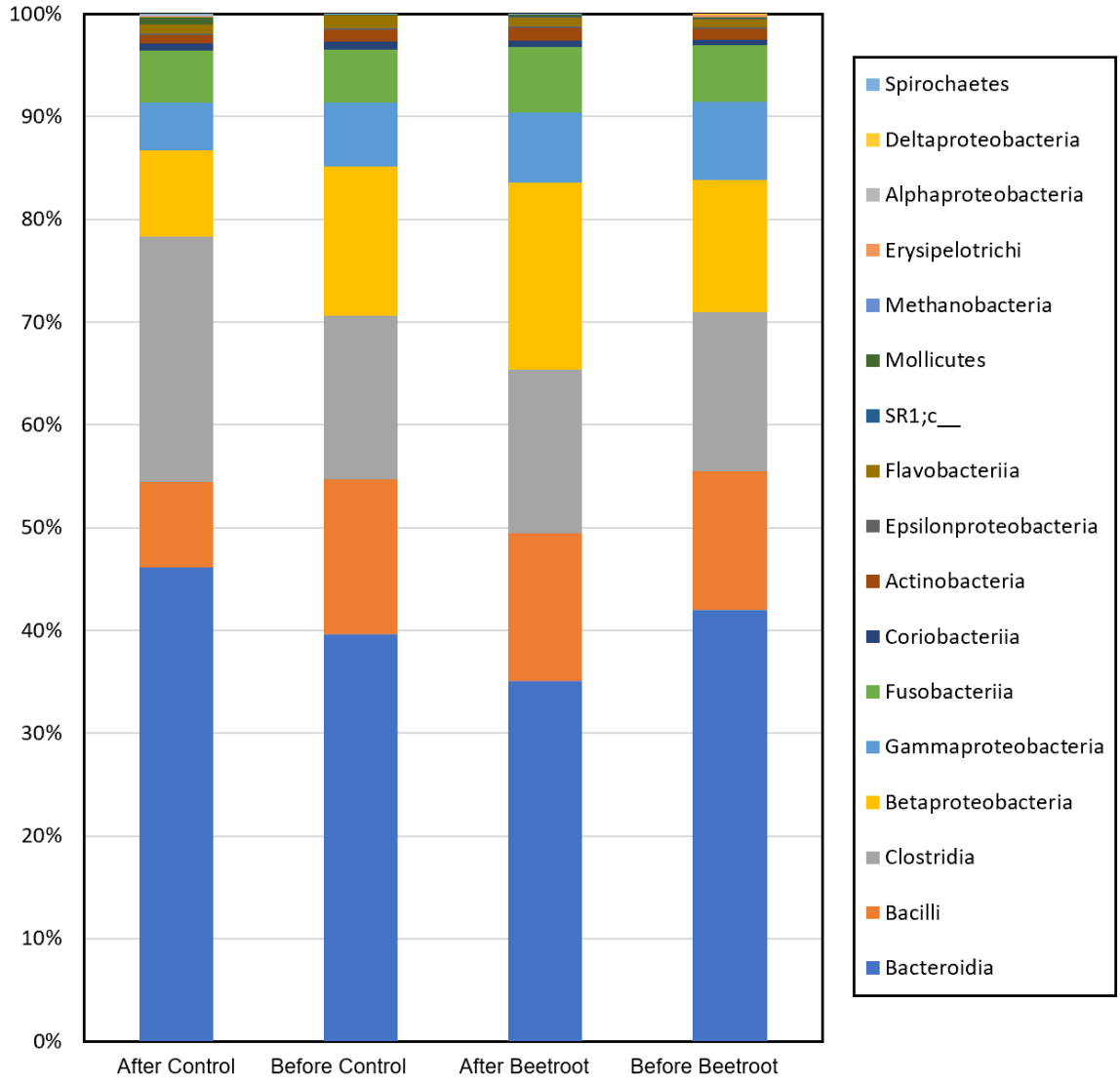


**Figure 6: Changes in relative abundance (%) distributions of microbial genus in the oral bacteria samples collected at baseline and after 8 weeks of consuming control and nitrate-rich beetroot juice. Each column represents before and after treatments and the colour represent bacteria genus which is listed on the right of the figure.**

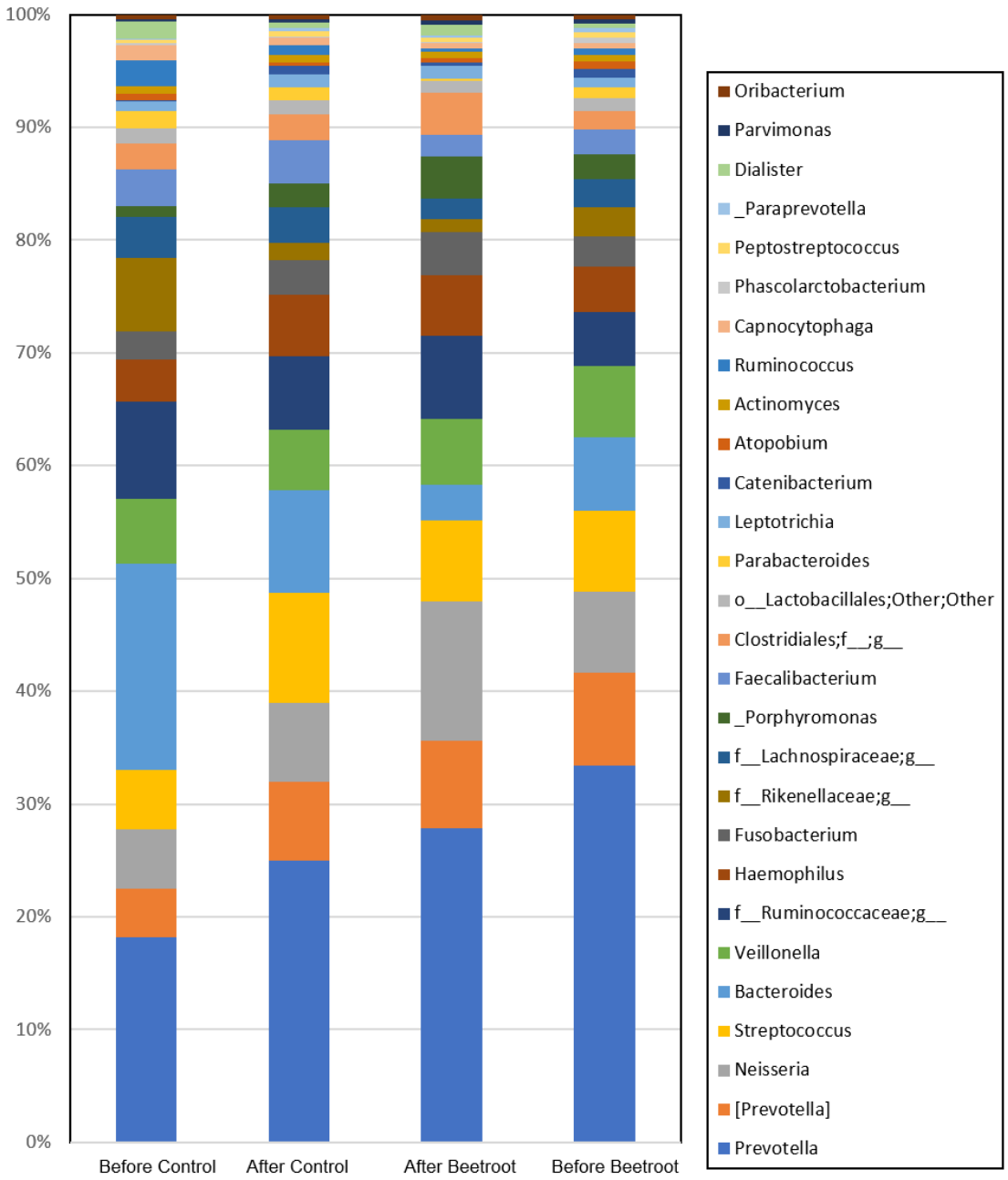


**Figure7: Alpha diversity (changes within the samples) for the gut microbiome.** The line inside the box represents the mean, the whiskers represent the smallest and largest values within the range. * indicate significant difference.

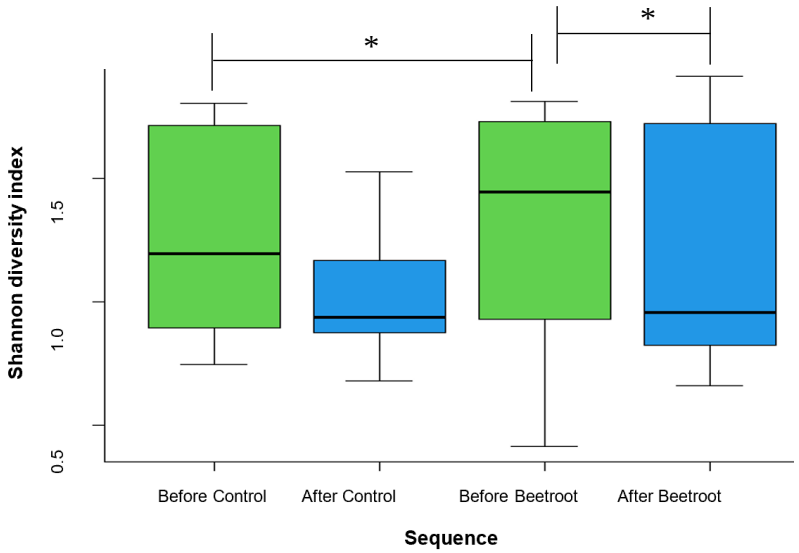



**Figure 8: Beta diversity (changes between the samples) for gut microbiome before and after the control and nitrate-rich beetroot juice. T**he line inside the box represents the mean, the whiskers represent the smallest and largest values within the range. * indicate significant difference.

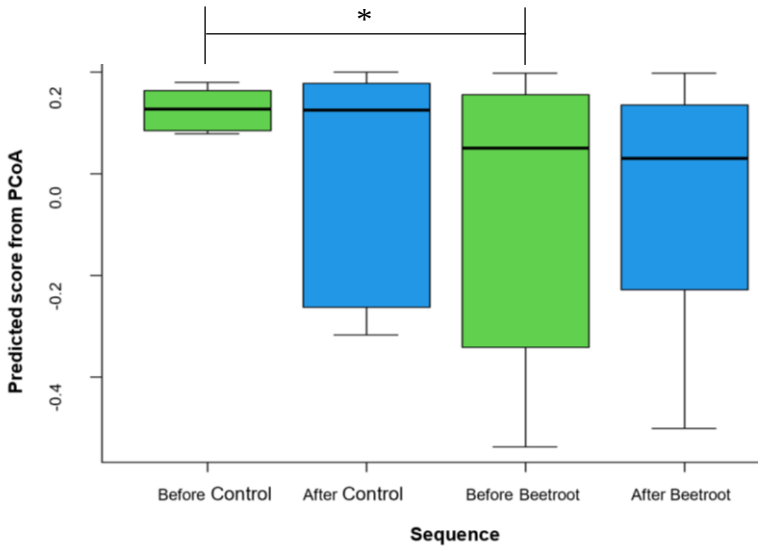


**Figure 9: Changes in relative abundance (%) distributions of gut microbial class at baseline and 8 weeks after consuming the control and nitrate-rich beetroot juice. Each column represents before and after treatments and the colure represent bacteria class which is listed on the right of the figure.**

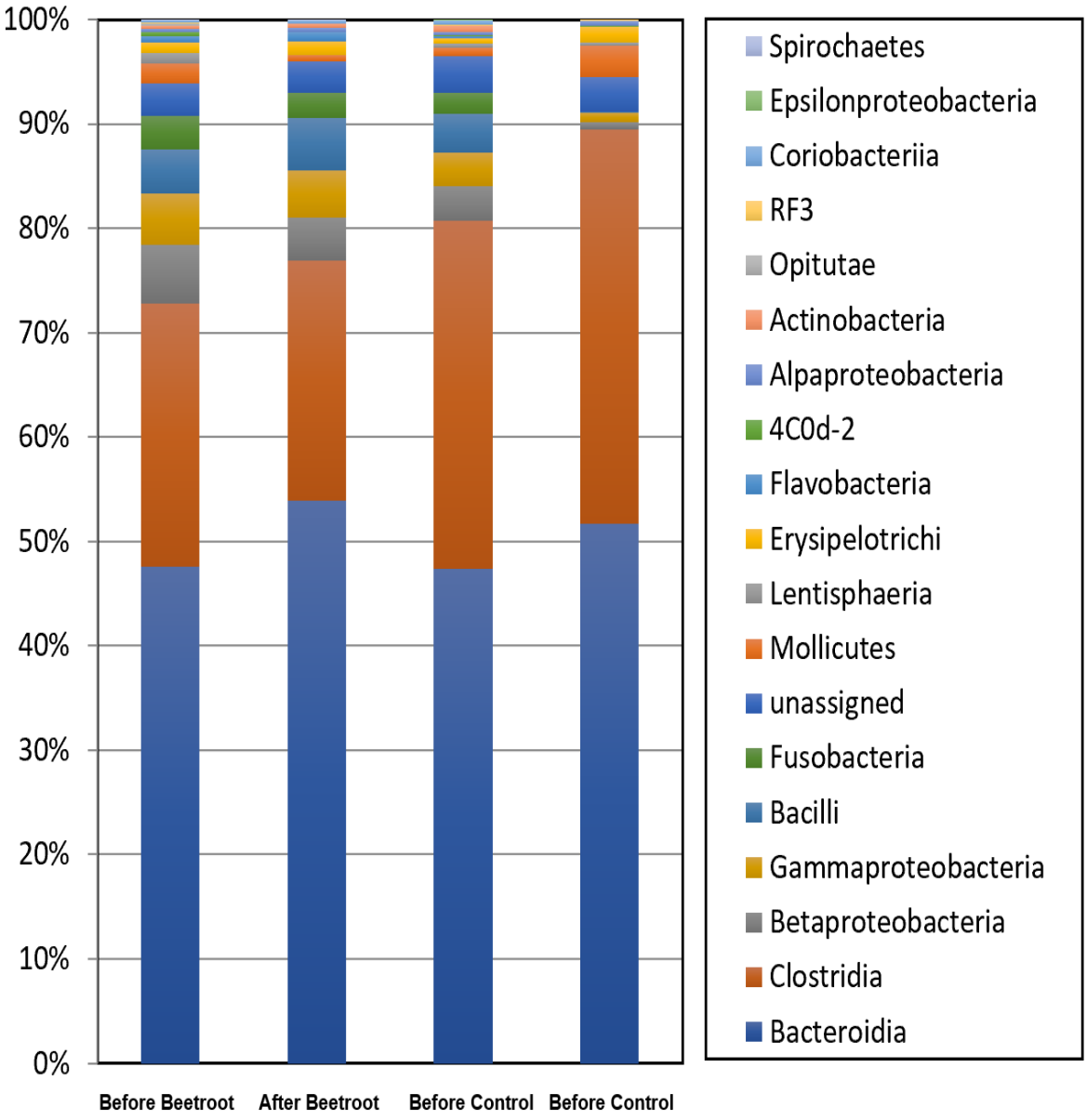
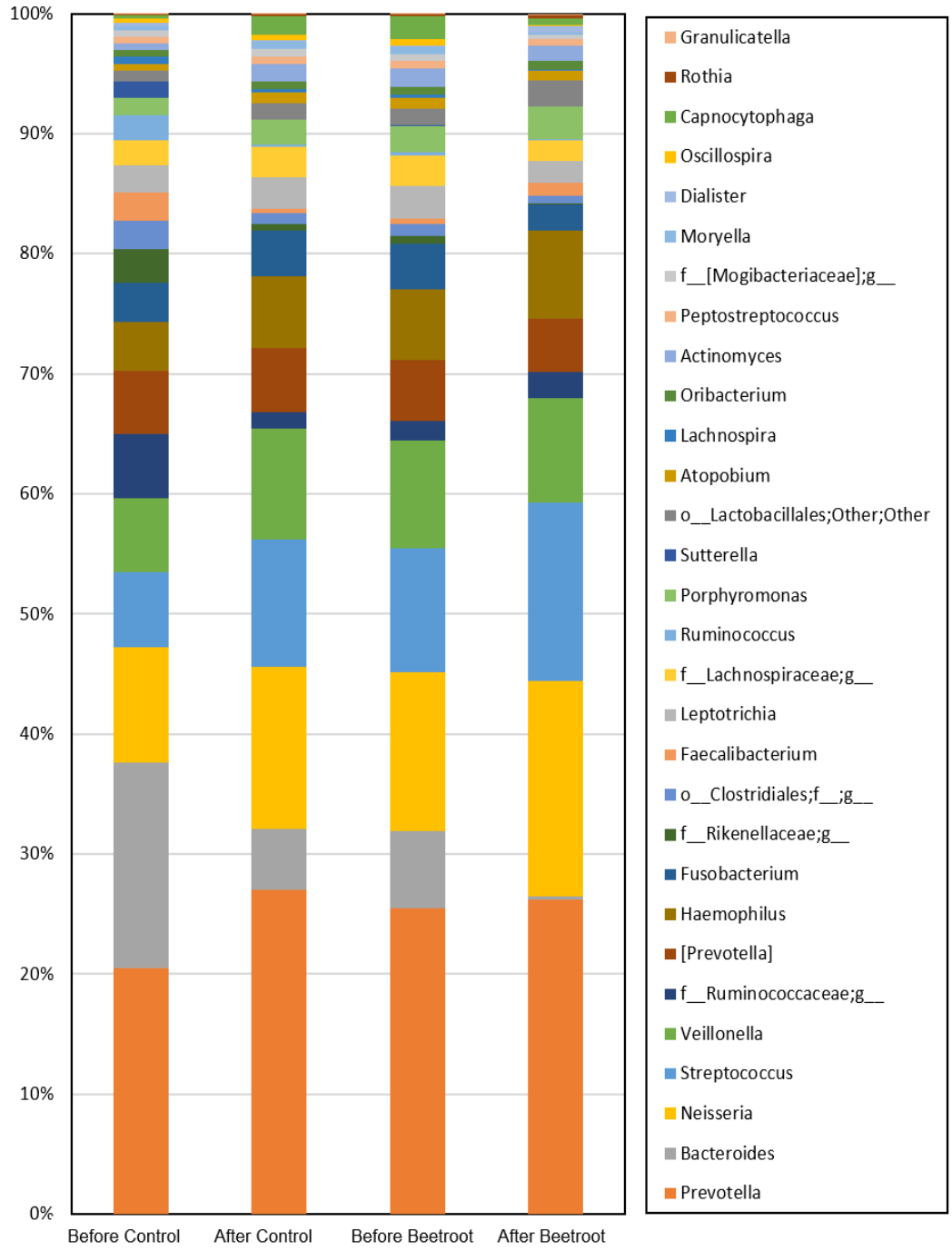


**Figure 10: Changes in relative abundance (%) distributions of gut microbial genus at baseline and after consuming the beetroot juice interventions. Each column represents before and after treatments and the colure represent bacteria class which is listed on the right of the figure.**



# CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

## 7.1. GENERAL DISCUSSION

Dietary nitrate is increasingly linked to a range of beneficial health effects including blood pressure (BP) lowering and improvement in vascular function. The cardioprotective effects of dietary nitrate, which after ingestion is reduced to nitrite by nitrate-reducing bacteria in the oral cavity and further to the vasodilator nitric oxide (NO) endogenously. NO is an important chemical recognised to be responsible for maintaining vascular homeostasis. Cardiovascular diseases (CVD) remain the leading cause of death worldwide with a substantial increase in risk with age. Despite this, much of the burden of CVD is preventable, with diet and lifestyle changes. One of the key recommendations is daily consumption of at least 5 portions of fruits and vegetables, which contain numerous bioactive compounds, including nitrates. Leafy green and some root vegetables, and drinking water are the main sources of dietary nitrate. In this thesis, the relationship between daily dietary nitrate consumption from all nitrate sources (vegetable, cured meat and water) with blood pressure and other CVD risk factors was determined using data from the UK NDNS cross-section cohort. The availability of urine samples in this cohort enabled the potential utility of urinary nitrate as a suitable biomarker of nitrate intakes to be assessed. Furthermore, we aimed to determine the effects of daily beetroot juice consumption for 8 weeks on blood pressure and vascular function in healthy volunteers and evaluate the impact on oral and gut microbiota composition.

### 7.1.1. Impact of daily nitrate consumption on blood pressure and CVDs factors

There is a lack of studies that have investigated the impact of daily dietary nitrate intake on BP and CVD risk factors, especially in the UK and Northern European

populations. To address this knowledge gap retrospective data analysis was performed in the UK representative NDNS cohort. In order to estimate the dietary nitrate intake of the NDNS participants the nutrient database was updated with the nitrate and nitrite content of vegetables, meats, drinks and drinking water, using published literature and data from the UK water authorities. It was observed that higher vegetable nitrate intake was associated with lower blood pressure and improvements in CVD risk markers, with association of higher nitrite intake from processed meat with higher systolic blood pressure (SBP). The latter associations support data from a cohort study that reported that processed and unprocessed red meat was associated with a higher hazard ratio of all-cause mortality (1), which may have been linked with higher dietary nitrite intake.

Our findings of the beneficial effects of nitrate from vegetables on CVD risk markers are supportive of data from a number of epidemiological studies. One example in elderly women reported a higher intake of nitrate from vegetables to be associated with a lower risk of atherosclerotic vascular disease mortality and all-cause mortality(2). Another, observed vegetable nitrate intake of more than 70 mg/day was associated with a significant reduction in CVD mortality, chronic heart disease and stroke hazard (3). A further study reported that vegetarians had a 24% lower risk of death due to ischemic heart disease and a 20% lower risk of death than occasional meat-eaters(4). However, excessive dietary nitrate intakes greater than 93.8 mg/d had no additional benefit(2). These findings agreed with our own analysis in which nitrate intakes of greater than 130 mg/day had no additional blood pressure lowering effect. This may represent a dose-effect of dietary nitrate on blood pressure which has been previously reported after acute ingestion of beetroot juice on postprandial SBP (5). Therefore, further chronic randomly controlled studies are warranted to determine

whether a dose dependent relationship exists between dietary nitrate and BP. Although many studies have investigated the impact of dietary nitrate on blood pressure, very few have also included real time measures of microvascular reactivity, an important vascular bed involved in BP regulation. The study described in chapter 6 aimed to address this research priority and, to our knowledge, is the first to investigate the effects of inorganic beetroot juice consumption for 8 weeks on blood pressure and microvascular vasodilation using LDI in healthy participants. The main finding of this double-blind, randomized, controlled, cross-over study was that chronic consumption of beetroot juice, matched to the acceptable daily intake (ADI) of nitrate (3.7mg nitrate /kg body weight/day), significantly lowered SBP and DBP by -4 and -2 mmHg, respectively compared to the control beetroot juice. These findings support other studies intervening with beetroot juice, which reported significant reductions in SBP of 7.7 mmHg(6) and 4.1 mmHg(2) and DBP of 2.4 mmHg (1). Furthermore, in our study, reductions in BP were associated with a significant increase in endothelium-independent microvascular reactivity in the 19 healthy volunteers. This improvement occurred in conjunction with significant increases in plasma (75%), salivary (78%) and urinary (53%) nitrate and nitrite concentrations.

Microvascular vasodilation was assessed using LDI, which measures responses to cutaneous perfusion of the forearm with Ach and SNP(7). Although a significant change in endothelial-dependent reactivity in response to Ach was not observed, our finding of a significant impact on endothelium-independent microvascular reactivity is an interesting phenomenon and is in agreement with other studies involving beetroot(8) and fish oil interventions (9). A previous study performed by our group reported a significant increase in endothelium-independent vasodilatation (SNP) after acute consumption of beetroot bread (containing 1.1 mmol of nitrate per 200 g) in

healthy men compared to control bread. However, other studies investigating the effects of beetroot juice or inorganic nitrate on vascular function, have used flow-mediated dilatation (FMD) and found significant improvements in brachial artery vasodilation after sodium nitrate supplementation (150 mmol/kg body weight) in mildly hypertensive elderly volunteers compared to controls (10). Also, FMD responses improved by 4% after beetroot juice consumption compared with the placebo control in hypercholesterolemic individuals, (11). Kapil et al. in 2015, reported a 20% improvement in FMD responses following four weeks of beetroot juice supplementation in a hypertensive cohort(6). These findings suggested that dietary nitrate could be an effective, affordable additional treatment for hypertensive patients. The improvements in endothelium-independent vasodilation and reduction in blood pressure after beetroot juice consumption in our study was associated with an increase in plasma nitrate concentrations. This is in agreement with our previous study investigating the acute effects of beetroot bread consumption on microvascular reactivity which proposed an endothelial-independent mechanism of action via direct effects of nitrate reduction to nitrite and nitric oxide(12). This is believed to promote activation of the nitric oxide-cyclic monophosphate-protein kinase G signalling pathway, thereby causing smooth muscle cell vasodilation (12,13). Analysis of the participants' food diaries confirmed similar nutrient intakes during the two intervention arms, apart from nitrate, further supporting our finding that the reduction in BP was due to nitrate-rich beetroot juice.

Diets containing nitrate-rich foods may contain other bioactive components which could also contribute to CVD risk reduction, including fibres, vitamins, minerals and flavonoids, whereas higher consumption of cured and processed meats have been associated with detrimental effects on CVD risk and some cancers(14). Diets high in

nitrate-rich vegetables may offer several advantages over nitrate/nitrite supplemental use, not only due to the availability of other bioactive components but also due to reported vascular adaptation and risk of marked acute hypotension after supplemental nitrate use, not found with nitrate-rich diets(15).

### 7.1.2. Impact of nitrate consumption on Oral and gut microbiome

The nitrate-reducing bacteria that reside in the oral cavity play an important role in the conversion of dietary nitrate to nitrite, which contributes to the systemic availability of nitrite and ultimately the potent vasodilator nitric oxide. In Chapter 2, the current evidence on the potential role of dietary nitrate and the oral microbiome on vascular function including blood pressure and vascular tone was presented. To achieve this, a systematic approach was used to identify the studies that have determined the impact of oral bacteria on blood pressure in response to nitrate intake, from dietary sources or supplements. However, only n=9 relevant human and n=3 animal studies were identified during the literature search. The use of anti-bacterial mouthwash was associated in a small number of studies with higher blood pressure even when accompanied by a high nitrate intake. However, some of the studies failed to report any effects, which may be due to the type of mouthwash used in human studies or the method of application of the mouthwash in animal studies(16). Some modifiable and non-modifiable factors such as sex, hypertension, and tongue cleaning regime were found to be important potential determinants of the variability in the responses between participants. However, the limited number of studies identified make it difficult to draw any firm conclusions from this literature review. There is an urgent need for further studies to investigate the role of the oral bacteria on cardiovascular health in response to dietary nitrate intake, and to determine the underlying mechanisms. Furthermore, determination of the changes in the abundance and composition of the

oral bacteria in response to the intake of dietary nitrate could help to identify important nitrate-reducing bacteria related to changes in vascular function and determine whether these bacterial groups are also evident in the gut microbiome, a proposed modulator of chronic disease risk.

Oral and gut microbiota composition can be influenced by many factors, including diet. Dietary inorganic nitrate has been reported to enhance the abundance of specific species of oral nitrate-reducing bacteria that are associated with increased plasma nitrite concentrations and reductions in blood pressure. REBOC 1 aimed to isolate and identify nitrate-reducing bacteria and to determine if the oral nitrate-reducing capacity was reduced in response to prior use of anti-bacterial mouthwash. The secondary aim of the REBOC2 study (Chapter 6) was to evaluate whether the identified nitrate-reducing bacteria were increased after chronic dietary nitrate intake. Our results indicated increased relative abundances of bacteria genus *Neisseria* (from both studies) and *Staphylococcus* (from REBOC1) and reduction in *Prevotella* (from both studies) and *Veillonella* (from REBOC1) after use of nitrate-rich beetroot juice compared to control. These findings support other studies that observed an increase in *Neisseria* and reduction in *Prevotella* after nitrate consumption for 10 days compared with placebo(17). However, most of these nitrate-reducing bacteria were abolished after prior use of anti-bacterial mouthwash. Accordingly, the oral nitrate-reducing capacity was seen to be increased after the beetroot juice in both human studies compared with the control. This increase in salivary nitrite concentration may be due to the presence and activity of nitrate-reducing bacteria in the oral cavity which converts dietary nitrate to nitrite. In agreement with our results, Govoni et al reported that nitrite was increased after ingestion of dietary nitrate, and removal of the nitrate-reducing bacteria by mouthwash use had a marked effect on systemic nitrite formation,

which was due to the removal of the nitrate-reducing bacteria(18). Further research should focus on understanding the role of the oral microbiome in salivary nitrate reduction and the impact of different dietary factor on their oral nitrate-reducing capacity.

In REBOC2 consumption of beetroot juice for 8 weeks was found to reduce the abundance of *Clostridia* and increase *Bacteroide* by 7% in the gut microbiome. This supported findings from another human study that observed increased Bacteroides and decrease Firmicutes in addition to higher plasma nitrite concentrations after 3 days of nitrate consumption with other components such as apple (19). Our study suggested that nitrate intake may improve the gut microbiome which could also modulator chronic diseases risk. Further studies are needed to confirm these findings.

### 7.1.3. Urinary nitrate as a biomarker of nitrate intake

There is increasing interest in the use of biomarkers to assess nutritional intake and link to chronic disease risk. In Chapter 4, the utility of urinary nitrate as a potential biomarker of total nitrate exposure was examined. To our knowledge, this is the first study conducted in a representative UK population to associate urinary nitrate and nitrite with total dietary nitrate intake. Therefore, in this study 24-h urine samples were obtained and analysed to determine total nitrate exposure. No associations were found between quartiles of increasing urinary nitrate or nitrite with blood pressure, which was in contrast with a cohort study using data from the Italian National Institute of Research and care on Aging (INRCA) in elderly people(20). In this study, urinary nitrate excretion of more than 1 mmol was associated with reductions in SBP and DBP of 4-7 and 2-3mm Hg respectively(20). However, 85% of the participants in this study were elderly over 65 years old and the concentrations of urinary nitrate was higher than all of the participants from the NDNS included in our analysis. Two further cohort studies using

data from the National Health and Nutrition Examination Survey (NHANES) found that high urinary nitrate execration was associated with a 32% lower prevalence of hypertension(21) and lower congestive heart failure(22). The nitrate urinary excretion presented as uM/L in NHANES study which make it difficult to compare with our urinary nitrate excretion in UK population.

Another study determined the impact of chronic dietary nitrate intake on salivary, plasma and urine levels after consuming 400 mg/day inorganic nitrate for 7 days, with a follow-up period of 14 days without nitrate intake. They found that salivary and plasma nitrate concentrations returned to baseline levels within 2 days of stopping nitrate consumption, whereas the urinary nitrate level was 7 fold lower after 14 days of completing the study(23). This study raises the possibility that urinary nitrate might reflect the short-term (48 hours) dietary nitrate exposure(23). In our analysis of the urine samples of NDNS participants, the period between dietary exposure and urine collection was not known, which represents a limitation of the analysis performed. Urinary nitrate and nitrite excretion are also influenced by factors such as health condition, medication use, sex, race, smoking and diets high in caffeine and vitamin C(24). However, it is worthy of noting that in our study urinary nitrate was only weakly associated with dietary nitrate intake, whereas there was no association with urinary nitrite. Furthermore, these potential biomarkers were not found to be associated with BP or other CVD risk markers, which is in contrast to positive associations with dietary nitrate estimated from the diet diaries. A previous study reported a moderate positive correlation between urinary nitrate and vegetable intakes only. This study did not include other sources of nitrate, such as water or processed meats, which may have impacted on the reported associations. Our result showed urinary nitrite to be more associated with other CVD risk factors. The mechanism underlying this association is

unknown and there were no studies that measured urinary nitrite separately and related the levels to BP and other CVD risk factors. Further work is needed to investigate the association between urinary nitrite and CVD risk markers and to determine whether it represents a potential biomarker of dietary nitrate exposure.

## 7.2. FUTURE WORK

The studies in the present thesis have addressed some important research questions while highlighting some opportunities for future research. The randomised controlled trial (RCT) in the thesis demonstrated beneficial effects of beetroot consumption in healthy people, however, the impact in people with mild hypertension is of further interest. Previous studies showed that patients with hypertension and hypercholesterolemia may further benefit from nitrate intake, however, findings are somewhat inconclusive and limited, therefore further chronic, suitably powered, controlled RCTs are required to assess both classic and novel CVD risk markers. Furthermore, although the results of this thesis suggest an association of daily nitrate consumption with lower BP, it would also be of paramount importance to further investigate and confirm these findings in large cohort studies. A key challenge in the investigation of dietary nitrate and health is the lack of availability of nitrate and nitrite data in dietary analysis databases. Therefore, there is an urgent need to update nutrient databases with accurate food and drink nitrate and nitrite concentrations to facilitate the estimation of nitrate and nitrite intake. Based on our findings in chapter 4, further studies are needed to determine the nitrate intake and urinary nitrate excretion to determine whether urinary nitrate excretion is a suitable biomarker of nitrate intake.

## 7.3. GENERAL CONCLUSION

This thesis has presented novel insights into the effects of dietary nitrate on BP, microvascular function and other CVD risk markers using data from the cross-sectional NDNS cohort and a chronic double-blind, randomised controlled dietary intervention study. Furthermore, data from the chronic study indicated a change in the oral and gut microbiome after the consumption of beetroot juice for 8 weeks in healthy people as well as improvement in microvascular reactivity. The weak association between urinary nitrate and dietary nitrate as well as a lack of association with blood pressure suggests urinary nitrate may not be an appropriate biomarker of dietary nitrate exposure in the general UK population. However, this might represent a limitation of the variable timing of the urine collection and dietary assessment in the NDNS cohort. Further studies are needed to assess the use of urinary nitrate as a biomarker of dietary nitrate intake. The findings from this thesis contribute to the increasing evidence base of the benefits of dietary nitrate, which could be used to refine public health guidance on types of vegetable consumption for CVD risk reduction. Further, research is required to confirm the types of oral and gut bacteria which facilitate nitrate reduction and mechanisms underlying the beneficial effects on BP and vascular function.

## 7.4. REFERENCES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# APPENDIX

## ACHIEVEMENTS DURING MY PHD JOURNEY

1. **Ten Conferences:**
    a. Internal conference:
        i. One Poster
        ii. Three Presentations
        iii. Five Abstracts
    b. External conference:
        i. Two Posters
        ii. Three Presentations
2. **Winning Prize:**
    a. Best abstract
    b. Paper of the Month over 5 journals in UK
3. **Publication:**
    a. Review paper in Nutrition Research Reviews journal – Q1
    **b.** Four Conference Abstracts
4. **Ethics application**
    a. Four Ethics application (Two Internal, One University committee, One NHS)
5. **Certificates:**
    a. PhD plus
    b. Leadership

**PUBLISHED PAPER**

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# The role of dietary nitrate and the oral microbiome on blood pressure and vascular tone

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## Abstract

There is increasing evidence for the health benefits of dietary nitrates including lowering blood pressure and enhancing cardiovascular health. Although commensal oral bacteria play an important role in converting dietary nitrate to nitrite, very little is known about the potential role of these bacteria in blood pressure regulation and maintenance of vascular tone. The main purpose of this review is to present the current evidence on the involvement of the oral microbiome in mediating the beneficial effects of dietary nitrate on vascular function and to identify sources of inter-individual differences in bacterial composition. A systematic approach was used to identify the relevant articles published on PubMed and Web of Science in English from January 1950 until September 2019 examining the effects of dietary nitrate on oral microbiome composition and association with blood pressure and vascular tone. To date, only a limited number of studies have been conducted, with nine in human subjects and three in animals focusing mainly on blood pressure. In general, elimination of oral bacteria with use of a chlorhexidine-based antiseptic mouthwash reduced the conversion of nitrate to nitrite and was accompanied in some studies by an increase in blood pressure in normotensive subjects. In conclusion, our findings suggest that oral bacteria may play an important role in mediating the beneficial effects of nitrate-rich foods on blood pressure. Further human intervention studies assessing the potential effects of dietary nitrate on oral bacteria composition and relationship to real-time measures of vascular function are needed, particularly in individuals with hypertension and those at risk of developing CVD.

**Key words:** **Nitrate: Nitrite: Nitric oxide: Oral microbiome: Blood pressure: Mouthwash**

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## Introduction

CVD, including CHD and stroke, are one of the leading causes of death globally. In 2017, the WHO reported that 18 million individuals had died from CVD worldwide which represents 31 % of deaths[1]. Abnormally raised blood pressure, defined as greater than 140 (systolic)/90 (diastolic) mmHg, is an independent risk factor for CVDs and this silent killer is associated with a three-fold higher risk of having a stroke or developing heart failure[2,3]. High blood pressure affects more than one in four adults in England, about 12·5 million individuals. However, the prevalence of hypertension appears to differ between the sexes, with 31 % reported amongst men and 26 % amongst women[4]. Dysfunction of the endothelium, which controls vascular tone and is strongly associated with hypertension, is now recognised as an early, but potentially reversible, step in the development of CVD[5].

The control of vascular function is known to be influenced by dietary factors, with nitrate-rich vegetables considered an important modulator[6–8]. This has been demonstrated in many observational and cohort studies which have shown consumption of nitrate- and nitrite-rich foods to significantly improve cardiovascular health[9] such as lowering blood pressure[10] in both healthy[11] and hypertensive individuals[12], reducing endothelial dysfunction[13–17] and inflammation[18], protection from ischaemia–reperfusion injury[19], and improved exercise performance in patients with heart failure[20]. A prospective cohort study has also concluded that an increased adherence to a diet high in nitrate is accompanied by a significant reduction in the risk of suffering both cardiovascular complications and death due to any cause[21]. Clinically, nitrate supplementation or use of nitrate as a medication to increase the bioavailability of nitrite and NO can reduce blood pressure[22]. The interest in using dietary nitrates as a treatment for lowering blood pressure is growing but mechanisms underlying the effects are unclear, which limits their current application as a dietary treatment for hypertension[22]. Furthermore, there is some evidence to suggest that high dietary nitrate intakes are associated with negative effects on health, which has led to the development of the acceptable daily intake for nitrate of 3·7 mg/kg body weight per d and for nitrite of 0·07 mg/kg body weight per d[23]. The acceptable daily intake for nitrate is based on the risk of methaemoglobinaemia commonly known as blue baby syndrome, which can occur following high nitrate intake in some babies, and can be fatal[24]. In addition, some epidemiological studies have reported an association

**Abbreviations:** eNOS, endothelial NO synthase; NOS, NO synthase.

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between dietary nitrite intake and colorectal cancer. However, the weight of evidence only supports a significant relationship between cancer and red and processed meat[25], with little known about vegetables and drinking water. The nitrate and nitrite within processed meat may be a contributing factor in the association with cancer, although this needs further confirmation.

Humans are naturally colonised by an array of micro-organisms, such as commensal or symbiotic communities, whose metabolic activity is important for host physiology and health. Commensal oral bacteria and those residing in the gastrointestinal tract play an important role in converting dietary nitrate to nitrite and the potent vasodilator NO[26–31]. Up to 85 % of ingested nitrate is reduced to nitrite by the nitrate-reducing bacteria in the oral cavity[32], raising the salivary nitrite concentration to 1000 times that of plasma[28]. A cohort study conducted in 281 volunteers found that the high abundance of nitrate-reducing bacteria was associated with blood pressure in normotensive individuals, although this association was not found in those with hypertension[33]. To date, very little is known about the role of these oral bacteria in the control of vascular function, and the variation in composition that exists between individuals. The aim of this review is to present the current evidence on the potential role of dietary nitrate and the oral microbiome on vascular function including blood pressure and vascular tone. Important determinants of the number and composition of the oral bacteria between individuals will also be described. However, the impact of dietary nitrate interventions on vascular function only will not be specifically addressed in this instance due to the large number of review articles which already exist in this research area[13–17]. Before presentation of the methodology and results of the literature review, we provide a general overview of dietary nitrate sources, the pathways for the conversion of dietary nitrate and nitrite to NO, location and type of nitrate-reducing bacteria in the oral cavity and their potential role in regulating vascular tone.

## Nitrate, nitrite and nitric oxide sources and nitric oxide pathway

NO, the most effective form of nitrate, was first recognised in 1998 as an important signalling molecule in the cardiovascular system[34]. NO plays a significant role in virtually all organs in the body, and higher circulating concentrations are associated with a lower CVD risk[35]. In additional to the dietary (exogenous) sources of nitrate and nitrite which leads to the production of nitrite, and subsequently NO, via the oral bacteria, the body can also derive NO endogenously (Fig. 1). The endogenous pathway can occur in a number of different tissues in the body using three forms of NO synthase (NOS) enzyme: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS was initially discovered in endothelial cells and is important in modulating vascular tone and upholding endothelial integrity. However, eNOS can also be expressed in various tissues and requires the presence of oxygen, Ca and calmodulin to be activated[36]. Within the endothelium, L-arginine undergoes a five-electron oxygen-dependent oxidation to produce NO and L-citrulline, catalysed by the synthase

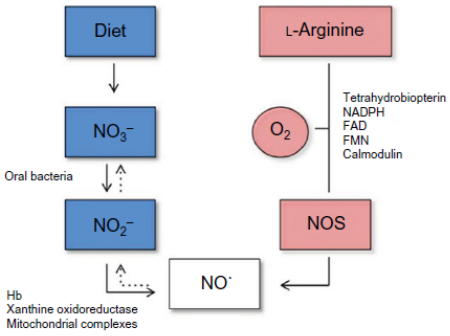


**Fig. 1.** Diagram of the endogenous generation of nitric oxide (NO) by NO synthase (NOS) (right panel), and exogenous generation of NO from the diet (left panel)[28]. In biological fluids, NO is oxidised to nitrite ($NO_2$) and nitrate ($NO_3$) (dashed arrows). For a colour figure, see the online article.

enzymes. Five cofactors required by the NOS enzymes are FAD, FMN, tetrahydrobiopterin (BH4), NADPH and haeme Fe[28]. Once produced in the endothelial cell, NO rapidly diffuses to the underlying smooth muscle layer where it mediates blood vessel vasodilation. Any NO remaining in the circulation is rapidly converted to nitrate by oxyHb or superoxide before it enters the enterosalivary pathway. Therefore, the NO produced has a relatively short half-life in the order of seconds to minutes[37].

## Nitrate metabolism, enterosalivary circulation and gastrointestinal tract

High levels of inorganic nitrate are found in vegetables (such as beetroot and spinach) as well as drinking water, and these dietary sources accounts for 80 % of the daily intake. In contrast, the intake of dietary nitrite is very low, being approximately 100 times lower[38] than that of nitrate[39]. Although the process of re-circulation of nitrates in the body has been known since the 1970s, the importance of the oral nitrate-reducing bacteria in the enterosalivary circulation has only recently been recognised[27] (Fig. 2). The key role that these bacteria play in nitrate reduction was supported by a previous human study in which a significant correlation was found between high abundance of oral nitrate-reducing bacteria and nitrite level in saliva[40]. Nitrate secretion from the salivary glands leads to a 10-fold rise in salivary nitrate levels[41] and this nitrate-enriched saliva appears to be a supportive environment for the growth of the oral bacteria particularly the nitrate-reducing bacteria on the tongue[42]. These bacteria are mostly facultative anaerobes which use nitrate as an alternative electron acceptor for their respiration[43]. A symbiotic relationship therefore exists between the oral commensal bacteria in which they receive nitrate from the host for their own respiration and in return produce nitrite required by the host[42]. This relationship is particularly important for nitrite bioavailability since humans are unable to complete this process independent of the nitrate-reducing bacteria, with 80 % of nitrates swallowed and present in the stomach produced by the oral commensals[44]. Once in the

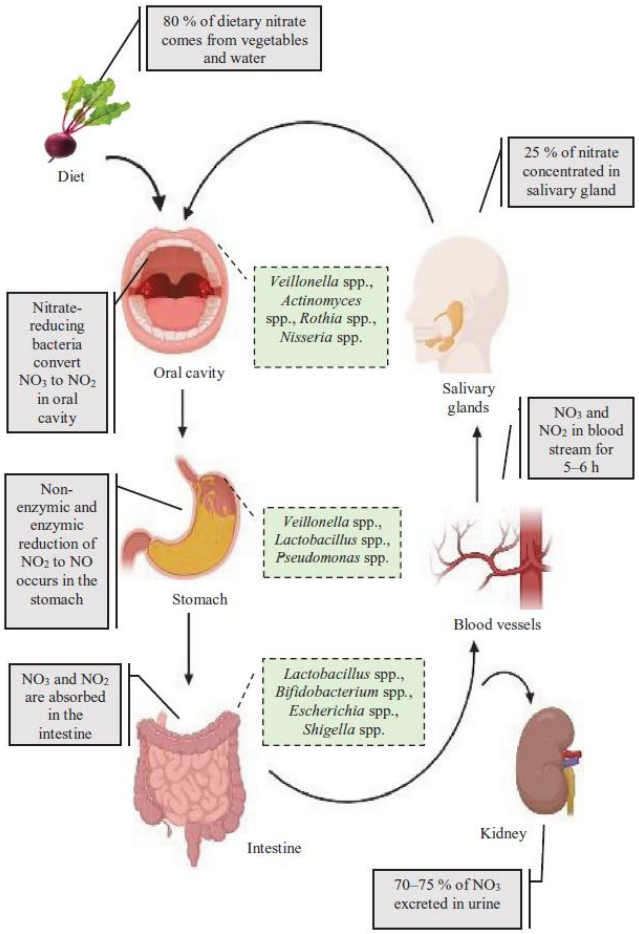


**Fig. 2.** Overview of the nitrate enterosalivary circulation and nitrate metabolism in humans. Ingested inorganic nitrate is converted to nitrite in the oral cavity by nitrate-reducing bacteria with reduction to nitric oxide (NO) and nitrogen oxides occurring within the acidic environment of the stomach. Remining nitrate and other nitrate components are then rapidly absorbed into the bloodstream via the small intestine. A large proportion of nitrate is then excreted by the kidneys into the urine, with up to 25 % being recycled by the salivary glands and then concentrated in saliva. For a colour figure, see the online article.

stomach, contact with the gastric acidity leads to the protonation of nitrites to form nitrous acid ($HNO_2$), which then decomposes into not only NO but also several other nitrogen oxides[45] which have localised benefits on maintaining the gastric mucosa layer[46] and enhancing mucosal blood flow[45] which increases the thickness of the mucosal layer[47]. This process is referred to as non-enzymic conversion which does not require bacteria. However, the presence of *Helicobacter pylori* can contribute to a more acidic environment within the stomach and increase non-enzymic conversion[48]. Residual nitrates and nitrites are then absorbed in the small intestine with the half-life of circulating nitrate in the bloodstream of about 5–6 h[49]. In contrast, plasma nitrite concentrations start to increase within 15 min of nitrate ingestion and reach a peak level in 2 h[50]. A large proportion, approximately 70–75 % of the plasma nitrate, is excreted in the urine whereas the remaining 25 % is stored in the salivary gland and then recycled in the enterosalivary pathway[51].

The role of the nitrate-reducing bacteria can persist past the oral cavity as most of these bacteria move into the stomach with both swallowed food and saliva. Limited studies have investigated the existence of these bacteria in the stomach and have confirmed that the gastric acidity is not a germ-free environment[52]. Although the gastric pH is below 5, some bacteria species can tolerate the stomach acidity, with a culture-based study

**Table 1.** Commonly reported nitrate-reducing bacteria species found in the oral cavity

| Bacteria species | Condition | Change in abundance in response to nitrate intake | Location in the oral cavity |
|---|---|---|---|
| *Veillonella dispar*[42,43] | Anaerobic | ↑ | Tongue |
| *Actinomyces odontolyticus*[42,43] | Facultative anaerobic | ↑ | Tongue |
| *Prevotella salivae*[42,43] | Anaerobic | ↑ | Tongue |
| *Rothia mucilaginosa*[14,42] | Aerobic | ↑↑ | Tongue |
| *Neisseria flavescens*[14,43] | Aerobic | ↑↑ | Tongue |

↑, Increased; ↑↑, highly increased.

reporting *Clostridium* spp., *Veillonella* spp. and *Lactobacillus* spp. as the most predominant gastric species[53], with *Veillonella* spp. identified as the most abundant nitrate-reducing bacteria[43]. There are many factors that can influence gastric acidity such as inflammation and long-term use of proton pump inhibitors. The pH level has been found to have a positive impact on nitrate and nitrite concentration in the gastric juice. In a study conducted in ninety-nine patients with dyspepsia, results showed that when the pH level of the mucosal surface increased there was a comparable increase in both nitrate and nitrite concentrations. Another study conducted in participants with achlorhydria, in which gastric pH ranged from 6 to 8, reported three genera of nitrate-reducing bacteria (*Streptococci*, *Neisseriae* and *Haemophili*) to be responsible for the nitrite accumulation in the gastric secretions[54].

The small intestine and colon contain many different species of bacteria including both facultative and obligate anaerobes which are involved in the bioconversion of nitrite to NO, although they are not necessarily the same as the nitrate-reducing bacteria found in the oral cavity[55]. A study conducted in germ-free and normal rats has shown that NO can be produced by the bacteria resident in the small intestine of normal rats, but not in germ-free rats[56]. Furthermore, two studies have identified lactobacilli, bifidobacteria[56], *Escherichia coli* and *Shigella* as the predominant nitrate-reducing bacteria in the large intestine[57]. However, an *in vitro* study which used pure strains of gut bacteria incubated in agar media with nitrate then nitrite, found that in the presence of nitrite, both bifidobacteria and lactobacilli generated large amounts of NO, up to 5000 parts per billion (ppb), but only approximately 35 ppb of nitrate[58]. Interestingly, Sobko *et al.*[46] reported that the NO formed was being utilised by *E. coli* and *Staphylococcus aureus*. These authors speculated that these gut bacteria may consume NO in order to help adapt to their environment in this *in vitro* experiment. Therefore, it appears that the presence of NO and other nitrate metabolites in the large intestine may be dependent on the relevant abundance of these bacteria species and their production and utilisation of NO[59]. Localised effects of the NO could include altering blood flow which could potentially increase the uptake of nitrate and nitrite in the proximal small intestine where the majority are absorbed[44]. However, the NO level in the gastrointestinal tract could also be influenced by other factors such as pH level, inflammation, oxygen tension and the level of dietary nitrate intake of an individual. Further studies are needed to determine the direct effects of nitrate and nitrite on gut bacteria composition and nitrate metabolism.

## Bacterial nitrate reduction in the oral cavity, composition and location

A continuous flow of saliva, specialised mucosal surfaces and teeth in the human oral cavity provide a unique microbial habitat for bacteria. Most of these bacteria are found on the dorsum (surface) of the tongue and around the teeth where a wash of 1 ml of saliva can contain up to $10^7$–$10^8$ micro-organisms[44]. However, only 700 species have currently been identified[44]. The majority of these bacteria shelter in the gingival crevices between teeth which represent a conducive anaerobic environment. Here, the gingival crevicular fluid bathes the bacteria within a nutritionally rich medium supporting their proliferation[60]. In contrast, the smoother surfaces of teeth have much lower levels of bacteria due to the forces that act on these areas during eating and drinking. However, the nitrate-reducing bacteria are found predominately on the rear dorsum of the tongue, with a higher proportion of Gram-negative bacteria found within the papillae of the tongue compared with the surface. Some studies have identified the genus and species of these bacteria that can produce nitrate reductases and nitrite reductases that aid in the production of NO. These include: *Veillonella atypica*, *V. dispar*, *Actinomyces eslumdii*, *A. odontolyticus*, *Staphylococcus epidermids*, *Neisseria flarescens*, *Haemophilus*, *Porphyromonas*, *Rothia mucilaginosa*, *R. dentocarisa*, *Prevotella* and *Leptotrichia*[42,43]. The two major groups of oral nitrate-reducing bacteria are the strict anaerobes such as *V. atypica* and *V. dispar* and the facultative anaerobes such as *A. odontolyticus* and *R. mucilaginosa*[42] (see Table 1). Facultative anaerobes are mostly prevalent on the surface of the tongue, with a study stratifying participants according to oral nitrate-reduction capacity observing a higher abundance of *Streptococcus*, *Granulicatella*, *Prevotella*, *Neisseria and Haemophilus* on the posterior surface of the tongue compared with *Actinomyces*[43]. Interestingly, although lower in prevalence, *Actinomyces* have been reported to be more efficient reducers of dietary nitrates under anaerobic conditions.

## Mechanisms by which bacteria may convert nitrate to nitrite

The three mechanisms through which nitrates are converted to nitrites and other components by bacteria are denitrification, assimilation and dissimilation. The first process, denitrification, occurs in the oral cavity under aerobic conditions[61] and is also called the respiratory nitrate reduction process. During microbial respiration, oxygen is replaced by nitrogen oxides as terminal electron acceptors and ultimately reduces nitrate to nitrous oxide or free nitrogen[62]. Most of the bacteria which have genes for respiratory nitrate reductases (*nirS* and *nirK*) prefer aerobic conditions[63] such as *Rothia* spp. and *Neisseria* spp. However, some denitrification species of bacteria also reside in anaerobic conditions[44], such as *Veillonella*. The specialised surface of the tongue dorsum therefore represents a microaerophilic environment which allows denitrification to occur under both aerobic and anaerobic conditions. In the oral cavity, nitrite ($NO_2$) is initially formed from salivary nitrate ($NO_3$) by some oral bacteria such as *Actinomyces*[43] that are considered to possess the nitrate reductase enzyme (*nar*) and further converts nitrite to NO through either enzymic (*nir*) or non-enzymic denitrification. The latter process is a well-established step in the gastric environment of the stomach. NO is then converted to nitrous oxide ($N_2O$) by NO reductase (*nor*) and finally to nitrogen ($N_2$) by nitrous oxide reductase (*nos*). The nitrogen oxides and enzymes that participate in the process of denitrification are as follows:

$$NO_3 \xrightarrow{nar} NO_2 \xrightarrow{nir} NO \xrightarrow{nor} N_2O \xrightarrow{nos} N_2$$

In the second pathway known as dissimilation, nitrate is reduced to ammonia ($NH_4^+$) by periplasmic nitrate reductase (*nap*), with the intermediate product being nitrite[64]. This two-step process is strictly anaerobic and occurs in the human gut by the facultative anaerobes[55]:

$$NO_3 \xrightarrow{nap} NO_2 \xrightarrow{nrf} NH_4^+$$

Assimilation, which occurs predominantly in plants, water and soil[65], is the third pathway. Similar to denitrification, the conversion of nitrate to ammonia occurs, but, during this pathway, the enzyme cytoplasmic nitrate reductase (*nas*) is used[65]. In this biosynthetic anabolic pathway, nitrite is further reduced to ammonia, which can then undergo ammonium assimilation by incorporating the amino acid glutamine[44]. The assimilation and dissimilation processes are therefore important in the utilisation of nitrates. Nitrifying bacteria (including *Nitrobacter*, *Nitrococcus* and *Nitrosomonas*)[66] are responsible for the dissimilation and ammonification of nitrates and oxidise ammonium salts and nitrites to nitrates in a process called nitrification. It has been hypothesised that this process might happen in the gut, but, to date, this has not been described[67].

In humans, nitrate reduction seems to occur either directly, such as in assimilatory nitrate reduction, or during a series of reactions during respiratory nitrate reduction. Notably, the latter process needs more than one enzyme for further reduction which is mediated by the bacterial communities[44]. This suggests that nitrate-reducing capacity of nitrate-reducing bacteria is related to the bacterial species, cellular location of enzymes and environmental conditions such as oxygen level. Therefore, dissimulation would occur more in the gut and denitrification in the oral cavity[67]. Although the role of oral bacteria in mediating the beneficial effect of nitrate on vascular function is poorly understood, this review aims to address this knowledge gap by focusing on studies that used antibacterial mouthwash and toothpaste to determine the importance of the presence of oral microbiome on blood pressure and vascular tone.

## Methods

A systematic approach was used to identify the relevant human and animal studies which investigated the role of dietary nitrate and the oral microbiome on blood pressure. PubMed and Web of Science were used for the literature search which included all relevant articles published in English from January 1950 until September 2019. There were three stages in the selection process. The combinations of the key terms used in the search strategy were as follows: ('Nitrate' OR 'Nitrite' OR 'Nitric Oxide') AND ('Oral Bacteria' OR 'Oral Microbiom' OR 'Nitrate-Reducing Bacteria') AND ('Blood Pressure' OR 'Hypertension' OR 'Cardiovascular' OR 'Vascular Function') AND ('Mouth Wash' OR 'Antiseptic' OR 'Antibacterial'). The titles and abstracts of the identified papers were screened by one member of the review team (H. S. A.) who identified potentially relevant papers. This review was restricted to animal studies and human studies which used antibacterial mouthwash or toothpaste to determine the effects on oral nitrate reduction on blood pressure and vascular tone. Only published peer-reviewed literature was considered and 'grey' literature such as dissertations, conference proceedings, reports, letters to editors and other non-peer-reviewed research was excluded. After duplicates were removed, the abstract and full papers were screened for eligibility. In addition, a hand-search of the bibliographies of the articles found from the electronic database searches was also conducted. An overview of the literature search is shown in Fig. 3.

The quality of the included randomised controlled trials in human subjects and animal studies were assessed for the risk of bias using the Cochrane risk of bias tool[68] for human studies and SYRCLE's tool[69] for animal studies.

## Results and discussion

The systematic search identified 160 publications. Of these, twelve relevant publications were included, with nine describing studies conducted in human subjects and three in animals. The risk of bias assessment summaries for each study are presented in online Supplementary Tables 1 and 2, respectively. Animal studies will be discussed before studies including human participants. This will be followed by discussion of the non-modifiable and modifiable factors affecting inter-individual variability in number and composition of oral bacteria, with potential mechanisms of action.

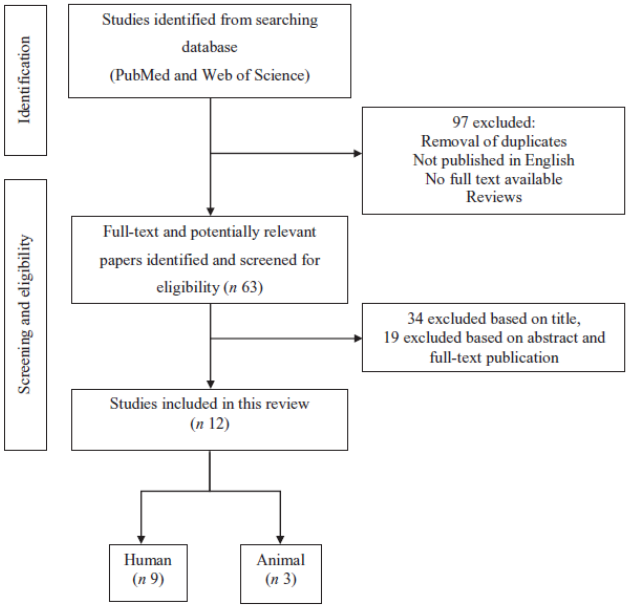


**Fig. 3.** Flow of information through the different phases of the literature review.

## *Animal studies*

Of the fourteen animal studies which have investigated the effect of nitrate on blood pressure, only three studies have determined whether oral bacteria are important in mediating the improvements in blood pressure and endothelial function (Table 2). Formation of bioactive NO takes place within the gastric environment of the stomach as a result of the enterosalivary circulation of nitrate, as well as systemically in the blood vessels. In 2009, Petersson *et al.*[70] reported daily mouthwash treatment for 7 d in rats to attenuate both the gastroprotection provided by NO and the diastolic blood pressure-lowering effect of sodium nitrate. A similar pattern was also evident for the mean arterial pressure in the rats treated with mouthwash and nitrate, but the lack of an effect in the rats treated with mouthwash and nitrite suggested that oral bacteria play an important role in the metabolism of nitrate to NO and mediated vasodilation. Furthermore, these rats also had reduced oral bacteria, suggesting that nitrite could bypass the reduction step by the oral bacteria and was being reduced in the circulation or within endothelial cells to NO, or via effects on the formation of the intermediate nitrosothiols[70]. However, dietary nitrite intake is generally lower than that of nitrate, and the half-life in plasma shorter (seconds *v.* hours), which suggests that even if nitrite directly stimulates NO signalling, the quantity and kinetics of nitrite *v.* nitrate indicate that the critical aspect of this mechanism is the reduction of nitrate. Therefore, the role that dietary nitrite plays in blood pressure lowering may be more limited relative to nitrate.

In agreement, Hyde *et al.*[29] also reported a significant reduction in diastolic blood pressure and increase in plasma nitrite concentrations following the addition of sodium nitrate to drinking water in male Wistar rats. However, in this study, mouthwash treatment was unable to diminish the blood pressure-lowering effects of the nitrate supplementation. The authors speculated that the direct application of the chlorhexidine-based mouthwash (Vedco) to the tongue surface using a swab might not have enabled sufficient time for the mouthwash to exert its full extent on the bacteria relative to mouthspray[29]. A novel aspect of this longer-term supplementation study was the focus on the changes in the microbiota composition on the rat tongue in response to the treatments. Compared with baseline, there was a greater relative abundance of nitrate-reducing bacteria (*Haemophilus* spp. and *Steptococcus* spp.) after 6 d of sodium nitrate consumption, and of these *Haemophilus parainfluenzae* has also been identified as one of fourteen species contributing to nitrate reduction in the oral cavity of healthy adults. Co-supplementation of mouthwash with nitrate was found to increase the diversity of the oral bacteria present relative to nitrate intake only, with increases found in the low abundance taxa such as Enterobacteriaceae, *Corynebacterium* and *Morganella*. Therefore, the use of mouthwash appeared to disturb the oral microbiome by reducing the abundance of the normally dominant taxa but not completely to impact nitrate reduction. These findings suggest that the lower abundance taxa which were evident after mouthwash treatment may be functionally important in the bioactivation of dietary nitrate. However,

**Table 2.** Animal studies investigating the importance of oral nitrate-reducing bacteria on blood pressure in response to nitrate intake

| Reference | Animals | Study design and duration | Intervention | Measurement | Outcome measures |
|---|---|---|---|---|---|
| Petersson *et al.* (2009)[70] | *n* 4–7 Male Sprague–Dawley rats in each group (190–360 g; B&K) | Parallel groups with 7 d treatment periods:<br>(1) No treatment (control)<br>(2) $NaNO_3$ only<br>(3) Mouthwash<br>(4) Mouthwash + $NaNO_3$ or $NaNO_2$ | Water supplemented with 10 mM $NaNO_3$ or 1 mM $NaNO_2$<br>Mouthwash groups: chlorhexidine mouthwash spray (0·3 ml), 2 × daily | Plasma | Δ $NO_2$ ↓ after mouthwash + $NaNO_3$ *v.* control ($P<0·05$) |
| | | | | HR | NS |
| | | | | SBP | NS |
| | | | | DBP | ↓ After $NaNO_3$. DBP lowering absent in mouthwash-treated rats |
| | | | | MAP | ↓ After $NaNO_3$ and mouthwash + $NaNO_2$ *v.* mouthwash only. MAP lowering absent in mouthwash + $NaNO_3$ rats |
| | | | | Oral bacteria | ↓ Viable bacteria on tongue after mouthwash |
| Hyde *et al.* (2014)[29] | *n* 8 Male Wistar rats 7 weeks old | 19 d sequential intervention:<br>Days 0–5: control (water)<br>Days 6–12: $NaNO_3$<br>Days 13–19: $NaNO_3$ + mouthwash<br>Blood collected on days 1, 5, 6, 12, 13 and 19. BP (telemetry) and tongue swab every day | $NaNO_3$ (1 g/l) in drinking water<br>Mouthwash regimen: 0·3 ml of chlorohexidine applied 2 × daily to tongue dorsal surface (days 13–19) | SBP | NS |
| | | | | DBP | ↓ After $NaNO_3$ and mouthwash + $NaNO_3$ *v.* control |
| | | | | Plasma NOx | NS |
| Pinheiro *et al.* (2016)[71] | *n* 10 Male Wistar rats in each group (190–210 g)<br>2K1C hypertensive group<br>Sham-operated control group | 6 weeks: 2 weeks baseline followed by 4 weeks treatment<br>Experiment 1<br>Vehicle<br>$NaNO_2$<br>Mouthwash<br>Mouthwash + $NaNO_2$<br>Experiment 2<br>Vehicle<br>$NaNO_3$<br>Mouthwash<br>Mouthwash + $NaNO_3$<br>6 h after last treatment, blood and tongue swab collected | 15 mg $NaNO_2$/kg or 140 mg $NaNO_3$/kg (oral administration)<br>Mouthwash groups: daily mouth clean with chlorhexidine (0·12 %) soaked swab | Plasma | Δ $NO_2$ ↓ 25–30 % after mouthwash *v.* $NaNO_2$ and $NaNO_3$ groups ($P<0·05$)<br>Δ $NO_3$ ↓ 45 % after mouthwash *v.* $NaNO_2$ group ($P<0·05$) |
| | | | | BP | ↓ SBP (40 mmHg) and MAP with $NaNO_2$ and $NaNO_3$ ($P=0·01$)<br>Mouthwash blunted MAP- and SBP-lowering effect of $NaNO_3$ ($P<0·05$) but not $NaNO_2$ |
| | | | | Oral bacteria | ↓ CFU 50–70 % with mouthwash |

$NO_2$, nitrite concentration; HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; BP, blood pressure; NOx, sum of nitrate and nitrite; 2K1C, 2 kidney, 1 clip; $NO_3$, nitrate concentration; CFU, colony-forming units (number of viable bacteria).

the authors did caution against translating these findings on the oral bacteria composition to humans since the oral human microbiome has been shown to be more diverse and of a differing composition compared with the rat[29].

The impact of mouthwash on chronic changes in blood pressure in response to nitrate or nitrite supplementation was further examined by Pinheiro *et al.*[71] in both control and hypertensive rats. After 4 weeks, significant reductions in mean arterial pressure and systolic blood pressure were evident in both the nitrate and nitrite groups, with concordant increases found in circulating plasma nitrate and nitrite levels. Interestingly, co-supplementation with mouthwash attenuated the rise in plasma nitrite levels by 25–30 % in both groups but was only found to blunt the blood pressure-lowering effect of nitrate, with little impact found on blood pressure in the mouthwash and nitrite group. In agreement with Petersson *et al.*[70], these findings suggested that anti-hypertensive effects of nitrite were potentially occurring via non-enzymic reactions within the gastric environment after swallowing this ion independently of the enterosalivary pathway. Analysis of the endogenously produced vasodilatory compound *S*-nitrosothiol and levels of vascular nitrosylation revealed mouthwash to reduce nitrosylation responses to nitrate only, leading the authors to speculate that *S*-nitrosylation was an important mediator of the blood pressure-lowering effects of both nitrate and nitrite[70,71]. Studies have also reported that the foods consumed with dietary nitrites, such as conjugated fatty acids, are also a target of nitrating species in the stomach leading to the formation of nitro-fatty acids (such as nitro-conjugated linoleic acid). These electrophiles have been shown to have anti-hypertensive effects independent of *S*-nitrosothiols, suggesting that they may also play a role in mediating the effects of nitrate and nitrite on blood pressure[72]. Antiseptic mouthwash was proposed to attenuate the beneficial effects of dietary nitrate intake on blood pressure by reducing the amount of nitrite formation by the oral bacteria and therefore reaching the stomach, inhibiting gastric formation of *S*-nitrosothiols. However, the positive benefits on blood pressure of raised *S*-nitrosothiols was only found in the hypertensive rats, supporting previous observations in both animals and human subjects that raised blood pressures often show a greater sensitivity to the anti-hypertensive effects of medication and/or dietary modification.

Studies performed in animals may provide useful insights into the mechanisms underlying the effects of oral bacteria in the bioactivation of nitrate. However, findings in rats and mice need to be interpreted with caution due to differences in physiology and dependence on nitrate as a source of NO between organisms. In contrast to human subjects, rats and mice do not recirculate nitrate in saliva[73] and so salivary nitrate concentrations never exceed those levels found in plasma[74] and they also have other nitrate-reducing mechanisms that may work in tandem with nitrate reduction by the oral bacteria to control nitrite and NO level[73].

### *Human studies*

The publications describing the human studies were divided into those which examined (1) the association between oral bacteria with nitrate/nitrite levels and/or blood pressure ($n$ 5; Table 3) and (2) the combined effects of nitrate ingestion and oral bacteria on nitrate/nitrite levels and/or blood pressure ($n$ 4; Table 4). The role of the oral bacteria in mediating systemic nitrite production after nitrate intake has been primarily investigated with the use of an antiseptic mouthwash to remove the bacteria before the measurement of the outcomes of interest. The type of mouthwash has been shown to be important, with the strong antibacterial chlorhexidine-based mouthwash (Corsodyl) found to be more effective at reducing *V. dispar* (nitrate-reducing bacteria) in the oral cavity than Listerine (mixture of essential oils), Isodine and Cepacol (antibacterial) in healthy adults[75]. In support of these findings, gargling with 10 ml of chlorohexidine mouthwash (Corsodyl) twice for 1 min was also found to reduce the bacterial count of nitrate-reducing bacteria by approximately 80 % and virtually abolish the oral nitrate-reducing capacity compared with no mouthwash in healthy subjects[27]. Although nitrate accumulated in saliva after ingestion of sodium nitrate in both studies, a significant reduction in the conversion of salivary nitrate to nitrite after mouthwash was associated with 30 % lower plasma nitrate concentrations at 3 h post-ingestion, compared with no prior use of mouthwash. In contrast, a randomised cross-over study found an antibacterial toothpaste to have no effect on salivary or plasma nitrate concentrations in sixteen women after consuming 400 mg of nitrate before brushing their teeth with antibacterial toothpaste (0·3 % triclosan) or toothpaste containing no antibacterial agent[76]. The lack of an effect observed with the antibacterial toothpaste may reflect either the lower prevalence of the nitrate-reducing bacteria on the surface of the teeth, relative to the tongue, or the less efficient removal of the bacteria sheltering within the gingival crevices between the teeth compared with mouthwash.

Four studies have determined the impact of mouthwash on changes in oral nitrate-reducing capacity and blood pressure (Table 4). Compared with no mouthwash, Kapil *et al.*[41] reported that using 0·2 % chlorhexidine twice daily for 7 d significantly increased systolic and diastolic blood pressure measured using three different techniques (clinic, ambulatory and home measurements) by approximately 3 and 2 mmHg, respectively, in nineteen healthy normotensive subjects. Interestingly, the effects of mouthwash treatment on blood pressure were evident after only a single use of the chlorhexidine mouthwash and was maintained for the following 6 d. The rise in blood pressure was significantly correlated with the significant reduction in plasma nitrite levels, with only a trend for a relationship with the salivary nitrite, highlighting the potential importance of the oral nitrate-reducing bacteria in blood pressure modulation.

In fifteen subjects treated with anti-hypertensive medication, the attenuation found in oral nitrate-reducing capacity after daily use of chlorhexidine mouthwash for 3 d was associated with an increase in systolic blood pressure of 2·3 mmHg, but only a trend for a decrease in plasma nitrite concentrations compared with the control (tap water)[77]. The lack of a significant effect on the plasma nitrite response relative to Kapil *et al.*[41] was thought to be due to the study visit being performed 12 h after prior use of the mouthwash treatment or related to the age or medication use of the hypertensive participants. In order to determine the mechanism underlying the effects of dietary nitrate intake on blood pressure, plasma cGMP (cyclic guanosine monophosphate), a mediator of NO-dependent smooth muscle relaxation

**Table 3.** Human studies determining the effects of oral bacteria on salivary and plasma nitrite concentrations, and/or blood pressure in response to nitrate intake

| Reference | Subject characteristics | Study design and duration | Nitrate dose | Type of mouthwash | Measurement | Significant outcomes |
|---|---|---|---|---|---|---|
| Acute studies | | | | | | |
| Mitsui & Harasawa (2017)[75] | *n* 12 (6 M/6 F)<br>Normotensive<br>Age 19–44 years<br>Non-smokers | Acute, RCT, CO<br>Four visits 10 h in duration with 1 week washout<br>Saliva and oral bacteria collected 0, 1 and 10 h | 100 g lettuce (110 mg $NO_3$) with breakfast. Lunch at 5 h | (1) Water (control)<br>(2) Listerine (antiseptic)<br>(3) Isodine (povidone-iodine, 0·35 %)<br>(4) Chlorhexidine (0·0025 %)<br>Treatment for 3 min before nitrate ingestion | Saliva<br>Oral bacteria | Relative to baseline:<br>↑ $NO_3$ and $NO_2$ after each treatment ($P<0·05$)<br>↓ Nitrate-reducing bacterium *Veillonella dispar* at 1 and 5 h after chlorhexidine |
| Govoni *et al.* (2008)[27] | *n* 7<br>Normotensive<br>Age 24–51 years<br>BMI 23 kg/m$^2$<br>Non-smokers | Acute, RCT, CO<br>Two visits of 3 h in duration<br>Blood and saliva samples collected before and for 3 h after nitrate intake<br>Oral bacteria collected in *n* 4 after mouthwash only | 10 mg/kg $NaNO_3$ in 100 ml water | Mouthwash *v.* no mouthwash<br>Corsodyl (chlorhexidine) gargled twice for 1 min, 15 min before nitrate ingestion | Saliva<br>Plasma<br>Oral bacteria | ↑ $NO_3$ on both visits<br>↓ $NO_2$ *v.* no mouthwash<br>$NO_3$ ↓ 29 nM and $NO_2$ ↓ 250 nM at 3 h *v.* no mouthwash<br>↓ Bacteria count (80 %) and nitrate-reducing capacity after mouthwash |
| Woessner *et al.* (2016)[30] | *n* 12 (M)<br>Normotensive<br>Mean age 36 years and BMI 24 kg/m$^2$<br>Non-smokers | Acute, RCT, CO<br>Four visits, 4 h in duration with 1 week washout<br>BP, blood and saliva collected before and for 4 h after juice consumption | 140 ml of concentrated beetroot juice (8·4 mmol nitrate) | (1) Water (control)<br>(2) Listerine (antiseptic)<br>(3) Cepacol (antibacterial)<br>(4) Chlorhexidine (0·12 %)<br>Treatment 15 min after beetroot juice for 60 s | SBP<br>DBP<br>Saliva<br>Plasma | ↓ Listerine and control *v.* Cepacol and chlorhexidine ($P\leq0·05$)<br>NS<br>↑ $NO_3$ all treatments<br>↑ $NO_2$ control *v.* all mouthwashes and ↓ $NO_2$ chlorhexidine and Cepacol *v.* antiseptic ($P\leq0·05$)<br>↑ $NO_3$ all treatments<br>↓ $NO_2$ chlorhexidine *v.* all treatments and Cepacol *v.* control ($P\leq0·05$) |
| Bondonno *et al.* (2012)[76] | *n* 16 F<br>Normotensive<br>Mean age 52±11 years (F)<br>Non-smokers | Acute, RCT, CO<br>Five visits of 3 h in duration<br>1 week washout<br>Blood and saliva samples collected before and for 3 h after nitrate intake | 0, 100, 200, 400 mg $NaNO_3$ in water | (1) Antibacterial toothpaste (0·3 % triclosan)<br>(2) Toothpaste without antimicrobial agent (control) | Saliva<br>Plasma | ↑ $NO_3$ all treatments<br>↑ $NO_3$ all treatments |

**Table 3.** (*Continued*)

| Reference | Subject characteristics | Study design and duration | Nitrate dose | Type of mouthwash | Measurement | Significant outcomes |
|---|---|---|---|---|---|---|
| Acute within chronic | | | | | | |
| McDonagh *et al.* (2015)[80] | *n* 12 (6 M/6 F)<br>Normotensive<br>Mean age 22±2 years (F) and 24±2 years (M)<br>Non-smokers | Acute within chronic, RCT, double blind<br>Six visits over 8 weeks<br>Each treatment 6 d, with acute visits (4 h) on days 0 and 6<br>Acute visits: rinse with mouthwash 15 min before ingesting 2 × 70 ml beetroot juice. Measurements at 0, 2 and 4 h. BP and PWA measured at rest and during 10 min of treadmill walking. Saliva and plasma samples collected | 70 ml of beetroot juice (6·2 mmol nitrate) twice per d | (1) Strong: Corsodyl (chlorhexidine)<br>(2) Weak: Vademecum med (non-chlorhexidine-containing antibacterial mouthwash)<br>(3) Deionised water (control)<br>3 × daily 15 min before beetroot juice and meals, for 6 d | SBP | Relative to baseline (0 h):<br>Resting: NS<br>After 10 min exercise, ↑ 3 mmHg after strong mouthwash *v.* control ($P$=0·07) 4 h after beetroot juice |
| | | | | | DBP | Resting and during exercise: NS |
| | | | | | MAP | Resting: NS<br>After 10 min exercise, ↑ after strong mouthwash *v.* control ($P$<0·05) at 4 h |
| | | | | | HR | During exercise ↑ after strong *v.* control and weak ($P$<0·05) |
| | | | | | PWA | NS |
| | | | | | Plasma | ↑ $NO_3$ all treatments<br>Δ NO2 ↓ after strong *v.* other treatments at 2 and 4 h, and weak *v.* control ($P$<0·05) at 2 h |
| | | | | | Saliva | Δ $NO_3$ ↑ and Δ $NO_2$ ↓ after strong *v.* weak and control ($P$<0·05) at 4 h |

M, male; F, female; RCT, randomised controlled trial; CO, cross-over; $NO_3$, nitrate concentration; SBP, systolic blood pressure; DBP, diastolic blood pressure; BP, blood pressure; PWA, pulse wave analysis; MAP, mean arterial pressure; HR, heart rate.

**Table 4.** Chronic human studies investigating the involvement of oral bacteria in the blood pressure-lowering effect of nitrate

| Reference | Subject characteristics | Study design and duration | Oral nitrate-reducing capacity | Mouthwash regimen | Measurement | Significant outcome between treatment |
|---|---|---|---|---|---|---|
| Tribble *et al.* (2019)[78] | *n* 26 (16 F/10 M)<br>Normotensive<br>Age 22–71 years | Sequential<br>Four visits over 14 d:<br>Days 1 (baseline), 7 (post-mouth wash), 10 (recovery) and 14 (recovery)<br>Clinic BP and oral bacteria at each visit. *n* 6 oral nitrate-reducing capacity for 8 h after 30 s mouthwash | Mouth rinse with 1 mM $NaNO_3$ for 2 min | Chlorhexidine (0·12 %)<br>2 × daily for 30 s | SBP<br>DBP<br>Oral bacteria<br><br><br>Oral nitrate-reducing capacity | In response to mouthwash, ↑ 5 mmHg (*n* 9) and ↓ (*n* 4)<br>NS<br>↓ Species diversity and abundance with mouthwash for 7 d. ↑ Bacterial metabolic activity at day 14<br>↓ $NO_3$:$NO_2$ ratio for 6–8 h after mouthwash |
| Sundqvist *et al.* (2016)[79] | *n* 17 (F)<br>Normotensive<br>Mean age 23 years<br>BMI 22 kg/m$^2$<br>Non-smokers | RCT, CO, double blind<br>Each treatment 3 d with a 28 d washout<br>Four visits (days 3 and 4 of each treatment)<br>24 h ABP and urine sample<br>Clinic BP, saliva and plasma samples and oral nitrate-reducing capacity | Mouth rinse with 10 mM $NaNO_3$ for 5 min | Chlorhexidine (0·2 %) or placebo mouth-wash<br>3 × daily after meals for 60 s | BP<br>Saliva<br>Plasma<br><br>Urine<br>Oral nitrate-reducing capacity | No difference in ABP or clinic BP<br>↑ $NO_3$ and ↓ $NO_2$ after mouthwash ($P \leq 0{\cdot}01$)<br>No change in $NO_3$ and $NO_2$ with mouthwash *v.* placebo<br>Excretion of $NO_3$ with mouthwash *v.* placebo<br>↓ $NO_2$ after mouthwash (8 μM) *v.* placebo (234 μM) ($P<0{\cdot}001$) |
| Bondonno *et al.* (2015)[77] | *n* 15 (8 M/7 F)<br>Hypertensives taking medication<br>BP 120–159/100 mmHg<br>Age 53–69 years and BMI 20–35 kg/m$^2$<br>Non-smokers | RCT, CO<br>Each treatment 3 d with a 10–12 d washout<br>Visits at day 0 and 3 of each treatment<br>Saliva sample, oral nitrate-reducing capacity and plasma sample. BP measured at home | Ratio of $NO_2$ and $NO_3$ measured in saliva | Chlorhexidine or tap water (control)<br>2 × daily with 20 ml for 30 s after brushing teeth | SBP<br>DBP<br>Saliva<br><br>Plasma<br><br>Oral nitrate-reducing capacity | ↑ 2·3 mmHg after mouthwash *v.* water ($P$=0·01)<br>NS<br>↑ NO3 and ↓ NO2 after mouthwash *v.* control ($P$=0·001)<br>↓ $NO_2$ after mouthwash *v.* control ($P$=0·09). $NO_3$ – NS<br>↓ Nitrate reductase ratio after mouthwash |
| Kapil *et al.* (2013)[41] | *n* 19<br>Normotensive<br>Age 18–45 years<br>BMI 18–40 kg/m$^2$<br>Non-smokers<br>No self-reported use of mouthwash or antibiotic | Sequential<br>Two visits (0 and 14 d)<br>At each visit, clinic BP, blood, urine and saliva samples and oral nitrate-reduction capacity<br>Fitted with ABP unit for 24 h and BP measured at home | Mouth rinse after holding three doses of $KNO_3$ (0, 0·8 and 80 μmol) in the mouth for 5 min | Chlorhexidine (0·2 %)<br>2 × daily on days 8–14 only | <br>Clinic SBP<br>Clinic DBP<br>A-SBP<br>A-DBP<br>Home SBP<br>Home DBP<br>HR<br>Saliva<br>Plasma<br>Urine<br>Oral nitrate-reducing capacity | Relative to baseline, use of mouthwash:<br>↑ 3·5 mmHg ($P$=0·003)<br>↑ 2·2 mmHg ($P$=0·038)<br>↑ 2·4 mmHg ($P$= 0·017)<br>↑ 2·2 mmHg ($P$= 0·014)<br>↑ 2·9 mmHg ($P<$ 0·001)<br>↑ 2·0 mmHg ($P<$ 0·001)<br>NS<br>↑ $NO_3$ and ↓ $NO_2$ 90 % ($P<0{\cdot}001$)<br>↑ $NO_3$ and ↓ $NO_2$ 25 % ($P$=0·001)<br>↑ $NO_3$ and ↓ $NO_2$<br>At baseline, $NO_2$ in mouth rinse dose dependent (0<0·8<80 μmol $KNO_3$)<br>After mouthwash, ↓ 90 % $NO_2$ in mouth rinse for 0·8 and 80 μmol $KNO_3$ |

F, female; M, male; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; $NO_3$, nitrate concentration; $NO_2$, nitrite concentration; RCT, randomised controlled trial; CO, cross-over; ABP, arterial blood pressure; A-SBP, ambulatory SBP; A-DBP, ambulatory DBP; HR, heart rate.

in the endothelium and a good marker of NO production, can be measured. Although increases in plasma nitrite and cGMP after dietary nitrate intake have been previously associated with blood pressure lowering, no effects were evident on cGMP concentrations after 3 d of using mouthwash. This may be related to the lack of a nitrate challenge on the study visit (which provides an important source of NO under hypoxic conditions) but could also suggest that dietary nitrate may have an impact on vascular tone via direct effects on smooth muscle function.

In contrast to these two studies, Tribble *et al.*[78] reported use of chlorhexidine mouthwash twice daily for 7 d to be associated with a highly variable effect on clinic systolic blood pressure (an increase of at least 5 mmHg found in nine subjects whereas a decrease was observed in four subjects) in an orally healthy cohort. *Post hoc* data analysis revealed the inclusion of tongue cleaning as part of the daily dental hygiene routine to play a significant role in the responses observed both on blood pressure and the diversity of the oral bacteria at baseline and during the study. Specifically, regular tongue cleaning was associated with a greater ability to reduce nitrite to NO whereas the lack of tongue cleaning resulted in an oral microbiome composition which favoured conversion of nitrite to ammonia and not NO. The authors speculated the use of chlorhexidine mouthwash was having a chemo-stimulatory effect on the oral bacteria, with the temporary loss of bacterial numbers proposed to stimulate a rapid population recovery and increase in bacterial nitrate reductase activity. However, these effects may also reflect a protective up-regulation of the nitrate-, nitrite- and NO-regulating mechanisms in the microbiota suddenly detached from their biofilms during tongue cleaning and warrants further investigation.

In a cross-over study, treatment with chlorhexidine (0·2 %) for 3 d was shown to have no effect on clinic or 24 h ambulatory blood pressure in seventeen young females compared with a placebo mouthwash[79]. Although a reduction in salivary nitrite- and oral nitrate-reducing capacity was found after the antibacterial mouthwash, comparable changes were not evident in either the plasma or urine samples collected. The lack of effects observed relative to other studies may reflect the short intervention time with the mouthwash treatments or inclusion of female participants only. Based on a previous study conducted by the same research group in athletes, they speculated that cross-talk may exist between the enterosalivary nitrate–nitrite–NO pathway and eNOS, with a greater intake of dietary nitrate associated with a lower eNOS activity. However, whether a reduction in nitrate–nitrite–NO with antibacterial mouthwash leads to an up-regulation in eNOS is yet to be established.

In the studies presented in Table 3, measures of blood pressure have been related to salivary and plasma nitrate/nitrite levels following nitrate intake and use of mouthwash. In agreement with previous findings, Woessner *et al.*[30] found antibacterial mouthwashes to attenuate postprandial salivary and plasma nitrite concentrations following dietary nitrate intake (concentrated beetroot juice) compared with the weaker antiseptic mouthwash and control. Although changes in clinic systolic blood pressure 0–3 h after the treatments were not related to plasma/salivary nitrite or nitrate levels, systolic blood pressure at 4 h was 2–5 mmHg higher after chlorhexidine and Cepacol mouthwashes compared with control and Listerine mouthwash. These findings potentially suggest an important role of the nitrate–nitrite–NO enterosalivary pathway, but should be interpreted with caution due to the small sample size, inclusion of male subjects only and the short duration of the study visit relative to the expected peak in plasma nitrite concentrations (approximately 3 h). Furthermore, these findings may have been influenced by the large inter-individual variability observed in blood pressure responses following the mouthwash treatments.

In the study of McDonagh *et al.*[80], consumption of 2 × 70 ml shots of concentrated beetroot juice and daily use of strong or weak antibacterial mouthwash for 6 d were found to have limited effects on baseline blood pressure and salivary and plasma nitrate/nitrite levels compared with the control (water). However, differences were evident 2–4 h after drinking the beetroot juice, with the rise in plasma nitrite found to be attenuated after use of the strong and weak mouthwash for 6 d. These changes were associated with a reduced oral nitrate-reducing capacity after the strong mouthwash, with lower nitrite levels compared with both the weak and placebo mouthwashes. Although changes in resting measures of blood pressure (supine and seated) and pulse wave analysis (arterial stiffness) after the juice were not influenced by the strength of the mouthwash used, differences were evident in blood pressure during low-intensity activity on the treadmill. In particular, there was a greater increase in systolic blood pressure and mean arterial pressure after rinsing with the strong (chlorhexidine) compared with the control (water) mouthwash. The lack of effect on arterial stiffness even in the presence of lower salivary and plasma nitrite levels after the strong mouthwash indicates that either the availability of NO was not altered sufficiently over the 4 h acute test period in these young active participants or that their higher physical active level may have masked any effects of the mouthwash on the vascular function measures. However, this is one of the only studies to incorporate a measure of blood vessel elasticity to determine the role of oral bacteria in mediating the beneficial effects of beetroot juice on vascular function, and so further studies are needed in which to compare these findings and determine the underlying mechanisms.

As highlighted in the human studies, oral bacteria composition appears to vary between individuals, with both non-modifiable (for example, age, sex, genetics and tongue physiology) and modifiable (for example, diet, health conditions, lifestyle and dental hygiene routine) factors considered to have an impact on the abundance and prevalence of nitrate-reducing bacteria in the oral cavity. These factors are important to consider during interpretation of the study findings and for informing the design of future studies exploring the role of oral nitrate-reducing bacteria on the regulation of vascular function. The following section summarises the main factors identified from the human studies.

*Inter-individual variability in number and composition of oral bacteria: non-modifiable factors.* Geographical location and culture have both been suggested to have an impact on oral bacteria composition. Findings from a study including participants from Northern and Southern Europe reported a higher abundance of *Rothia* and unclassified Gemellaceae in Finnish populations compared with Spanish while *Lactococcus*, *Fusobacterium* and

*Porphyromonas* genera were significantly higher in Spanish compared with Finnish groups[81]. Comparing findings of this study with another study which investigated the differences in oral bacteria between individuals living in Africa, Alaska and Germany showed that oral bacteria composition is highly variable between countries[82]. These differences may represent the sex and age distributions of these different populations, genetic make-up and habitual food preferences[82,83].

Moreover, the dorsal surface of the tongue plays a major role in nitrate reduction and represents a highly papillated surface area. The papillary structure of the human tongue is unique in nature and supports a higher bacterial density than the mucosal surface, accumulating oral debris and anaerobic bacteria on the rear of tongue[42]. There are three kinds of papillae on the tongue: fungiform, circumvallate and foliate papillae. The fungiform papillae have a mushroom shape and are found predominately on the dorsal surface of the tongue covering up to two-thirds of the surface. Their shape supports a higher bacterial density[84]. However, the shape and number of papillae varies between individuals which has been related to differences in oral bacteria composition. Studies have shown that a number of factors can affect the papillary number on the tongue including ageing (with lower number of papillae observed in those individuals over 60 years), genetic make-up, ethnicity[81], demographics and environment[84].

Within the oral cavity, the presence of teeth increases the bacterial density compared with those with permanent tooth loss since the gingival crevices between teeth represent a greater surface area and environment for bacterial growth[85]. Other important factors considered to make an impact on the variety of nitrate-reduction bacteria present in the oral cavity are ageing and sex. However, in a recent human study conducted in nine participants < 22 years and nine participants > 70 years, a similar salivary microbiome at baseline and after placebo beetroot juice was found in both groups. Comparable changes in bacterial composition (increases in *Rothia* and *Neisseria*) were also evident in both age groups in response to consuming 70 ml of beetroot juice (≈ 6·2 mmol nitrate) daily for 10 d[86], suggesting that age was not an important modulator of the oral bacteria composition in this study. Few studies have determined differences in oral bacteria composition between men and women. In order to address this knowledge gap, Kapil *et al.*[87] examined the impact of sex on nitrate-reducing bacteria abundance in thirteen male and thirteen females aged 18–45 years. Oral bacteria samples were collected before and after nitrate supplementation and all samples were analysed by 16S ribosomal RNA sequencing. Significant sex-dependent effects on oral nitrate-reducing bacteria composition were not found in this study. However, subgroup analysis indicated females to have a non-significant tendency for a higher activity of nitrate-reducing bacteria than men[74,87], but these findings need to be confirmed in a suitably powered study.

*Inter-individual variability in number and composition of oral bacteria: modifiable factors.* Several modifiable factors have been reported to influence and change the oral nitrate-reducing bacteria composition, with dietary nitrate intake considered to be one of the most important factors[27,88]. In a recent cross-over study conducted in eighteen volunteers assigned to receive a nitrate supplement or a placebo for 10 d, an increase in the abundance of some nitrate-reducing bacteria, particularly *Rothia* and *Neisseria*, was linked with the ability of an individual to reduce the nitrate supplement. However, changes were not observed with the *Prevotella* and *Veillonella* species[86]. Interestingly, these results corroborate findings from another study which reported the reduction in *Prevotella* and *Veillonella* species in the oral cavity of elderly adults following dietary nitrate intake to be associated with a lower mortality risk in this population[88]. Furthermore, the increased prevalence of *Rothia* and *Neisseria* species relative to the *Prevotella* and *Veillonella* species was linked to higher NO bioavailability in both saliva and plasma[86]. These findings imply that the oral bacteria community is responsive to changes in the level of dietary nitrate intake[89]. However, the authors also reported that individuals with a higher abundance of *Campylobacter concisus* and *Prevotella melaninogenica* in their oral cavity at baseline may not be as responsive to dietary nitrate intake than those with a lower proportion of these bacteria[86]. This might reflect the fact that both *C. concisus* and *P. melaninogenica* are predominately nitrite, but not nitrate, reducers in the oral cavity. Therefore, dietary nitrate availability may affect the growth and composition of particular groups of oral bacteria which can be related to improved cardiovascular health[89]. Of particular note, drinking beetroot juice rich in dietary nitrate can increase the oral cavity pH from 7·0 to 7·5, which is close to the optimal pH of 8 required for nitrate reductase activity[90]. Therefore, the effect of pH is also important in terms of the proliferation and inhibition of different populations within the oral bacterial community[86].

In a similar fashion, some health conditions have also been reported to influence the oral bacterial composition, with a lower density of nitrate-reducing bacteria and a different bacterial composition found in individuals with raised blood pressure (hypertensives) than normotensive subjects[31]. A recent novel study has provided further evidence on the relationship between differences in oral bacteria composition with hypertension in postmenopausal women ($n$ 446). This study analysed oral bacterial samples by using 16S RNA sequencing and found that the abundance of *Prevotella oral species 317* and *Streptococcus oralis* were significantly lower in women with elevated blood pressure compared with those with normal blood pressure[91]. Furthermore, the differences in the oral bacteria communities between groups also seemed to be associated with the severity and progression of the hypertension[33]. Conversely, a higher abundance of nitrate-reducing bacteria were observed in individuals who suffer from migraines (a vascular-driven process associated with changes in NO). Interestingly, the dominant nitrate-reducing bacteria in these individuals were *Pseudomonas* and *Streptococcus* which were not common in subjects who did not suffer with migraines. Oligotyping (the technique for differentiation between closely related microbial taxa)[92] was performed for both genera to investigate the strain-level differences across the bacterial population. *Pseudomonas* decompose to two oligotypes (different strains of the same species) and have differential abundance patterns with significantly higher abundance in oligotype 2 in those suffering from migraines compared with non-sufferers[93]. These

results suggest that the type of these oral bacteria may be more prevalent in individuals with migraines. However, more work is needed to find the link and the mechanism to explain how these bacteria adapt genetically to their host environment. Therefore, there may be an optimum number and composition of nitrate-reducing bacteria which has beneficial effects, and a greater level may have a negative impact on conditions associated with blood vessel dilation such as migraine. However, it should be acknowledged that nitrate reduction and metabolism cannot be attributed to a single bacterial species as they are unlikely to express all of the enzymes required to decompose nitrate simultaneously. More likely, these individual nitrate-reducing bacteria are considered to work in synergy with other members of the microbial community. This has been demonstrated by Hyde *et al.*[43] who found that mixed colonies of high and low nitrate reducers showed a greater capacity for nitrate reduction than mixes of either multiple high reducers or individual nitrate-reducing bacteria. This highlights the complexity of the oral microbiome and the impact on dietary nitrate metabolism.

Cardiometabolic diseases including obesity, the metabolic syndrome and type 2 diabetes are major contributors to global CVD disease burden. Whilst some studies have reported plasma nitrate/nitrite levels to be negatively associated with waist circumference[94], obesity[95] and blood pressure, others have observed positive associations between plasma nitrite and BMI, fasting blood glucose[96], systolic blood pressure and the fasting lipid profile. In support of these findings, Akram *et al.*[97], found plasma nitrite levels to be higher in individuals with both obesity and the metabolic syndrome followed by those with obesity alone, with the lowest levels in those with normal weight. Whether high plasma nitrite levels play a role in the worsening of the cardiometabolic risk markers is a public health issue since higher dietary nitrate intakes may also cause higher levels of plasma NOx (sum of nitrate and nitrite levels). Furthermore, these data are associations, which do not indicate whether cardiometabolic risk markers change in response to varying nitrate/nitrite intakes and do not prove cause and effect. Interestingly, a review of the evidence suggests the contrary, with dietary nitrate supplementation found to reverse or improve some of the features of the metabolic syndrome and be protective against the development of CVD[98]. Although these beneficial effects may be related to improvements in NO metabolic pathways and glucose control, we cannot discount that favourable changes in the gut microbiota in response to dietary nitrate intake may also represent an important mechanism since dysbiosis (a term to describe microbial imbalance) is a common feature of the cardiometabolic diseases. However, very few studies have determined the impact of dietary nitrate supplementation on the gut microbiota in humans, with a very short-term study with nitrate-rich fruit and vegetable juice suggesting a reduction in the Firmicutes:Bacteroides ratio after 3 d which was related to higher plasma nitrate/nitrite levels[99]. Furthermore, a 1-year intervention with the Mediterranean diet, rich in vegetables, was associated with increased abundance of specific taxa that were inversely associated with inflammatory markers[100]. More studies are needed to address this research gap which also include analysis of the oral microbiome to determine whether increases in the abundance of nitrate-reducing bacteria are related to improvements in cardiovascular health.

Oral hygiene habits, including daily use of an antibacterial mouthwash or tongue scraper, have been found to not only reduce acute bacterial infection, but also numbers of bacteria present[30]. On the other hand, poor oral hygiene contributes to dysbiosis by accumulating a plaque biofilm which contains large number of microbes including nitrate-reducing bacteria[101]. This can cause dental infections and gingivitis by increasing pathogenic bacteria such as *Porphyromonas gingivalis*[85]. Studies have shown that patients with periodontal disease to have higher levels of salivary nitrite which may be partly derived from the reduction of nitrates by the oral bacteria. Since nitrite has been shown to have an antimicrobial effect against gastrointestinal and oral pathogens, it has been speculated that the salivary glands may respond to the periodontal infection by enhancing the secretion of nitrate and production of nitrite by the nitrate-reducing bacteria as a host defence mechanism[102]. This is thought to reduce the prevalence of the acidogenic bacteria which contribute to the development of dental caries. In agreement, Doel *et al.*[42] has reported a significant reduction in dental carries in study participants with high salivary nitrate concentration. Epidemiological studies have reported an association between periodontal disease with CVD. Although the cause-and-effect relationship has not been proven, studies have suggested that inflammation caused by the oral infection may contribute to the development and progression of the atherosclerotic plaque. Interestingly, periodontal pathogens have been identified in the atherosclerotic plaque, suggesting a direct role in CVD. However, to date, periodontal disease has not been considered to be a CVD risk marker[103]. Lifestyle habits such as smoking can also influence oral bacteria composition[74]. In a study conducted in nine non-smokers aged 20–45 years and five healthy active smokers (>20 cigarettes per week) aged 30–60 years, nitrate reduction activity was found to be over 80 % lower in smokers compared with non-smokers[74]. However, the low numbers of individuals within each group may have influenced the results observed.

As previously mentioned, dietary nitrates have been shown to interact with other food components such as lipids[72], with similar reports for polyphenols[104], alcohol[105] and proteins[25]. In particular, foods and beverages rich in polyphenols including apple, tea and orange juice have been shown to lead to a 3-fold increase in NO production in the stomach[106] and reduce endogenous N-nitrosamine formation. Along with polyphenols, the content of ethanol in red wine can also interact with nitrite forming ethyl nitrite which works as a nitrosation agent and may mediate NO effects. These interactions with other dietary components may therefore play a role in modulating the circulating NO levels and bioavailability of the nitrate and nitrite contained within foods.

In summary, a systematic approach was used to identify the studies that have determined the impact of oral bacteria on blood pressure in response to nitrate intake, from dietary sources or supplements. However, only a very limited number of human ($n$ 2) and animal ($n$ 3) studies have addressed this research question, with the remaining studies examining the importance of the

oral bacteria on the nitrate-reducing capacity on circulating nitrite concentrations and blood pressure. Based on our observations from these studies, there is accumulating evidence to suggest that absence of nitrate-reducing oral bacteria was associated with increasing blood pressure even when accompanied by a high nitrate intake. However, some of the studies failed to see any effects, which may be due to type of mouthwash used in the human studies or the method of application of the mouthwash in the animal study[29]. Sex, hypertension, and tongue cleaning were all found to be important potential determinants of the variability in the responses between participants. Of these, the dental hygiene practice of tongue cleaning, which is recommended by the American Dental Association, appeared to promote oral microbiota diversity and be associated with a greater ability to recover the tongue microbiome after mouthwash use. Potential mechanisms to explain the blood pressure-lowering effects of dietary nitrates included increases in plasma nitrite, *S*-nitrosothiols, nitro-fatty acids and vascular nitrosylation and cross-talk between the enterosalivary nitrate–nitrite–NO pathways and eNOS activity in the endothelial cells. However, the limited number of studies performed make it difficult to draw any firm conclusions from this literature review.

## Conclusions

With the increasing prevalence of non-communicable diseases there is an urgent need for further studies to investigate the role of the oral bacteria on cardiovascular health in response to dietary nitrate intake, and to determine the underlying mechanisms. With vascular function now recognised as an important prognostic marker for future CVD risk, studies incorporating real-time measures of vascular reactivity and tone are required. Furthermore, the use of rigorous methods to determine changes in the abundance and composition of the oral bacteria in response to intake of dietary nitrate would help to identify important nitrate-reducing bacteria related to changes in vascular function and determine whether these bacterial groups are also evident in the gut microbiome, a proposed modulator of chronic disease risk. Diets containing nitrate-rich foods may contain other bioactive components which could also contribute to CVD risk reduction, including fibres, vitamins, minerals and flavonoids. Such diets may offer a number of advantages over nitrate/nitrite supplemental use, not only due to the availability of other bioactive components, but also because of reports of vascular adaptation and risk of marked acute hypotension after supplemental nitrate use, not found with nitrate-rich diets[107]. With hypertension a major risk factor for CVD, more studies are needed to determine whether diets higher in nitrate-rich foods can be recommended for blood pressure lowering and disease prevention in healthy individuals and those at greater CVD risk.

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## Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0954422420000281

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## PUBLISHED ABSTRACT


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## Association of daily nitrate consumption with blood pressure and other risk factors for cardiovascular diseases in a representative UK population


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ABSTRACT

Dietary inorganic nitrate has been shown to lower blood pressure (BP) and improve endothelial function[1]. The main sources of dietary nitrate are vegetables (root and green leafy varieties) as well as drinking water but data available on dietary analysis software on nitrate levels in vegetables and vegetable-based foods is very limited. To date, very few studies have investigated the relationship between the level of consumption of dietary nitrate on BP and other cardiovascular disease (CVD) risk factors in a representative UK population. The aim of the study was to address this knowledge gap using data from the National Diet and Nutrition Survey (NDNS) years 1-8, a cross-sectional study conducted in 3339 men and women aged 19-64 y between 2008/09-2011/12. A comprehensive database was first developed to evaluate the nitrate and nitrite levels in vegetables, cured meats and composite dishes to more accurately estimate the dietary nitrate intakes of the NDNS participants. The population was then classified into quartiles of daily nitrate intake, with quartile 1 (Q1: 26-106 mg/d) and quartile 4 (Q4: 183-559 mg/d) representing diets with the lowest and highest intakes, respectively. ANCOVA analysis was performed to determine the relationship between the level of daily nitrate intake with available data on biomarkers of CVD risk (including BP (systolic, diastolic and pulse pressure), lipid profile, (total, high-density lipoprotein and low-density lipoprotein (LDL-C) cholesterol), C-reactive protein, anthropometric measures (body mass index and waist to hip ratio) and glycaemic control (glucose and glycated haemoglobin). There were significant differences in systolic (*P-trend*=0.008) and diastolic (*P-trend*=0.025) BP across increasing quartiles of dietary nitrate intake, with BP significantly lower in Q3 than all other quartiles. Pulse pressure (calculated as systolic–diastolic BP) was also found to be significantly different across quartiles (*P-trend*=0.001), with diets of participants in Q3 and Q4 being associated with significantly lower pulse pressures than those in Q1 (*P*-Q1 vs. Q3=0.005, *P*-Q1 vs. Q4=0.007). All of the other CVD risk markers were not different between quartile groups. Our preliminary results suggest that the level of dietary nitrate intake may be significantly associated with BP, a key independent CVD risk factor. There is an urgent need to more accurately estimate the dietary nitrate intake in the UK population and to determine whether the source of dietary nitrate (vegetables vs cured meats) impacts on the significant relationship with BP.

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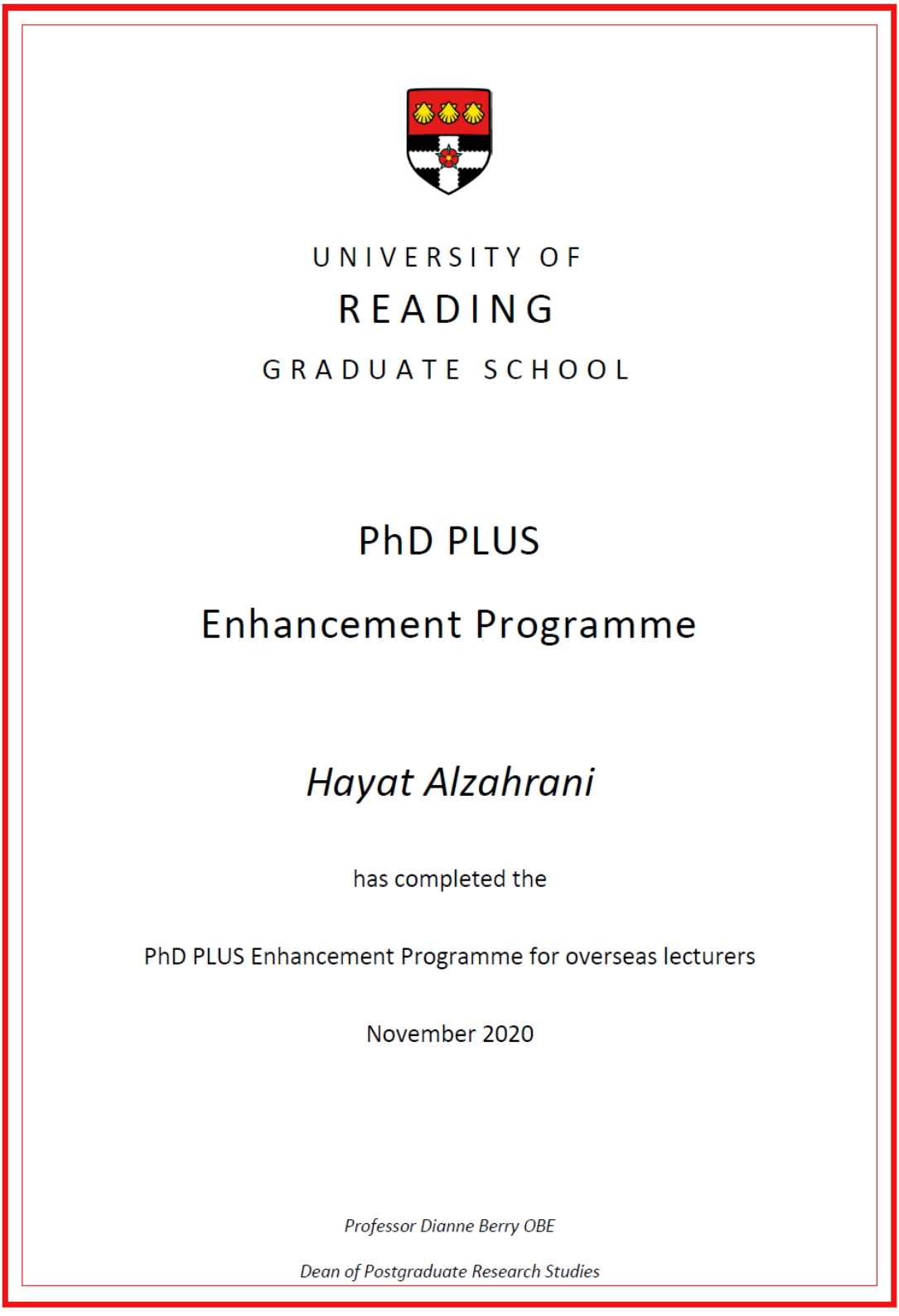

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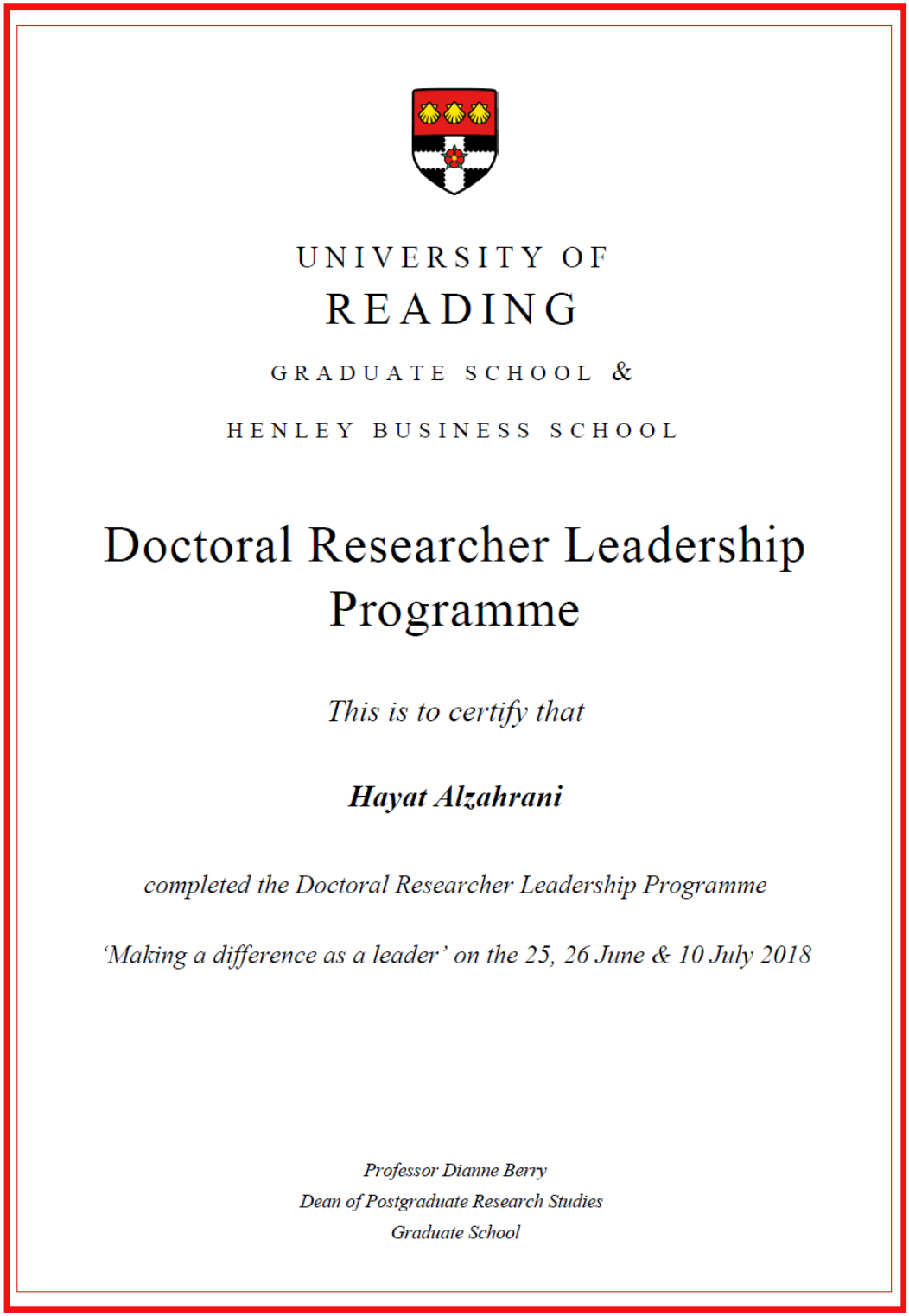

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