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PHD

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PRESSURE IN HEALTHY HUMANS

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UNIVERSITY OF PLYMOUTH

EFFECTS OF DIETARY NITRATE FROM VEGETABLES ON BLOOD PRESSURE IN HEALTHY HUMANS

by

ELIZABETH ANN ASHWORTH

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

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At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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Publications

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Signed.....

A handwritten signature in black ink, appearing to be 'K. A. A.', written over a horizontal line.

Date..... 20th February 2019

Abstract

Elizabeth Ann Ashworth

Effects of dietary nitrate from vegetables on blood pressure in healthy humans

Dietary nitrate is a naturally occurring component of vegetables and nitrate salts have been used for centuries as preservatives in processed meats. Up until recently, nitrate has been considered as a harmful contaminant. However, more recent research suggests that dietary nitrate supplements can reduce blood pressure (BP) and could be beneficial to cardiovascular health. This is important as 1 in 4 adults world-wide have hypertension, the largest single risk factor for cardiovascular disease. Three studies were conducted in order to assess the effects of dietary nitrate, obtained from vegetables, on BP in healthy humans.

The first study evaluated the effect of supplementing the diets of healthy young men with high-nitrate vegetables following a randomised crossover trial. They received vegetable boxes of either high-nitrate or low-nitrate vegetables over a two-week period. BP was measured and blood samples were obtained to analyse nitrate and nitrite concentrations prior to a moderate exercise test at the beginning and end of each two-week period.

The second study evaluated the same question but in a group of healthy young women. They were randomised in a crossover trial to receive boxes of high-nitrate vegetables, or to a control diet avoiding high-nitrate vegetables for one week. BP was measured and blood samples were obtained.

A third study was conducted to estimate dietary nitrate intake and its effect on BP and resting metabolic rate (RMR) in a group of healthy vegetarians, previously reported to have high nitrate intakes, compared to a similar group of omnivores. Following a single blind and non-randomised design, participants were provided with placebo mouthwash for seven days and then antibacterial mouthwash for a further seven days. BP and RMR were measured after both interventions. Dietary nitrate intake was estimated in both groups and blood and salivary samples were obtained.

This thesis concludes that consumption of high-nitrate vegetables lowered BP in healthy young women. However, this effect was attenuated in healthy young men. Additionally, there were no differences in nitrate intake, BP or RMR between vegetarians and omnivores.

Further research is required before dietary nitrate can be considered as a nutrient beneficial to cardiovascular health.

349 words

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List of abbreviations

ADI Acceptable daily intake

ANOVA Analysis of variance

BP Blood pressure

CVD Cardiovascular disease

DASH Dietary Approaches to Stop Hypertension

DBP Diastolic blood pressure

eNOS Endothelial NO synthase

FAO Food and Agriculture Organization

FFQ Food frequency questionnaire

FSA Food Standards Agency

GET Gas exchange threshold

MAFF Ministry of Agriculture, Fisheries and Food

MAP Mean arterial pressure

NO Nitric oxide

NOS Nitric oxide synthase

ONRC Oral Nitrate Reducing Capacity

RER Respiratory exchange ratio

RMR Resting metabolic rate

ROS Reactive oxygen species

RNI Reference Nutrient Intake

SBP Systolic blood pressure

UK United Kingdom of Great Britain and Northern Ireland

USA United States of America

VCO₂ Carbon dioxide production

VO₂ Oxygen consumption

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1 INTRODUCTION

1.1 Rationale

This thesis investigated the role of dietary nitrate consumption on blood pressure (BP) as a marker of cardiovascular health. Dietary nitrate is currently regarded as a contaminant, with an Acceptable Daily Intake (ADI) of 0-3.7 mg nitrate/kg body weight (~260 mg/70 kg person) due to previous health concerns. However, in the last decade, studies have shown that dietary nitrate in the form of supplements such as beetroot juice (~500 mg nitrate/dose) may enhance exercise capacity as well as cardiovascular health. Although the initial research interest was directed into sports performance, this thesis will mainly focus on the effects of dietary nitrate on BP. This is of particular interest to dietitians, who are not only involved in health promotion but also with the nutritional care of patients who have hypertension, a major public health problem in both the UK and globally.

1.2 Epidemic of hypertension: health and financial implications.

In 2017, there were over 9.5 million people in the UK registered with hypertension and a further estimated 5.5 million people in England alone with undiagnosed hypertension (Stroke Association, 2017). This is relevant to public health as hypertension is a major risk factor for cardiovascular diseases such as heart attack, stroke and heart failure; and contributes to ~50% of stroke (Stroke Association, 2017). Promotion of normal BP levels in the community could significantly reduce the risk of cardiovascular disease such as stroke (Seshadri *et al.*, 2006). For example, reducing SBP by 10 mmHg reduces the risk of cardiovascular events by ~20% (Stroke Association, 2017). A small reduction of ~3 mmHg across the population could result in a reduction in stroke in the UK by 11% and ischaemic heart disease by 6% (He, Pombo-Rodrigues & MacGregor, 2014). This reduction in SBP

has been achieved in various population groups by using various forms of nitrate supplements (Table 1.6, pp. 47). Not only would this BP reduction save lives and prevent significant disabilities, it would also represent a major cost saving to health services. For example, in 2008-2009, stroke cost the NHS at least £3 billion/year in direct care costs (Department of Health, 2010) within a wider economic cost of about £9 billion/year (including lost economic productivity and increased benefits payments) (Stroke Association, 2015).

Existing UK public health advice includes quantitative advice to eat 'at least 5-A-DAY' of a variety of fruit and vegetables, with 80 g portion sizes (NHS Choices, 2015a) in order to achieve the WHO target of 400 g/day (World Health Organisation, 1990). Similarly, advice to reduce BP includes increasing consumption of fruit and vegetables in line with the DASH diet (which encourages 3-6 portions of vegetables daily) as a key approach (Public Health England, 2014). However, there is no specific guidance on which fruit and vegetables should be eaten and this amount of fruit and vegetables seems to be greater than current consumption in the UK. According to data from the UK National Diet and Nutrition Survey, only about one third of adults meet the 'at least 5-A-Day' target (Public Health England, 2016). This is significantly more than in Canada where it has been estimated that nearly 90% of the adult population consume inadequate amounts of fruit and vegetables (Ekwaru *et al.*, 2016). The economic costs of not eating enough fruit and vegetables in Canada (which has a population size 50% lower than the UK) has been estimated at ~£2 billion/year (Ekwaru *et al.*, 2016) which is below the economic cost of stroke in the UK, but still represents a significant cost to their economy.

In summary, the UK has a high prevalence of hypertension, costing the health and care services billions of pounds per year, as well as wider costs to the economy. Evidence suggests poor compliance with public health guidance to eat fruit and vegetables and there is scant evidence to suggest that this guidance has any effect on high BP levels in

the UK. However, a variety of studies (Table 1.6) as well as systematic reviews (Jackson *et al.*, 2018; Lara *et al.*, 2016; Siervo *et al.*, 2013) have indicated that dietary nitrate could contribute towards reducing population BP, which in turn could reduce the incidence of CVD such as stroke. Therefore, there is a need to investigate whether the BP lowering effects demonstrated in studies using dietary nitrate supplements such as beetroot juice can be duplicated by fresh, whole high-nitrate vegetables.

1.3 What is dietary nitrate?

‘Dietary nitrate’ can be defined as a naturally occurring, unreactive, inorganic ion (NO_3^-) with a relative molecular mass of 62.005 (World Health Organization, 2010). It is produced by plants, bacteria and other microorganisms in the soil as part of the nitrogen cycle (Figure 1.1). The nitrogen cycle enables plants and animals to ‘fix’ atmospheric nitrogen which is necessary for the formation of amino acids and subsequent protein synthesis (Berg, Tymoczko & Stryer, 2002).

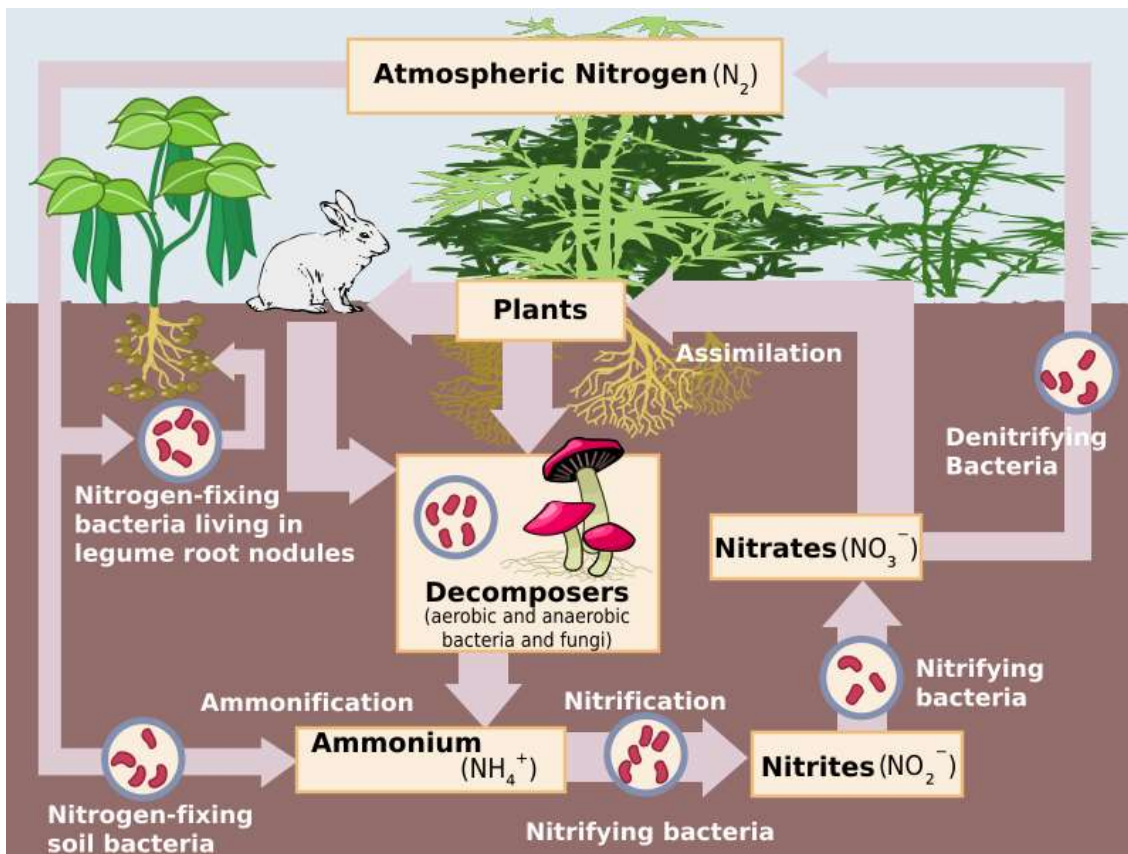


Figure 1.1. Schematic representation of the nitrogen cycle through the land environment illustrating assimilation of nitrate by plants (Environmental Protection Agency, 2004) (Figure reproduced under Creative Commons license).

Microorganisms in the roots of plants convert atmospheric nitrogen (N_2) into ammonia (NH_3) and subsequently nitrite (NO_2^-) and nitrate (NO_3^-) in a series of reactions (Figure 1.2) (Bernhard, 2012):

Figure 1.2. Chemical reactions of nitrogen fixation, ammonia oxidation and nitrite oxidation to form nitrate (Bernhard, 2012).

Inorganic dietary nitrate should not be mistaken for organic nitrates commonly used in cardiovascular medicine, such as glyceryl trinitrate (GTN) (Lundberg *et al.*, 2011; Parker & Gori, 2001). In addition, inorganic nitrate salts, such as potassium and sodium nitrate (KNO_3^- and NaNO_3^-) are food additives used in the production and preservation of cured meat and in some countries, fish. They have been used in some clinical trials as nitrate supplements (Section 1.9).

1.4 Sources of dietary nitrate

This section will mainly focus on dietary nitrate obtained from vegetables as well as nitrate supplements. Furthermore, nitrates from water supplies and food preservatives are important from a historical perspective and will be briefly discussed.

1.4.1 Dietary nitrate in vegetables

Vegetables, which can be defined as plants or part of plants used for food, are the main source of dietary nitrate. Green leafy vegetables such as rocket, spinach, kale, pak choy, certain types of lettuce and beetroot have the highest levels of nitrate, with mean values up to 468 mg/100g for rocket (European Food Safety Authority, 2008). In contrast, the nitrate content of legumes such as peas can be as low as 1 mg/100g (European Food Safety Authority, 2008).

Environmental factors significantly affect nitrate content of vegetable crops. For example, nitrate levels increase in crops grown in low light conditions, such as in dull or cloudy weather or when grown under cover. This is because light activates the enzyme nitrate reductase which stimulates reactions converting nitrate to glutamate (Noctor & Foyer, 1998; Weightman *et al.*, 2012). For example, a significant negative relationship was demonstrated between nitrate content of rocket and daily solar radiation, so that nitrate

content was reduced from ~7000 mg/kg to 2000 mg/kg as solar radiation increased (Weightman *et al.*, 2012).

Other environmental factors which impact on nitrate content include the amount of water received by the vegetable crops: too little water restricts nitrate uptake whereas too much dilutes nitrate in the soil (European Food Safety Authority, 2008). The available nitrogen supply also influences nitrate content and this is derived from existing soil mineral nitrogen, existing organic matter in the soil, breakdown of previous crops and fertiliser applied (Weightman *et al.*, 2012). For example, nitrate content of rocket increased from ~800 mg/kg to ~6000 mg/kg with increasing amounts of fertiliser (Weightman *et al.*, 2012). Due to all of these variables, vegetables grown under 'organic' conditions, even where no chemical fertilizer is applied, can have either higher or lower nitrate content than conventionally grown vegetables (Matallana González, Martínez-Tomé & Torija Isasa, 2010).

The many factors which can affect nitrate content are reflected in the results of different analyses. For example, one study sampled 25 different vegetables obtained from a market in Italy at nine sampling dates over a 15 month period (n=327) (Santamaria *et al.*, 1999). Vegetables were classified according to their nitrate content from 'very low' (< 200 mg nitrate/kg) to 'very high' (> 2500 mg nitrate/kg). Nitrate content showed large variations over the period of study; spinach was reported as having a mean nitrate content of 1845 mg/kg (range 547-3350 mg/kg) (Santamaria *et al.*, 1999) suggesting that this classification is arbitrary.

A subsequent risk assessment by the European Commission received over 40,000 analytical results using standardised assessments from 20 member states and Norway. This confirmed that nitrate content varies significantly in vegetables and between countries (European Commission, 2006; European Food Safety Authority, 2008) (Table 1.1). This risk assessment did not classify vegetables into 'low' or 'high' nitrate, but noted that 'leafy

vegetables exhibited high-nitrate levels' (European Food Safety Authority, 2008). In this case, spinach was reported as having a mean nitrate content of 1066 mg/kg (range 64-3048 mg/kg) (European Food Safety Authority, 2008).

Table 1.1. Nitrate content of raw vegetables (mg/kg) and estimated nitrate content per 80 g portion (mg/portion).

Vegetable (raw)	Nitrate content (mg/kg) ^a		Mean nitrate content per adult (80g) portion (mg) ^b
	Mean	Range (5 th - 95 th percentile)	
Rocket (rucola)	4677	1528-7340	374
Mixed Lettuce	2062	281-5242	165
Swiss chard	1690	178-3685	135
Beetroot	1379	110-3670	110
Celery	1103	18-3319	88
Spinach	1066	64-3048	85
Fennel	1024	25-3047	82
Carrots	296	21-1574	24
Cucumber	185	22-409	15
Onion	164	1-638	13
Cauliflower	148	7-390	12
Peppers (capsicum)	108	1-476	8
Tomatoes	43	1-144	3
Peas	30	1-100	2

^aData from European Food Safety Authority (European Food Safety Authority, 2008).

^bData for 80g portion sizes (NHS Choices, 2015b) based on WHO recommendations to eat at least 400g fruit and vegetables daily (World Health Organisation, 1990b).

Once harvested, nitrate content can be further affected by storage and cooking. For example, refrigeration or freezing of vegetables prevents nitrate loss (European Food Safety Authority, 2008). Storage at ambient temperature for more than 3 days virtually eliminated nitrate content of spinach and Chinese cabbage (Chung, Chou & Hwang, 2004), presumably due to nitrate reductase enzymes as there was a concomitant increase in nitrite content. As nitrate is highly soluble, it can be lost by washing, peeling and cooking. Boiling vegetables can reduce nitrate content by up to 75% (Ministry of

Agriculture Fisheries and Food, 1998a) whereas steaming retains nitrate compared to other cooking methods (Mozolewski & Smoczynski, 2004).

In summary, it is widely accepted that green leafy vegetables and beetroot have higher mean nitrate levels than other vegetables, but levels vary according to environmental conditions, storage and cooking.

1.4.2 Dietary nitrate supplements

In the last decade, various beetroot products (juice, concentrated 'shots', gels etc.) have been marketed primarily as ergogenic aids to enhance athletic performance. These dietary nitrate supplements are readily available online and contain varying amounts of nitrate (200-1500 mg nitrate/portion) (Table 1.2).

Table 1.2. Examples of beetroot containing products marketed as ergogenic aids to the athletic community with potential nitrate dose compared to ADI for 70kg person (259 mg nitrate/day).

Supplement name	Presentation /Supplier	Composition	Nitrate content (mg/portion)	Dosage instructions	Potential nitrate dose (mg/day)	% ADI for 70kg person
Beet It Organic – Original	75 cl bottle/James White Drinks, United Kingdom.	90% Beetroot juice, not from concentrate and 10% apple juice	600-1500*	None	Up to 1500	579%
Beet it Sport Nitrate 3000 (7 servings)	250 ml bottle/James White Drinks, United Kingdom.	Concentrated beetroot juice	429	1-2 X 35 ml servings before training and for up to 6 days prior to competition	~860	332%
Beet It Sport Nitrate 400 (1 serving)	70 ml bottle/James White Drinks, United Kingdom.	Concentrated beetroot juice and lemon juice	400	1-2 shots before training and for up to 6 days prior to competition	800	308%
Beet It Sport Beetroot Flapjack (1 serving)	40 g bar/James White Drinks, United Kingdom.	Oat flakes, beetroot juice concentrate, raisins, apple juice extract, sunflower oil	200	1 bar for training days, 2 for competition days	400	154%
BeetElite (20 servings)	200g Canister/ Human ⁿ , USA	Beetroot powder, flavourings, magnesium ascorbate	Not stated, equivalent to '6 beets'**	2 scoops (10g). 'Do not exceed 10g in 24 hours'	unknown	?

*ADI: Acceptable Daily Intake. *Obtained from James White Drinks Ltd (James White, 2108). **Obtained from HumanN (humanⁿ, 2018)*

1.4.3 Nitrate in water

To a much lesser extent, drinking water is a source of nitrate. In the UK, it has been estimated that tap water contributes 10-20 mg nitrate/day (compared to ~52-73 mg/day from food) (Ministry of Agriculture Fisheries and Food, 1992; Ministry of Agriculture Fisheries and Food, 1998a). Nitrate content of bottled 'mineral' waters varies according to brand, with some containing up to 8 mg/l (Table 1.3). Therefore, nitrate intake from water can vary according to volume consumed and whether tap or bottled water is consumed.

Table 1.3. Nitrate content (mg/l) of various mineral waters available from supermarkets in the UK.

Brand of bottled water*	Nitrate content (mg/l)
Buxton (still)	< 0.1
San Pellegrino (sparkling)	2.6
Highland Spring (still)	3.1
Brecon Carreg (still)	3.5
Evian (still)	3.7
Volvic (still)	7.3
Perrier (sparkling)	7.8

*Source: www.Ocado.com (Ocado.com, 2018)

1.4.4 Nitrate in food additives

Inorganic nitrate and nitrite salts have traditionally been used as food additives due to their antimicrobial properties (Ministry of Agriculture Fisheries and Food, 1998b). For example, nitrate (and nitrite) salts are used as preservatives in processed meats, such as bacon and sausages and other foods, due to their ability to inhibit the growth of *Clostridium botulinum* spores (European Food Safety Authority, 2008). The contribution of nitrates as preservatives is comparatively small in the UK diet at ~7 mg/day (European Food Safety Authority, 2008).

1.5 Dietary nitrate as a contaminant - historical perspective

Nitrate has been viewed as a harmful dietary contaminant since early research in rodents suggested that consumption could lead to the formation of *N*-nitroso compounds such as nitrosamines (Magee & Barnes, 1956; Terracini, Magee & Barnes, 1967). Further findings suggested a link between nitrate and development of gastric cancer in animals due to exposure to these *N*-nitroso compounds (Correa *et al.*, 1975). Subsequent evaluation of evidence by the WHO led to a conclusion that 'there is *inadequate evidence* in humans for the carcinogenicity of nitrate in food' (World Health Organization, 2010). However, 'inadequate evidence' suggests that results from the available evidence (up until 2006) cannot confirm the presence or absence of a carcinogenic effect and therefore there is still an element of doubt. A critical review of animal toxicology research and epidemiological studies challenges the view that dietary nitrate has potentially carcinogenic effects (Bryan *et al.*, 2012). This review concludes that the available epidemiological evidence does not support the hypothesis of an association between dietary nitrate intake and gastric cancer (Bryan *et al.*, 2012), although few figures regarding actual dietary nitrate intake of participants were reported. Recent data from a meta-analysis seems to confirm this view indicating an inverse relationship between nitrate intake (66-220 mg nitrate/day) and gastric cancer in humans (Song, Wu & Guan, 2015).

As well as associations with cancer, nitrate has also been linked to the development of infant methaemoglobinaemia or cyanosis (Comly, 1987). This condition can be caused by nitrite (derived from bacterial conversion of nitrate in water) oxidising ferrous iron in haemoglobin (Fe^{2+}) to the ferric form (Fe^{3+}), leading to the formation of methaemoglobin, which is unable to release oxygen to tissues (Fewtrell, 2004). In response to reports of methaemoglobinaemia, worldwide guidelines on nitrate content of drinking water were implemented. Drinking water cannot contain > 50 mg nitrate/l, as levels above 100 mg/l can give rise to this condition in bottle fed infants < 1 year old who have a gastrointestinal

infection (World Health Organization, 1958; World Health Organization, 2004). As a consequence, the agriculture industry must adhere to legislation to minimize the use of nitrate fertilizers in order to avoid contaminating the water supply, for example, by providing storage for animal waste (Council of the European Communities, 1991).

As a result of perceived health risks of both gastric cancer and methaemoglobinaemia, an Acceptable Daily Intake (ADI) for adults of 0-3.7 mg nitrate ion/kg body weight was established by the Joint FAO/WHO Expert Committee on Food Additives (Joint FAO/WHO Expert Committee on Food Additives, 2002). In 2008, and with a lack of new data, the European Food Safety Authority accepted the previous ADI (European Food Safety Authority, 2008). This is reflected in current UK and EC legislation (European Commission, 2011; Food Standards Agency, 2004). A recent re-evaluation of sodium and potassium nitrate food additives concluded that there was insufficient evidence to withdraw this ADI (European Food Safety Authority, 2017). Although the toxicity of nitrate is low, an oral lethal dose of nitrate for humans has been reported at ~330 mg/kg/day (equivalent to 23 g nitrate/70kg person) (Walker, 1990) and more recently at > 440 mg/kg/day (equivalent to 31g nitrate/70kg person) (European Food Safety Authority, 2017).

To ensure that intakes from fresh vegetables remain below the ADI, maximum levels of nitrate in crops such as lettuce, spinach and rocket are defined by legislation (European Commission, 2011) (Table 1.4). Similar restrictions apply to the addition of nitrate salts to meat products, such as bacon and ham (150 mg/kg sodium or potassium nitrate) (Food Standards Agency, 2015).

Nitrate salts are often associated with foods such as processed meats and sausages (Section 1.3.4) (Knekt *et al.*, 1999). Therefore, it is important to differentiate between sources of nitrate i.e. that which occurs naturally in vegetables and nitrate salts added to processed meats. This thesis will focus on dietary nitrate obtained from vegetables.

In summary, dietary nitrate has been viewed historically as a harmful, potentially carcinogenic contaminant. This view has reverberated down the decades with significant implications for food, agriculture and water industries today.

Table 1.4. European Commission Regulations on maximum levels of nitrate in foods (adapted from Commission Regulation (EU) No 1258/2011 (European Commission, 2011)).

Food	Harvesting conditions	Maximum nitrate content (mg/100g)
Fresh spinach		350
Frozen spinach		200
Lettuce (excluding 'Iceberg' type)	Harvested 1 st October to 31 st March:	500
	Grown under cover	400
	Grown in the open air	
	Harvested 1 st April to 30 September:	400
	Grown under cover	300
	Grown in the open air	
'Iceberg' type	Grown under cover	250
	Grown in the open air	200
Rocket	Harvested 1 st October to 31 st March:	700
		600
	Harvested 1 st April to 30 September:	
Processed cereal- based foods and baby foods for infants and young children		20

1.6 Estimation of dietary nitrate intake

Nitrate does not appear in UK Food Composition Tables (Food Standards Agency, 2014) or in the standard text book of Dietary Reference Values (Department of Health, 1991) because it is not considered a 'nutrient' (defined as 'a substance that provides nourishment essential for the maintenance of life and for growth' (Oxford Dictionaries, 2018)). Nevertheless, exposure analyses have been carried out in order to confirm the ADI is not exceeded (< 260 mg nitrate/day/70 kg person) (Table 1.5).

Table 1.5. Comparison of estimated intakes of dietary nitrate (mg/d) as exposure analyses, with brief description of methodology and limitations of each assessment method.

Methodology	Mean/median (mg/d)	Upper limit (mg/d)	Limitations	Authors
Population estimate using data from 1997 Total Diet Study	49	52	Food groups used instead of individual items. No correction for preparation losses.	Ministry of Agriculture Fisheries and Food, 1998a
Nitrate content of 11 different vegetables (raw and cooked) applied to 1994 Total Diet Study	104	151	Double counting of vegetable items. No correction for losses from cooking.	Ministry of Agriculture Fisheries and Food, 1998b
Duplicate diet study of vegetarians	52	178	Significant deterioration of samples. Underreporting.	Ministry of Agriculture Fisheries and Food, 2000
Scenario 1: Exposure estimate 400g vegetables/day	157	457	Regional and individual intake variations. No correction for preparation losses.	European Food Safety Authority, 2008
Scenario 1 amended: Exposure estimate 400g mix of fruit and vegetables/day	81	106	Regional and individual intake variations. No correction for preparation losses.	European Food Safety Authority, 2008
FFQ	98	125	Low correlation for vegetable intake in validation of FFQ.	Keszei <i>et al.</i> , 2014

Web based 24-hr dietary recall and questionnaire	106	525	Inaccurate completion of food diaries. No correction for preparation losses.	Jonvik <i>et al.</i> , 2017
FFQ	72	105	17.4 mg/day lower than 24 hr dietary recall.	Blekkenhorst <i>et al.</i> , 2017b
24 hr dietary recall	89	154	17.4 mg/day higher than FFQ.	Blekkenhorst <i>et al.</i> , 2017b
Mean	91	206		

FFQ, Food Frequency Questionnaires

Some estimates of daily nitrate intake have included nitrate intake from all food items consumed, whereas others have assessed nitrate from vegetables only. Each assessment has limitations, according to the method used. For instance, the Total Diet Study used food samples purchased at intervals from different locations around the UK (Table 1.5, pp. 32-33) (Ministry of Agriculture Fisheries and Food, 1998b). These foods were placed into broad groups, such as 'other vegetables', therefore losing data on individual variation of foods within each group. This is important as nitrate content of vegetables can vary significantly (Section 1.4.1). Most of the estimates were based on data using nitrate content of raw foods. Nitrate content can be significantly reduced by different methods of cooking (Section 1.4.1) and this level of detail was not captured in surveys and FFQ (Food Frequency Questionnaires). Therefore, total nitrate intakes from cooked vegetables could be significantly different than reported.

Certain population groups such as vegetarians may have higher nitrate intakes than the rest of the population (Ministry of Agriculture Fisheries and Food, 1992). Vegetarian and vegan diets have been studied due to potentially containing more vegetables and therefore higher nitrate content. The term 'vegetarian diet' can be defined as 'a diet of grains, pulses, legumes, nuts, seeds, vegetables, fruits, fungi, algae, yeast. A vegetarian does not eat meat, poultry, fish, shellfish' (Vegetarian Society, 2016). Within this definition, there are further classifications of vegetarian diets, such as lacto-ovo vegetarians and vegans. A longstanding UK study estimated that lacto-ovo vegetarians consumed almost five times more nitrate from vegetables (~190 mg nitrate/day) than omnivores (~40 mg nitrate/day) (Ministry of Agriculture Fisheries and Food, 1992). More recent research suggests that total dietary nitrate intake is nearly three times higher in vegetarians living in Poland (248 mg nitrate/day) than omnivores (91 mg nitrate/day) (Mitek, Anyzewska & Wawrzyniak, 2013). In contrast, a duplicate diet study of UK vegetarians did not suggest significantly higher intakes of nitrate from food than in the general population (vegetarians: 52 mg

nitrate/day, range 5.9–178 mg/day; general population 52 mg nitrate/day, 97.5 percentile 105 mg/day) (Ministry of Agriculture Fisheries and Food, 2000). However, this study noted that significant deterioration of the samples on arrival at the laboratory could have contributed to lower nitrate levels, as well as under-reporting by participants (Ministry of Agriculture Fisheries and Food, 2000).

Another population group with potentially high intakes of nitrate are athletes as nitrate supplements marketed as ergogenic aids contain significant amounts of nitrate (Table 1.2, p. 26). Conversely, a Dutch study has suggested that athletes may consume the majority of dietary nitrate from vegetables (106 mg nitrate/day, range 19-525 mg nitrate/day) (Jonvik *et al.*, 2017). Beetroot juice users tended to consume more vegetables, but no data was provided on nitrate intake from supplements (Jonvik *et al.*, 2017). There is no data available on dietary nitrate consumption of UK athletes and it has been suggested that supplementation with 310-558 mg nitrate/d may be necessary to improve exercise performance (Jones, 2014). Thus, it is possible that amounts higher than the ADI are being regularly consumed, but little research has been carried out in this area. In addition, beetroot can cause a known side effect of beeturia (pink colouration of urine). This can occur after consumption of 100g beetroot (Watson, Luke & Inall, 1963), which could provide 11-367 mg nitrate/100g (European Food Safety Authority, 2008).

In summary it is difficult to obtain valid and reliable data on nitrate intake from vegetables, as numerous methods of dietary assessment together with different databases for nitrate content have been used. Certain groups have been identified as potentially having higher nitrate intakes, such as vegetarians and athletes taking nitrate supplements.

1.7 Recognition of the role of nitric oxide

This section will discuss the important physiological role of nitric oxide (NO) and how it came to be recognized. Until relatively recently NO was regarded as an environmental contaminant, similarly to dietary nitrate.

Early research into the prevention of hypertension and cardiovascular disease was directed towards drugs which promote vasodilation (dilatation of blood vessels decreases BP). Acetylcholine is a potent vasodilator and in 1980 it was demonstrated that its activity was dependent on the presence of endothelial cells lining the blood vessels (Furchgott & Zawadzki, 1980). This suggested that the endothelial cells produced a substance which caused vasodilation and this substance became known as endothelium-derived relaxing factor (EDRF) (Furchgott & Martin, 1985). Following a separate line of experiments, earlier research had shown that the effects of nitrates (such as nitro-glycerine, often used to relieve angina) to cause relaxation of vascular smooth muscle were mediated by NO (Arnold *et al.*, 1977; Rodeberg *et al.*, 1995). NO was found in *in vitro* animal experiments to activate guanylate cyclase and increase concentrations of cyclic GMP (cGMP) associated with relaxation of smooth muscle (Arnold *et al.*, 1977; Katsuki & Murad, 1977). Finally, in 1987, two separate groups of researchers published the suggestion that EDRF and NO was one and the same molecule (Ignarro *et al.*, 1987; Palmer, Ferrige & Moncada, 1987). Before this, it had not been recognized that NO had any specific role or that it was even synthesized in mammalian physiology as it was considered as an atmospheric pollutant (Moncada & Higgs, 2006).

The source of NO was identified to be the terminal guanidine nitrogen atoms of the amino acid L-arginine, suggesting that L-arginine was the precursor for NO synthesis (Palmer, Ashton & Moncada, 1988). Furthermore, the formation of NO from L-arginine is catalysed by the enzyme endothelial NO synthase (eNOS), activated by sheer stress (force) of blood

passing over the endothelium (Moncada & Higgs, 2006; Palmer, Ashton & Moncada, 1988).

The results from these *in vitro* experiments were then extended to *in vivo* animal studies, where NO production was inhibited by administration of an L-arginine analogue, N-monomethyl-L-arginine (L-NMMA) (Rees, Palmer & Moncada, 1989). This resulted in an increase in mean arterial BP in rabbits in a dose dependent manner, which could be reversed by administration of L-arginine (Rees, Palmer & Moncada, 1989). This was the first evidence that L-arginine and NO were significantly involved in the regulation of BP (Rees, Palmer & Moncada, 1989).

NO formed in the endothelial cells diffuses to the adjacent smooth muscle cells, increasing production of cGMP, which leads to relaxation of the smooth muscle around the periphery of the blood vessels and increased blood flow (Figure 1.3) (Sandoo *et al.*, 2010). Further effects of NO derived from the endothelium include inhibition of platelet adhesion (attachment of platelets to non-platelet surfaces, such as after trauma) and aggregation (progressive accumulation of platelets) both of which are implicated in the development of atherosclerosis (Radomski & Salas, 1995).

NO is recognized as having key physiological roles in the maintenance of vascular tone, neurotransmitter function in the central and peripheral nervous systems as well as immunological cellular defence (Moncada & Higgs, 2006). In addition, NO inhibits cytochrome c oxidase, the terminal enzyme in the mitochondrial oxidative phosphorylation pathway, therefore regulating cell respiration (Moncada & Erusalimsky, 2002). Reduced generation or activation of NO, eNOS or cGMP may contribute to the development of pulmonary arterial hypertension (Klinger & Kadowitz, 2017). It is widely accepted that decreased production of NO is associated with the development of hypertension with ageing (Heilpern, 2008).

NO was designated Molecule of the Year in 1992 in recognition of its physiological importance as a signalling molecule (Koshland, 1992) and scientists involved in its discovery were awarded the 1998 Nobel Prize for Physiology and Medicine (SoRelle, 1998).

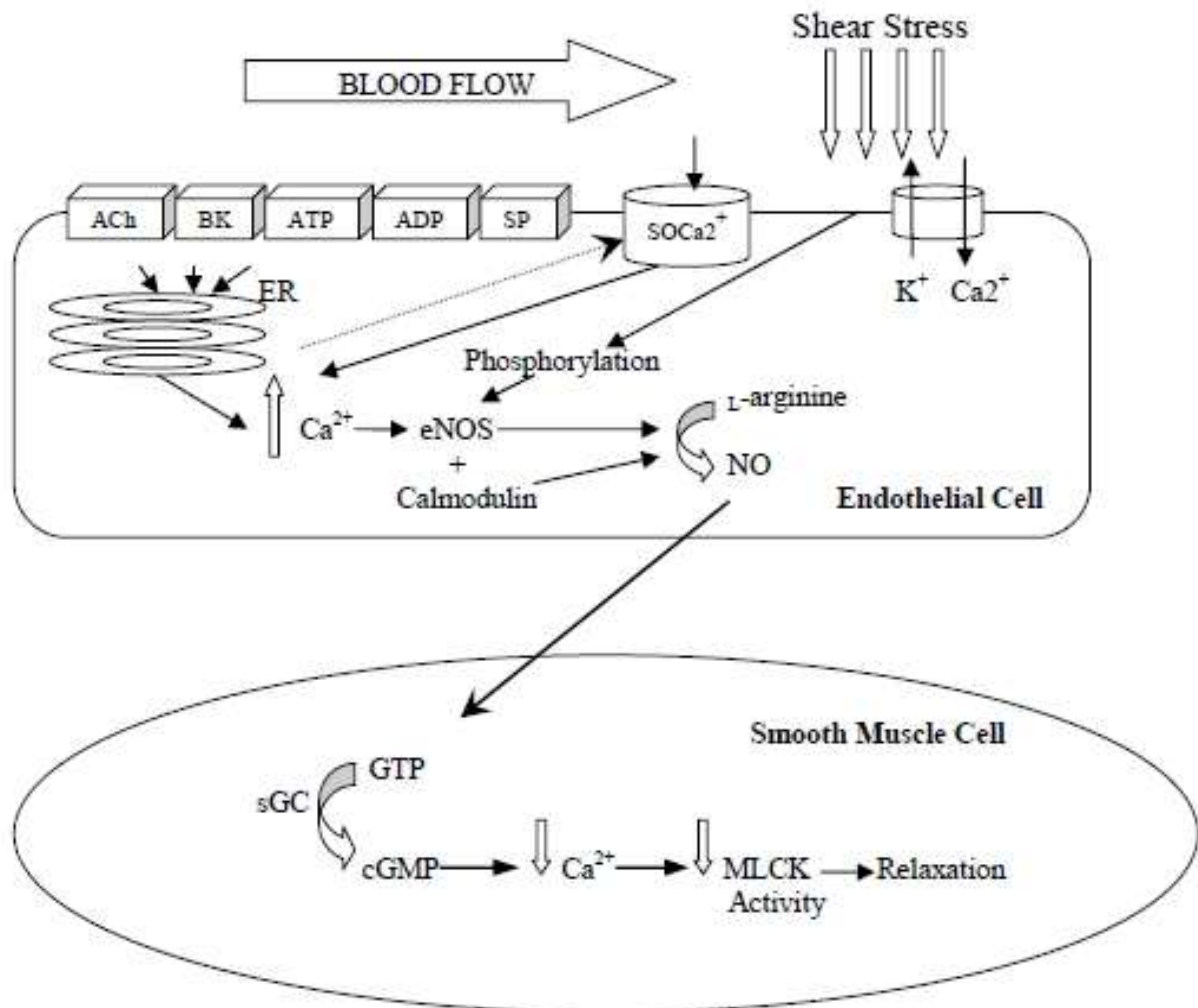


Figure 1.3 Endothelial NO production and its actions in the vascular smooth muscle cell.

Figure reproduced from (Sandoo et al., 2010) under Creative Commons license. ACh, acetylcholine; BK, bradykinin; ATP, adenosine triphosphate; ADP, adenosine diphosphate; SP, substance P; SOCa²⁺, store-operated Ca²⁺ channel; ER, endoplasmic reticulum; sGC, soluble guanylyl cyclase; cGMP, cyclic guanosine-3', 5-monophosphate; MLCK, myosin light chain kinase.

1.8 The nitrate-nitrite-nitric oxide pathway

In 1994, two research groups in the UK and Sweden simultaneously published the discovery that NO could be generated in the stomach from salivary nitrite and linked this production to inorganic nitrate in the diet (Benjamin *et al.*, 1994; Lundberg *et al.*, 1994). This changed the previous view of nitrate as it was initially assumed that nitrate was an inert end product from the L-arginine-NOS-NO pathway.

Animal experiments suggested that NO was generated systemically from nitrite in acidotic conditions (Zweier *et al.*, 1995) and correlated with vasodilation (Modin *et al.*, 2001). Furthermore, human studies suggested that plasma and red blood cells could be 'loaded' with NO, by using NO solutions, NO gas or NO donors (Cosby *et al.*, 2003). This was demonstrated by infusing 75 mg sodium nitrite into forearms of healthy volunteers which increased blood flow by 175% before and after handgrip exercise (Cosby *et al.*, 2003). This vasodilation was associated with a mean reduction in systemic BP of ~7 mmHg (Cosby *et al.*, 2003). Importantly, this study reported that the production of NO still occurred after inhibition of the activating enzyme, endothelial NO synthase (eNOS) by L-NMMA infusion, suggesting that the infused nitrite was reduced to NO during exercise-induced physiological stress (Cosby *et al.*, 2003). These authors suggested that nitrite, present in plasma, erythrocytes and tissues, could act as a major vascular storage pool of NO, so that NO is generated along the physiological oxygen gradient in conditions of low oxygen tension or hypoxia (Cosby *et al.*, 2003).

It was recognized that after a sodium nitrate load, plasma nitrate and nitrite bioavailability¹ were significantly increased (Lundberg & Govoni, 2004). Further experiments confirmed this hypothesis showing that nitrate and nitrite can be physiologically recycled in blood and tissues to form NO through an entero-salivary pathway, (Lundberg, Weitzberg & Gladwin,

¹ Bioavailability can be defined as the degree to which a substance becomes available to tissues.

2008). This pathway requires the reduction of nitrate to nitrite. As mammalian cells do not contain nitrate reductase enzymes, it was proposed that the oral microbiome was mainly responsible for this NOS independent pathway (Lundberg & Govoni, 2004). Once nitrate is reduced to nitrite, numerous pathways exist in tissues to further reduce nitrite to NO and these pathways are increasingly activated in conditions of hypoxia and acidosis, thus complementing the arginine-NOS-NO pathway, which is oxygen dependent (Lundberg, Weitzberg & Gladwin, 2008). The effects of increased NO bioavailability from dietary nitrate have recently been reviewed and include vasodilation, improved endothelial function and vascular integrity (Carlström, Lundberg & Weitzberg, 2018). These may be beneficial in conditions of reduced NO bioavailability, such as hypertension and cardiovascular disease (Carlström, Lundberg & Weitzberg, 2018).

Dietary nitrate from vegetables is absorbed very effectively and has an absolute bioavailability of ~100% (van Velzen *et al.*, 2008). After consumption of high-nitrate vegetables, nitrate is absorbed from the duodenum into the bloodstream, rapidly increasing plasma nitrate concentrations (Gangolli *et al.*, 1994; Spiegelhalder, Eisenbrand & Preussmann, 1976). Plasma nitrate concentrations increase within 30 minutes, peak at 1.5-1.8 hours and remain high for several hours, returning to baseline after 24 hours (van Velzen *et al.*, 2008). Once absorbed into plasma, nitrate is actively taken up by the salivary glands. Although this mechanism was previously unknown, it has been suggested that a protein (sialin) acts as a nitrate transporter in salivary cells (Qu *et al.*, 2016). Salivary glands concentrate nitrate so that saliva levels are at least 10-20 times higher than plasma nitrate concentrations (Lundberg & Weitzberg, 2009). This concentrated salivary nitrate is then secreted into the oral cavity, where up to 25% is reduced to nitrite (Spiegelhalder, Eisenbrand & Preussmann, 1976). The key step of this entero-salivary pathway is performed by facultative anaerobic bacteria (i.e. bacteria which switch from aerobic to anaerobic respiration depending on oxygen availability) which use nitrate as an alternative

electron acceptor to gain ATP in the absence of oxygen (Lundberg, Weitzberg & Gladwin, 2008). The reaction is confined to a specialised area on the posterior surface of the tongue, heavily colonised by bacteria, such as *Veillonella* species, *Actinomyces* species, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Corynebacterium* (Duncan *et al.*, 1995; Duncan *et al.*, 1997; Hyde *et al.*, 2014). Evidence for the importance of oral bacteria (known as the oral microbiome) has been provided by various studies using antibacterial mouthwash. For example, the effects of antibacterial mouthwash resulted in increased BP (systolic BP 3.5 ± 1.0 mm Hg, $P = 0.003$; diastolic BP 2.2 ± 1.0 mm Hg, $P = 0.038$), and the abolition of a rise in plasma nitrite after a dietary nitrate supplement (Govoni *et al.*, 2008; Kapil *et al.*, 2013). It has been suggested that the oral microbiome plays an important role in the development of chronic diseases such as diabetes (Long *et al.*, 2017), with exposure to certain antibiotics as well as antibacterial mouthwash associated with increased risk (Boursi *et al.*, 2015; Joshipura *et al.*, 2017). Therefore, the importance of the oral microbiome should not be underestimated.

When swallowed, some salivary nitrite is rapidly protonated to nitrous acid, which decomposes to NO and other nitrogen oxides in the stomach (Lundberg, Weitzberg & Gladwin, 2008). Gastric NO has an important role in maintaining gastric integrity, by being part of an antimicrobial effect on pathogenic organisms such as *Salmonella* and *Shigella* sp. as well as increasing gastric mucosal flow and mucus generation (Lundberg & Weitzberg, 2013).

An acidic environment is essential for reduction of nitrite to NO, as inhibition of acid secretion by proton pump inhibitors can virtually abolish NO production (Lundberg *et al.*, 1994). However, most salivary nitrite is rapidly absorbed into the circulation, elevating plasma nitrite concentrations within 15 minutes of a nitrate dose (Lundberg & Govoni, 2004). The mechanism for nitrite absorption is, as yet, unknown (Lundberg & Govoni, 2004), but appears to have an absolute bioavailability of 95-98% (Hunault *et al.*, 2009).

Once absorbed, nitrite reduction to NO in blood and tissues can be catalysed by various proteins and enzymes, such as xanthine oxidase, cytochrome P450, deoxyhemoglobin and deoxymyoglobin, and NO production is enhanced in acidic/hypoxic conditions (Lundberg & Govoni, 2004; Lundberg & Weitzberg, 2009) (Figure 1.4).

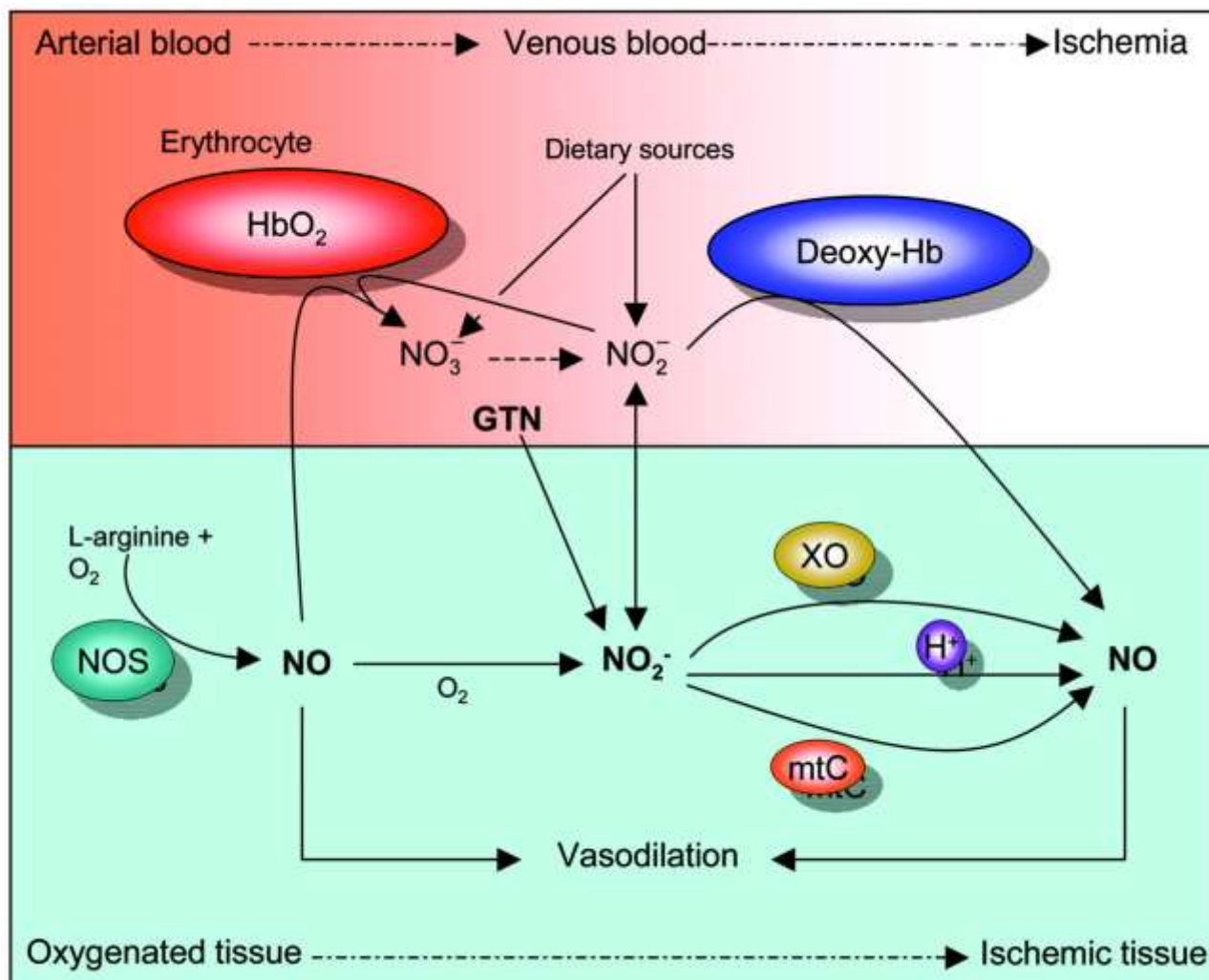


Figure 1.4 NO generation in oxygenated and ischaemic tissue.

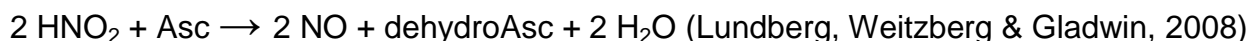
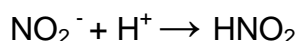
Image reproduced with permission of the rights holder, RightsLink. HbO₂, oxyhaemoglobin; deoxy-Hb, deoxyhaemoglobin; XO, xanthine oxidase; mtC, mitochondrial cytochromes; H⁺, protons (Lundberg & Weitzberg, 2005).

This may be important in conditions such as myocardial infarction and ischemic stroke; under ischaemic conditions, intracellular acidosis occurs and as pH reduces from 7 to 6, NO generation increases twelve-fold (Li *et al.*, 2008; Lundberg *et al.*, 2011). Recent research suggests that patients with pulmonary arterial hypertension have about a third of the levels of plasma nitrate and nitrite compared to healthy controls (Zhang *et al.*, 2016),

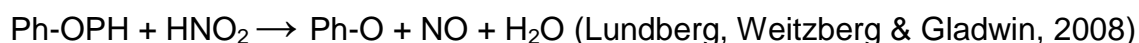
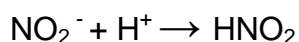
further suggesting that impaired NO synthesis is associated with the development of hypertension (Klinger, 2016).

The entero-salivary pathway accounts for ~25% of ingested nitrate. The remaining ~75% is excreted in the urine (Pannala *et al.*, 2003). However, recent animal research suggests that skeletal muscle tissue acts as a large storage pool for nitrate in mammals, as well as the liver (Gilliard *et al.*, 2018). These authors suggests that in times of starvation, skeletal muscle nitrate stores are used to support physiological function, especially under hypoxic conditions (Gilliard *et al.*, 2018).

Other dietary components are involved in NO production. Vitamin C, an antioxidant found in fruit and vegetables, enhances NO production from nitrite (Lundberg *et al.*, 2006). After a meal, Vitamin C is secreted into the stomach lumen with gastric juice and the reduced form, ascorbate (Asc), reacts with nitrite to generate NO (Pereira *et al.*, 2013), as follows:



Vitamin C also inhibits the formation of nitrosating compounds, such as carcinogenic nitrosamines. It has been suggested that this is the reason why high-nitrate vegetable consumption is not associated with an increased incidence of cancer (Lundberg & Weitzberg, 2013), contrary to previous dogma (Section 1.5). Similarly, it has been recognised that polyphenols (Ph-OPH) such as quercetin, also found in fruit and vegetables, increase NO production in the stomach (Khoo *et al.*, 2010):



Furthermore, mono-unsaturated fatty acids, such as in olive oil, react with NO to form nitrated fatty acids, which act as anti-inflammatory agents (Borniquel *et al.*, 2010). In addition, other dietary components may contribute to NO production. For example, ethanol

from alcoholic drinks is nitrosated to ethyl nitrite, which releases NO (Rocha *et al.*, 2015). Vitamin E given as alpha-tocopherol supplements in animal studies increased NO availability (Newaz *et al.*, 1999). These dietary components are important when considering epidemiological data on protective aspects of diets as a whole, rather than individual nutrients (Figure 1.5) (Section 1.13 and 1.14).

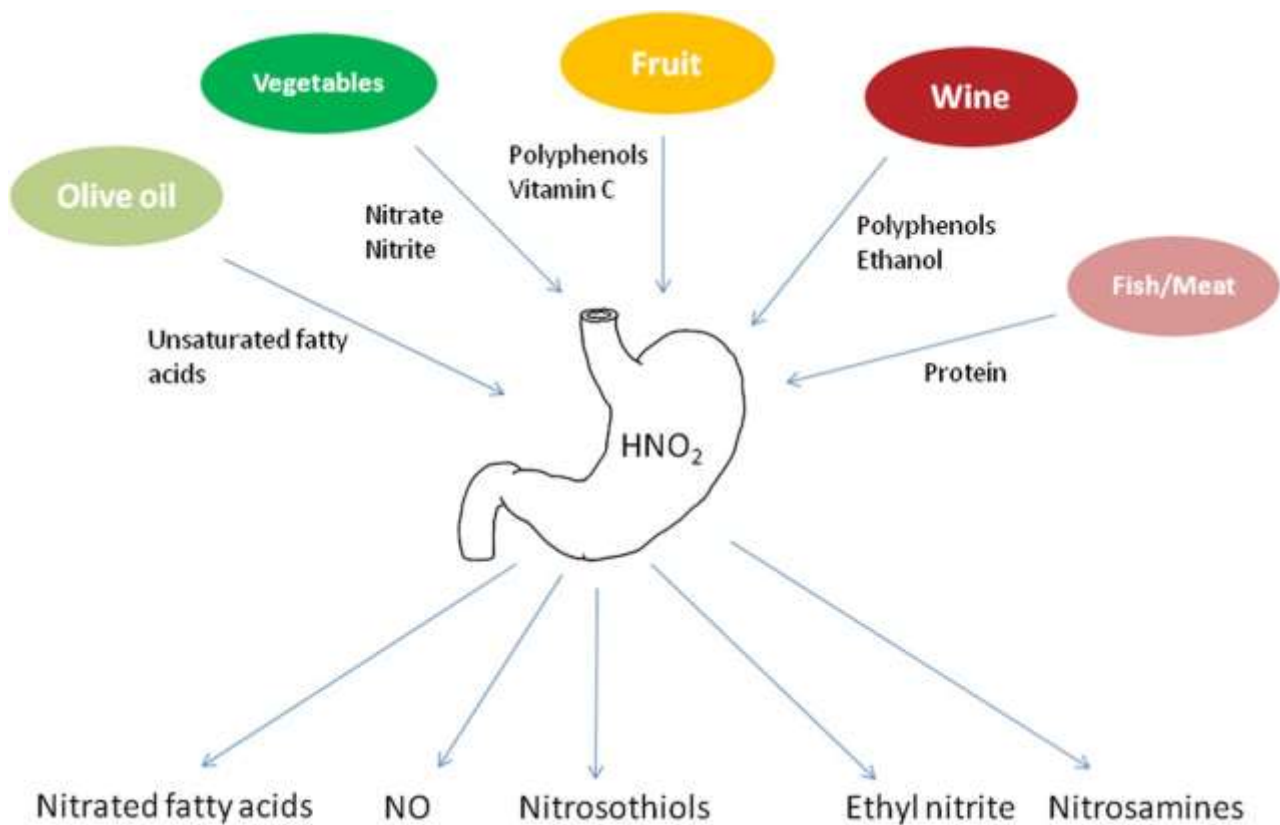


Figure 1.5. The interaction between various dietary components and acidified nitrite in the stomach to form novel biologically active compounds. Image reproduced with permission of the rights holder, RightsLink. HNO_2 , acidified nitrite. (Lundberg & Weitzberg, 2013).

In summary, this section has described the entero-salivary circulation of the nitrate-nitrite-NO pathway. Ingested dietary nitrate, primarily from vegetables, is rapidly absorbed from the gastrointestinal tract, concentrated by the salivary glands and secreted into the oral cavity. Bacteria in the oral cavity reduce salivary nitrate to nitrite, which is then swallowed and reacts with acid in the stomach to generate nitric oxide (Figure 1.6). The nitrate-nitrite-NO pathway is enhanced when oxygen availability is reduced and may be especially

beneficial in conditions of hypoxia and ischaemia. The role of dietary nitrate and the oral microbiome has been recognized as integral to this pathway.

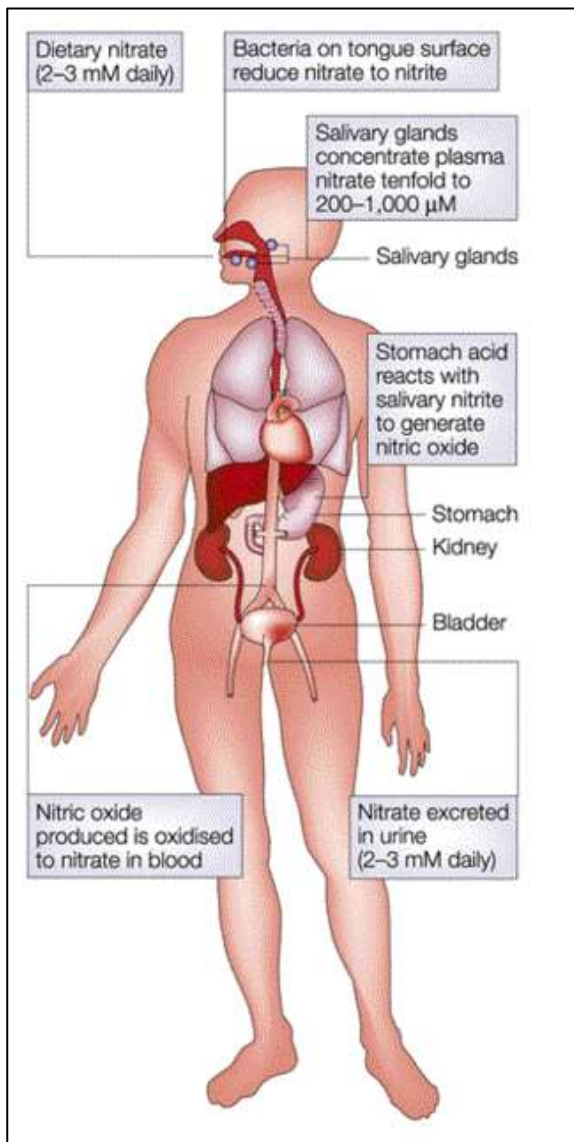


Figure 1.6. Entero-salivary circulation of nitrate in humans.

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(Correction advised by author: 2-3 mM should read 2-3 mmol). (Lundberg et al., 2004).

1.9 The effect of dietary nitrate on blood pressure

The first evidence showing that dietary nitrate could play an important role in vascular control was published by Larsen *et al.* (2006). This study demonstrated that a dose of sodium nitrate (0.1 mmol/kg/day, equivalent to ~500 mg nitrate) reduced diastolic BP (DBP) by ~4 mmHg compared to a placebo dose of sodium chloride in young, healthy individuals. This dose of nitrate was chosen as equivalent to '150-250 g of a nitrate-rich vegetables, such as beetroot or lettuce' (Larsen *et al.*, 2006), but no figures were provided regarding the nitrate content of these vegetables. This study significantly changed the view on the physiological effects of dietary nitrate. Multiple studies investigating the role of dietary nitrate have been published since, showing a potential role of dietary nitrate on BP regulation. A systematic review and meta-analysis of such studies published from 2006-2013 concluded that inorganic nitrate and beetroot juice supplements reduced systolic BP (SBP) by 4.4 mmHg (95% CI: -5.9, -2.8, $P < 0.001$) and DBP by 1.1 mmHg (95% CI: -2.2, 0.1, $P = 0.06$) in a mixed sample of subjects with and without health comorbidities (Siervo *et al.*, 2013). The nitrate dose ranged from 157-2790 mg and changes in SBP significantly correlated with the nitrate dose (Siervo *et al.*, 2013). Similarly, a further review by Hobbs *et al.* (2013) of studies from 2006-2012 concluded that there is a significant inverse relationship between nitrate dose and SBP (Hobbs, George & Lovegrove, 2013). This review suggested that doses of nitrate as low as 186 mg reduced SBP by 3 mmHg (Hobbs, George & Lovegrove, 2013).

In agreement with these results, 26 further intervention studies have been published (Table 1.6). These studies found significant reductions in BP after various forms of nitrate supplementation in a wide range of participants, including patients with hypertension, chronic obstructive pulmonary disease (COPD), chronic kidney disease (CKD) and heart failure.

Table 1.6. Summary of nitrate supplement research studies (2013-2018) with a significant ($P < 0.05$) effect on systolic or diastolic blood pressure, with nitrate dose, participant characteristics and other significant findings.

Supplement	Nitrate dose (mg/d)*	Reduction SBP (mmHg)	Reduction DBP (mmHg)	Participant characteristics & mean age (years)	Other significant findings	Authors
Beetroot juice	217	11	10	HT (53)	↑ vasodilation	Ghosh <i>et al.</i> , 2013
Beetroot juice	595	5	3	Healthy (64)	↑ respiratory response during treadmill walking	Kelly <i>et al.</i> , 2013
Beetroot juice (3 doses)	260	5	NS	Healthy (23)	↓ O ₂ consumption of moderate intensity exercise	Wylie <i>et al.</i> , 2013b
	521	10	3			
	1042	9	4			
Beetroot bread	139	NS	7	Healthy (31)	↑ vasodilation	Hobbs <i>et al.</i> , 2013
Spinach	220	8	NS	Healthy (59)	↑ vasodilation	Liu <i>et al.</i> , 2013
Beetroot juice	750	5	NS	Healthy (21)	↑ cerebral blood flow	Bond <i>et al.</i> , 2013
					↓ O ₂ . Consumption	
Beetroot juice	165	7	NS	Overweight/obese (62)		Jajja <i>et al.</i> , 2014
Sodium nitrate	~7mg/kg**	8	NS	Risk of CVD (63)	↑ vasodilation	Rammos <i>et al.</i> , 2014
High nitrate vegetables	339	4	NS	Healthy (20)		Ashworth <i>et al.</i> , 2015b
Beetroot juice	470	8	NS	COPD (70)	↑ exercise capacity	Berry <i>et al.</i> , 2015
Spinach soup	845	3	3	Healthy (25)	↑ vasodilation	Jovanovski <i>et al.</i> , 2015
Beetroot juice	400	8	2	HT (57)	↑ vasodilation	Kapil <i>et al.</i> , 2015

Beetroot juice	450	NS	12	Healthy (24)	↑ vasodilatation	Keen <i>et al.</i> , 2015
Beetroot juice	800	12	2	COPD (69)	↑ exercise capacity	Kerley <i>et al.</i> , 2015
Beetroot juice	800	NS	6	COPD (68)	↓ O ₂ Consumption	Curtis <i>et al.</i> , 2015
Potassium nitrate	360	4 (NS)	5	Healthy (23)	↑ vasodilation	Alsop & Hauton, 2016
Beetroot gel	397	6	5	Healthy (27)		Silva <i>et al.</i> , 2016
Beetroot juice	378	7	NS	Heart failure (69)	↑ exercise capacity	Eggebeen <i>et al.</i> , 2016
Beetroot juice	800	5	↓***	Healthy (28)		Jonvik <i>et al.</i> , 2016
Rocket drink	800	6				
Spinach drink	800	7				
Raw beetroot	**	7	5	HT (25-68)	↑ vasodilation	Asgary <i>et al.</i> , 2016
Cooked beetroot		5	4		↓ TC and LDL	
Beetroot juice	304	8	9	CKD (72)	↓ renal resistance index	Kemmner <i>et al.</i> , 2017
Beetroot juice	800	8	6	Pre-HT (64)	↓ granulocyte and platelet activation	Raubenheimer <i>et al.</i> , 2017
Beetroot juice	500	10	NS	Healthy (28)	↑ NO species after beetroot juice	Mcllvanna <i>et al.</i> , 2017
Chard gel	500	12	NS			
Beetroot juice	357	5	3	Healthy (24)		McDonagh <i>et al.</i> , 2018
Beetroot flapjack	357	NS	4			
Beetroot juice	769	CI 1-6	CI 1-4	Healthy (27)	Nitrate reducing bacteria correlated with salivary nitrite	Burleigh <i>et al.</i> , 2018
Beetroot juice	800	8	4	Obese/HT (63)		Kerley <i>et al.</i> , 2018

*NS, non-significant; ↑ improved or increased; ↓ reduced or decreased; SBP, systolic blood pressure; DBP, diastolic blood pressure; HT, hypertension; COPD, Chronic obstructive pulmonary disease; CVD, cardiovascular disease; CKD, chronic kidney disease; CI, confidence interval. *Mean reported nitrate dose of all studies ~ 500 mg/d, range 139-1042 mg/d. **Mean dose not reported. ***Absolute values not reported.*

Thus, according to all the previous evidence, it seems that dietary inorganic nitrate consumption within a range of 139-1042 mg can induce reductions in SBP (3-12 mmHg) and DBP (2-12 mmHg) in both healthy and clinical populations. Accordingly, it has been suggested that guidelines limiting the acceptable intake of nitrate should be reviewed (Lundberg *et al.*, 2009). Furthermore, it has been suggested that nitrate supplementation in the diet may help reduce the world-wide prevalence of CVD (Kapil *et al.*, 2010) and nitrate obtained from vegetables should be recognised as a nutrient necessary for cardiovascular health (Bryan & Ivy, 2015; Hord, Tang & Bryan, 2009).

However, there are also studies which have failed to see positive effects of dietary nitrate on BP in both healthy young and old subjects as well as patients with cardiovascular risk factors, hypertension, heart failure, type 2 diabetes, obesity and COPD (Table 1.7). This difference in results after dietary nitrate supplementation may be related to several factors (Table 1.8). For example, a recent study found that 70 ml of concentrated beetroot juice, which was expected to provide between 300-400 mg of nitrate, in fact yielded only 165 mg (Jajja *et al.*, 2014). This could be due to environmental factors, soil mineral nitrogen and storage conditions (Section 1.4.1). In addition, some studies included participants taking anti-hypertensive medications or hypoglycaemic medications which could affect NO metabolism (Bondonno *et al.*, 2015b; Gilchrist *et al.*, 2013; Shepherd *et al.*, 2015). Furthermore, the recent use of antibiotics or routine use of antibacterial mouthwash was not reported in some studies (Aamand *et al.*, 2013; Gilchrist *et al.*, 2013; Haldar *et al.*, 2015; Kim *et al.*, 2014; Lara *et al.*, 2015; Wightman *et al.*, 2015). These factors as well as experimental conditions such as nitrate source can affect the response of BP to dietary nitrate and could help to explain the non-significant findings (James *et al.*, 2015). Other potential confounding factors should be considered, such as exposure to ultraviolet radiation, which has previously been shown to reduce BP (Feelisch *et al.*, 2010; Monaghan *et al.*, 2018). Skin exposure to ultraviolet radiation stimulates the release of NO

and increases plasma nitrite concentrations (Feelisch *et al.*, 2010) but subsequent effects on BP have not been confirmed (Monaghan *et al.*, 2018). As outlined in Section 1.8, the composition of the oral microbiome is key to the nitrate-nitrite-NO pathway and can be affected by nitrate intake (Velmurugan *et al.*, 2016). Few of the studies which found non-significant changes in BP (Table 1.7) have investigated the oral microbiome and further studies are needed to clarify the lack of positive outcomes. Finally, reported differences in BP may depend on the method of BP measurement itself. In the diagnosis of hypertension, 24-hour ambulatory (ABPM) and home BP measurements are recommended as more accurate than clinic BP measurement (Williams *et al.*, 2018). This is due to potential bias caused by the phenomenon of ‘white coat’ hypertension, whereby BP of patients increases in a clinical setting. A recent systematic review and meta-analysis of trials comparing the effects of beetroot juice or inorganic nitrate to placebo suggested that the significant effect size was driven by BP measurements in clinic settings (Ashor, Lara & Siervo, 2017). This meta-analysis suggested that the effect size became non-significant in studies using more accurate methods, such as 24-hour ambulatory and daily home monitoring (Ashor, Lara & Siervo, 2017). However, clinic measurements have been more widely used in dietary nitrate research. In the 26 ‘significant’ studies described in Table 1.6, 22 used clinic BP measurements, three used 24-hour ABPM and only one study used all three methods (Jajja *et al.*, 2014). In comparison, in the 19 studies described in Table 1.7 reporting non-significant findings, 15 studies used clinic BP, one used ABPM alone and 3 used combinations of home, clinic and ABPM. Clearly there is a need to standardise BP measurement methodology used in future research trials assessing the effects of dietary nitrate.

The conclusion that could be drawn from studies reporting non-significant changes to BP, notwithstanding the methodological differences, is that the overall understanding of the efficacy of nitrate supplementation is still unclear, especially in older patients with pre-

existing conditions, such as obesity and diabetes. This is supported by a meta-analysis and systematic review of trials (2008-2014) to examine the effects of dietary nitrate on endothelial function (Lara *et al.*, 2016). Whilst dietary nitrate (97-1460 mg) was associated with significant improvements in endothelial function, ageing (> 60 years), BMI (> 25 kg/m²) and baseline SBP were associated with a reduced effect (Lara *et al.*, 2016). These results are similar to those reported by Jackson *et al.* (2018) who state that inorganic nitrate was more effective at reducing SBP in healthy participants (-5.8 mmHg; 95% CI, -7.6-4.1), than in patient populations (-2.6 mmHg; 95% CI, -6.5-1.3) (Jackson *et al.*, 2018). Similarly, the eight research studies which included participants > 60 years found non-significant effects of nitrate supplementation on BP, despite significant increases in plasma or salivary nitrate or nitrite (Table 1.7). Accordingly, it seems that both the ageing process and the presence of pre-existing medical conditions are important factors in respect to the effectiveness of dietary nitrate intake (Lara *et al.*, 2016; Siervo *et al.*, 2018).

Table 1.7. Summary of nitrate supplement research studies (2011-2018) with a non-significant ($P > 0.05$) effect on systolic or diastolic blood pressure, with nitrate dose, participant characteristics and other significant findings.

Supplement	Mean nitrate dose (mg/d)*	Mean reduction SBP (mmHg)	Mean reduction DBP (mmHg)	Participant characteristics & mean age (years)	Other significant findings	Authors
Hawthorn & beetroot	**	7 (NS)	5 (NS)	↑ risk of CVD (56)	↑ plasma nitrate and nitrite ↓ triglycerides	Zand <i>et al.</i> , 2011
High-nitrate diet ± beetroot juice	682	NS	NS	Healthy (72)	↑ plasma nitrate and nitrite	Miller <i>et al.</i> , 2012
Beetroot juice	500	NS	NS	Healthy (31)	↓ O ₂ consumption during sub-maximal exercise, improved exercise capacity	Cermak, Gibala & van Loon, 2012
Sodium nitrate	**	NS	NS	Healthy (25)	↑ blood flow in brain	Aamand <i>et al.</i> , 2013
Beetroot juice	480	NS	NS	T2DM/HT (67)	↑ plasma nitrite	Gilchrist <i>et al.</i> , 2013
Beetroot juice	600	NS	NS	Healthy (21)	↑ contractile properties skeletal muscle	Haider & Folland, 2014
Beetroot juice	800	NS	NS	Healthy (22)	↑ plasma nitrate and nitrite ↓ pulse wave velocity	Kim <i>et al.</i> , 2014
High-nitrate vegetables	345	NS	NS	High-normal SBP (61)	↑ plasma and salivary nitrate and nitrite	Bondonno <i>et al.</i> , 2014
Beetroot juice	434	NS	NS	HT (63)	↑ plasma, salivary and urinary nitrite and nitrate	Bondonno <i>et al.</i> , 2015b
High-nitrate vegetables	420	3 (NS)	3 (NS)	Healthy (25)	↑ plasma nitrate and nitrite correlated with SBP	Ashworth <i>et al.</i> , 2015a

Beetroot juice	600	NS	NS	Overweight/ obese (62)	↑ plasma nitrate	Lara <i>et al.</i> , 2015
Beetroot juice	840	NS	NS	COPD (65)	↑ plasma nitrate	Shepherd <i>et al.</i> , 2015
Beetroot juice	340	NS	NS	Healthy (21)	↑ plasma nitrite	Wightman <i>et al.</i> , 2015
High-nitrate vegetables	140	NS	NS	HT (58)	↑ plasma nitrate ↑ salivary nitrate and nitrite	Haldar <i>et al.</i> , 2015
Beetroot juice	744	2 (NS)	NS	Healthy (65)	↑ plasma nitrate	Siervo <i>et al.</i> , 2016
Beetroot juice	738	5 (NS)	NS	Healthy (2 cohorts: 27 & 59)	↑ plasma nitrate and nitrite	Shepherd <i>et al.</i> , 2016
Beetroot juice	372	4 (NS)	2 (NS)	↑ TC (53)	↑ plasma, salivary and urinary nitrite and nitrate, ↑ vasodilation	Velmurugan <i>et al.</i> , 2016
Beetroot juice	380	NS	NS	HT/HF (65)	↑ plasma nitrate and nitrite	Shaltout <i>et al.</i> , 2017
Beetroot juice	400	NS	NS	Healthy (21)	↑ exhaled nitric oxide	Kroll <i>et al.</i> , 2018

*SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, non-significant; CVD, cardiovascular disease; HT, hypertension; T2DM, type 2 diabetes; COPD, Chronic obstructive pulmonary disease; TC, total serum cholesterol; HF, heart failure. *Mean nitrate dose of all studies ~520 mg/d, range 140-840 mg/d. **Mean dose not reported.*

Table 1.8 Summary of potential factors in studies reporting effects of dietary nitrate on BP that may explain differences in results.

Factor	Detail	Implications	Reference
Nitrate dose	Insufficient nitrate dose	Lower amounts of nitrate in beetroot juice than expected.	Jajja <i>et al.</i> , 2014
	Source and timing of nitrate dose	Beetroot juice and high-nitrate vegetables may have different pharmacokinetic properties.	James <i>et al.</i> , 2015
Participant characteristics	Ageing	Ageing may affect the oral microbiome, production of NO and sensitivity to NO.	Lara <i>et al.</i> , 2016
	Inclusion of participants taking medications	Anti-hypertensive and hypoglycaemic medications affect NO metabolism.	Siervo <i>et al.</i> , 2018
	Antibiotic and mouthwash use not reported	Both antibiotic and antibacterial mouthwash use could affect the oral microbiome and therefore the nitrate-nitrite-nitric oxide pathway.	Bondonno <i>et al.</i> , 2015b; Gilchrist <i>et al.</i> , 2013; Shepherd <i>et al.</i> , 2015
	Differences in the oral microbiome	May be the reason for individual variation i.e. for 'responders' and 'non-responders'.	Aamand <i>et al.</i> , 2013; Gilchrist <i>et al.</i> , 2013; Haldar <i>et al.</i> , 2015; Kim <i>et al.</i> , 2014; Lara <i>et al.</i> , 2015; Wightman <i>et al.</i> , 2015
	Sunlight exposure during the study period	Sunlight affects NO production.	James <i>et al.</i> , 2015
Methodological differences	Differences in processing plasma samples for nitrite determination	May explain differences in reported plasma nitrite concentrations.	Feelisch <i>et al.</i> , 2010
	Differences in measurement of BP	Clinic BP measurements after beetroot juice compared with placebo were associated with significant results. The effect size became non-significant when 24-hour ambulatory and daily home BP methods were used.	James <i>et al.</i> , 2015
			Ashor, Lara & Siervo, 2017

1.10 Effect of dietary nitrate on exercise performance and resting metabolic rate

Nitrate supplementation has been reported to improve exercise efficiency and time to exhaustion in various types of exercise tests (Bailey *et al.*, 2010; Bailey *et al.*, 2009; Bescós *et al.*, 2011; Bond, Morton & Braakhuis, 2012; Cermak, Gibala & van Loon, 2012; Lansley *et al.*, 2011a; Lansley *et al.*, 2011b; Larsen *et al.*, 2007; Larsen *et al.*, 2010; Masschelein *et al.*, 2012; Vanhatalo *et al.*, 2010; Wylie *et al.*, 2013a; Wylie *et al.*, 2013b). Although the precise mechanisms are not fully understood, nitrate supplementation may reduce the ATP cost of muscle force production (Bailey *et al.*, 2010) and perhaps increase mitochondrial efficiency (Larsen *et al.*, 2011). This research has driven the current popularity of dietary nitrate supplements in sport (Jones, 2014). There is currently some controversy about the effect of nitrate supplements on exercise performance, in that optimal dosing strategies and the exact populations who might benefit most have yet to be determined (McMahon, Leveritt & Pavey, 2017).

It has been suggested that some of the above benefits on exercise performance may be due to changes in mitochondrial function. The exact mechanism is still unknown, but a major contributing factor could be a reduction in proton leakage, thereby improving mitochondrial respiratory efficiency (Lundberg *et al.*, 2011).

Testing the hypothesis that these mitochondrial effects could be reflected by changes in RMR, a study by the same group found that inorganic nitrate supplementation for 3 days induced an ~4% reduction in RMR (Larsen *et al.*, 2014). These findings were strongly associated with a significant increase in salivary nitrate but not with salivary or plasma nitrite (Larsen *et al.*, 2014). This group suggested that the reduction in RMR was similar to that achieved by energy restriction, which in turn is linked to positive metabolic adaptations and reduced risk of CVD (Larsen *et al.*, 2014). This could, in theory, help to promote healthier ageing and improve public health, which is important in the context of an ageing population (Government Office for Science, 2016). A systematic review and meta-analysis

of effects of nitrate on metabolic rate during rest and exercise noted that this study has not been replicated (Pawlak-Chaouch *et al.*, 2016). Other studies reporting metabolic rate at rest have not used the required methodology of overnight fasting plus a ventilated hood (Pawlak-Chaouch *et al.*, 2016). Also, using these different methodologies, there was no significant effect of dietary nitrate compared to placebo on oxygen consumption at rest prior to exercise tests (Pawlak-Chaouch *et al.*, 2016).

Furthermore, results from two other recent studies failed to support the hypothesis that nitrate improves mitochondrial efficiency and this hypothesis is therefore subject to controversy. Whitfield *et al.* (2016) did not find changes in mitochondrial function after providing ~1600 mg nitrate/day for 7 days in healthy, young subjects. Instead, these authors suggest that NO may either improve excitation or contraction efficiency via Ca^{2+} uptake or ATPase activity. Alternatively, redox signalling and the production of Reactive Oxygen Species (ROS) might be alternative mechanisms whereby the response to exercise is enhanced (Whitfield *et al.*, 2016). Similarly, a second study which examined the effects of ~500 mg nitrate on hand grip exercise, found no effects on basal muscle oxidative efficiency and reached a similar conclusion (Papadopoulos *et al.*, 2018).

Additionally, it might be expected that blocking the nitrate-nitrite-NO pathway might affect RMR, based on previous research findings (Larsen *et al.*, 2014). However, antiseptic mouthwash appears to have no effect on RMR (Sundqvist, Lundberg & Weitzberg, 2016). These conflicting results suggest that the role of dietary nitrate in the nitrate-nitrite-NO pathway and its effects on RMR requires further investigation.

To conclude, the results of experimental research into dietary nitrate, exercise performance and resting metabolic rate are controversial.

1.11 Observational studies suggesting that consumption of green leafy vegetables is protective against cardiovascular disease.

Evidence from observational studies suggests that green leafy vegetables specifically protect against CVD, either as individual dietary components or as part of specific diets, such as the Mediterranean diet.

For instance, daily consumption of raw salad and fresh fruit has been reported to be associated with a 24-26% reduced risk of overall mortality from ischaemic heart disease (Key *et al.*, 1996). Moreover, consumption of green leafy vegetables, cruciferous vegetables and citrus fruit has been reported to be protective against stroke (Joshi *et al.*, 1999) and coronary heart disease (Joshi *et al.*, 2001). In addition, an increase of just one serving of green leafy vegetables/day is associated with an 11% lower risk of CVD (Hung *et al.*, 2004). In the UK, both fresh vegetables and salad showed significant associations with reduced mortality² and to a greater extent than fresh or dried fruit (Oyebode *et al.*, 2014).

Further epidemiological data suggests that the Mediterranean diet, which includes the consumption of fresh fruits and vegetables together with whole grain cereals, extra-virgin olive oil and nuts, has been related to lower cardiovascular risk and improved life expectancy (Nestle, 1995). Recent reviews have suggested that the Mediterranean diet can provide large quantities of nitrate (estimated at 340 mg/day) and this could explain some of the cardiovascular benefits (Bryan & Hord, 2010; Lundberg *et al.*, 2006). It should be noted that other dietary compounds may induce similar effects such as monounsaturated fatty acids and polyphenols (Capurso *et al.*, 2014; Scoditti *et al.*, 2014). Intervention studies based on the Mediterranean diet are described in Section 1.12.

² Hazard Ratio per portion: Vegetables 0.85 (95% CI 0.81 to 0.89) $P < 0.001$, salad 0.87 (95% CI 0.82 to 0.92) $P < 0.001$.

However, in agreement with previous epidemiological evidence, a recent Australian cohort study of free-living, older women (70-85 years) found that an intake of 64 mg nitrate/day from vegetables was associated with a 21% lower risk of mortality from atherosclerotic vascular disease (HR 0.58, 95% CI 0.42, 0.82) (Blekkenhorst *et al.*, 2017a). Benefits plateaued at intakes of 53-76 mg per day and no additional benefit was found for participants with intakes > 76 mg per day (Blekkenhorst *et al.*, 2017a). This study used post-hoc data from a previous prospective cohort study investigating the relationship between calcium intake and fracture and so was not specifically set up to investigate nitrate intake and cardiovascular risk, therefore providing suggestive and not conclusive data. Also, the authors developed their own database for nitrate content of vegetables and noted that the FFQ used in the cohort study measured ~17 mg nitrate/day less than a 24-hr dietary recall method. In addition, the study population included women only and therefore more research is required into nitrate intake and cardiovascular outcomes in men.

Nevertheless, in support of the Australian study, another prospective cohort study of free-living Iranian men and women (20-70 years) suggests that higher dietary nitrate intakes protect against development of hypertension (Golzarand *et al.*, 2016). Participants consuming large amounts of high-nitrate vegetables (median 438 g/day) had a 37% lower risk of developing hypertension after 3 years. Limitations of this study include using an arbitrary 'low', 'medium' and 'high' classification of nitrate content of vegetables which can be inaccurate (Section 1.4.1). Although the authors corrected for possible confounders, such as potassium and fibre intake, it is possible that other components of vegetables, such as polyphenol intake, also affected the outcome (Khoo *et al.*, 2010).

Vegetarian diets are another interesting way to investigate the cardiovascular effects of nitrate. Vegetarian diets have been reported to be higher in nitrate (Section 1.6) and have been associated with lower mortality rates compared to omnivore diets (Huang *et al.*,

2012; Orlich & Fraser, 2014). This could be due to reported lower SBP and DBP in vegetarians than non-vegetarians (Acosta-Navarro *et al.*, 2017; Wang *et al.*, 2015; Yokoyama, Tsubota & Watanabe, 2016), which is only partly accounted for by their lower body mass (Pettersen *et al.*, 2012). Further studies suggest that vegetarians have significantly lower risks of ischaemic heart disease than non-vegetarians (Crowe *et al.*, 2013; Key *et al.*, 2009). The mechanisms of this protection could also be explained by other reasons such as higher intakes of dietary fibre, antioxidants and other bioactive compounds that can reduce levels of oxidative stress and NO availability (Rathod, Velmurugan & Ahluwalia, 2016). Other factors in vegetarian diets that have been proposed as protective against CVD are potassium (Orlich & Fraser, 2014), which has been associated with a reduction in BP (Haddy, Vanhoutte & Feletou, 2006) and plasma antioxidant levels (Szeto, Kwok & Benzie, 2004). Nonetheless, hard evidence to support the use of either potassium or antioxidants as therapeutic agents is lacking. Indeed, antioxidant supplements may increase mortality (Bjelakovic *et al.*, 2012) and potassium supplements are not recommended in the treatment of hypertension (Dickinson *et al.*, 2006).

It has been suggested that vegetarian diets can reduce levels of CVD biomarkers (myeloperoxidase and metalloproteinase (MMP)) compared to omnivores (Navarro *et al.*, 2016). There is a significant negative correlation between MMP levels and plasma nitrite (Demacq *et al.*, 2008) which suggests that nitrite attenuates the production of MMP by endothelial cells (Meschiari *et al.*, 2016). From this viewpoint, it has been proposed that the health benefits of vegetarian diets are partly due to their high-nitrate content (Lundberg *et al.*, 2006).

Most of the studies described in this section are observational and provide correlations between intake of vegetables and outcomes such as CVD. However, these observational studies cannot prove causation. Other factors are linked with eating more fruit and

vegetables, such as a healthier lifestyle, better education levels and reduced saturated fat intakes, which could confound the results (Dauchet *et al.*, 2006).

In summary, epidemiological evidence suggests that diets containing green leafy vegetables, vegetarian diets and the Mediterranean diet are protective against CVD. The factors behind this cardiovascular protection are not fully understood and it has been suggested that dietary nitrate might be partially responsible for these health benefits.

1.12 Experimental studies suggesting consumption of vegetables is protective against diseases of the cardiovascular system.

Following the previous discussion on the Mediterranean diet (Section 1.11), the PREDIMED (Prevencion con Dieta Mediteranea) trial found a link between lower SBP (6-7 mmHg) and DBP (3 mmHg) with total polyphenol excretion in urine (TPE) and plasma nitrate and nitrite availability (Medina-Remón *et al.*, 2015). The authors concluded that the Mediterranean diet may help to reduce BP in participants who are at high cardiovascular risk via an increase NO availability. Unfortunately, there is no data indicating that the Mediterranean diet in this study contained significantly more nitrate than the control diet, although participants did eat more vegetables on the Mediterranean diet (Medina-Remón *et al.*, 2015)³. Previous estimates of nitrate intakes on the Mediterranean diet (~340 mg nitrate/day) have been based on theoretical dietary patterns (Bryan & Hord, 2010). The reported increase in plasma nitrate and nitrite was only ~9 µM, which is lower than the increase in plasma nitrate observed after a Japanese traditional diet (~65 µM) (Sobko *et al.*, 2010) and can also be associated with other factors such as sunlight exposure and physical activity levels (Feelisch *et al.*, 2010; Rassaf *et al.*, 2007). Therefore, there is

³ 491 ± 176 g on the Mediterranean diet, compared to 333 ± 129 g on the Control diet

currently a lack of evidence to support the hypothesis that nitrate plays a key role in the cardiovascular benefits associated with the Mediterranean diet.

In the USA, the Dietary Approaches to Stop Hypertension (DASH) diet was devised with the knowledge that diets based on vegetables are associated with lower BP (Sacks *et al.*, 1995). Specifically, it was noted that vegetarians tend to have lower BP than non-vegetarians and it was believed at that time that this was due to higher intakes of fibre, potassium, magnesium and reduced fat intake (Appel *et al.*, 1997; Sacks *et al.*, 1995). Using this data, and noting that intervention studies using single nutrients produced inconsistent results, this intervention study (DASH) compared three different interventions; 1) a control diet, typical of an American diet, 2) a fruit and vegetable diet, providing more fruit and vegetables and fewer snacks and sweets than the control diet, and 3) a 'combination' diet, which was rich in fruits, vegetables, low fat dairy foods and with reduced amounts of saturated fat, total fat and cholesterol (Appel *et al.*, 1997). On diets 2 and 3, participants consumed 3-4 servings⁴ of vegetables/day. The sodium content of the diets was similar at approximately 3 g per day. Diet 3, subsequently termed the 'DASH' diet, was most effective in reducing SBP and DBP (–5.5 and –3 mmHg respectively), compared to the other two diets (Appel *et al.*, 1997). The authors noted that the BP reductions occurred in the context of stable bodyweight, intake of 3 g sodium/day (7.5 g salt) and ≤ 2 alcoholic drinks per day (Appel *et al.*, 1997). Participants who had previous hypertension had greater reductions in SBP and DBP (–11.4 mmHg and –5.5 mmHg respectively). In an attempt to explain these results, it has been suggested that high-nitrate vegetables, which could provide more than 1200 mg nitrate daily, contribute to the effectiveness of the DASH diet (Hord, Tang & Bryan, 2009). However, this analysis assumes that 1 cup raw spinach provides 926 mg nitrate. Assuming a cup is equivalent to a 'serving size' for vegetables of 85 g (U.S. Food and Drug Administration, 2014), the

⁴ 1 serving = 1 cup raw leafy vegetables or ½ cup cooked vegetables

above estimate suggests that spinach contains 1089 mg nitrate/100g, roughly 10 times the EFSA figure. Nevertheless, the DASH diet encourages consumption of 4-6 servings of vegetables daily, depending on energy requirements, so is likely to contain more nitrate than the average American diet depending on the types of vegetables chosen.

An additional randomised trial specifically encouraged consumption of high-nitrate vegetables for 10 days as part of a traditional Japanese diet, which included vegetables, mushrooms and seaweed (Sobko *et al.*, 2010). The authors estimated that this traditional Japanese diet contained 18.8 mg nitrate/kg/day (equivalent to ~1100 mg nitrate/day/participant) (Sobko *et al.*, 2010). This was compared to 10 days on a control diet, where both high-nitrate and nitrite foods were excluded. Mean DBP was 4.5 mm Hg lower after the Japanese diet although there were no differences in SBP even with significantly higher plasma and salivary nitrate and nitrite concentrations (Sobko *et al.*, 2010). The authors concluded that the Japanese diet was easy to follow by their participants and that the results might explain why traditional Japanese diets can be beneficial to health (Sobko *et al.*, 2010). However, it is unlikely that the traditional Japanese diet would be acceptable to the majority of people in the UK, as it contains many unfamiliar foods including seaweed. In addition, this study had some limitations such as a small sample size ($n = 25$), no washout period between the diets, no information given on sodium content of the diets, dependence on food and drink diaries for assessment of nitrate intake and little information provided on the method of BP measurement.

In contrast to these studies, other randomised controlled trials in the UK have shown mixed results. One trial increased fruit and vegetable intake via intensive counselling by research nurses and after six months showed a significant reduction in SBP (-4 mmHg, 95% CI, -2.0 to -6.0 mmHg) (John *et al.*, 2002). Physical activity was not assessed but there was no significant difference in weight change between the groups, implying that the reduction in BP was not due to changes in body mass. Unfortunately, the type of fruit and

vegetables consumed was not specified in this study and therefore it is not possible to estimate nitrate intake. However, this study found that increasing fruit and vegetable intake reduced BP in this group of patients.

Based on this, a Food Standards Agency (FSA) study aimed to increase fruit and vegetable intake to similarly reduce BP by 4 mmHg (Berry *et al.*, 2010; Sanders, 2007). This trial increased intake by 3, 5 or 10 portions of fruit and vegetables/day together with personalised dietary advice in 48 participants, with 'early stages' of hypertension (baseline BP 137/89 mmHg) (Berry *et al.*, 2010; Sanders, 2007). Body weight remained stable throughout the study although no measurements of physical activity were reported. No significant changes in ambulatory BP were observed. A possible explanation for this could be that participants chose fruit and vegetables that were convenient to carry and consume at work, such as apples, pears, bananas, oranges, dried fruit and tomatoes, which are all low in nitrate. This study concluded that simple dietary advice to increase fruit and vegetable consumption to at least five portions a day to increase potassium intake is unlikely to have any useful effect on BP in the UK (Sanders, 2007).

In summary, some intervention studies using diets high in fruit and vegetables such as the DASH and Mediterranean diets, have demonstrated that significant reductions in BP can be achieved in both healthy participants and those with cardiovascular risk factors, such as hypertension. It has been speculated that these BP reductions may be partially due to the nitrate content of these diets.

2 HYPOTHESIS

The overall hypothesis that this thesis set out to test was as follows; increased consumption of high-nitrate vegetables will reduce BP and will enhance cardiovascular function in healthy individuals. Further hypotheses were developed over time to provide evidence for the main hypothesis:

- A diet supplemented with high-nitrate vegetables for 7-14 days will increase plasma nitrate and nitrite concentrations in healthy men and women.
- Higher plasma nitrate and nitrite concentrations resulting from high-nitrate vegetables will reduce oxygen demand of moderate-intensity exercise in healthy men.
- Increased bioavailability of plasma nitrate and nitrite resulting from consumption of high-nitrate vegetables will be associated with lower BP in healthy men and women.
- Vegetarians will consume more dietary nitrate compared to a similar group of omnivores and this will be associated with lower BP (and improved markers of metabolic health, such as blood lipid and glucose levels).
- The inhibition of oral bacteria with antibacterial mouthwash will increase BP and resting metabolic rate more in vegetarians compared to omnivores due to higher chronic intakes of dietary nitrate.

3 METHODS

Three separate studies were carried out and these will be described in detail.

3.1 Effects of vegetables on health and exercise performance (Study 1)

3.1.1 Introduction

This research started by aiming to replicate the results of a previous study which demonstrated both reduced SBP (by 6 mmHg) and oxygen demand of moderate-intensity exercise (by ~20%) in healthy men after 6 days of beetroot juice compared to placebo (Bailey *et al.*, 2009). Fresh, whole high-nitrate vegetables were selected as these were felt to be more available and acceptable to the majority of the UK population than supplements.

3.1.2 Aims and Objectives

The aims of the study were to supplement the diets of young healthy men with both high-nitrate and low-nitrate vegetables over 14 days.

The primary outcome measurements were BP, plasma nitrate and nitrite and oxygen consumption of moderate-intensity exercise. The secondary outcome measurements were height, weight, BMI, dietary intake and physical activity.

3.1.3 Participants

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter (Appendix 1). All participants who were recruited to the study provided written, informed consent to participate (Appendix 2).

Participants were recruited by poster and word of mouth from the University of Exeter and surrounding area (Appendix 3). All participants who responded were given a detailed information sheet (Appendix 4). Participants were included if they were 18-40 years old, non-smoking, healthy, physically active men willing to perform one high-intensity exercise session as well as moderate-intensity exercise sessions on a cycle ergometer. In addition, participants were free from pathological or physiological impairments, pre-existing medical conditions such as hypertension and diabetes or any other limitation that restricted their ability to perform one high-intensity cycling session. Participants were requested to avoid using mouthwashes during the study, as chlorhexidine antibacterial mouthwash has been shown to decrease the conversion of nitrate to nitrite (Govoni *et al.*, 2008).

3.1.4 Study design

The study used a randomised, repeated measures cross-over design. Participants were randomised, using a random allocation sequence (Altman & Bland, 1999) to receive either high- or low-nitrate vegetables for 2 weeks, followed by a 2-week 'wash out' period, before receiving further vegetables for 2 weeks, so that each participant received both dietary interventions in approximately 6 weeks.

3.1.5 Study protocol

Participants were invited to attend the Exercise Physiology Laboratory at Sport and Health Sciences, University of Exeter over ~7 weeks (Figure 3.1). Prior to the first visit, participants were requested to avoid strenuous exercise, alcohol and caffeine for 24 hours and to eat not less than 3 hours beforehand.

At the first visit, participants were weighed on digital scales sensitive to 100 g (XWM-105K, Hampel Electronic Co. Ltd, Taiwan), their height measured (Harpenden Portable

Stadiometer, Holtain Ltd, UK) and their BMI calculated as weight/height² (kg/m²). They were advised to maintain their normal body mass throughout the study, to avoid the effects of weight loss on BP (Blumenthal *et al.*, 2010). Apart from vegetables, participants were advised to continue with their normal diets and physical activity levels throughout the study. They were requested to record their exercise pattern using an exercise diary (Appendix 5) and to maintain their normal exercise pattern throughout the study, in order to avoid exercise-induced reduction in BP (Blumenthal *et al.*, 2010). They completed a Physical Activity Readiness questionnaire (Canadian Society for Exercise Physiology, 2017) and their BP was measured. Then, participants performed an incremental exercise test to determine the peak oxygen consumption and gas exchange threshold (GET) on an electronically braked cycle ergometer (Lode Excalibur Sport V2, Lode BV, Groningen, The Netherlands). The method used for the determination of the GET was similar to that used by Bailey *et al.* (2009) and has been described in detail elsewhere (Beaver, Wasserman & Whipp, 1986). Once their GET had been determined, participants were invited to return for their second visit, when they started their 14-day supplementation period.

Before and after each 14-day supplementation period, at visits 2, 3, 4 and 5, participants underwent tests to assess changes in BP, plasma nitrate and nitrite levels, weight and exercise performance. During the 14-day wash out period, participants returned to their normal diet, with no restrictions. Participants were not informed which diets were high or low in nitrate or about the study hypotheses until after data collection was complete.

Figure 3.1 Timeline of study to investigate the effects of two weeks supplementation with either high or low nitrate vegetables in men.

3.1.6 Blood pressure

On arrival at the laboratory, participants were instructed to relax in a comfortable chair for at least five minutes, undisturbed, not moving or speaking, before BP was measured. Participants attended at the same time of day (either morning or afternoon according to preference) to avoid potential diurnal variation in BP. Guidelines from the British Hypertension Society (British Hypertension Society, 2014) and the European Society of Hypertension (O'Brien *et al.*, 2003) were used to ensure accurate measurement. A validated, automated sphygmomanometer (Dinamap PRO 100V2, GE Medical Systems Information Technology, Tampa, USA) was used to avoid measurement bias of the observer with accuracy criteria of 'meets or exceeds American National Standards Institute/Association for the Advancement of Medical Instrumentation standard SP-10 (mean error ≤ 5 mmHg, standard deviation ≤ 8 mmHg)'. Four readings of SBP, DBP and mean arterial pressure (MAP) were obtained. The first reading was discarded and the mean taken of the final three readings. The arm was supported at the level of the heart to avoid error, and participants were advised to wear short sleeves so that tight clothing did not constrict the arm. The same arm was used in each participant, as studies have demonstrated significant differences between arms. The cuff was placed on the upper arm and secured by Velcro®.

3.1.7 Plasma nitrate and nitrite

Directly after BP measurement, venous blood samples (>3 hours post-prandial) were obtained by venepuncture by a trained phlebotomist into lithium-heparin tubes (BD Vacutainer Plasma tube, 6 ml, Becton Dickinson, Plymouth, UK). Samples were centrifuged immediately to avoid oxidation of nitrite at 4 °C, at 4000 rpm for 10 minutes (Sorvall ST16R Thermo Scientific, Hemel Hempstead, UK). Plasma was subsequently withdrawn into 3 separate micro centrifuge tubes (Fisher Scientific, Loughborough UK).

They were frozen at -80°C for later analysis of plasma nitrate and nitrite by an experienced technician, who was blinded to the test conditions, using a modified chemoluminescence technique (Bailey *et al.*, 2009; Bateman, Ellis & Freeman, 2002).

All glassware was rinsed with nitrite-free, deionized water before use to minimize contamination. After thawing at room temperature, 200 μl of sample was placed in a micro centrifuge tube and mixed with 400 μl 0.5 N NaOH and 400 μl 10 % by weight aqueous ZnSO_4 . This mixture was vortexed then left to stand for 15 minutes. Then, the deproteinized samples were centrifuged at 4000 rpm for 5 min (Hettich Mikro 120 Centrifuge, Hettich GmbH & Co, Germany) and the supernatant removed for analysis. Samples were injected into a gas-sealed purging vessel at room temperature. This technique is based on a gas-phase chemiluminescent reaction between NO and ozone, yielding nitrogen dioxide (NO_2). The NO_2 content of the deproteinized plasma samples was determined by its reduction to NO, in the presence of 5 ml glacial acetic acid and 1% NaI under nitrogen at room temperature. The NO generated was quantified by a chemiluminescence NO analyser (Sievers Nitric Oxide Analyzer NOA 280i, GE Instruments, US) and the NO_2 was derived from the NO signal compared to NaNO_2^- standards previously measured.

3.1.8 Exercise performance and gas exchange

Participants attended the exercise physiology laboratory for a moderate-intensity cycle exercise test (80% GET), using the same settings on the ergometer for saddle position (Lode Excalibur Sport V2, Lode BV, Groningen, The Netherlands) and the same portable metabolic cart (Cortex Metalyzer 3B, Cortex Biophysik gmbh, Leipzig, Germany) as previously. Pulmonary gas exchange and ventilation were continuously measured using the portable metabolic cart, which had been calibrated before use. Participants wore a nose clip and breathed through a mouthpiece securely attached to a turbine.

After a 4-minute baseline cycle (20 W), the work rate was increased to a level consistent with 80% of their individual GET, as used by Bailey *et al.* (2009), for 3 bouts of 6 minutes duration. In between each 6-minute bout, participants rested on the bike for 7 minutes, followed by a 3-minute baseline cycle at 20 W. The mean pulmonary oxygen uptake (VO_2), carbon dioxide output (VCO_2) and respiratory exchange ratio (RER) from the final 60 seconds of the baseline and moderate-intensity exercise periods were calculated.

3.1.9 Dietary supplementation and nutritional analysis

As nitrate content of vegetables can vary considerably, vegetables were obtained from one supplier (Riverford Organic Farms Ltd, Buckfastleigh, Devon, UK). High-nitrate vegetable boxes were designed to provide approximately 500 mg of nitrate daily and approximately 300-400 g of vegetables daily (Table 3.1). Fruit (which contains little dietary nitrate, apart from rhubarb) was included to equate the carbohydrate content of the boxes as high-nitrate vegetables tend to be lower in carbohydrate than low-nitrate vegetables.

Table 3.1. Example of 7-day high-nitrate and low-nitrate vegetable boxes (varieties and quantities varied according to availability).

High-nitrate vegetable box	Weight (g) or portions
Leeks	600
Broccoli	450
Fennel	400
Salad pack (not iceberg)	400
Rocket	200
Satsumas	7 medium
Bananas	4 medium
Low-nitrate vegetable box	
Green pepper	400
Tomatoes	400
Carrots	400
Onions	400
Cucumber	400
Garlic	3 medium
Bananas	3 medium

On Days 1 and 7, vegetable boxes (Riverford Organic Farms Ltd, Buckfastleigh, Devon, UK) were delivered to participants. Whilst on the low-nitrate vegetable diet, participants were requested to avoid high-nitrate vegetables (Appendix 6). During each 2-week supplementation period, participants were requested to record their dietary intake using a daily food diary (Appendix 7). They were not asked to weigh their food intake, as experience from a previous feasibility study suggested this would be too inconvenient for participants. After each 14 days supplementation they were asked to recall if they had eaten all of the vegetables and if not, to recall the quantity uneaten by direct questioning after each post-supplementation period. The quantity of high-nitrate vegetables consumed was estimated as:-

Total vegetables given (g) – wastage reported (g) x (edible portion factor) where ‘edible portion factor’ refers to the ‘edible material remaining after the inedible waste has been trimmed away’ (Food Standards Agency, 2014), for example, outer leaves or stalks of vegetables.

Quantitative nutritional assessments of the edible portions of fruit and vegetables eaten from the vegetable boxes were carried out using a nutritional analysis software programme (Microdiet, Downlee Systems, Chapel-en-le-Frith, UK). Total polyphenol (determined by folin assay) and quercetin content was obtained using an on-line database, Phenol-Explorer (Neveu *et al.*, 2010). Nitrate, total polyphenol and quercetin figures were uploaded to the Microdiet database prior to analysis. Direct measurement of nitrate content of the vegetables consumed was beyond the scope of this study.

Previous studies have suggested that the peak action time for dietary nitrate to reduce BP is ~2.5-3 hours after ingestion (Lidder & Webb, 2013). Participants were therefore asked to consume some of the vegetables as part of their last meal 3 hours before the test, where possible.

3.1.10 Physical activity

Exercise diaries were used to assess the total time of physical activities, such as running, going to the gym, swimming, cycling etc. The total amount of time over the supplementation period was estimated in order to check that it was similar in both conditions.

3.1.11 Statistical analysis

A power calculation was performed (G*Power statistical analysis programme) using results from a previous study where systolic BP was significantly reduced by a mean of 6 mmHg ($P < 0.01$) (Bailey *et al.*, 2009). Assuming a power of 80%, at the 5% level of significance and a standard deviation of 5 mmHg (as reported by Bailey *et al.*, 2009), to detect a difference of 6 mmHg between the two groups, resulted in an estimated sample size of 22 subjects. Data analysis was performed using the Statistical Package for Social Sciences (Version 19, IBM® SPSS Statistics), with statistical significance accepted when $P < 0.05$. Results were reported as means \pm SD, unless stated otherwise. Paired sample t-tests were conducted to compare differences in body mass before and after each supplementation period, exercise reported during each supplementation period, and nutritional analyses of vegetables consumed during each supplementation period. An analysis of variance (ANOVA) (diet by time) was used to assess the impact of the high- and low-nitrate diets on plasma nitrate, nitrite, BP and exercise performance variables before and after each supplementation period. Post hoc (Fisher's Least Significant Difference) tests were carried out to identify significant differences. Relationships between changes in the variables were assessed using Pearson's product-moment correlation coefficients, where correlation was accepted as significant at the 0.05 level (one-tailed). A one-tailed test was used to test the directional hypothesis that BP would be reduced, as previously reported (Bailey *et al.*, 2009).

3.2 Effects of vegetable consumption on blood pressure in women (Study 2).

3.2.1 Introduction

As outlined in the Introduction (Section 1.7), plasma nitrite concentrations rise in response to a dietary nitrate dose (Lundberg & Govoni, 2004). A gender difference in response was shown by Kapil *et al.* (2010), who showed higher plasma nitrite concentrations in women compared to men, with the same nitrate dose. These authors suggested that this different response could contribute to the prevalence of lower BP levels and rates of CVD in premenopausal women than in men (Kapil *et al.*, 2010). However, one limitation of the findings by Kapil *et al.* (2010) is that their study did not control for stages of the menstrual cycle, which can cause changes in BP (Dunne *et al.*, 1991). This study aimed to explore the relationship between dietary nitrate doses in women, taking into account the menstrual cycle. It was decided to make this study shorter (7 day's supplementation only) as it was difficult to both recruit and retain participants to the previous study in men. Measurement of the oxygen consumption of moderate-intensity exercise was not included as there was a lack of evidence from the first male study that this was effective and the commitment to 5 exercise tests placed an additional burden on participants.

3.2.2 Aims and Objectives

The aims of the study were to supplement the diets of young healthy women with high-nitrate vegetables for 7 days compared to a control diet for 7 days, where high-nitrate vegetables were avoided.

The primary outcome measurements were BP and plasma nitrate and nitrite levels. The secondary outcome measurements were height, weight, BMI, dietary intake and physical activity.

3.2.3 Participants

Healthy, adult women were recruited via a poster and word of mouth from the University of Exeter and surrounding area (Appendix 8) and assessed for eligibility. Participants were non-smoking, premenopausal women aged 18-40 years with self-reported, regular (28-day) menstrual cycles, who were not taking any medications. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the local institutional Ethics Committee, Sport and Health Sciences, College of Life and Environmental Sciences (Appendix 9). All participants provided written informed consent (Appendix 10) and were provided with a written information sheet about the study (Appendix 11) prior to being enrolled in the study. The study took place between September and December 2012.

3.2.4 Study design

The study had a randomised, crossover design. Each participant consumed both high-nitrate (HN) and control (Control) diets during a five-week period, with a 'wash-out' of three weeks between diets. The study was designed so that participants would be at the same stage of their 28-day menstrual cycle for both the HN and Control diets in order to control for potential BP fluctuation (SBP \sim -0.65 mmHg during days 17-26) (Dunne *et al.*, 1991).

3.2.5 Study protocol

Participants visited the Exercise Physiology Laboratory at Sport and Health Sciences, University of Exeter five times over approximately six weeks (Figure 3.2). At the first visit, participants were weighed on digital scales (XWM-105K, Hampel Electronic Co. Ltd, Taiwan). Body height was also measured (Harpenden Portable Stadiometer, Holtain Ltd, UK) to calculate their body mass index (BMI) as weight/height^2 (kg/m^2). The timeline of the

study was explained and they were given 7-day food and exercise diaries to complete (Appendix 5 & 7). They were advised to maintain their normal body mass, maintain their normal exercise pattern and avoid using mouthwash as in the previous study. Participants were randomised using a sealed envelope technique to receive a box of high-nitrate vegetables (HN diet) for one week or were given written instructions to avoid high-nitrate vegetables for one week (Control diet) (Appendix 6). At the second visit, participants were re-weighed, their BP measured and a venous blood sample (~6 mL) was subsequently obtained for analysis of plasma nitrate and nitrite. Participants were asked to confirm that they had taken no recent medications, including the contraceptive pill (Appendix 12). This was repeated at visits 3, 4 and 5. Participants were not informed of the study hypothesis until the end of the study.

Figure 3.2 Timeline of study to investigate the effects of one-week supplementation with high nitrate vegetables or Control diet in women.

3.2.6 Blood pressure

The same method for measuring BP was used as in the previous study 'Effects of vegetables on health and exercise performance' (Section 3.1.6).

3.2.7 Plasma nitrate and nitrite

The same method for obtaining samples and for analysis was used as in the previous study in men, titled 'Effects of vegetables on health and exercise performance' (Section 3.1.7).

3.2.8 Dietary supplementation and nutritional analysis

Participants on the HN diet were requested to avoid high-nitrate vegetables (Appendix 6) for three days before their initial laboratory visit and this was confirmed on arrival. The HN diet consisted of a vegetable box from the same supplier (Section 3.1.9) and the trial was completed in a four-month period. Vegetable box contents were weighed, using digital kitchen scales (Hanson Scale Company, Chicago, Illinois) and given directly to each participant. On the Control diet, participants were requested to avoid high-nitrate vegetables, but were permitted to consume low-nitrate vegetables (Appendix 6) and were requested not to alter their normal diet in any other way. Specifically, after the 7-day high-nitrate intervention participants were asked to recall if they had eaten all of the vegetables provided in the high-nitrate vegetable box and if not, to recall the quantity uneaten by direct questioning after each period. The quantity of high-nitrate vegetables consumed was estimated as:-

Total vegetables given (g) – wastage reported (g) x (edible portion factor) where 'edible portion factor' refers to the 'edible material remaining after the inedible waste has been trimmed away' (Food Standards Agency, 2014), for example, outer leaves or stalks of

vegetables. This data, together with 7-day food diaries, was used to calculate the nutritional analysis of fruit and vegetables consumed during the HN and Control diets, as in the previous study in men (Section 3.1.9).

3.2.9 Physical activity

The same method for measuring physical activity was used as in the previous study in men (Section 3.1.10).

3.2.10 Statistical analysis

The same statistical analysis was used as in the previous study in men (Section 3.1.11), as follows: A power analysis was performed (G*Power statistical analysis programme) using results from a previous study where systolic BP was significantly reduced by a mean of 6 mmHg ($P < 0.01$) (Bailey *et al.*, 2009). Assuming a power of 80%, at the 5% level of significance and a standard deviation of 5 mmHg (as reported by Bailey *et al.*, 2009), to detect a difference of 6 mmHg between the two groups, resulted in an estimated sample size of 22 subjects. Data analysis was performed using the Statistical Package for Social Sciences (Version 19, IBM® SPSS Statistics), with statistical significance accepted when $P < 0.05$. Results were reported as means \pm SD, unless stated otherwise. Paired sample t-tests were conducted to compare differences in body mass before and after each intervention period (HN diet or Control diet), exercise reported during each intervention period, and nutritional analyses of vegetables consumed during each intervention period. A two-way ANOVA (diet by time) was used to assess the impact of the HN and Control diets on plasma nitrate, nitrite and BP before and after each intervention period. Post hoc (Fisher's Least Significant Difference) tests were carried out to identify significant differences. Relationships between changes in the variables were assessed using

Pearson's product-moment correlation coefficients, where correlation was accepted as significant at the 0.05 level (one-tailed). A one-tailed test was used to test the directional hypothesis that BP would be reduced, as previously reported (Bailey *et al.*, 2009).

3.3 Dietary nitrate intake in vegetarians and omnivores and impact on blood pressure and resting metabolic rate (Study 3).

3.3.1 Introduction

The previous studies described (Section 3.1 and 3.2) supplemented diets of healthy young men and women with high-nitrate vegetables for 1-2 weeks only. The effects of higher, chronic intakes of dietary nitrate from vegetables is unknown, although epidemiological data suggest it may be beneficial to cardiovascular health (Blekkenhorst *et al.*, 2017a; Smallwood *et al.*, 2017). From this viewpoint, a key population to study may be vegetarians, particularly since previous evidence suggests that vegetarians have a higher intake of dietary nitrate than omnivores (Mitek, Anyzewska & Wawrzyniak, 2013). This may also explain higher salivary nitrate and nitrite concentrations found in vegetarians (Traczyk & Szponar, 2000). Vegetarians might also have greater nitrate-reducing capacity of oral bacteria and these factors could explain, at least partially, lower levels of SBP (by ~5-7 mmHg) (Yokoyama *et al.*, 2014) of this group compared with omnivores.

A lower resting metabolic rate (RMR) (~110 kcal/day) has been linked to better health outcomes with ageing (Schrack *et al.*, 2014). Dietary nitrate has been shown to reduce RMR (by ~82 kcal/day) and this reduction was associated with salivary nitrate concentration (Larsen *et al.*, 2014). The third study of this thesis aimed to investigate the relationship between nitrate intake, BP and RMR in vegetarians and omnivores.

3.3.2 Aims and Objectives

The aims of the study were to compare dietary nitrate intakes, BP, RMR, plasma and salivary nitrate and nitrite levels, oral nitrate reducing capacity, salivary pH, blood glucose

and lipid levels of a group of vegetarians to a similar group of omnivores before and after one-week of twice daily antibacterial mouthwash.

The primary outcome measurements were dietary nitrate intake, BP, RMR plasma and salivary nitrate and nitrite levels, oral nitrate reducing capacity, salivary pH, blood glucose and lipid levels. The secondary outcome measurements were height, weight, BMI, macronutrient dietary intake and physical activity.

3.3.3 Participants

This study was approved by Ethics Committee of Faculty of Health & Human Sciences, University of Plymouth (Appendix 13). All participants provided written consent to participate (Appendix 14).

Healthy vegetarians (vegans and lacto-ovo vegetarians) following this dietary pattern for at least a year and healthy omnivores between 18 and 45 years of age were invited to participate in this study. Both groups were matched by age, gender and BMI. Matching was achieved by recruiting the vegetarian group first, then recruiting omnivores retrospectively. Participants were recruited by poster and word of mouth from the University of Plymouth and the surrounding area (Appendix 15). The study was advertised on the University of Plymouth website. All participants who responded were sent a detailed information sheet (Appendix 16) and medical questionnaire (Appendix 17) to complete and return in order to assess if they meet the inclusion criteria for this study. Respondents were excluded if they were smokers, taking any medications or recreational drugs that might have affected the study outcomes, or had pre-existing medical conditions, for example, hypertension, diabetes or dental conditions such as gingivitis.

3.3.4 Study design

The study used a single blinded, non-randomised, cross over design.

3.3.5 Study protocol

Participants visited the laboratory on three different occasions (Figure 3.3). At the first visit, the participants were informed about the main aims of the study and instructed by a researcher to complete the food and physical activity diaries. In addition, they were given 14 placebo (water) mouthwash tubes of 10 mL in order to rinse their mouth for one minute, twice a day for 7 days. They were also given a small tube of toothpaste (Colgate Total®) to standardise toothpaste use throughout the study. This was to avoid potential use of other toothpastes which might have different antibacterial activity or contain other substances which might affect study outcomes, such as arginine.

Participants returned to the laboratory after one week (second visit). At least 24 hours prior to their visit, they were given written instructions (via email) to avoid caffeine containing drinks such as tea or coffee before the test and to refrain from strenuous exercise for at least 24 hours before the test. They were also requested to have a good night's sleep before the trial. Participants arrived at the laboratory between 8 and 9 am having fasted overnight. Body weight and height were measured using a mechanical bathroom scale (Salter, Tonbridge, United Kingdom) and stadiometer (Seca, Birmingham, UK) respectively, in order to calculate BMI as weight/height^2 (kg/m^2). Then, participants were asked to lie on a medical couch for 30 minutes in order to measure resting metabolic rate (RMR) using indirect calorimetry (Jaeger® Oxycon Pro, CareFusion, Germany). Following this measurement, participants were kept in the supine position in order to measure BP using an automated sphygmomanometer, (manufacturer's accuracy; ± 5 mmHg mean error, 8 mmHg standard deviation) (Welch Allyn Connex ProBP 3400, Welch Allyn UK Ltd.). Three successive readings were taken (four if variation of 4 mmHg was found), with

a one minutes rest between readings. After completing these measurements, a venepuncture was performed on the antecubital vein to obtain a blood sample (~12 mL) to analyse plasma nitrate and nitrite (lithium-heparin tubes, BD Vacutainer[®], 6 ml, Becton Dickinson, Plymouth, UK), blood glucose and blood lipids (serum separator tubes, BD Vacutainer[®], 5 ml, Becton Dickinson, Plymouth, UK). Then, a non-stimulated salivary sample (3-4 mL) was taken into a sterile tube in order to analyse nitrate and nitrite and pH. Finally, the nitrate-reducing capacity of oral bacteria was measured. Participants were asked to hold 10 ml of sodium nitrate (80 µmol) and nitrite free water in the oral cavity for 5 min after which time the mouth rinse was collected into a sterile tube. The volume of the sample was measured and centrifuged (4000 rpm, 4⁰ C, 10 min). The supernatant was collected and stored at -80 °C until analysis of nitrate and nitrite concentrations was performed. At the end of the visit, the participant was given breakfast and the food and activity diaries from the previous seven days were collected. A dietitian checked the seven-day food diaries in order to confirm the foods and portion sizes consumed, preparation methods, recipes and any brand names. This was to ensure that adequate detail was captured. The food and activity diaries were then photocopied and returned to the participant who was requested to replicate the previous week's food intake and activity levels as closely as possible. The participant was given a further one week supply (14 tubes of 10 mL) of antibacterial mouthwash containing 0.2% chlorhexidine (Corsodyl, GlaxoSmithKline, UK), encouraged to use it as per the previous mouthwash (one minute, twice a day) and requested to return to the laboratory in 7 days to repeat all measurements in the same order (third visit).

Figure 3.3 Timeline of study to investigate the effects of one-week placebo mouthwash followed by one-week antibacterial mouthwash in vegetarians and omnivores.

3.3.6 Resting metabolic rate (RMR)

A respiratory analyser (Jaeger® Oxycon Pro, CareFusion, Germany) was calibrated before each test using a reference gas (15.8% O₂, 4.9% CO₂). Oxygen uptake (VO₂), carbon dioxide production (VCO₂) and the Respiratory Exchange Ratio (RER) were measured continuously during 30 minutes using a ventilated hood connected to the respiratory analyser. Data from the first 20 minutes was discarded and the RMR was calculated as the average measurements of the final 10 minutes of the test by using the following modified equation (Weir, 1949):

$$\text{RMR (kcal/day)} = [3.941 \times (\text{VO}_2/1000) + 1.106 (\text{VCO}_2/1000)] \times 1440$$

(VO₂ and VCO₂ in mL/min)

3.3.7 Blood pressure

BP was measured following British Hypertension Guidelines (British Hypertension Society, 2014). Three successive supine readings were taken (four if variation in SBP or DBP of \geq 4 mmHg was found) using an automated sphygmomanometer (Welch Allyn Connex ProBP 3400, Welch Allyn UK Ltd.), with a one-minute rest between readings. The second and third readings were averaged to determine mean clinic BP.

3.3.8 Plasma and salivary nitrate and nitrite

Plasma nitrate and nitrite was measured using the same methodology as previously described (Section 3.1.7). For the analysis of salivary nitrate and nitrite, the reagents and standard curve used for plasma nitrate and nitrite analysis were used again. For both metabolites, saliva samples were thawed as previously described and then diluted at a ratio of 1:100 with deionised water due to the large expected concentrations. Subsequently, 100 μ L of the salivary nitrite sample and between 25-50 μ L of the salivary

nitrate sample was injected into the purge vessel in duplicate and calculated as previously described, after accounting for the water in the diluted sample.

3.3.9 Blood lipids

A blood sample (~5 ml) was collected into a serum separator tube (serum separator tubes, BD Vacutainer[®], 5 ml, Becton Dickinson, Plymouth, UK), inverted several times, and then stored at room temperature for 30 minutes. Total cholesterol, triglycerides, high density lipoproteins (HDL) and low density lipoproteins (LDL) were analysed with enzymatic methods using the Roche 702 spectrophotometric module of a Cobas 8000 analyser (Roche Diagnostics Ltd, UK), as follows:

3.3.9.1 Cholesterol

Cholesterol esters were hydrolysed by cholesterol esterase to yield cholesterol and free fatty acids. Cholesterol oxidase then catalysed the conversion of cholesterol to Cholest-4-ene-3-one and hydrogen peroxide. Hydrogen peroxide combines with hydroxybenzoic acid (HBA) and 4-aminoantipyrine to produce a quinoneimine dye, which was measured spectrophotometrically.

3.3.9.2 HDL and LDL Cholesterol

Cholesterol esters were converted under the influence of polyethylene glycol cholesterase to form HDL and LDL cholesterol. HDL and LDL cholesterol and oxygen were converted to delta-4-cholestenone and hydrogen peroxide under the catalytic effect of cholesterol oxidase. Hydrogen peroxide then reacted with 4-aminoantipyrine and hydrobenzoic acid to give quinoneimine dye, which was measured spectrophotometrically.

3.3.9.3 Triglycerides

Triglycerides were measured using a Wahlefield modified Trinder method, as follows: Triglyceride and water were broken down by lipoprotein lipase to yield glycerol and free fatty acids. In the presence of glycerokinase, glycerol and ATP formed glycerol-3-phosphate and ADP. Glycerol-3-phosphate and oxygen in the presence of glycerol-3-phosphate oxidase formed dihydroxyacetone and hydrogen peroxide. Hydrogen peroxide reacted with 4-aminoantipyrine and 4-chlorophenol to give a red dye, which was measured spectrophotometrically.

3.3.10 Blood glucose

Whole blood glucose was measured using a glucose analyser (YSI 2300 Stat Plus, YSI Life Sciences, USA). Samples were analysed twice and the mean value estimated.

3.3.11 Salivary pH

Salivary pH was measured using a single electrode digital pH meter (Lutron Electronic Enterprise Co Ltd., Model PH-208, Taiwan). The pH meter was calibrated every day using freshly prepared buffers of pH 7 and pH 4. Prior to dipping the electrode in the sample, it was gently dried using fresh sterile filter papers each time. After analysing the pH, the electrode tip was again washed with a gentle stream of distilled water and then dipped in the double distilled water.

3.3.12 Oral nitrate-reducing capacity

Participants were instructed to hold 10 ml of sodium nitrate (80 μ mol) and nitrite-free water for 5 minutes in the oral cavity after which time the mouth rinse was collected into tubes

and centrifuged (4,500 rpm, 4 °C, 10 minutes) and the supernatants were collected and stored at -80 °C until nitrite and nitrate determination as described in Section 3.3.8.

3.3.13 Nutritional analysis

Seven-day food diaries were analysed using the same software as previously (Section 3.1.9). In addition, a standard protocol was adapted to ensure consistency of coding from food diaries (Airwave Health Monitoring Study, Imperial College, London). A previously validated FFQ was given to participants to complete in order to assess intake of fruit and vegetables over the previous year (Mulligan *et al.*, 2014).

3.3.14 Physical activity

The same method was used as previously described (Section 3.1.10).

3.3.15 Statistical analysis

This study was powered to detect a 3 mmHg increase in systolic blood pressure when oral nitrate reducing bacteria are inhibited using antibacterial mouthwash. This increase is deemed to be clinically significant (Kapil *et al.*, 2013). Thus, the study was powered at 80%, using a type I error rate of 0.05, to detect a treatment effect of 3 mmHg. A minimum sample size of 22 participants was calculated on the primary outcome of mean clinic systolic BP.

Analysis was carried out using the SPSS software (SPSS Statistics, IBM® Version 24) and statistical significance was taken as $P < 0.05$. Independent sample t-tests were used to assess differences between vegetarians and omnivores at baseline (after placebo mouthwash). Paired sample t-tests were used to assess differences within the groups after antibacterial mouthwash. Results are presented as means \pm standard deviation (SD).

An analysis of variance (ANOVA) was performed to determine the influence of mouthwash (placebo and antibacterial mouthwash) on BP in vegetarians and omnivores.

Relationships between changes in the variables were assessed using Pearson's product-moment correlation coefficients (two-tailed), where correlation was accepted as significant at the 0.05 level.

4 RESULTS

4.1 Effects of vegetables on health and exercise performance (Study 1)

The hypothesis tested in this study was that supplementation with high-nitrate vegetables over a 14-day period will increase plasma nitrate and nitrite bioavailability. This increased bioavailability will lead to lower BP and reduce oxygen consumption at moderate-intensity exercise in healthy men. In contrast, 14 days supplementation with low-nitrate vegetables will have no effects on plasma nitrate, nitrite, BP or oxygen consumption at moderate-intensity exercise.

4.1.1 Participants

Twenty-one physically active, non-smoking male participants were recruited from January 2011 to March 2012. Five participants withdrew due to various reasons including illness and pressures of work. Data from one participant was subsequently excluded due to non-adherence to the study protocol, resulting in high plasma nitrite concentrations (Figure 4.1). The main factors affecting participation therefore included; being healthy throughout the six weeks of the study, availability to attend all 5 laboratory visits at the same time of day and ability to follow the study protocol. Every attempt was made to replace participants lost to achieve the target sample size, by continually advertising the study and running the study for over 12 months. Data were analysed on the remaining 15 participants, age 25 ± 6 years, BMI 24 ± 4 kg/m². Three participants had a BMI of >25 kg/m² but actively participated in weight training or rugby and therefore were deemed to be 'healthy'. There was no significant difference between the mean body mass of participants at the beginning compared to the end of the supplementation period (beginning: 75.3 ± 11.4 kg; end: 75.6 ± 11.6 kg, $t_{(14)} = -1.21$, $P = 0.246$). There was no significant difference between mean

reported physical activity during the two week supplementation periods (first: 12.8 ± 7.5 hours; second: 11.1 ± 6.6 hours, $t_{(9)} = 1.38$, $P = 0.20$).

Figure 4.1. CONSORT diagram to illustrate study 1 design.

4.1.2 Diet

The nutritional analysis of the content of the vegetable boxes reported as consumed is shown in Table 4.1. (example; Appendix 19). Participants consumed significantly more nitrate and total polyphenols from the high-nitrate diet than from the low-nitrate diet, although the mean daily intake of vegetables was lower ($P < 0.05$ for all; Table 4.1). However, there was no significant difference in total energy, carbohydrate, quercetin, or antioxidant vitamins, A, C and E between diets. Food diaries revealed that during the 2-week low-nitrate diet, 7/15 subjects did consume some high-nitrate vegetables (e.g. as spinach and ricotta pasta or salad leaves in a sandwich). There were no adverse side effects reported by the participants.

Table 4.1. Nutritional analysis of edible portion of high-nitrate and low-nitrate vegetable boxes with mean \pm SD daily intake during 14 days supplementation.

Intake from vegetables per day	Low-nitrate diet	High-nitrate diet	<i>P</i> value*
Vegetables consumed (g)	350 \pm 100	266 \pm 58	0.005
Nitrate (mg)	26 \pm 11	417 \pm 139	< 0.001
Energy (kcal)	136 \pm 54	146 \pm 47	0.173
Energy (kJ)	574 \pm 226	620 \pm 199	0.14
Carbohydrate (g)	28 \pm 10	28 \pm 10	0.482
Total polyphenols (mg)	463 \pm 256	718 \pm 368	0.001
Quercetin (mg)	6 \pm 2	6 \pm 3	0.432
Vitamin A (μ g) ^a	999 \pm 742	732 \pm 369	0.281
Vitamin C (mg)	11 \pm 50	101 \pm 43	0.544
Vitamin E (mg) ^b	2 \pm 1	2 \pm 1	0.769

**P* values are for paired sample *t*-tests (2-tailed), ^aretinol equivalent, ^b α -tocopherol equivalent

4.1.3 Plasma nitrate and nitrite

Changes in plasma nitrate and nitrite are shown in Figure 4.2 and Figure 4.3, respectively.

The high-nitrate diet significantly raised plasma nitrate and nitrite concentrations.

There was a significant main effect of two weeks dietary supplementation on mean plasma nitrate ($P = 0.001$; ANOVA) and a significant main effect for time on plasma nitrate ($P = 0.001$). Moreover, there was a significant interaction effect of dietary supplementation and time on plasma nitrate ($P = 0.001$). Post-hoc tests showed that two weeks supplementation with high-nitrate vegetables significantly increased plasma nitrate ($P =$

0.001). There was no significant difference after supplementation with low-nitrate vegetables for two weeks ($P > 0.05$) (Figure 4.2).

Figure 4.2. Effects of two weeks supplementation with high and low-nitrate vegetables on mean plasma nitrate in 15 participants. Plasma nitrate was significantly higher after eating high-nitrate vegetables (* $P = 0.001$)⁵.

⁵ Error bars indicate SD

Responses in plasma nitrite are shown in Figure 4.3. There were significant main and interaction effects of diet and time ($P < 0.05$; ANOVA) There was a significant main effect of dietary supplementation on mean plasma nitrite ($P = 0.037$) and there was a significant main effect for time on plasma nitrite ($P = 0.007$). There was a significant interaction effect for supplementation and time on plasma nitrite ($P < 0.001$). Post-hoc tests showed that two weeks supplementation with high-nitrate vegetables significantly increased plasma nitrite. There was no significant difference after supplementation with low-nitrate vegetables for two weeks ($P > 0.05$).

Figure 4.3. Effects of high and low-nitrate vegetables on mean plasma nitrite in 15 participants. Plasma nitrite was significantly higher after eating high-nitrate vegetables (* $P < 0.001$)⁶.

Group mean values for plasma nitrate and nitrite at baseline and after 14 days supplementation are shown in Appendix 20.

In order to investigate the large variability indicated by the SD in plasma nitrate and nitrite levels after the high-nitrate diet, a sensitivity analysis was performed whereby participants were split into those who ate high nitrate vegetables before the post-supplementation test ($n=11$) compared with those that did not ($n=4$). There was a greater increase in mean

⁶ Error bars indicate SD

plasma nitrite (126 vs 58 nmol/l) and nitrate levels (107 vs 79 μ mol) in the 11 participants who ate high nitrate vegetables before the test compared to the 4 participants who did not eat high nitrate vegetables before the test. This could explain the large variation in the results.

4.1.4 Blood pressure after high and low-nitrate diets

Mean SBP was reduced by 3 ± 8 mmHg and DBP and MAP by 3 ± 7 mmHg after supplementation with the high-nitrate vegetables. The results of the ANOVA suggested no significant main or interaction effects between diet (high-nitrate and low-nitrate) and time (baseline and post-supplementation) ($P > 0.05$). Paired sample t-tests show that reductions were not statistically significant ($P > 0.05$) (Table 4.2). This was possibly because the study was underpowered to detect BP differences, due to recruitment failures. A sensitivity analysis was performed whereby participants were split into those who ate high nitrate vegetables before the post-supplementation test ($n=11$) compared with those that did not ($n=4$). The sensitivity analysis revealed that there was no change in the results i.e. no significant reduction in BP in either group.

Similarly, no significant differences were found in mean SBP, DBP or MAP after supplementation with low-nitrate vegetables (Table 4.2).

Table 4.2. Systolic blood pressure, diastolic blood pressure and mean arterial pressure measured at pre-supplementation baseline and after 14 days of high-nitrate diet and low-nitrate diet (mean \pm SD).

BP (mmHg)	Low-nitrate diet				High-nitrate diet			
	Baseline	Post supplementat ion	Δ	<i>P</i>	Baseline	Post supplement ation	Δ	<i>P</i>
SBP	118.3 \pm 9	118.6 \pm 10	0.3	.882	121.4 \pm 9	118.3 \pm 9	-3.1	.174
DBP	64.6 \pm 9	65.9 \pm 9	1.3	.487	68.4 \pm 8	65.3 \pm 8	-3.1	.106
MAP	85.7 \pm 6	86.5 \pm 7	0.8	.562	88.7 \pm 5	85.6 \pm 7	-3.1	.086

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure

4.1.5 Correlations between plasma nitrate and nitrite concentrations and blood pressure after high and low-nitrate diets

To determine whether there was any linear association between BP and plasma concentrations, Pearson's product-moment correlation coefficient, *r*, was used. One-tailed tests were used to test the directional hypothesis that plasma nitrite (and therefore plasma nitrate) levels would be increased after dietary nitrate supplementation, as previously reported (Bailey *et al.*, 2009). A moderate but significant negative correlation was found between SBP and change in plasma nitrate after supplementation with high-nitrate vegetables, indicating that increased plasma nitrate was associated with lower SBP ($r = -0.49$, $P = 0.033$) (Figure 4.4).

Figure 4.4. Pearson's correlation between change in individual systolic blood pressure and the change in plasma nitrate following two weeks supplementation with high-nitrate vegetables.

A similar effect was found between MAP and changes in plasma nitrate ($r = -0.44$, $P = 0.049$) (Figure 4.5) and nitrite ($r = -0.50$, $P = 0.028$).

Figure 4.5. Pearson's correlation between change in individual mean arterial pressure and the change in plasma nitrate following two weeks supplementation with high-nitrate vegetables.

No significant correlations were found between SBP and changes in plasma nitrite ($r = -0.27$, $P = 0.164$) or between DBP and plasma nitrate ($r = -0.38$, $P = 0.083$) after supplementation with high-nitrate vegetables. However, significant correlations were found between DBP and changes in plasma nitrite after high-nitrate vegetables ($r = -0.56$, $P = 0.015$) (Figure 4.6). In addition, there were significant correlations between DBP and change in plasma nitrite after supplementation with low-nitrate vegetables ($r = -0.63$, $P = 0.006$) indicating that lower plasma nitrite was associated with increased DBP (Figure 4.6). Similarly, there was a significant negative correlation between change in MAP and change in plasma nitrite ($r = -0.51$, $P = 0.027$).

Figure 4.6. Pearson's correlations between change in diastolic blood pressure and change in plasma nitrite following two weeks supplementation with low nitrate (dashed line) and high-nitrate vegetables (dotted line).

There were no significant correlations between SBP and changes in plasma nitrate ($r = -0.11$, $P = 0.352$) or nitrite ($r = -0.08$, $P = 0.39$) after low-nitrate vegetables. There were no significant correlations between DBP ($r = 0.18$, $P = 0.262$) or MAP ($r = 0.93$, $P = 0.37$) and changes in plasma nitrate after low-nitrate vegetables.

4.1.6 Gas exchange after moderate exercise

There were no significant main or interaction effects of either high or low-nitrate vegetables on VO_2 , VCO_2 or RER after the moderate exercise tests (80% GET) (Table 4.3). Comparison by paired sample t-tests show that changes in oxygen consumption (VO_2) carbon dioxide production (VCO_2) or respiratory exchange ratio (RER) were not statistically significant ($P > 0.05$), apart from VCO_2 ($P = 0.038$).

Table 4.3. Gas exchange variables before and after moderate exercise measured at baseline and after 14 days of high-nitrate diet and low-nitrate diet (mean \pm SD).

	Low-nitrate diet				High-nitrate diet			
	Baseline	Post supplementation	Δ	P	Baseline	Post supplementation	Δ	P
(VO_2) (L/min)	1.36 \pm 0.24	1.34 \pm 0.20	0.02	.270	1.38 \pm 0.24	1.36 \pm 0.21	0.02	.448
(VCO_2) (L/min)	1.21 \pm 0.22	1.20 \pm 0.21	0.02	.038	1.20 \pm 0.20	1.21 \pm 0.19	0.01	.596
RER	0.90 \pm 0.04	0.89 \pm 0.05	0.01	.491	0.88 \pm 0.03	0.89 \pm 0.04	0.015	.265

VO₂; oxygen consumption, VCO₂; carbon dioxide production, RER; respiratory exchange ratio

4.1.7 Summary

The main findings of this first study in males were that supplementation with high-nitrate vegetables resulted in significant increases in plasma nitrate (430%) and nitrite (191%) compared to supplementation with low-nitrate vegetables. This was in agreement with results of a previous study by Bailey *et al.* (2009), who reported an increase of plasma nitrite of 195% after 6 days of beetroot juice (~350 mg nitrate/day) compared to placebo. However, in contrast to Bailey *et al.*, this was not associated with significant changes in SBP, DBP or MAP or in the respiratory response to exercise. However, the study was underpowered to detect statistically significant changes in BP and therefore subject to

Type II error. Similarly to Kapil *et al.* (2010), changes in plasma nitrate and nitrite concentrations were significantly correlated with SBP, DBP or MAP after consumption of high-nitrate vegetables. In contrast, consumption of low-nitrate vegetables resulted in significant negative correlations between plasma nitrite, DBP and MAP. Kapil *et al.* suggest that the correlation between plasma nitrite concentrations and BP reflects, at least in part, the bioactivity⁷ of nitrite.

4.1.8 Study limitations

This study was underpowered and therefore the main limitation is the risk of type II error. Recruitment was difficult, possibly due to the length of the study, maximal exercise tests and the requirement to eat vegetables. This was reflected in the fact that it took 15 months to complete. In addition, supplementation was over a comparatively short period in a small group of young healthy men. Therefore, extrapolation of the main results of this study to the general population is limited.

Another limitation of this study was that the consumption of nitrate was determined by indirect estimation and the nitrate content of the vegetables provided may have been higher or lower than estimated. Recent estimates of nitrate content of vegetables are not readily available and future research should address this. However, the significant changes in plasma nitrate and nitrite suggest that the high-nitrate diet effectively increased the circulatory concentrations of this anion.

In addition, the limitations of using food diaries are that the burden of completion rests on the participants, who might delay entering data, thereby relying on memory which could lead to error (Lovegrove *et al.*, 2015). Completion of food diaries is time consuming and

⁷ Bioactivity can be defined as the property of a substance to exert an effect on, or response from, a living organism

this could have predisposed the participants who withdrew from the study due to lack of time. Also, the burden of completion of the food diary can cause participants to alter eating habits and intakes can be under-reported (Lovegrove *et al.*, 2015). These factors can affect the accuracy of the dietary data. However, alternative dietary assessment methods are also subject to measurement error. For example, FFQ can be difficult for participants to complete and do not accurately capture portion sizes (Lovegrove *et al.*, 2015).

Participants with a BMI > 25 kg/m² were included as they had a high muscle mass and participated in regular weight training and physical activity. BMI is a crude measure of body composition and does not reflect muscle mass and a more accurate method of body composition measurement could have clarified that these participants were healthy and not overweight.

The acceptability of high-nitrate vegetables and possible adverse side effects were not formally investigated. Due to low recruitment, it could be assumed that the acceptability of eating vegetables in this population group was poor. However, despite low recruitment, participants reported no adverse side effects. Due to the very low toxicity of nitrate, there are few reported cases of adverse effects even of massive doses of nitrate (up to 7000 mg/day) in the literature, apart from methaemoglobinaemia in infants (L'Hirondel & L'Hirondel, 2002). One study which administered over 8000 mg nitrate orally to adults reported that one subject vomited after 20 minutes and another had diarrhoea after 7 hours (Ellen *et al.*, 1982).

Compliance was assessed by completion of food diaries by participants, direct questioning by the investigator as well as plasma nitrate and nitrite levels. However, nearly 50% participants ate high-nitrate vegetables whilst on the low-nitrate diet and just over 25% did not eat vegetables before the final test as requested. Similarly, compliance can be a major problem in pharmacological trials as some participants fail to take medications. It has been suggested that direct observation of participants could improve compliance (Czobor &

Skolnick, 2011), however this was not possible during the two-week interventions of this study. Future studies could use shorter interventions to improve compliance.

Other variables that can significantly affect BP but are not easily controllable in free-living participants include stress levels, alcohol and salt intake. Many of the participants were students and it is possible that they experienced increased stress levels during the study due to pressures of academic work. Although participants were requested to adhere to their normal diets apart from their vegetable intake, it is possible that participants altered their alcohol and salt intake during the study.

A contamination effect can occur when participants learn about possible beneficial effects of an experimental diet and adopt it during the control phase. At the time of the study, there was little information available about high-nitrate vegetables, apart from beetroot juice experiments being conducted in the same facility. For this reason, beetroot was not included in the vegetable boxes. Although 7/15 participants ate some high nitrate vegetables during the low nitrate diet, plasma nitrate and nitrite concentrations did not alter on the low-nitrate diet, suggesting that contamination did not occur at a significant level.

Notwithstanding its limitations, it can be concluded from this study that supplementing the diets of physically active, healthy young males with ~3 portions/day of a variety of high-nitrate vegetables resulted in significant changes in plasma nitrate and nitrite concentrations, some of which correlated with BP. These correlations suggest that a relationship exists between plasma nitrate and nitrite concentrations and BP in humans and are in agreement with previous findings by Kapil *et al.* (2010).

4.2 Effects of vegetable consumption on blood pressure in women (Study 2)

The hypothesis tested in this study was that supplementation with high-nitrate vegetables over a 7-day period will increase plasma nitrate and nitrite bioavailability, which in turn, will lead to lower BP in healthy women, compared to 7 days of a control diet, avoiding high-nitrate vegetables.

This study has been published (Ashworth *et al.*, 2015b) and cited by ~30 publications.

4.2.1 Participants

Thirty healthy, non-smoking female participants were enrolled and assessed for eligibility between September and December 2012. Ten participants were either excluded or withdrew after enrolment due to personal reasons, time constraints or illness unrelated to the study, or difficulties in obtaining vascular access. One of the participants allocated to the HN diet was excluded following analyses of primary outcome variables based on the criterion that her plasma nitrite measurement was >3 standard deviations (SD) beyond the group mean (Figure 4.7). The principal results of the study would not be altered if this participant had not been excluded. Data analysis was performed on the remaining 19 participants (20 ± 2 years old; BMI 22.5 ± 3.8 kg/m²). Similar levels of physical activity were performed during both the Control and HN diets (Control 4.0 ± 2.6 hours/wk; HN 4.2 ± 2.6 hours/wk).

Figure 4.7. CONSORT diagram to illustrate Study 2 design.

4.2.2 Nutritional analysis

Nutritional analysis of the fruit and vegetables eaten suggested that participants consumed significantly more nitrate, total polyphenols, protein, fat, potassium, calcium and magnesium during the HN diet than the Control diet (Table 4.4), whereas energy and carbohydrate intakes were similar on both diets. Compliance with the request to not alter their normal diet in any way apart from the amount of vegetables eaten was indicated by the lack of changes in body mass of participants from baseline (62.7 ± 10.3 kg) to the end of the study period (62.4 ± 10.4 kg). However, six participants consumed small portions of high-nitrate vegetables during the Control diet and five participants did not consume high-nitrate vegetables 2-3 hours prior to the laboratory visit as requested.

Table 4.4. Estimated quantitative nutritional analysis of daily intakes of fruit and vegetables eaten during high-nitrate (HN) and Control diets (mean \pm SD).

Intake per day	Control diet	HN diet	<i>P</i> Value
Vegetables eaten (g)	177 \pm 116	181 \pm 75	0.90
Nitrate (mg)	8 \pm 6	339 \pm 133	< 0.001
Total polyphenols (mg)	227 \pm 198	432 \pm 206	< 0.001
Energy (kcal)	79 \pm 55	82 \pm 31	0.79
Energy (kJ)	351 \pm 227	357 \pm 137	0.89
Protein (g)	2.0 \pm 1	4.0 \pm 1	<0 .001
Fat (g)	0.5 \pm 0.3	1.1 \pm 0.3	<0.001
Carbohydrate (g)	18.2 \pm 14	15.0 \pm 8	0.31
Potassium (mg)	362 \pm 231	835 \pm 218	<0.001
Calcium (mg)	29 \pm 18	150 \pm 72	< 0.001
Magnesium (mg)	22 \pm 17	43 \pm 23	< 0.001

Mean nitrate intake on the HN diet was approximately 340 mg/day (5.4 mg nitrate/kg/day) (Table 4.4). This is in agreement with the previous study in men, as well as other research findings which indicate that it is possible to consume amounts of nitrate through dietary means in excess of the ADI of 3.7 mg nitrate/kg/day. Findings from this study support the suggestion that dietary nitrate intakes can easily exceed the ADI, primarily through the consumption of high-nitrate vegetables.

4.2.3 Blood pressure

Baseline SBP and DBP were in line with mean UK population levels in 16-24 year old women of 110 mmHg and 66 mmHg respectively. Values of SBP, DBP and MAP before and after both diets are shown in Table 4.5. Results of the ANOVA suggested there was a significant main effect by time on SBP ($P = 0.037$). There was no significant main effect by

diet ($P = 0.103$) and little evidence of an interaction ($P = 0.082$). Follow up tests (Least Significant Difference) revealed that SBP was significantly reduced after the HN diet ($P = 0.008$) but not after the Control diet (Figure 4.8).

Comparisons by paired sample t-tests demonstrate significant reductions in SBP, DBP and MAP after the high-nitrate diet (Table 4.5). Using a sensitivity analysis to exclude participants who did not eat vegetables before the final test, a further ANOVA suggested a significant effect of diet on SBP ($P = 0.037$).

Table 4.5. Systolic blood pressure, diastolic blood pressure and mean arterial blood pressure at baseline and post Control and post high-nitrate diets (mean \pm SD).

BP (mmHg)	Control				High-nitrate			
	Baseline	Post diet	Δ	P	Baseline	Post Diet	Δ	P
SBP	105.9 \pm 8	106.0 \pm 8	0.1	.944	106.5 \pm 9	102.7 \pm 6	-3.9	.018
DBP	62.4 \pm 5	61.3 \pm 6	-1.1	.446	63.4 \pm 7	60.9 \pm 5	-2.6	.046
MAP	78.2 \pm 6	77.9 \pm 6	-0.3	.782	79.4 \pm 7	76.4 \pm 4	-3.0	.027

HN, high-nitrate; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

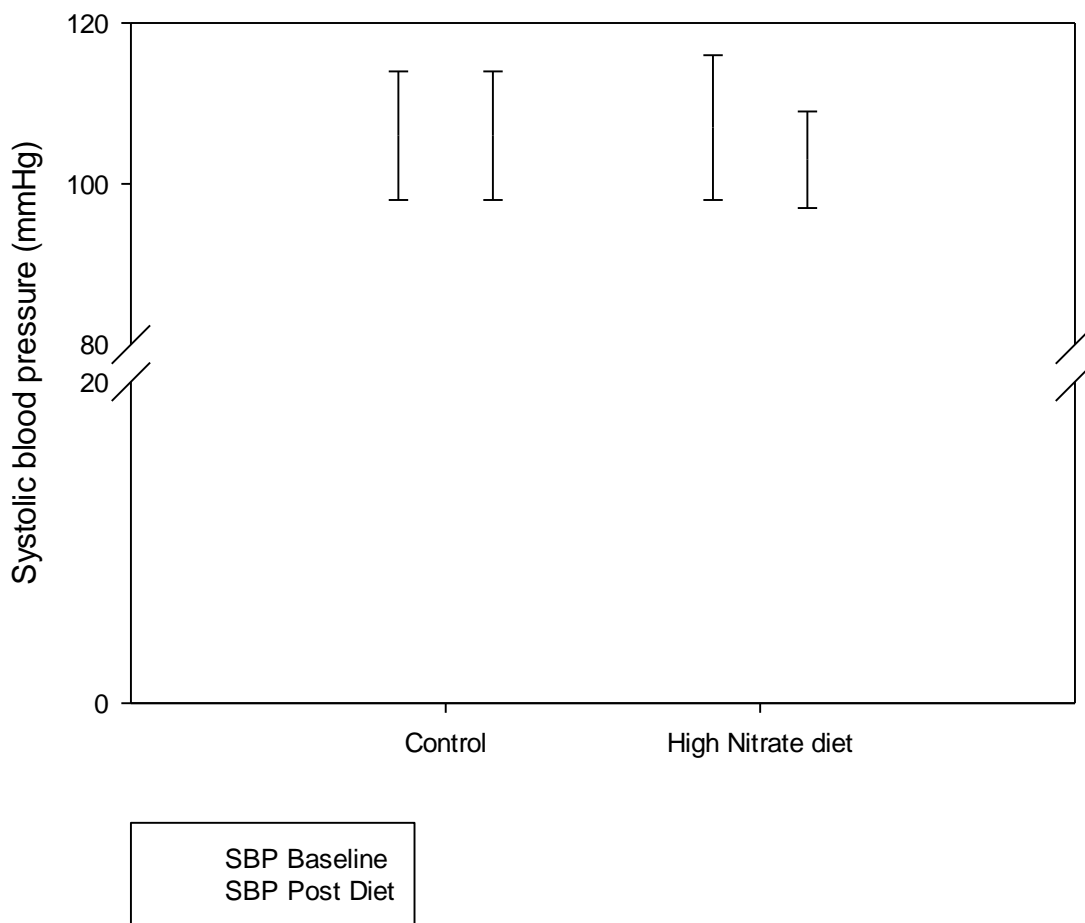


Figure 4.8. Effects of 7-days Control and high-nitrate diets on mean systolic blood pressure (SBP) (* $P = 0.008$)⁸.

There was no significant difference between SBP before the HN diet and the Control diet ($P = 0.68$). There was no significant change in SBP, DBP or MAP after the Control diet (Table 4.5).

There was a significant and negative correlation (two-tailed) between the baseline SBP and change in SBP following the HN diet ($r = -0.74$, $P < 0.001$) (Figure 4.9). This remained significant when the outlier was removed ($r = -0.603$, $P = 0.008$). There was no correlation between change in SBP and baseline SBP following the Control diet ($r = -0.345$, $P = 0.148$). There was no correlation between estimated nitrate intake and change in SBP ($r = 0.002$, $P = 0.99$).

⁸ Error bars indicate standard deviation.

Figure 4.9. The relationship between baseline systolic blood pressure and the change in systolic blood pressure following the high-nitrate diet ($r = -0.74$, $P < 0.001$).

4.2.4 Plasma nitrate and nitrite

Having completed the majority of the study, one participant had a vasovagal response before the final venous sample collection, resulting in one missing data value. There were significant main effects by diet ($P = 0.002$) and by time ($P = 0.003$) as well as an interaction effect ($P = 0.001$) on mean plasma nitrate. Follow up tests revealed that the HN diet significantly increased plasma nitrate ($61.0 \pm 44.1 \mu\text{M}$) ($P = 0.002$), whereas the Control diet had no significant effect ($26.0 \pm 9.8 \mu\text{M}$) ($P = 0.52$) (Figure 4.10).

Figure 4.10. Effects of the Control diet and the high-nitrate (HN) diet on mean plasma nitrate after one-week compared to baseline (* $P = 0.002$).⁹

⁹ Error bars indicate standard deviation.

There was a significant main effect by time ($P = 0.015$), little evidence of a main effect by diet ($P = 0.06$) and no interaction effect on plasma nitrite ($P = 0.12$). Follow up tests revealed that plasma nitrite increased after the HN diet ($P = 0.027$), whereas the Control diet had no significant effect ($P = 0.23$) (Figure 4.11).

Figure 4.11. Effects of the Control diet and the high-nitrate (HN) diet on mean plasma nitrite after one-week compared to baseline (* $P = 0.027$).¹⁰

In order to investigate the large variability indicated by the SD in plasma nitrate and nitrite levels after the high-nitrate diet, a sensitivity analysis was performed whereby participants were split into those who ate high nitrate vegetables before the post-supplementation test ($n=14$) compared with those that did not ($n=5$). There was a greater increase in mean plasma nitrite (+111 vs 28 nmol/l) and nitrate levels (+47 vs 12 μ mol) in the 14 participants who ate high nitrate vegetables before the test compared to the 5 participants who did not eat high nitrate vegetables before the test. This could explain the large variation in the results.

¹⁰ Error bars indicate standard deviation.

4.2.5 Summary

The main finding of this study was that consumption of approximately two portions daily of a variety of high-nitrate vegetables over seven days significantly reduced SBP (by ~4 mmHg) in healthy young women. DBP and MAP were not significantly affected by the HN diet. No significant changes in SBP, DBP or MAP were observed when participants avoided high-nitrate vegetables during the Control diet.

In summary, this study was novel in nitrate research in that it used fresh, whole, high-nitrate vegetables readily available in the UK instead of nitrate supplements and found a reduction of SBP of ~4 mmHg in normotensive women.

4.2.6 Study limitations

Food diaries were used to estimate consumption of vegetables and fruit on both diets, but they were not analysed for total intake of macro- and micro-nutrients of all foods eaten, due to limited resources. The effect of eating two portions of HN vegetables, which can add bulk to the diet, on normal dietary intake was not assessed. However, there was no significant change in body mass throughout the study, suggesting that overall energy intake was not significantly affected. A potential limitation in dietary control was that nitrate intake was estimated based on self-reported vegetable consumption and indirect estimation of nitrate content of vegetables, which may vary according to factors such as crop variety, season and light conditions. However, compliance with the HN diet was confirmed by significant changes in plasma nitrate and nitrite. Future research should consider direct measurement of nitrate concentrations in the HN diet, as well as assessment of effects of high-nitrate vegetables on macro- and micro-nutrient intake.

The main outcome of the study was to assess the effects of 7 days consumption of high-nitrate vegetables in free-living participants. It could be argued, however, that the acute

effect was measured as subjects were requested to eat vegetables on the final day 2-3 hours before testing, in order to reflect the metabolism of dietary nitrate (Section 1.8). Indeed, a sensitivity analysis suggested that the 5 participants who did not eat high-nitrate vegetables before the final test had lower increases in plasma nitrate and nitrite levels, compared to the 14 participants who did eat vegetables before the final test. However, despite this, these 5 participants also experienced reductions in SBP, DBP and MAP.

The possibility of a 'placebo effect' should be considered because participants were given high-nitrate vegetables to consume in the experimental arm but asked to maintain their normal diet during the control arm. However, participants were blind to the purpose of the study, i.e. they were not informed that the vegetables they were given were high in dietary nitrate nor that this might influence BP.

BP is known to vary during the menstrual cycle (Dunne *et al.*, 1991). The current study design relied on recruitment of participants having a 28-day menstrual cycle, with testing timetabled such that the participants would be at the same stage of their cycle for both the HN and Control diets. As the length of the menstrual cycle is subject to variability (21-35 days), a sliding washout period might have allowed for shorter or longer cycle length and could be included in the design of further studies.

The present findings suggest that high-nitrate vegetables represent a promising intervention to improve cardiovascular health in young women and warrant further investigation.

4.3 Dietary nitrate intake in vegetarians and omnivores and impact on blood pressure and resting metabolic rate (Study 3).

4.3.1 Aims and objectives

The aims of the study were to assess dietary nitrate intake, selected physiological variables and the effects of anti-bacterial mouthwash on these variables in a group of vegetarians compared to a similar group of omnivores.

The objectives were to;

- measure dietary nitrate intake, BP, RMR, plasma and salivary nitrate and nitrite levels, oral nitrate reducing capacity, salivary pH, blood glucose and lipid levels after one-week of placebo mouthwash (water) in a group of vegetarians compared to a similar group of omnivores.
- Assess the effects of a further one-week of antibacterial mouthwash on these variables.

4.3.2 Participants

Twenty-two vegetarians (5 vegans and 17 lacto-ovo vegetarians) and nineteen omnivores successfully completed this study between May 2016 and August 2017 (Figure 4.12). Participants were matched as closely as possible for age, gender and BMI (Table 4.6). Participants were mainly under-graduate and postgraduate students together with some staff members of the University of Plymouth. Approximately 14% (6/41) participants were students, researchers or lecturers in Nutrition and Dietetics.

Figure 4.12. CONSORT diagram to illustrate Study 3 design.

Table 4.6. Baseline characteristics of vegetarians and omnivores after 7 days placebo mouthwash (mean \pm SD).

	Vegetarians (n = 22)	Omnivores (n= 19)	P value
Age (years)	26 \pm 6	26 \pm 7	0.995
Gender	16 female, 6 male	11 female, 8 male	0.346
BMI (kg/m ²)	22.9 \pm 4	22.1 \pm 3	0.459
Physical activity (min/wk)	315 \pm 221	334 \pm 216	0.785
SBP (mmHg)	104 \pm 8	103 \pm 6	0.572
DBP (mmHg)	64 \pm 6	61 \pm 6	0.104
MAP (mmHg)	77 \pm 6	75 \pm 5	0.225
HR (bpm)	59 \pm 8	59 \pm 11	0.982
RMR (kcal)	1175 \pm 208	1202 \pm 267	0.642
Plasma nitrate (μ M)	43.4 \pm 32.8	39.9 \pm 18.3	0.673
Plasma nitrite (nM)	84.1 \pm 40.8	74.5 \pm 20.8	0.962

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; RMR, resting metabolic rate; HR, heart rate.

4.3.3 Nutritional analysis

Both vegetarians and omnivores consumed similar amounts of dietary nitrate and total polyphenols, despite results from the FFQ indicating that vegetarians ate more total vegetables (343 g/day) and fruit (262 g/day) than omnivores (240g and 157 g/day respectively). In addition, energy and total fat intake were similar between the groups. However, saturated fat and protein intakes were higher in omnivores (Table 4.7).

Table 4.7. Nutritional analyses of 7-day food diaries of vegetarians and omnivores (mean \pm SD).

	Vegetarians (n = 22)	Omnivores (n= 19)	<i>P</i> value
Energy (kcal)	1827 \pm 526	2021 \pm 560	0.234
Energy (kJ)	7680 \pm 2209	8506 \pm 2356	0.229
Protein (g)	60.5 \pm 19	90.9 \pm 36	0.001
Fat (g)	71.1 \pm 27	78.3 \pm 24	0.335
Saturated fat (g)	21.2 \pm 10	27.9 \pm 11	0.036
Carbohydrate (g)	234.9 \pm 71	246.3 \pm 76	0.594
Nitrate (mg)	97 \pm 79	78 \pm 47	0.348
Polyphenol (mg)	182 \pm 124	178 \pm 116	0.840

4.3.4 Blood pressure and heart rate

Opposing the original hypothesis, independent-samples *t*-tests showed no significant differences between baseline BP in vegetarians and omnivores (Table 4.6).

There were no significant main or interaction effects of antibacterial mouth on BP in vegetarians and omnivores (Figure 4.13). Paired samples *t*-tests showed no significant changes in either group (Table 4.8).

Similarly, no significant differences in heart rate were observed either between groups or between treatments (Table 4.8).

Table 4.8. Blood pressure and heart rate in vegetarians and omnivores after using placebo (water) and antibacterial mouthwash for 7 days (mean \pm SD).

	Vegetarians (n = 22)			Omnivores (n = 19)		
	Placebo	Mouthwash	<i>P</i>	Placebo	Mouthwash	<i>P</i>
SBP (mmHg)	104 \pm 8	105 \pm 8	0.259	103 \pm 6	104 \pm 7	0.221
DBP (mmHg)	64 \pm 6	63 \pm 5	0.480	61 \pm 7	61 \pm 6	0.928
MAP (mmHg)	77 \pm 6	77 \pm 5	0.923	75 \pm 5	75 \pm 6	0.631
HR (bpm)	59 \pm 8	59 \pm 10	0.573	59 \pm 11	59 \pm 10	0.663

SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

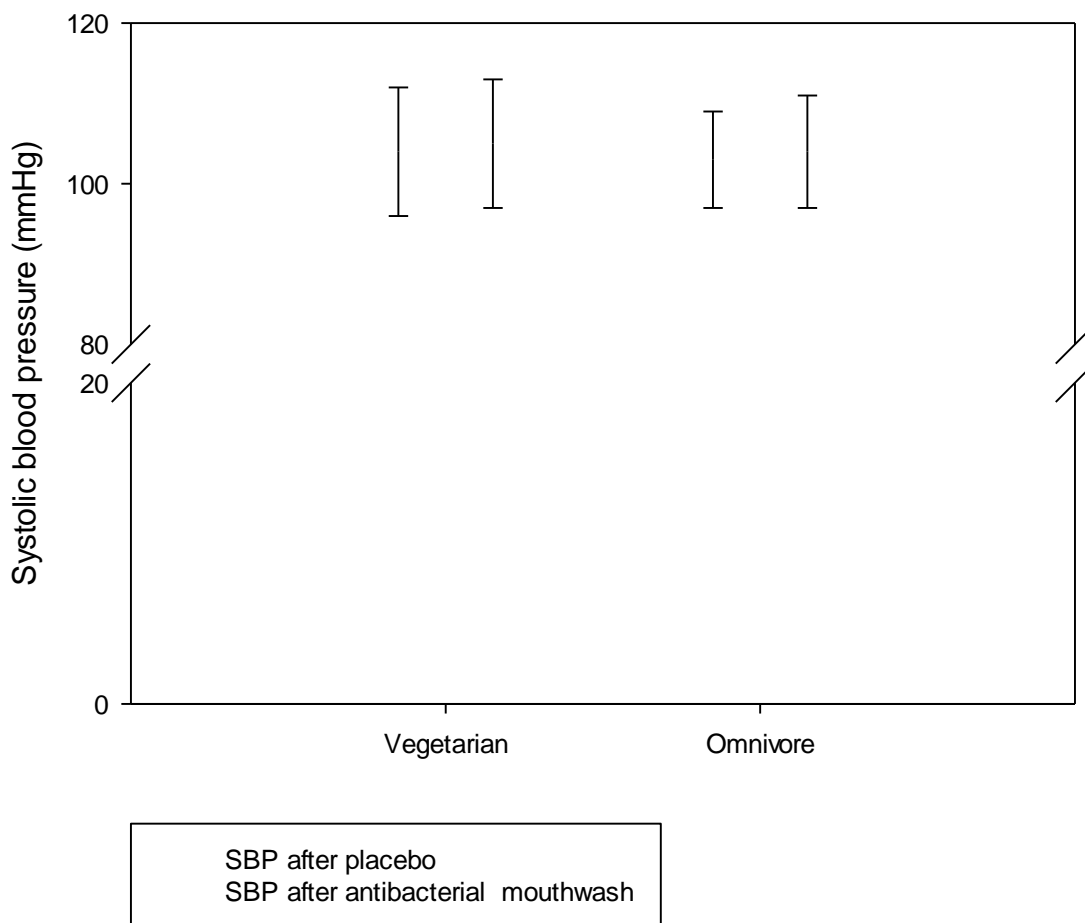


Figure 4.13. Effects of placebo and antibacterial mouthwash on systolic blood pressure (SBP) in vegetarians and omnivores ($P > 0.05$; ANOVA)¹¹.

4.3.5 Resting metabolic rate (RMR)

Independent sample t -tests showed no significant differences in RMR between groups after 7 days placebo mouthwash (Table 4.6). In addition, there were no significant main ($P = 0.806$) or interaction ($P = 0.719$) effects of antibacterial mouthwash on RMR in either group. Paired sample t -tests showed no significant differences in either group after antibacterial mouthwash (Table 4.9).

¹¹ Error bars indicate standard deviation.

Table 4.9. Resting metabolic rate (RMR) in vegetarians (n = 22) and omnivores (n = 19) using antibacterial mouthwash for 7 days (mean \pm SD).

Vegetarians			Omnivores		
Placebo (Kcal/d)	Mouthwash (Kcal/d)	<i>P</i>	Placebo (Kcal/d)	Mouthwash (Kcal/d)	<i>P</i>
1175 \pm 208	1185 \pm 218	0.599	1202 \pm 267	1200 \pm 272	0.948

4.3.6 Plasma and salivary nitrate and nitrite

Independent samples *t*-tests showed no significant differences in plasma concentrations of nitrate and nitrite between vegetarians and omnivores after using placebo mouthwash for one week (Table 4.6).

There were no significant main or interaction effects of antibacterial mouthwash on plasma nitrate ($P = 0.678$ and $P = 0.671$ respectively) in vegetarians and omnivores (Figure 4.14).

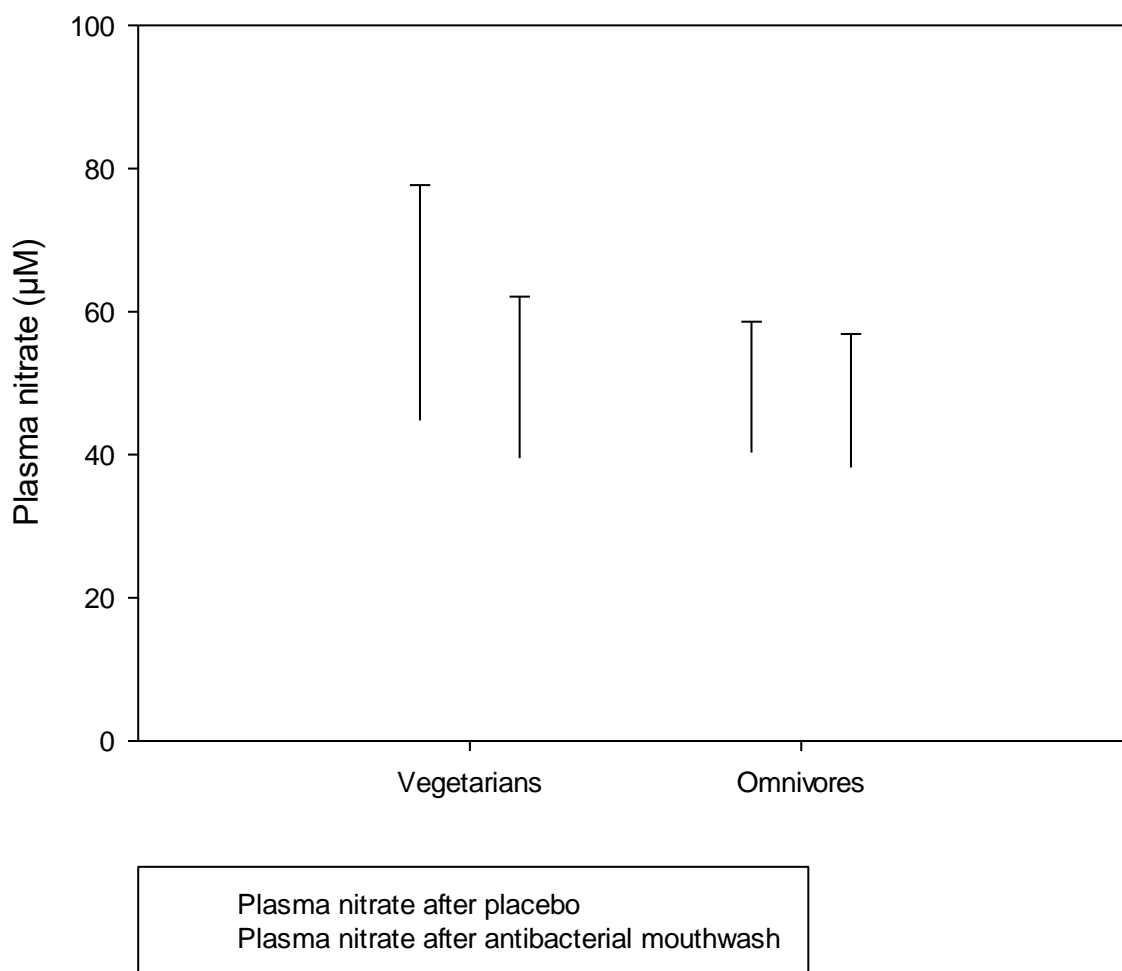


Figure 4.14. Effects of placebo and antibacterial mouthwash on plasma nitrate in vegetarians and omnivores ($P > 0.05$; ANOVA)¹².

¹² Error bars indicate standard deviation.

There was a significant main effect of mouthwash on plasma nitrite in vegetarians and omnivores ($P = 0.002$). There was no significant interaction effect ($P = 0.375$) and the response was similar in both groups after using antibacterial mouthwash (Figure 4.15).

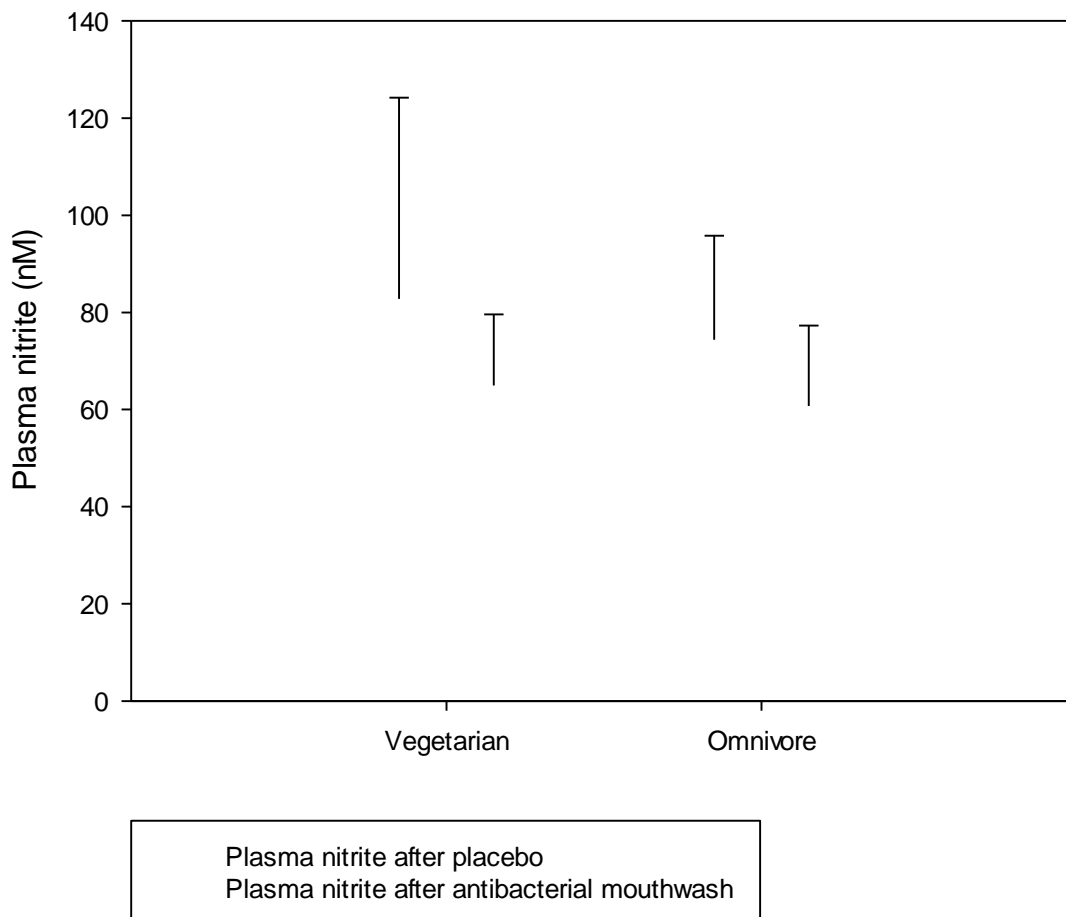


Figure 4.15. Effects of placebo and antibacterial mouthwash on plasma nitrite in vegetarians and omnivores ($*P < 0.05$; ANOVA)¹³.

¹³ Error bars indicate standard deviation.

Paired sample *t*-tests showed significant reductions in plasma nitrite concentrations in both vegetarians ($P = 0.033$) and omnivores ($P = 0.005$) (Appendix 23).

Regarding saliva, independent samples *t*-tests showed no significant differences in salivary nitrate or nitrite between vegetarians and omnivores after placebo mouthwash (Table 4.10).

Table 4.10. Salivary nitrate, nitrite and oral nitrate-reducing capacity after placebo mouthwash in vegetarians and omnivores (mean \pm SD).

	Vegetarians	Omnivores	<i>P</i> value
Nitrate (μM)	525.0 \pm 698.3	542 \pm 571.0	0.929
Nitrite (μM)	329.1 \pm 340.0	319.2 \pm 282.8	0.919
Oral nitrate reducing capacity (μM)	222.6 \pm 171.1	247.8 \pm 155.3	0.624

There were no significant main or interaction effects of mouthwash on salivary nitrate ($P = 0.187$ and $P = 0.336$ respectively) in vegetarians and omnivores (Figure 4.16).

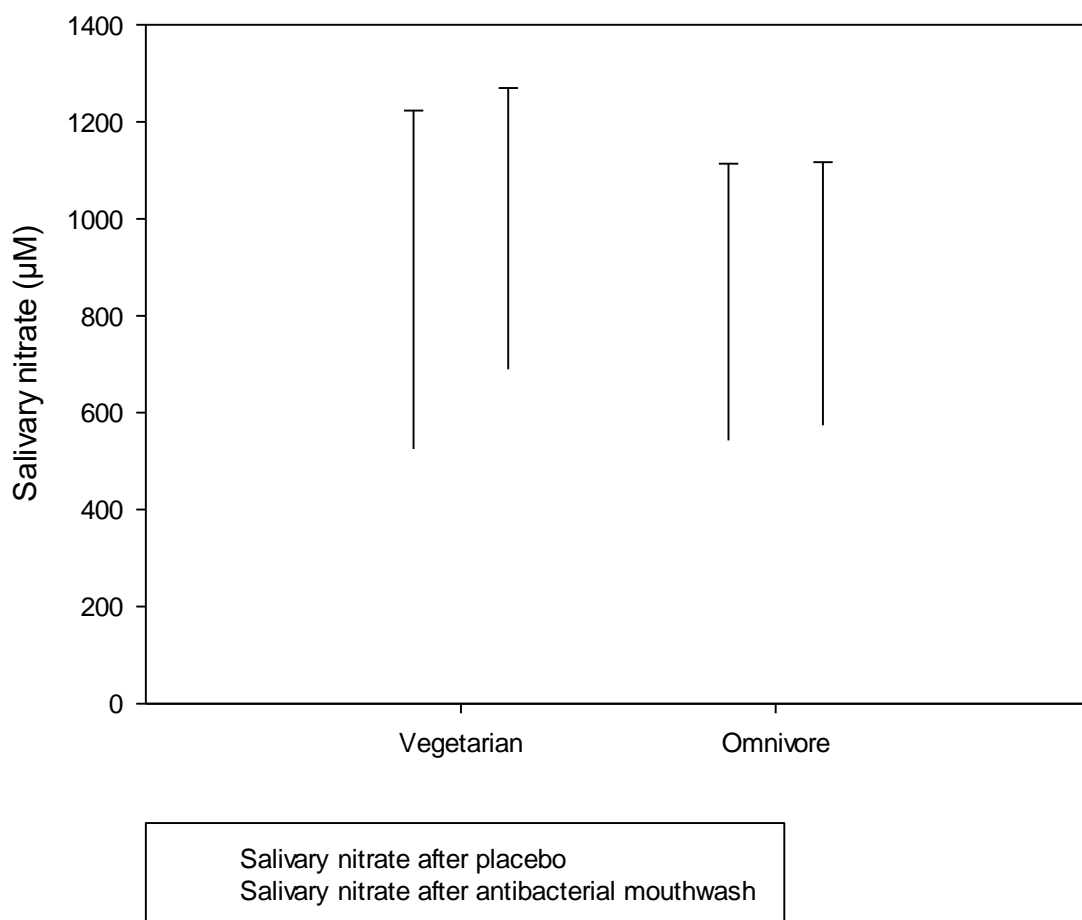


Figure 4.16. Effects of placebo and antibacterial mouthwash on mean salivary nitrate ($P > 0.05$; ANOVA).¹⁴

There was a significant main effect of mouthwash on salivary nitrite in vegetarians and omnivores ($P = 0.001$). There was no significant interaction effect ($P = 0.907$) and the response to antibacterial mouthwash was similar in both groups (Figure 4.17).

¹⁴. Error bars indicate standard deviation.

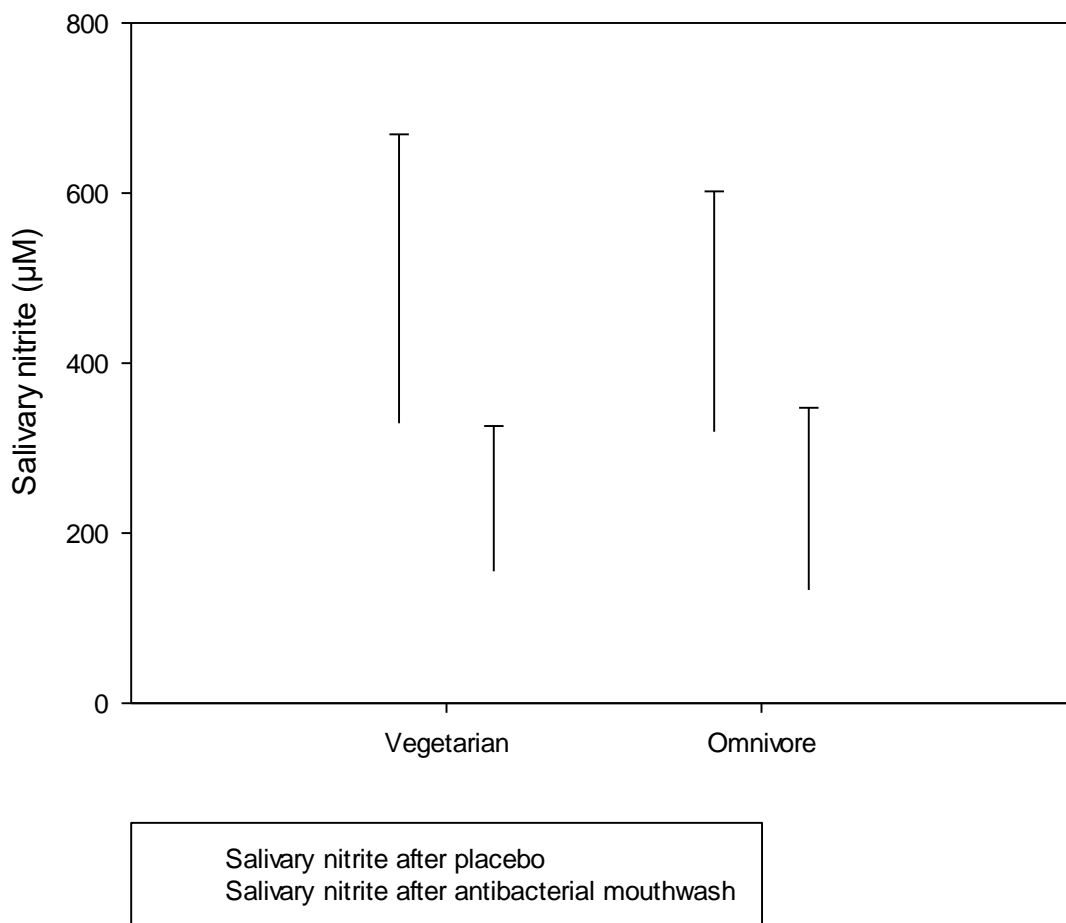


Figure 4.17. Effects of placebo and antibacterial mouthwash on mean salivary nitrite (* $P < 0.05$; ANOVA).¹⁵

Paired sample t -tests suggest statistically significant decreases in salivary nitrite after antibacterial mouthwash in both vegetarians ($P = 0.009$) and omnivores ($P = 0.039$) (Table 4.11).

¹⁵ Error bars indicate standard deviation.

Table 4.11. Salivary nitrate, nitrite and oral nitrate-reducing capacity after placebo and after antibacterial mouthwash in vegetarians and omnivores (mean \pm SD).

	Vegetarians			Omnivores		
	Placebo	Mouthwash	<i>P</i>	Placebo	Mouthwash	<i>P</i>
Nitrate (μ M)	525.0 \pm 698.3	720 \pm 575.9	0.027	542.9 \pm 571.0	574.0 \pm 543.2	0.841
Nitrite (μ M)	329.1 \pm 340.0	155.0 \pm 171.0	0.009	319.2 \pm 282.8	133.1 \pm 214.4	0.039
Oral nitrate reducing capacity (μ M)	222.6 \pm 171.1	49.5 \pm 69.3	0.000	247.8 \pm 155.3	38.0 \pm 68.9	0.000

4.3.7 Oral nitrate-reducing capacity (ONRC)

Independent samples *t*-tests showed no significant differences in oral nitrate-reducing capacity after placebo mouthwash (Table 4.10).

There was a significant main effect of mouthwash on oral nitrate-reducing capacity ($P < 0.001$). There was no significant interaction effect ($P = 0.466$) and antibacterial mouthwash significantly impaired oral nitrate-reducing capacity in both omnivores and vegetarians (Figure 4.18). Paired sample *t*-tests suggest that oral nitrate-reducing capacity was significantly reduced in both groups ($P < 0.001$) (Table 4.11).

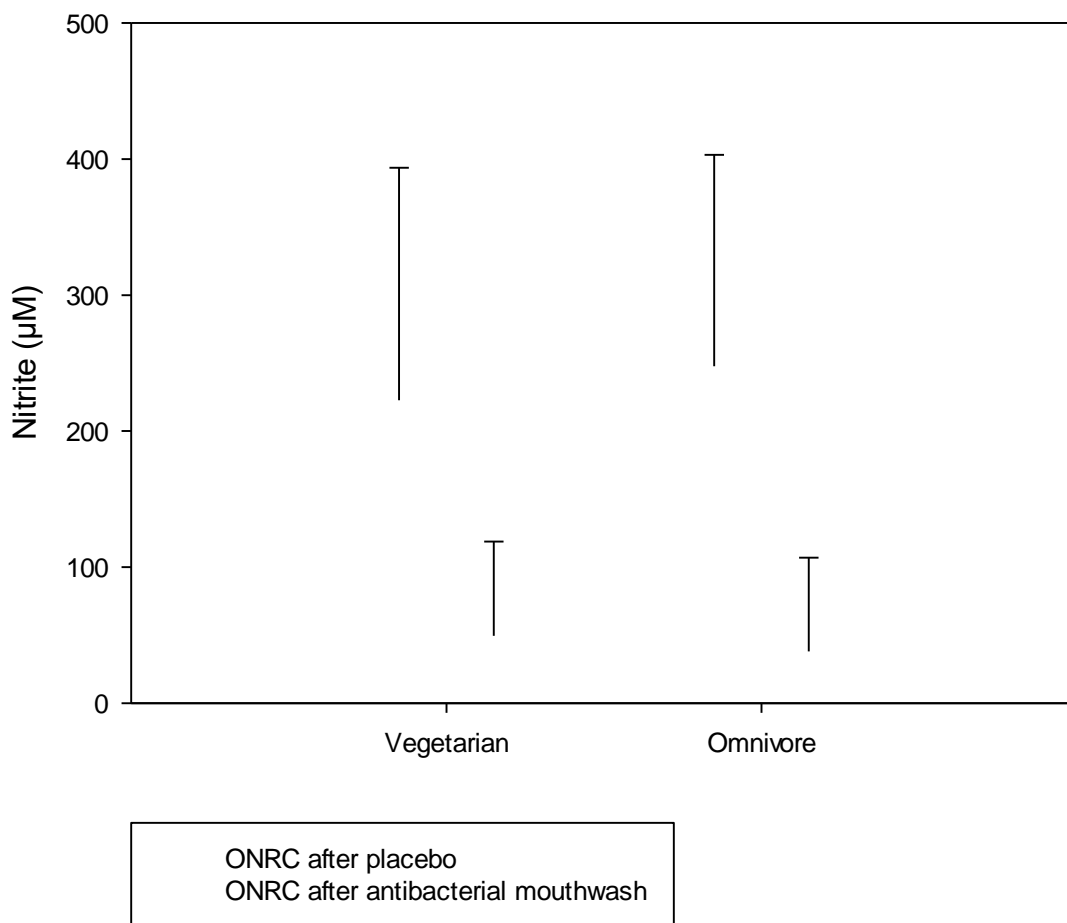


Figure 4.18. Mean oral nitrate-reducing capacity measured after 10 ml sodium nitrate solution (80 μmol) held in the mouth for 5 min ($P < 0.001$; ANOVA). 16.**

4.3.8 Salivary pH

Independent samples *t*-tests showed no significant differences in salivary pH after placebo mouthwash ($P = 0.375$) (Table 4.12).

Table 4.12 Salivary pH after placebo mouthwash in vegetarians and omnivores (mean \pm SD).

	Vegetarians	Omnivores	<i>P</i> value
Salivary pH	6.83 \pm 0.19	6.88 \pm 0.14	0.375

16 Error bars indicate standard deviation.

There was a significant main effect of antibacterial mouthwash on salivary pH ($P < 0.001$). There was no significant interaction effect ($P = 0.687$) and antibacterial mouthwash reduced salivary pH in both groups (Figure 4.19). Paired sample t -tests suggest that antibacterial mouthwash significantly reduced salivary pH in both groups ($P < 0.001$).

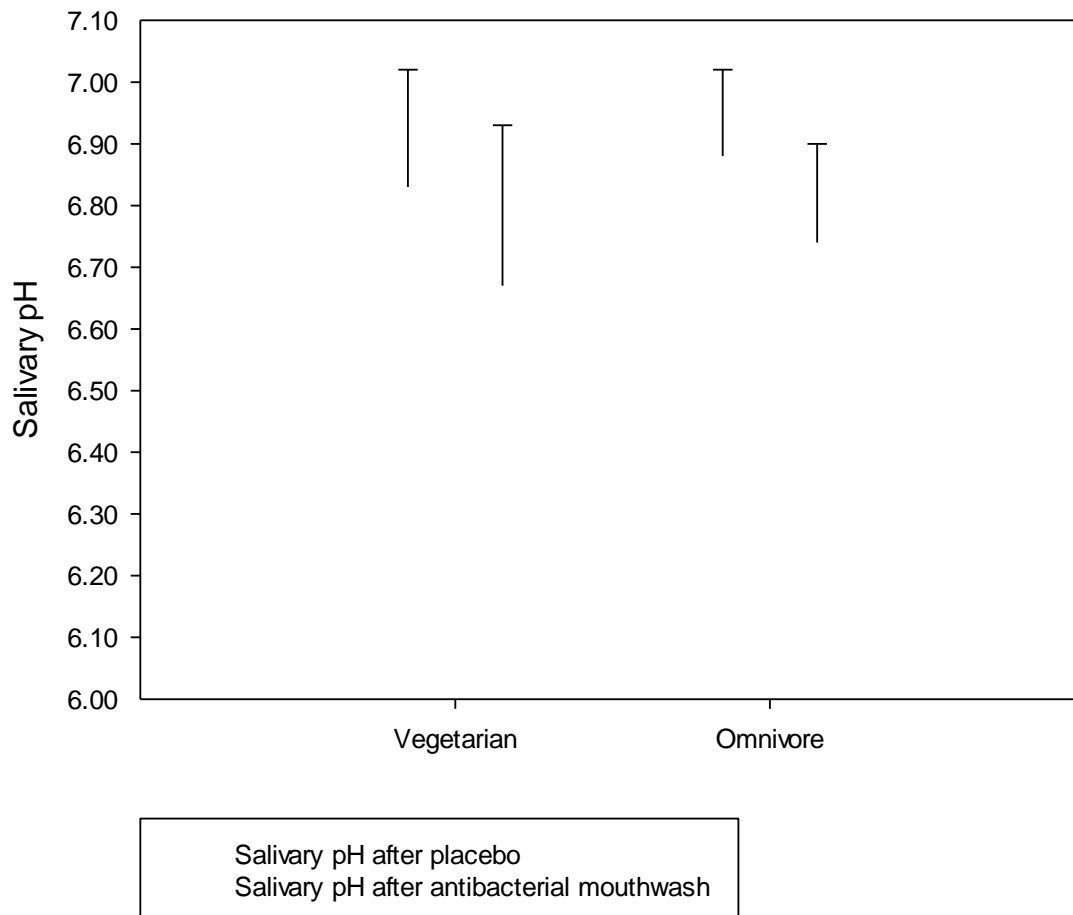


Figure 4.19. Mean salivary pH in vegetarians and omnivores after placebo and antibacterial mouthwash (* $P < 0.001$; ANOVA)¹⁷.

4.3.9 Blood lipids and glucose

Using independent sample t -tests, blood lipid profile did not differ ($P > 0.05$) between vegetarians and omnivores after placebo mouthwash, apart from unexpectedly lower

¹⁷ Error bars indicate standard deviation.

concentrations of serum triglycerides in omnivores ($P = 0.005$). Similarly, no differences were found between groups in blood glucose levels after placebo mouthwash ($P = 0.078$). There were no significant main ($P = 0.182$) or interaction ($P = 0.309$) effects of antibacterial mouthwash on blood cholesterol levels. There were significant main ($P = 0.047$) and interaction effects ($P = 0.007$) of antibacterial mouthwash on serum triglyceride concentrations. Paired sample t -tests suggest that antibacterial mouthwash significantly increased serum triglyceride levels in omnivores, but not in vegetarians (Table 4.13).

Table 4.13. Lipid and glucose results in vegetarians and omnivores after placebo and after antibacterial mouthwash (mean \pm SD).

	Vegetarians				Omnivores			
(mmol/l)	Placebo	ABMW	Δ	<i>P</i>	Placebo	ABMW	Δ	<i>P</i>
TC	4.14 \pm 0.76	3.99 \pm 0.66	0.15	0.092	4.31 \pm 0.61	4.31 \pm 0.65	0.01	0.905
HDL	1.55 \pm 0.35	1.52 \pm 0.33	0.03	0.381	1.66 \pm 0.43	1.65 \pm 0.42	0.01	0.860
LDL	2.21 \pm 0.5	2.10 \pm 0.5	0.13	0.08	2.32 \pm 0.5	2.28 \pm 0.51	0.05	0.396
Non-HDL	2.59 \pm 0.57	2.47 \pm 0.50	0.12	0.11	2.63 \pm 0.54	2.63 \pm 0.49	0	1.00
TG	0.83 \pm 0.23	0.84 \pm 0.22	0.01	0.777	0.59 \pm 0.13	0.71 \pm 0.2	0.11	0.029
BG	4.29 \pm 0.31	4.19 \pm 0.38	0.1	0.279	4.45 \pm 0.31	4.36 \pm 0.31	0.09	0.233

ABMW, antibacterial mouthwash; Δ , change; TC, total cholesterol; HDL, HDL cholesterol; LDL, LDL cholesterol; Non-HDL, non-HDL cholesterol; TG, triglycerides; BG, blood glucose.

4.3.10 Correlations

There was a significant correlation ($r = 0.57$, $P = 0.001$) between changes in salivary nitrite and changes in plasma nitrite in vegetarians and omnivores grouped together ($n = 41$) (Figure 4.20). This was expected as nitrite formation was inhibited by antibacterial mouthwash.

Figure 4.20. Change in salivary nitrite (μM) versus change in plasma nitrite (nM) in vegetarians and omnivores ($n = 41$) after antibacterial mouthwash ($r = 0.57$, $P = 0.001$).

Furthermore, for the whole group ($n = 41$) there were no significant correlations between dietary nitrate intake and change in SBP ($r = -0.008$; $P = 0.959$) or DBP ($r = -0.037$, $P = 0.820$) respectively. There were no significant correlations between changes in ONRC and changes in SBP ($r = -0.085$, $P = 0.598$) or DBP ($r = 0.104$, $P = 0.519$).

4.3.11 Summary

The main findings of this study were that estimated dietary nitrate intake was similar in both vegetarians and omnivores, contrary to previous research (Ministry of Agriculture Fisheries and Food, 1992; Mitek, Anyzewska & Wawrzyniak, 2013). In addition, no significant differences were found in BP, blood glucose or cholesterol levels between groups under baseline conditions. This is in contrast to previous research suggesting that vegetarians have better cardiovascular health compared to omnivores, although physical activity levels were not reported (Navarro *et al.*, 2016; Wang *et al.*, 2015; Yokoyama, Tsubota & Watanabe, 2016). Antibacterial mouthwash effectively impaired the nitrate-reducing capacity of oral bacteria, limiting salivary nitrite bioavailability, but this was not associated with changes in BP and RMR in this group of vegetarians and omnivores. In addition, a significant decrease in salivary pH was found in both groups after the antibacterial mouthwash treatment. This finding requires further attention as it may cause substantial changes in the oral microbiome and may increase the risk of oral diseases (Marsh, 2018; Radcliffe *et al.*, 2002). Data from previous studies suggest that reduced salivary pH is associated with hypertension (Kagawa *et al.*, 2013; Nimma *et al.*, 2016). Although no significant differences in BP were noted in this third and final study, it could conceivably be hypothesised that salivary pH, the oral microbiome and BP are interconnected.

4.3.12 Study limitations

The sample size was relatively small with the majority of participants recruited from the student population of the University of Plymouth. Therefore the results could be subject to selection bias and not generalisable to the general population. In addition, approximately 14% had varying levels of knowledge of nutrition and dietetics. However, these 6 participants were evenly divided between both groups, so it is less likely that this prior

knowledge affected the results. It was not possible to randomise participants to different dietary conditions, as a key goal of this study was to analyse dietary nitrate intakes of participants following vegetarian diets for at least one year. Another objective was to compare plasma and salivary nitrate and nitrite concentrations of vegetarians with a group of omnivores of similar characteristics. In addition, treatments (placebo mouthwash versus antibacterial mouthwash) were not randomised as it is unknown how long the oral microbiome takes to recover after using antibacterial mouthwash for several days. A placebo mouthwash was not available, and so a similar approach to a previous study by Kapil *et al.* (2013) was used in order to choose the placebo (water).

In this study, the EFSA database and recent figures from the FSA were used to estimate nitrate content of vegetables as they contained the largest number of samples with a wide range of vegetables and an approved methodology was used for analysis (European Food Safety Authority, 2008; Food Standards Agency, 2004). Ideally, duplicate diets could be analysed for nitrate content by using HPLC methods to obtain a more accurate assessment of nitrate intake. However, this approach would have been very time consuming and would require additional laboratory resources and funding but could be considered for future research. As discussed in Section 4.1.8, methodological limitations apply for nutritional analysis of food diaries, which can be inaccurate for various reasons such as the participants forgetting to record foods eaten or incorrect interpretation of portion sizes and this could affect the validity of estimated nitrate intake (Lovegrove *et al.*, 2015). In particular, the relatively low energy intakes of both vegetarians and omnivores suggest that underreporting could have occurred. However, there were no significant differences between the groups in salivary or plasma nitrate concentrations at baseline, suggesting that dietary nitrate intake corresponded to these biomarkers.

5 DISCUSSION

5.1 The effects of a high-nitrate vegetable diet on plasma nitrate and nitrite concentrations.

An initial objective of this thesis was to explore previous findings reported by Bailey *et al.* (2009) which showed a significant decrease in SBP and increased plasma nitrite concentrations after supplementation with 500 ml of beetroot juice (~350 mg nitrate/day for 6 days). In particular, whether nitrate obtained from whole high-nitrate vegetables would have the same effect as beetroot juice.

The results from the first two studies in this thesis support the hypothesis that the nitrate content of vegetables is a key factor in the bioavailability of nitrate, confirming findings from other studies using high-nitrate vegetables (Bondonno *et al.*, 2014; Jonvik *et al.*, 2016; Sobko *et al.*, 2010). The results suggest that whole fresh vegetables (providing ~300-400 mg nitrate/day) can significantly increase plasma nitrate and nitrite concentrations to a similar degree as supplements such as beetroot juice. As outlined in Section 1.8, the metabolism of dietary nitrate is complex and can be affected by many factors, such as dose and nitrate source (James *et al.*, 2015). However, most of this work has been based on the effect of nitrate supplements such as sodium nitrate and beetroot juice, not whole, fresh vegetables. For example, early landmark studies investigating the effects of dietary nitrate on BP used sodium nitrate, in a dose 'equivalent to 300g lettuce or spinach' (~496 mg nitrate) (Lundberg & Govoni, 2004). Beetroot juice was used in the study by Webb *et al.* (2008) as it has the highest nitrate concentration (2790 mg/l) of any commercially available juice and it was felt impractical to ask participants to eat 2-3 lettuces. Also, it has been reported that supplements have ~100% greater effects on plasma nitrate concentrations than vegetables, despite the higher nitrate contents of the

vegetables used¹⁸ (van Velzen *et al.*, 2008). A further study suggested that the consumption of high-nitrate vegetables (providing 155 mg nitrate/day for 3 days) failed to show any impact on plasma nitrate and nitrite concentrations of 8 participants¹⁹ (Miller *et al.*, 2012). These authors suggested this was because the nitrate dose was distributed throughout the day and therefore was not large enough to raise plasma levels (Miller *et al.*, 2012). Similarly, a recent UK FSA report concluded that 8 weeks of a high-nitrate diet (providing ~140 mg/day) increased plasma nitrate but not nitrite in 66 participants²⁰ (Haldar *et al.*, 2015). The nitrate dose (300-400 mg nitrate/day) in the first two studies of this thesis was approximately twice as much as that reported by Miller *et al.* (2012) and Haldar *et al.* (2015). Therefore, the findings from the first two studies of this thesis suggest that more than 140-155 mg nitrate/day from vegetables is needed in order to significantly increase plasma concentrations of nitrate and nitrite. This is consistent with findings of two systematic reviews of dietary nitrate (mainly supplements), which concluded that doses of nitrate from 124 mg/day (Hobbs, George & Lovegrove, 2013) and from 157 mg/day (Siervo *et al.*, 2013) were effective in reducing systolic BP. In addition, the results suggest that a variety of whole fresh high-nitrate vegetables have similar effects on plasma nitrate and nitrite concentrations as supplements such as beetroot juice (Table 5.1).

¹⁸ Sodium nitrate supplement (500 mg nitrate) had greater effect on plasma nitrate concentrations than spinach (564 mg nitrate), lettuce (1014 mg nitrate) or cooked beetroot (643 mg nitrate).

¹⁹ 3 men, 5 women, mean age 73 ± 5 years, BMI 29 ± 4 kg/m², baseline SBP 132 (95% CI; 112-153) mmHg.

²⁰ 34 men and 32 women; mean age 57 ± 9 years, BMI 26 ± 3 kg/m², baseline SBP 130 ± 9 mmHg.

Table 5.1. Plasma nitrite (mean \pm SD) in first male study and second female study compared with other relevant studies, post placebo and post supplement.

	Plasma nitrite Post placebo (nM)	Plasma nitrite Post supplement (nM)	% increase	Supplement	Nitrate dose (mg)
Bailey <i>et al.</i> (2009)	140 \pm 142	273 \pm 124	195	Beetroot juice	350
First male study	119 \pm 35	227 \pm 89	191	High-nitrate vegetables	417
Second female study	101 \pm 76	185 \pm 146	183	High-nitrate vegetables	340
Sobko <i>et al.</i> (2010)	132 \pm 75	204 \pm 102	154	High-nitrate vegetables	1100
Bondonno <i>et al.</i> (2014)	2000 \pm 1500	8000 \pm 6500	400	High-nitrate vegetables	400
McDonagh (2018)	76 \pm 16	371 \pm 136	488	Beetroot flapjack	360

The results from the first two studies support the idea that the availability of nitrate is not affected by the higher fibre content of fresh vegetables compared to juice extracts. Theoretically, the higher fibre content of vegetables could alter absorption due increasing the viscosity of gastric contents (Thomas & Bishop, 2007) and this could explain the lesser effects of vegetables on plasma nitrate concentrations reported by Van Velzen *et al.* (2008). In addition, eating vegetables involves extra chewing and an associated increase in saliva production, which could decrease salivary nitrate and nitrite concentrations (Granli *et al.*, 1989; McDonagh *et al.*, 2018). Nevertheless, a recent study reported similar plasma nitrite concentrations after beetroot flapjack compared to concentrated beetroot shots (McDonagh *et al.*, 2018). These results together with findings from the first two studies suggest that the availability of nitrate from high-nitrate vegetables and subsequent

effects on plasma nitrate and nitrite concentrations is equivalent to dietary nitrate supplements, such as beetroot juice and sodium nitrate.

Further studies in different population groups have provided evidence to support the hypothesis that high-nitrate vegetables significantly increase plasma nitrate and nitrite concentrations. The effects of 10 days on a traditional Japanese diet (providing ~1100mg nitrate/day) were compared to a control diet (avoiding high-nitrate vegetables) in a group of 25 healthy participants²¹ (Sobko *et al.*, 2010). The traditional Japanese diet, which included seaweed and common Japanese vegetables, increased plasma nitrate and nitrite concentrations by 356% and 154% respectively (Sobko *et al.*, 2010). This is comparable to increases in plasma nitrite found in the first two studies of this thesis (Table 5.1), even though the nitrate content of the Japanese diet was much higher. Similarly, Bondonno *et al.* (2104) supplemented the diets of 38 older Australian participants²² with frozen spinach and fresh, green leafy vegetables for 7 days compared to a low-nitrate diet. The high-nitrate diet (providing 400 ± 98 mg nitrate/day) resulted in increases in plasma nitrate by 600% and plasma nitrite by 400% (Bondonno *et al.*, 2014). A gas chromatography and mass spectrometry technique was used for measurement of plasma nitrate and nitrite, instead of chemiluminescence, which could explain the higher plasma nitrite concentrations reported (Table 5.1) (Bondonno *et al.*, 2014). Therefore, even though the characteristics of participants in the first two studies were different to Sobko *et al.* and Bondonno *et al.* with respect to diet, age, baseline SBP, smoking status and BMI, the results from the first two studies of this thesis confirm that consumption of high-nitrate vegetables significantly increases plasma nitrate and nitrite concentrations.

²¹ 10 men and 15 women; mean age 36 ± 10 years, BMI < 18.5 kg/m², 2 smokers.

²² 12 men and 26 women; mean age 61 ± 7 years, BMI 27 ± 4 kg/m², baseline SBP 120-139 mmHg.

As outlined in Section 1.8, the nitrate-nitrite-nitric oxide pathway has important biological functions, especially in conditions of hypoxia (Lundberg, Weitzberg & Gladwin, 2008). The bioavailability of nitrite is a key part of this pathway (Lundberg & Weitzberg, 2009). The results from the first two studies of increased plasma nitrate and nitrite concentrations are important because both plasma nitrate and nitrite have been used in the past to indicate the bioavailability of NO (Lundberg & Govoni, 2004; Lundberg & Weitzberg, 2013). However, it is recognised that plasma nitrite is the most important marker for NO metabolism (Kevil *et al.*, 2011), evidenced by concomitant increases in cGMP (Kapil *et al.*, 2010). The results from the first two studies suggest that high-nitrate vegetables can increase plasma nitrite concentrations (Table 5.1) and therefore increase nitrite bioavailability and subsequent NO bioactivity²³ (Section 1.7). This could be important for the future treatment and prevention of CVD, as reduced NO bioactivity is a central feature of CVD (Carlström, Lundberg & Weitzberg, 2018; Lundberg, Gladwin & Weitzberg, 2015). Previous studies have suggested that a gender difference may exist in the response to nitrate, which might account for lower BP and incidence of CVD in premenopausal women in the general population (Forte *et al.*, 1998; Kapil *et al.*, 2010). Although the nitrate dose in the first male study was higher²⁴ than the second female study²⁵ the mean dose/kg was similar at 5.6 mg and 5.4 mg nitrate/kg/day respectively. Beetroot juice (4.3 mg nitrate/kg/day) resulted in similar increases of plasma nitrite, with similar baseline plasma nitrite concentrations compared to the first male study (Bailey *et al.*, 2009) (Table 5.1). In contrast, previous studies evaluating a gender difference have noted higher plasma nitrite concentrations in premenopausal women at baseline and after supplementation with

²³ Bioactivity can be defined as the property of a substance to exert an effect on, or response from, a living organism

²⁴ 420 mg from ~3 portions of vegetables/day

²⁵ 340 mg from ~2 portions of vegetables /day

potassium nitrate (Kapil *et al.*, 2010). The findings of Kapil *et al.* are consistent with earlier studies where greater whole body production of NO was observed in healthy premenopausal women after L-arginine administration (Forte *et al.*, 1998). According to the results of Kapil *et al.*, higher baseline concentrations of plasma nitrite in women and greater increases after supplementation (~twofold more than men) would have been expected, but this was not the case. One explanation for the lower baseline plasma nitrite concentrations seen in women might be that many of the male participants were highly trained athletes (including triathletes, a cross country skier, a rower and rugby players). A higher training status has been associated with higher baseline concentrations of plasma nitrite (Christensen *et al.*, 2017), suggesting that this could be the reason for higher plasma nitrite concentrations in the first male study.

In summary, results from the first two studies support the hypothesis that the nitrate content of vegetables is a key factor involved in increasing plasma nitrite concentrations. Previous studies have associated increased bioavailability of nitrite with bioactivity of NO, which is associated with cardiovascular health. Therefore, increased plasma nitrite concentrations could have important implications for the future prevention and treatment of CVD, by increasing NO bioactivity.

5.2 Relationship between plasma nitrate and nitrite concentration and respiratory response to exercise

Much of the recent research on dietary nitrate has reported that nitrate supplements reduced the oxygen cost of exercise (Bailey *et al.*, 2009; Larsen *et al.*, 2007; Vanhatalo *et al.*, 2010). A further objective was to explore the findings of Bailey *et al.* (2009) on the oxygen cost of exercise after beetroot juice, but using whole, fresh high-nitrate vegetables

instead. In this section, the respiratory response to moderate exercise after supplementation with high-nitrate vegetables will be discussed.

Current physiological knowledge suggests that consumption of oxygen at a given workload on different occasions should be nearly identical (Larsen *et al.*, 2010). However, studies by Larsen *et al.* (2007) and Bailey *et al.* (2009) showed that supplementation with sodium nitrate and beetroot juice respectively²⁶ significantly reduced the oxygen cost of exercise in young, healthy males. These results were surprising because they challenged current thinking. The authors hypothesized that their findings were due to the reduction of dietary nitrate to nitrite and NO (Bailey *et al.*, 2009; Larsen *et al.*, 2007) although the precise mechanism is subject to debate (Section 1.10). The first study (in males only and using whole vegetables) attempted to replicate the results obtained by Bailey *et al.* (2009). However, the results did not replicate the reduction in oxygen consumption during exercise, even though a higher dose of dietary nitrate was provided in the form of high-nitrate vegetables for two weeks. The reasons for the discrepancies between the first male study and that by Bailey *et al.* could be due to various factors.

Firstly, the dosing protocol used by Bailey *et al.* (2009) was different in that participants were instructed to sip beetroot juice throughout the day when exercise testing took place and therefore was an acute supplementation study. In the first male study, participants were requested to eat high-nitrate vegetables for two weeks, and as part of their last meal at least three hours before attending the lab, meaning there was a greater delay between nitrate intake and exercise tests compared to subjects in the study by Bailey *et al.* As plasma nitrite tends to peak ~2-3 hours after nitrate consumption, with a steady decline after 4 hours (McDonagh *et al.*, 2018; Webb *et al.*, 2008), it is possible that plasma nitrite concentrations were already decreasing by the time participants attended the laboratory. In addition, the sensitivity analysis suggests that the 4 participants who did not eat high-

²⁶ Bailey *et al.* (2009); 350 mg nitrate/day for 6 days.

nitrate vegetables before the test had lower increases in plasma nitrate and nitrite levels. These factors could explain the lower plasma nitrite concentrations after supplementation obtained in the first male study (227 ± 89 nM) compared to those reported by Bailey *et al.* (273 ± 124 nM). Other factors such as storage and treatment of plasma samples could have affected plasma nitrite concentrations, although the same laboratory was used in both studies.

Secondly, and possibly more importantly, as noted in Section 5.1, many of the participants in the first male study were trained athletes in contrast to the 'recreationally active' participants in the study by Bailey *et al.* (2009). Prior studies have noted reduced physiological effects of dietary nitrate supplementation in highly trained athletes (Christensen *et al.*, 2017; Porcelli *et al.*, 2015; Rassaf *et al.*, 2007). In an attempt to explain these lesser effects, it has been suggested that NO bioactivity is greater in well-trained subjects compared to less trained individuals (Bescos *et al.*, 2012), and therefore the effect of dietary nitrate is reduced. This finding is consistent with that of Green *et al.* (2004) who suggested that short-term exercise increases NO bioactivity, but if exercise is maintained, short-term functional adaptations become normalized (Green *et al.*, 2004). This normalization is reflected in lower plasma nitrite concentrations after nitrate supplementation in well-trained individuals (Porcelli *et al.*, 2015). In support of this normalization hypothesis, plasma nitrite concentrations in the first male study (227 ± 89 nM) were similar to other studies which compared plasma nitrite concentrations of well-trained athletes (266 ± 164 nM) to less-trained individuals (462 ± 250 nM) (Porcelli *et al.*, 2015). A possible explanation for the normalisation process might be that the production of nitrite and subsequent nitroso-species are precisely regulated in the body, and furthermore, that large quantities of dietary nitrate supplements provide no further benefit in well trained athletes. The results from the first male study seem to support the

hypothesis that the effects of nitrate supplements may be associated with the fitness level of participants.

Thirdly, the duration and intensity of the exercise tests were different. Bailey *et al.* (2009) used a combination of moderate- and severe-intensity exercise tests on the final two days of testing, whilst moderate-intensity exercise tests were used throughout the first male study. The nitrate-nitrite-NO pathway is mainly activated when oxygen tension falls, such as under anaerobic conditions caused by severe-intensity exercise (Lundberg, Weitzberg & Gladwin, 2008). Thus, during the moderate-intensity exercise tests in the first male study, the nitrate-nitrite-NO pathway may not have been fully activated, which might partly explain the non-significant results.

The findings from the first male study are in contrast to previous studies, which reported that nitrate supplements improve exercise efficiency and time to exhaustion in various types of exercise tests (Section 1.10). However, they are in agreement with other studies showing no significant effect on the oxygen cost of submaximal exercise (Bescós *et al.*, 2012; Betteridge *et al.*, 2016; Cermak, Gibala & van Loon, 2012; Peacock *et al.*, 2012). These contrasting findings in the literature could not only be due to methodological differences already discussed, such as the training status of participants, but also due to individual differences in response to dietary nitrate. The concept of 'high responders' and 'low responders' has been suggested in several studies, where significantly different individual responses to nitrate were noted (Coggan *et al.*, 2018; Wilkerson *et al.*, 2012). A possible explanation for differences in response could be due to individual differences in the oral microbiome, which will be discussed further in Section 5.5.

In summary, these findings do not support the hypothesis that consumption of high-nitrate vegetables reduces the oxygen cost of moderate-intensity exercise. Having discussed the effects of high-nitrate vegetables on plasma nitrite concentrations and the oxygen cost of moderate-intensity exercise, the next section discusses the effects of dietary nitrate on

blood pressure, as this was a key finding of early research into the effects of dietary nitrate supplements (Larsen *et al.*, 2006).

5.3 Plasma nitrate and nitrite concentration and blood pressure regulation in healthy men and women

A strong relationship between dietary nitrate and BP has been reported in the literature (Section 1.9). However, no significant change in SBP was detected in the first male study after supplementation with high-nitrate vegetables. This outcome is contrary to two previous systematic reviews (Hobbs, George & Lovegrove, 2013; Siervo *et al.*, 2013), subsequent studies (Table 1.6,) and a recent systematic review which assessed BP in addition to other cardiovascular risk factors (Jackson *et al.*, 2018).

One explanation is that the nitrate dose was insufficient. Approximately 420 mg/day was provided for 14 days to the participants in the first male study. The average nitrate dose used in recent human studies, using a variety of forms of nitrate supplements, is ~500 mg/day (range 139-1042 mg), resulting in corresponding mean reductions in SBP of ~7 mmHg (range 3-12 mmHg) (Table 1.6). A recent systematic review estimated that as little as 130-259 mg nitrate/day effectively reduced SBP (-5.5 mmHg; 95% CI, -9.5 – -1.5; P = 0.007) (Jackson *et al.*, 2018). Therefore, it seems unlikely that the nitrate dose used in the first male study was insufficient. However, despite an adequate dose, BP changes (3 mmHg) were only half of the values reported by other studies and not statistically different. Secondly, the discrepancy between the results obtained and other studies could be attributed to baseline SBP. For example, even though participant characteristics were similar²⁷, SBP after the low-nitrate diet was lower (119 ± 10 mmHg) than reported by Bailey *et al.* (2009) after placebo (132 ± 5 mmHg) and also lower than the population

²⁷ Age; 25-26 years and BMI; 24-25 kg/m²

average for men of that age (125 mmHg) (Health and Social Care Information Centre, 2011). Consequently, the lower than average SBP of the participants in the first male study could have attenuated the effect of dietary nitrate. This is consistent with previous studies including participants with low baseline SBP; 115 ± 9 mmHg (Haider & Folland, 2014), 119 ± 3 mmHg (Cermak, Gibala & van Loon, 2012), 119 mmHg (Kim *et al.*, 2014), 115 ± 3 mmHg (Wightman *et al.*, 2015), 121 ± 12 (Shepherd *et al.*, 2016) and 110 ± 14 mmHg (Kroll *et al.*, 2018) which reported non-significant reductions in BP after beetroot juice supplementation, despite significant changes in plasma nitrate or nitrite concentrations.

Prior studies have noted that participants with higher baseline SBP respond to a greater extent to interventions to reduce BP. For example, participants following the DASH diet with higher BP at baseline experienced greater reductions (Appel *et al.*, 1997) similar to the effects of BP-lowering drugs (Law *et al.*, 2003). The participants in the study by Bailey *et al.* were pre-hypertensive (i.e. with SBP in the range of 120-139 mmHg, predisposing to hypertension) so they could potentially respond better to a BP-lowering treatment such as dietary nitrate. Therefore, it is possible that participants in the study by Bailey *et al.* experienced a greater reduction in SBP because their baseline BP levels were higher. Consistent with these results, individuals with higher baseline SBP values showed a greater decrease after nitrate supplementation (Figure 4.9) (Ashworth *et al.*, 2015b; Kapil *et al.*, 2010).

Under powering of the study is the most probable explanation for the lack of significant change in SBP in the first male study. Even though 21 participants were recruited (with some difficulty), subsequent drop outs and exclusions meant that data was only available from 15 participants. A retrospective sample size calculation²⁸ showed a sample size of 43 would be required to determine whether the difference seen (~3 mmHg) was true. A

²⁸ Assuming a power of 80%, at the 5% level of significance and a standard deviation of 8 mmHg, to detect a difference of ~3 mmHg between the two groups resulted in an estimated sample size of 43 subjects.

decision was made in view of the difficulties of recruiting this population that it was not feasible to carry out the study to the required sample size.

A further important factor which could have impacted on the results of the first male study and other studies reporting non-significant findings (Table 1.7) is the key role of the oral microbiome. Until recently, there has been a lack of evidence to suggest whether differences in the oral microbiome could be related to effects of dietary nitrate. However, one new study compared the response to beetroot juice with the prevalence of oral nitrate-reducing bacteria (Burleigh *et al.*, 2018). This study found that a higher prevalence of oral nitrate-reducing bacteria was associated with higher salivary nitrite concentrations following consumption of beetroot juice, but not with changes in plasma nitrite or BP (Burleigh *et al.*, 2018). In an attempt to explain these findings, it was suggested that plasma nitrite concentrations might not increase further than a 'saturation threshold', whereby excess nitrite is excreted in urine, thus avoiding potentially excessive drops in BP. The authors note that physiological effects of other storage forms of NO, such as s-nitrosothiols and nitrated fatty acids should be taken into account i.e. that plasma nitrite may act as a marker for NO availability but not be directly responsible for the BP lowering effect (Burleigh *et al.*, 2018). This observation is supported by previous studies. For example, nitrated fatty acids have been reported to modify plasma nitrate and nitrite concentrations (Hughan *et al.*, 2017). Animal studies also suggest that there is an unrecognized but significant source that contributes to NO bioavailability, with plasma nitrite being an intermediary in the process (Milsom *et al.*, 2012). Nitrated fatty acids and s-nitrosothiols are formed from unsaturated fatty acids, such as oleic and linoleic acid, and activate NO pathways, as well as affecting the microbiome (Koch *et al.*, 2017). It is possible, therefore, that differences in unsaturated fatty acid intake could explain why the first study in males and other studies found no significant change in BP, despite significant changes in plasma nitrate and nitrite (Table 1.7). This links to previous findings of the

health benefits of the Mediterranean diet (Section 1.11), which is high in olive oil, a good source of oleic acid, and clearly needs further investigation.

The results from the first male study appear to corroborate with findings from a large UK FSA study which found no significant effect of supplementation with high-nitrate vegetables on BP in older participants (58 ± 1 years) with mild hypertension (Haldar *et al.*, 2015). However, this study failed to achieve the target intake of 100g high-nitrate vegetables daily and also failed to control for the use of antibacterial mouthwash or antibiotics, which could explain the negative results (Haldar *et al.*, 2015). Therefore, both the first male study of this thesis and the study by Haldar *et al.* found no significant effect of high-nitrate vegetables on BP but both studies had methodological limitations. In the light of previous systematic reviews (Hobbs, George & Lovegrove, 2013; Jackson *et al.*, 2018; Siervo *et al.*, 2013), these findings suggest that the effect of high-nitrate vegetables in reducing BP requires further research.

In contrast, the results from the second, female study of this thesis showing a reduction of SBP of ~ 4 mmHg confirm the findings of a previous systematic review indicating a drop of SBP of ~ 4 -5 mmHg after inorganic nitrate and beetroot juice supplementation (Siervo *et al.*, 2013). However, most of the study populations in the review consisted of healthy, young men (Siervo *et al.*, 2013) and therefore the second female study adds novel data that were previously lacking. As discussed in Section 5.1, there is evidence to suggest that a gender difference exists in NO synthesis with females showing higher production of NO than a similar group of men²⁹ (Forte *et al.*, 1998). This might explain, at least partially, lower rates of CVD in premenopausal women than in men of a similar age (Forte *et al.*, 1998). This gender difference in response to nitrate supplementation has been confirmed by comparing the effects of nitrate supplements on BP (Kapil *et al.*, 2010), where increased plasma nitrite concentrations were noted in women. A further gender difference

²⁹ 13 men aged 22 to 40 years and 11 women aged 23 to 42 years

was reported in platelet aggregation (an important factor in CVD) after supplementation with potassium nitrate (Velmurugan *et al.*, 2013). Consistent with the literature concerning gender difference, the results of the first two studies of this thesis could also suggest the possibility of a gender difference in the BP response to high-nitrate vegetables, in that the first male study did not find a significant reduction in BP, whereas the second female study did. However, the studies had different designs, so direct comparison of the results is not possible.

Furthermore, the results support the hypothesis that the consumption of whole, high-nitrate vegetables may be as effective as inorganic nitrate in pharmacological form (sodium or potassium nitrate) and beetroot juice in reducing SBP in healthy, young women. These findings are unique, as no other studies reporting reduced BP after nitrate supplementation have used a variety of fresh, whole vegetables commonly available in the UK in males or females. Instead, previous studies have used a traditional Japanese diet to reduce DBP by 4.5 mmHg (Sobko *et al.*, 2010) or frozen spinach (250g/day) and fresh salad (120g/day), with no effect on BP (Bondonno *et al.*, 2014). As mentioned in Section 1.9, different approaches have been used to increase nitrate intake, such as beetroot bread (Hobbs *et al.*, 2013), spinach soup (Jovanovski *et al.*, 2015), beetroot gel (Silva *et al.*, 2016), drinks made with rocket and spinach (Jonvik *et al.*, 2016), chard gel (McIlvenna *et al.*, 2017) and beetroot flapjack (McDonagh *et al.*, 2018). It has been questioned whether nitrate absorption in the form of whole vegetables differs to supplements such as inorganic nitrate salts and beetroot juice (McIlvenna *et al.*, 2017; Pannala *et al.*, 2003). Prior studies have noted that a high-nitrate meal of lettuce (222 mg nitrate) did not increase plasma nitrite, despite increases in plasma nitrate and salivary nitrite and nitrate (Pannala *et al.*, 2003). However, findings from the second study in females suggest absorption of nitrate from high-nitrate vegetables can be as effective as nitrate supplements.

The findings of an association between the reduction in SBP and baseline SBP in the second female study corroborate the findings by Kapil *et al.* (2010) ($r = -0.728$, $P < 0.001$). This occurred despite participants having a lower than average SBP (107 ± 9 mmHg) compared to the general population (16-24 yrs; 111 mmHg) (Ashworth *et al.*, 2015b). However, in contrast, no correlations were found between changes in plasma nitrite and changes in SBP as reported by Kapil *et al.* This could have been due to the much higher concentrations of plasma nitrite reported (~ 2.0 μmol) compared to the second female study (~ 200 nmol) as a result of using large nitrate doses of 1488 mg (Kapil *et al.*, 2010). This dose is nearly sixfold higher than the ADI (3.7 mg nitrate/kg/day, ~ 260 mg/d for a 70 kg adult).

This result of a significant reduction in SBP in young healthy females suggests that high-nitrate vegetables might be effective in reducing BP in this population group. This finding is consistent with recent research (Table 1.6) and previous systematic reviews (Ashor *et al.*, 2016; Hobbs, George & Lovegrove, 2013; Lara *et al.*, 2016; Siervo *et al.*, 2013).

One of the issues emerging from the findings of this thesis is the unknown consequences of exceeding the current ADI, outlined in the Introduction (Section 1.5). Both the male and female studies of this thesis have demonstrated that it is possible to achieve nitrate intakes in the short term of ~ 340 - 420 mg/d, by consumption of 2-3 portions of green leafy vegetables daily. This intake exceeded the ADI without any reported side effects, at least acutely, but the effects of this level of consumption over the long term are unknown. This is in contrast to beetroot juice which has commonly been reported to cause beeturia (Jackson *et al.*, 2018). The current ADI acts as an upper safe limit of intake, in order to restrict intake of large amounts of dietary nitrate over a life time. There have been suggestions that the ADI should be reviewed to reflect emerging evidence that nitrate is important for cardiovascular health in adults (Hord, Tang & Bryan, 2009; Kapil, Webb & Ahluwalia, 2010; Lidder & Webb, 2013; Lundberg *et al.*, 2011). Although epidemiological

evidence suggests that moderate intakes of dietary nitrate (53-76 mg/day) are protective against CVD (Blekkenhorst *et al.*, 2017a), there is further epidemiological evidence to suggest that high plasma nitrate concentrations³⁰ are associated with increased all-cause mortality (Maas *et al.*, 2017). In this latter prospective cohort study, plasma nitrate was found to be higher in participants with impaired kidney function, smoking, hypertension and diabetes, which are all known to contribute to increased mortality (Maas *et al.*, 2017). However, elevation of plasma nitrate can be caused by inflammation and the authors note that ideally, the elevated plasma nitrate resulting from impaired renal function and smoking should be differentiated from plasma nitrate derived from dietary nitrate intake (Maas *et al.*, 2017). Future research therefore should be undertaken to address the long-term effects of high-nitrate intakes and measure both plasma nitrate and nitrite concentrations.

The epidemic of hypertension both in the UK and world-wide has been outlined (Section 1.2). A reduction in SBP of ~3 mmHg across the population could result in significant reductions in stroke and ischaemic heart disease (He, Pombo-Rodrigues & MacGregor, 2014). The results from the second study in females suggest that a reduction in SBP of ~4 mmHg could be achieved by eating 2 portions of high-nitrate vegetables daily. As these findings were limited to young, healthy females, extrapolation to the general population is limited. However, this is an important issue for future research, including the practical aspects of eating two portions of high-nitrate vegetables daily.

Government guidelines to eat at least '5 A Day' specifies quantities (NHS Choices, 2015a) but does not, however, specify which types of fruit and vegetables should be consumed (NHS Choices, 2015b). The second female study confirms that consumption of low-nitrate vegetables (with avoidance of high-nitrate vegetables) resulted in no significant changes in BP. This is consistent with previous findings which demonstrated that supplementing diets

³⁰ Lowest plasma nitrate tertile; 26 µmol/l, IQ range; 22-29 µmol/l. Highest plasma nitrate tertile; 61 µmol/l, IQ range; 53-77 µmol/l.

with fruit and low-nitrate vegetables do not induce BP lowering effects (Berry *et al.*, 2010). Therefore, taking into account the results from the second female study, together with previous research (Table 1.6), in order to obtain effects on BP it may be necessary to include high-nitrate vegetables as part of the '5 A Day' guidelines. In this respect, guidance already exists recommending daily intake of high-nitrate vegetables (Lidder & Webb, 2013). An average 70 kg person could eat two portions of high-nitrate vegetables (equivalent to four 'nitrate units') and be within the ADI (Lidder & Webb, 2013). Therefore, similar to the second female study, sufficient dietary nitrate could be obtained from two portions of high-nitrate vegetables daily and this could be incorporated into existing '5 A Day' guidance.

In summary, results from the second female study support the hypothesis that increased plasma nitrate and nitrite resulting from consumption of fresh, whole, high-nitrate vegetables is associated with lower BP in healthy young women, but in trained male participants using moderate exercise testing the hypothesis was rejected. The potential to reduce BP using high-nitrate vegetables to improve cardiovascular health and the effects of eating this amount of nitrate over the long term require further research.

5.4 Dietary nitrate intake in vegetarians compared to omnivores and its potential link to blood pressure control

Following the first two studies of this thesis into short-term supplementation, a remaining question was to investigate the effects of long-term intakes of dietary nitrate on BP. However, clinical trials examining this question in humans can be challenging for different reasons, such as adherence to diet, diseases that can affect the intervention and different seasons of the year. Previous studies in animals have found none of the adverse effects historically linked with nitrate, such as cancer (Hezel *et al.*, 2015).

Indeed, long term supplementation with dietary nitrate at levels equivalent to a human dose of 350 mg/day was associated with improved insulin responsiveness, reduced inflammatory markers and improved survival (control group; 600.5 days, vs nitrate group; 640.5 days, $P < 0.05$), although the authors suggested this should be confirmed in a larger study (Hezel *et al.*, 2015). In addition, animal studies suggest that a nitrate deficiency state can be induced by feeding a low-nitrate diet for 18 months which is associated with increased body weight, hypertension and insulin resistance as well as alterations in the gut microbiome (Kina-Tanada *et al.*, 2017). These metabolic abnormalities were reversed by feeding sodium nitrate whereas animals fed a low-nitrate diet for 22 months died mainly due to CVD (Kina-Tanada *et al.*, 2017). A feasible approach to investigate the effect of chronic ingestion of dietary nitrate in humans was to analyse subjects following vegetarian diets and to compare them with similar subjects following an omnivorous diet. As discussed in Section 1.6, previous studies reported that vegetarians consume greater quantities of nitrate (Ministry of Agriculture Fisheries and Food, 1992; Mitek, Anyzewska & Wawrzyniak, 2013). This is supported by the findings from another study which found higher concentrations of salivary nitrite in vegetarians compared to people on a traditional diet in Poland (Traczyk & Szponar, 2000). Furthermore, some studies have reported that vegetarian diets can lead to significant reductions in SBP and DBP (–5 and –2 mmHg respectively) (Yokoyama, Tsubota & Watanabe, 2016), values which are very similar to those reported in a meta-analysis on the effect of dietary nitrate supplementation on BP (Siervo *et al.*, 2013). Thus, it was speculated that the low BP associated with vegetarian diets could be associated with higher intakes of dietary nitrate and higher nitrite bioavailability. This was an original question that has not been investigated to date and could explain, at least partially, how vegetarian diets promote cardiovascular protection.

However, the findings of the third study of this thesis do not support the previous research concerning nitrate intake of vegetarians. Contrary to expectations, vegetarians had a slightly, but not significantly, higher consumption of dietary nitrate than omnivores (97 ± 79 and 79 ± 47 mg/day respectively). This was surprising, as FFQ data indicated that vegetarians ate more vegetables (343 g/day) than omnivores (240 g/day), but had similar energy intakes. However, it was subsequently found that the FFQ was not validated for use with vegetarians, explaining why the estimates of vegetable intakes could be inaccurate. Production of a validated dietary assessment tool to estimate nitrate intake in the UK would be an important requirement to enable future research in this area. As there is no standard database for nitrate content of vegetables, analysis was based not only on data from a comprehensive list of vegetables from the EFSA (European Food Safety Authority, 2008), but also from selected UK data on salads, which provided more detailed information (Food Standards Agency, 2004). The validity of estimates of dietary intake relies on the availability of an appropriate database, whether using 24-hr dietary recalls, 7-day diaries or FFQ (Table 1.5). For example, FFQ to estimate nitrate intake have been designed in the USA (Griesenbeck *et al.*, 2009; Inoue-Choi *et al.*, 2016) and in Australia (Blekkenhorst *et al.*, 2017b) although data for nitrate content of vegetables vary due to different sunlight exposures etc. (Bondonno, 2018). A UK nitrate database would be challenging to develop due to large variations in nitrate content as a result of the environmental factors (Section 1.4.1). Nevertheless, the development of a standard nitrate database for vegetables commonly eaten in the UK could then be used to develop a validated dietary assessment tool.

Despite the lack of a standardised database, comparison of the findings of nitrate intake from the third study (vegetarians; 97 ± 79 , omnivores; 79 ± 47 mg/day) with those of other studies confirms similar estimates of dietary nitrate intake (Table 1.5). For example, an Australian study estimated average nitrate intake to be $\sim 90 \pm 65$ mg/day, although this

was in an older population (62 years) and energy consumption was not reported (Blekkenhorst *et al.*, 2017b). A Dutch study used a web-based 24-hr dietary recall method to assess nitrate intake of omnivore athletes (106 mg/day), although energy intake was higher in this population, indicating higher overall nutritional intakes³¹ (Jonvik *et al.*, 2017). Another study from the USA exploring the relationship between nitrate intake and thyroid cancer reported mean nitrate intake from plant sources as 83 mg/day (range 19-95 mg/day) (Kilfoy *et al.*, 2011), again in an older omnivore population (62 years) but with similar energy intakes to the participants in the third study of this thesis. This is in agreement with a further USA cohort in elderly participants with similar energy intakes (61-64 years, 77-280 mg nitrate/day) (Kang *et al.*, 2016). All these studies suggest that the nitrate intakes of omnivores in the third study were consistent with estimates for different population groups from other countries, supporting the validity of the results.

In contrast, dietary nitrate intake in the vegetarian group differed considerably to the values reported by a previous study in the UK (~190 mg/day) (Ministry of Agriculture Fisheries and Food, 1992). The reasons behind the different results obtained could have been due to methodological differences in estimation of dietary intake. Whereas 7-day food diaries were used in the third study, the UK estimate was derived from 3-day weighed intakes of 127 vegetarians and used a different nitrate database (Ministry of Agriculture Fisheries and Food, 1992). Similarly, vegetarians from Poland were reported to consume 248 mg/day, 2.5 times more than vegetarians in the third study, but this estimate was based on an electronic survey using a Polish database (Mitek, Anyzewska & Wawrzyniak, 2013) and so could either be an overestimate or a true reflection of the Polish vegetarian diet, which tends to be high in beetroot, cabbage and herbs (Berkoff, 2001). No information is available in either the MAFF report (Ministry of Agriculture Fisheries and

³¹ Female athletes; 2056 kcal/d (IQ range 1792-2462 kcal/d); male athletes; 2653 kcal/d (IQ range 2270-3155 kcal/d (Jonvik *et al.* 2017). Vegetarians; 1827 ± 526 kcal/d. Omnivores; 2021 ± 560 kcal/day.

Food, 1992) or the Polish study (Mitek, Anyzewska & Wawrzyniak, 2013) concerning energy intake, so it is not possible to comment on total nutritional intake of the vegetarian participants. It is possible that the vegetarian group, who were mainly students, might be unusual in that they did not eat larger quantities of high-nitrate vegetables than has been found previously. This could be due to a less healthy diet, with reliance on convenience foods.

The values for dietary nitrate intake in the third study of this thesis were within the ADI and significantly below the amount provided in both the first male and second female study of this thesis. Therefore, data from the third study does not support the hypothesis that vegetarian diets necessarily provide large quantities of nitrate. Instead, data on nitrate intake from all three studies of this thesis suggest that it is the amount of high-nitrate vegetables in the diet which primarily determines nitrate intake. In addition, studies using nitrate supplements can achieve considerably higher intakes, often in excess of the ADI (Table 1.6 and 1.7).

Notwithstanding the lack of a standard database, the validity of estimated nitrate intake in this study was also supported by plasma and salivary results, which showed no significant differences between the two groups. This is in contrast with a previous study that analysed plasma and salivary nitrate and nitrite concentrations in vegetarians and reported greater values in this population compared to individuals eating a traditional Polish diet (Traczyk & Szponar, 2000). Plasma nitrate and nitrite concentrations after placebo mouthwash in the third study were also similar to those found at baseline in the first two studies, which also supports the validity of the results. In addition, SBP in all three studies were comparable at baseline. Circulating nitrite concentrations are not only affected by dietary nitrate intake but also by endogenous NO production (Tsikas, 2015) and exercise (Rassaf *et al.*, 2007). Therefore, it is possible that the results could be influenced by these factors and may not accurately reflect long term intake.

In contrast to previous literature, BP in vegetarians was not lower compared to omnivores in this study. This was surprising considering the main body of literature suggests that vegetarian diets are associated with a reduction in BP compared to omnivores³² (Yokoyama, Tsubota & Watanabe, 2016). The observed similarity in BP between vegetarians and omnivores could be due to similar nitrate intakes, but BP can be affected by other confounders, such as BMI and training status. However, using a crude measure of physical activity (time), this was not significantly different between the groups and neither was BMI. In addition stress levels (Vrijkotte, van Doornen & de Geus, 2000) and dietary factors such as total and insoluble fibre intake (Aljuraiban *et al.*, 2015; Yokoyama, Tsubota & Watanabe, 2016) total polyphenol intake (Medina-Remón *et al.*, 2015), sodium and potassium (He, Pombo-Rodrigues & MacGregor, 2014; Yokoyama, Tsubota & Watanabe, 2016) and alcohol intake (Puddey & Beilin, 2006) can all affect BP and therefore may have impacted on the reported results. Stress levels were not assessed and this is an important issue for future research, as stress is associated with an average increase in SBP of 4 mmHg (Vrijkotte, van Doornen & de Geus, 2000).

Although participants were requested to adhere as closely as possible to the previous week's intake, in practice this was difficult due to circumstances beyond their control, such as having to go abroad for a job interview. Nutritional analysis suggested that vegetarians and omnivores consumed similar amounts of macronutrients and total polyphenols, but unsaturated fatty acids, sodium, potassium, total and insoluble fibre intakes were not assessed due to lack of available data on many of the processed foods consumed and so was beyond the scope of this study.

These factors could be addressed in future research, for example, by using a validated questionnaire or salivary cortisol to assess perceived stress levels, measuring urinary sodium, potassium and total polyphenol levels to indicate salt, potassium and polyphenol

³² SBP -4.8 mmHg; 95% CI -6.6-3.1; DBP -2.2 mmHg; 95% CI -3.5-1, $P < 0.001$

consumption, measuring liver function tests to assess alcohol use and using physical activity monitors and body composition scales to more accurately reflect training status and percentage lean body mass.

Other important parameters of cardiovascular health were also measured, such as cholesterol and blood glucose concentrations and showed no significant differences between vegetarians and omnivores, which were all within physiologically normal ranges. This in contrast to the findings of a systematic review which found lower cholesterol concentrations (Wang *et al.*, 2015) and an epidemiological study (Acosta-Navarro *et al.*, 2017) which demonstrated lower fasting blood glucose concentrations in vegetarians. In the studies by Acosta-Navarro *et al.* (2017) and Wang *et al.* (2015), participants were older (47 years and 28-56 years respectively) and plasma cholesterol increases with age (Félix-Redondo, Grau & Fernández-Bergés, 2013). Physical activity was assessed by questionnaire and the protective effect of vegetarian diets remained after adjusting for physical activity (Acosta-Navarro *et al.*, 2017). However, unlike the third study, physical activity was not assessed in the review by Wang *et al.* (2015). Serum triglycerides were lower in the omnivore group in contrast to previous studies which have reported higher serum triglycerides in omnivores than vegetarians (1.35 ± 0.57 and 1.06 ± 0.45 mM respectively) (Szeto, Kwok & Benzie, 2004). On the other hand, a recent systematic review and meta-analysis did not find a significant lowering effect of vegetarian diets on triglycerides compared to omnivores (Wang *et al.*, 2015). These conflicting results suggest further research is required in this area.

In summary, results from the third and final study of this thesis did not support the hypothesis that this group of vegetarians consumed more dietary nitrate than a similar group of omnivores. In addition, plasma and salivary nitrate and nitrite concentrations of vegetarians did not differ to the values of omnivores. BP, cholesterol and glucose of omnivores were also similar in both groups. This suggests that an omnivorous diet with

similar energy content and including animal protein may have similar effects to a vegetarian diet in controlling the circulatory concentrations of lipids and glucose in young, healthy subjects.

5.5 Oral nitrate-reducing capacity and blood pressure

There has been little research into the effects of antibacterial mouthwash on nitrate metabolism in vegetarians compared to omnivores. It was hypothesized that using antibacterial mouthwash for one week would have a more pronounced effect on nitrate metabolism in vegetarians than in omnivores because of their reported higher nitrate intakes (Section 1.6). Higher nitrate intakes could be associated with changes in the oral microbiome which could lead to enhanced nitrate-reducing capacity of oral bacteria. However, this was not the case, as oral nitrate-reducing capacity, plasma and salivary nitrite were not significantly different at baseline and were all significantly reduced after using antibacterial mouthwash in both groups. This is in line with results from other studies using a similar approach (antibacterial mouthwash) in order to inhibit the activity of oral bacteria (Bondonno *et al.*, 2015a; Govoni *et al.*, 2008; Kapil *et al.*, 2013; McDonagh *et al.*, 2015; Woessner *et al.*, 2016). Therefore, this study adds to the evidence base supporting the hypothesis that the oral microbiome is a key factor influencing plasma and salivary nitrite concentrations and that mouthwash can significantly inhibit the activity of oral bacteria.

However, the lack of changes in BP in the third study after using antibacterial mouthwash are in contrast to a similar study indicating that use of the same mouthwash resulted in a significant increase in SBP of 3.5 ± 1 mmHg and significant reductions in oral nitrate-reducing capacity in a similar group of participants³³ (Kapil *et al.*, 2013). Comparable

³³ 24 ± 1 years, BMI 23 ± 1 kg/m²

increases in SBP of 2.3 mmHg (95% CI: 0.5-4 mmHg) were found using the same mouthwash in older and overweight individuals³⁴ (Bondonno *et al.*, 2015a). However, in the latter study, although they found a significant increase in SBP, there was no significant reduction in plasma nitrite (Bondonno *et al.*, 2015a). These authors suggested that the non-significant effects on plasma nitrite could be related to the timings of the blood samples, 12-14 hours after the last mouthwash use. However, this is in contrast to the results from the third study, which found a significant drop in plasma nitrite, which could be due to younger participants. In this regard, the findings reflect those of Sundqvist *et al.* (2016) who also found a lack of effect of antibacterial mouthwash on BP in 17 healthy young women³⁵ (Sundqvist, Lundberg & Weitzberg, 2016). However, their study did not find significant changes in plasma nitrite, similar to Bondonno *et al.* (2015a). The authors hypothesised that this could be related to an upregulation of eNOS expression in their group of participants as they were young healthy women (Sundqvist, Lundberg & Weitzberg, 2016). This explanation is unlikely to fully explain the lack of changes in BP as the study by Kapil *et al.* (2013) used a similar population of participants. Further factors which might affect the response to mouthwash include control of dietary nitrate before the study, whereby in the study by Sundqvist *et al.* dietary nitrate intake was limited. In addition, physical activity levels may have influenced the lack of difference in BP, but similar to the third study, these were not significantly different between interventions (Sundqvist, Lundberg & Weitzberg, 2016). A more likely reason is that prior use of antibacterial mouthwash was not reported by Sundqvist *et al.* and therefore, the condition of the oral microbiome prior to the intervention may have been compromised. This is in contrast to the third study where recent use of both mouthwash and antibiotics precluded participation, which could certainly explain the different findings. It is possible that the

³⁴ 53-69 years, BMI 27 kg/m²

³⁵ 23 years, BMI 22 ± 3 kg/m²

differences in results between the third study and results reported by Kapil *et al.*, Bondonno *et al.* and Sundqvist *et al.* could be related to the variations in the oral microbiome and further studies are needed to investigate this in more depth.

Salivary pH plays an important role not only in oral health but has also recently been linked to metabolic syndrome and high blood pressure. Normal saliva pH is maintained at near neutral levels of 6.7-7.3 (Aoyama *et al.*, 2017). There were no differences in salivary pH between groups after using placebo for one week, which were within the normal range. Similar to results from the third study, no differences in pH were found under baseline conditions between 29 vegetarians and 28 omnivores (Johansson & Raval, 1995). In addition, a more recent study reported no differences in the oral microbiome profile between vegetarians and omnivores suggesting that the effect of diet in the modulation of oral pH and the oral microbiota may be lower than expected (De Filippis *et al.*, 2014). In contrast, previous studies have suggested that saliva in omnivores is less acidic than that of vegans due to increased urea production in omnivores (Laffranchi *et al.*, 2010). These conflicting reports in the literature suggest that measurement of salivary pH should be included in future research in this area.

An interesting finding of this study was the significant reduction in salivary pH in both groups after 7 days of using mouthwash. This is important as an acidic environment can lead to dental caries and other periodontal diseases (Radcliffe *et al.*, 2002). Sodium nitrite appears to protect against reduction of pH by being bactericidal against *Streptococcus mutans*, a cariogenic species of bacteria (Radcliffe *et al.*, 2002). Therefore, according to the results of the third study, antibacterial mouthwash, instead of reducing dental caries, could be promoting conditions whereby cariogenic bacteria can multiply, by reducing both pH and salivary nitrite concentrations. In addition, salivary pH may be a marker for metabolic syndrome (obesity, dyslipidaemia, hyperglycaemia and hypertension), as lower pH in women has been associated with reduced metabolic health (Tremblay, Brisson &

Gaudet, 2012). Moreover, lower salivary pH has been related to hypertension in older adults (Kagawa *et al.*, 2013), suggesting that salivary pH levels have a significant association with BP regulation, although this was not confirmed in the third study. However, further studies are needed to investigate this question more in detail.

One unanticipated finding was that antibacterial mouthwash caused triglyceride concentrations to increase in the omnivore group, but not in the vegetarian group. This could have occurred by chance and is in contrast to previous findings, where 0.12 % chlorhexidine mouthwash, used as part of periodontal treatment, had no effect on triglyceride concentrations in non-obese participants (Altay, Gürkan & Ağbaht, 2013). The possible relationship between mouthwash and serum triglycerides should be investigated in future research, as it has been suggested that there is a link between the oral microbiome and metabolic syndrome (Si, Lee & Ko, 2017).

5.6 Dietary nitrate intake and oxygen consumption

Over the last decade multiple studies (Bailey *et al.*, 2009; Lansley *et al.*, 2011b; Larsen *et al.*, 2010; Tan *et al.*, 2018; Vanhatalo *et al.*, 2010; Wylie *et al.*, 2016), but not all (Bescós *et al.*, 2012; Breese *et al.*, 2013; Rienks *et al.*, 2015), have reported lower oxygen consumption during exercise after the ingestion of moderate or high amounts of inorganic nitrate. Following this, a further study by Larsen *et al.* (2014) also found lower oxygen consumption under resting conditions (RMR) in healthy subjects after dietary nitrate supplementation. This may be of interest from a metabolic perspective as it may significantly reduce overall energy expenditure and improve longevity. However, a second study by the same research group did not confirm these findings when they inhibited the nitrate-nitrite-NO pathway using antibacterial mouthwash (Sundqvist, Lundberg & Weitzberg, 2016). Using a similar method, no changes in resting metabolic rate in

vegetarians and omnivores were observed. Thus, according to these results, it seems that the oral nitrate-nitrite-NO pathway plays a limited role in the regulation of the oxygen consumption and energy production under resting conditions in young healthy individuals. This can be explained as the nitrate-nitrite-NO pathway seems to be gradually activated under physiological situations where oxygen tension falls, such as during exercise (Lundberg, Weitzberg & Gladwin, 2008), but this does not occur during resting conditions. Currently, an intense debate exists in an attempt to explain these effects as some studies have indicated that this could be associated with lower ATP demand of skeletal muscle (Bailey *et al.*, 2010) and perhaps improved mitochondrial efficiency (Larsen *et al.*, 2011). In contrast, other recent studies have not found similar results (Betteridge *et al.*, 2016; Whitfield *et al.*, 2016).

In summary, the results of the third study did not support the hypothesis that dietary nitrate intake of vegetarians is higher than omnivores or that this is associated with lower BP levels or glucose and lipid concentrations. In addition, the inhibition of the oral nitrate-nitrite-NO pathway and lower nitrite availability and salivary pH had no effect on BP or RMR in vegetarians and omnivores.

5.7 Conclusions

This thesis set out to investigate the effects of high-nitrate vegetables on BP in healthy adults. The first and second studies have shown that supplementation with high-nitrate vegetables increases nitrate and nitrite bioavailability in healthy young men and women. However, this was only associated with a significant reduction in SBP in healthy young women. No significant effects in SBP were found in men, but the small sample size and subsequent under powering of the study may mean this negative result is incorrect. The third study identified that dietary nitrate intake of vegetarians was no different to that of omnivores but was within the ADI for nitrate and significantly below levels provided by dietary nitrate supplements in previous studies. The effect of a vegetarian diet on BP, blood cholesterol and glucose did not differ from an omnivore diet in subjects with similar age, BMI and physical activity levels, but this may be because nitrate intakes were similar. In addition, no changes were observed in BP and RMR in both groups despite successful inhibition of the oral nitrate-nitrite-nitric oxide pathway, as shown by the lower nitrate-reducing capacity of oral bacteria and nitrite concentration in saliva and plasma after using antibacterial mouthwash for one week.

The results of the third study suggest that vegetarian diets are not necessarily associated with large intakes of dietary nitrate, compared to the amounts used in studies using dietary or pharmacological supplements. Consumption of ~2-3 portions/day of high-nitrate vegetables are required to achieve the amount of inorganic nitrate provided by these studies as shown in the first and second study of this thesis, and these may not be routinely consumed by free-living vegetarians. Future studies could investigate if such intakes of high-nitrate vegetables are feasible in the general population. Such amounts can significantly exceed the ADI for nitrate, and there is a lack of studies in humans analysing the impact of consumption of large quantities of dietary nitrate over long periods of time.

The generalisability of these results is subject to certain limitations. Being limited to young healthy women, the findings of a significant reduction in systolic BP lacks generalisability to the whole population. A further limitation of this study is that supplementation was for one-week only and therefore it is unknown if the BP reductions would continue with longer term intakes of high-nitrate vegetables. Also, BP reductions were not confirmed in men, as the study was underpowered and there was no change in BP after interruption of the nitrate-nitrite-nitric oxide pathway in the third study. However, the third study contributes to the understanding of bioavailability of nitrite after antibacterial mouthwash and lays the groundwork for future research into the importance of the oral microbiome.

Small reductions in systolic BP across a population have the potential to significantly reduce the incidence of cardiovascular disease, such as stroke. However, before recommending that high-nitrate vegetables have the potential to reduce BP, further research is required in both men and women with prehypertension, hypertension and other cardiovascular risk factors, to confirm whether or not dietary nitrate has a similar effect in these populations.

The findings from this thesis have also raised important questions about potential high intakes of nitrate from supplements and the lack of research into the effects of long-term high intakes. The findings of the third study will be of interest to future researchers as they do not confirm the previous findings of higher nitrate intakes in vegetarians. Therefore, this population group should be used with care in future studies examining the long-term effects of high-nitrate intakes from vegetables. The findings of reduced salivary pH levels after antibacterial mouthwash also merit further investigation. Whilst this study did not examine intake of unsaturated fatty acids, the effect of antibacterial mouthwash on triglyceride levels in omnivores suggests that there is the possibility of adverse effects on metabolic health.

6 FUTURE RESEARCH

Further research is needed to determine whether nitrate is in fact a nutrient rather than a contaminant and the research for this thesis has thrown up many questions which need additional investigation. This includes the development of a validated and accepted database for nitrate content of vegetables commonly eaten in the UK, using recent estimates of nitrate content where available. The addition of this new data to nutritional software and other applications will help to assess nitrate intake more accurately and to compare nitrate intake with other populations around the world. The construction of a nitrate database would use a similar methodology to that described by Blekkenhorst *et al.* (2017) to construct an Australian nitrate database (Appendix 21).

The development of a validated tool to assess dietary nitrate intake in the UK would be highly relevant to future research. Using this new tool, a potential future study could investigate whether high-nitrate intakes over a longer timeframe improve cardiovascular health, for example in older populations with cardiovascular risk factors such as prehypertension.

A further study could assess the optimum amounts and practical aspects of eating high-nitrate vegetables daily, including feasibility, safety, availability and palatability, which were not fully explored in this thesis. For example, future intervention trials investigating the role of dietary nitrate intake in vascular control should investigate whether it is possible and safe to regularly consume amounts of nitrate over the ADI as well as control for use of substances such as antibacterial mouthwash and antibiotics, which affect the nitrate-nitrite-NO pathway. In addition, confounders such as differences in the oral microbiome, stress levels, alcohol, sodium, potassium, total polyphenols, fibre and fatty acid intakes should be considered. More accurate and objective methods to assess vascular function after dietary intervention should be considered as well as BP (McCall *et al.*, 2011). This is particularly important as the widely-used method of clinic BP may not accurately reflect the

effectiveness of dietary nitrate, due to potential problems with observer bias and lack of standardised protocols (Ashor, Lara & Siervo, 2017).

A future research trial could investigate the emerging role of nitrated fatty acids in context of the protective effects of the Mediterranean diet, which is reported to be high in both vegetables and olive oil (Appendix 22).

Future research should also examine the effects of diet on the oral microbiome, salivary pH and nitrate and nitrite metabolism, both in healthy populations and in people with prehypertension and with pre-existing clinical conditions related to chronic metabolic abnormalities, such as type 2 diabetes and obesity. This is important since the effect of dietary nitrate may rely on the status and type of oral bacteria (Burleigh *et al.*, 2018). Assessment of the oral microbiome must also include an oral health screening as nitrate and nitrite levels in the mouth can be associated with periodontal disease (Sánchez *et al.*, 2014). There are, therefore, many opportunities for further research into the effects of dietary nitrate on cardiovascular health. The results of this future research may lead to a change in the policies which currently limit the intake of dietary nitrate in the form of an ADI. Thus, further research is necessary before dietary nitrate can be considered as a nutrient essential to cardiovascular health.

APPENDICES

Appendix 1.

Study 1 Ethical approval



SCHOOL OF SPORT AND
HEALTH SCIENCES

St Luke's Campus
Heavitree Road
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EX1 2LU

Telephone: +44 (0)1392 264603
Fax: +44 (0)1392 264728
Email: ssha-school-office@exeter.ac.uk
Web: www.exeter.ac.uk/sshs

Certificate of Ethical Approval

Proposal 7 (07/07/10)

Title: Effects of vegetables on health and exercise performance.

Applicants: Prof Andy Jones (staff) with Dr Anni Vanhatalo (staff), Prof Paul Winyard (staff), Stephen Bailey (PG research student) and Ann Ashworth (PG research student).

The proposal was reviewed by the Ethics Committee on 07/07/2010 and following amendments has now been approved until December 2010.

Decision: The proposal was approved from August 2010 to December 2010.

Signature:

A handwritten signature in black ink, appearing to read "ATaylor".

Date: 06/08/2010

Name/Title of Ethics Committee Reviewer: Prof. Adrian Taylor

Your attention is drawn to the attached paper which reminds the researcher of information that needs to be observed when Ethics Committee approval is given.

Appendix 2.

Study 1 Consent form

Effects of vegetables on health and exercise performance.

CONSENT FORM FOR PARTICIPANTS

I understand that:

My participation in the project is entirely voluntary

I am free to withdraw from the project at any time without any disadvantage.

The data collected will be coded (assigned a number to allow the identification of each subject) and stored securely in the principal investigator's filing cabinet. Following the date of collection, the data will be retained for a seven-year period. It will be destroyed following this time.

I will be required to perform 1 incremental exercise test and 4 sessions of 3 step cycle tests, before and after two different diets containing different mixtures of vegetables.

There are some risks associated with performing incremental exercise, and high-intensity constant-work-rate exercise to exhaustion, as outlined in the information sheet.

I will be required to provide a venous blood sample and understand the blood sampling procedure:

Yes, I understand the blood sampling procedure:	(please tick)
---	---------------

The results of the project may be published, but my anonymity will be preserved.

I have read and understand the Information Sheet concerning this project. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I agree to take part in this project.

..... (Signature of participant)

..... (Date)

This project has been reviewed and approved by the Ethics Committee of the School of Sport and Health Sciences

Professor Andrew. M. Jones

A.M.Jones@exeter.ac.uk

Appendix 3.

Study 1 Recruitment poster.

Free vegetables for 4 weeks!
Are you male, healthy, physically active and aged 18 - 40 years?

If so, please read on....

You are needed for a 6 week study into the effects of different vegetables on health and exercise performance. The study will include:

Measurement of blood pressure
One VO2 Max test
4 moderate exercise tests
4 blood samples
4 free vegetable boxes

Interested? Please e-mail Ann Ashworth, MPhil/PhD student, Sport and Health Sciences, at the address below:
aaa207@exeter.ac.uk

Appendix 4.

Study 1 Information sheet



SPORT AND HEALTH SCIENCES,
College of Life and Environmental Sciences

**St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom**

Effects of vegetables on health and exercise performance.

INFORMATION SHEET FOR PARTICIPANTS

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate, we thank you in advance for the time and effort you have decided to devote to our investigation. If you decide not to take part, there will be no disadvantage to you of any kind and we thank you for considering our request.

What is the aim of the project?

This project is being undertaken as part of the requirements for the PhD in Sport and Health Sciences. The aim of the current investigation is to determine the effects of dietary supplementation with different types of vegetables, on your ability to tolerate exercise and your oxygen uptake (VO_2) response to exercise. Supplementation with beetroot juice has been shown to improve endurance exercise performance and a diet high in fruit and vegetables can reduce blood pressure, but it is not known which component of fruit and vegetables is responsible. This will provide an interesting model to see if eating certain vegetables has an effect on your VO_2 response, blood pressure and your ability to tolerate exercise.

What types of participants are needed?

We seek young (18-40 year old), non-smoking, physically active men willing to perform moderate intensity as well as challenging exercise bouts on a cycle ergometer. Participants should also be free from disease, or any other limitation that restricts their ability to perform both moderate and sustained high-intensity cycling. Participants must be willing to eat commonly available vegetables.

What will participants be asked to do?

You will be asked to report to the School of Sport and Health Sciences on 5 occasions over about 6-8 weeks.

You will be asked to attend the exercise testing laboratory in the CHERC building at an agreed time for your exercise tests. During the first visit to the laboratory;

the project will be explained and you will be asked to sign a consent form
your blood pressure will be measured four times

your height and weight will be measured
you will be given food and exercise diaries to complete
you will perform a 'ramp' incremental exercise test to will assess your baseline aerobic fitness. You will cycle at a very light work rate at first. The work rate will gradually increase over a period of 10-15 minutes and you will be encouraged to continue for as long as possible, until exhaustion.

For each test you will be asked to arrive in the laboratory having not consumed food during the previous 3 hours, or caffeine and alcohol during the previous 24 hour period.

The second stage of the testing will require you will to perform 4 sessions of step cycle tests. A step cycle test is where you cycle at a baseline work rate which is very light for 4 minutes followed by an immediate increase in work rate. At each of the 4 tests, you will attend the exercise testing laboratory in the CHERC building. You will be tested as follows:

a blood sample will be taken from your arm
your blood pressure will be measured four times
your weight will be measured
you will be asked about your food and exercise diary
you will be asked to perform the step cycle test, which will consist of 3 moderate-intensity (light exercise) cycle exercise bouts of 6 minutes in duration.

You will be provided with a 10 minute rest period between each bout to ensure you are completely recovered. It will be necessary for you to wear a mouthpiece and nose-clip during these cycle bouts for the collection of inhaled and exhaled gases.

You will be asked to eat a certain amount of specific vegetables every day for the next two weeks. It is important that you eat the vegetables, including outside leaves when possible and eat your normal foods apart from the vegetables. **Please do not try to lose weight on this diet.** After 14 days on the diet, you will be tested again as above.

Following the first 14 day diet period you will be asked to return to your normal diet for 14 days to allow the effects of the first diet to 'wash out'. You will be then be asked to eat a different selection of vegetables for another 14 days and repeat the testing before and after as above.

Cycle exercise performed at high intensity workloads are challenging and involve some discomfort. This discomfort should create no lasting harm and will simply represent the feelings of effort that individuals typically experience in an intense exercise training session. Please note that any concerns you have about the degree of effort that you will be asked to expend will be discussed with you in full by the researchers prior to you giving your consent to participate.

If at any time you decide that you no longer want to take part in this project (because of the discomfort associated with intense exercise, unable to tolerate the diet or any other reason) you will be allowed to withdraw without disadvantage to yourself of any kind.
--

Are there any side effects of the diets?

The vegetables which will be supplied to you have not been treated in any way. They are normal vegetables as sold to the general public. The portion sizes and amounts advised are in line with Department of Health recommendations to eat 'at least 5 portions of fruit and vegetables a day'.

What data or information will be collected and what use will be made of it?

Any data collected during these exercise tests will be used to establish response profiles across the group of subjects involved. These cumulative scores may be made available for public inspection in research journals and/or at seminars and conferences. In addition, individual response profiles indicative of the typical response may also be presented. However, in all cases, anonymity will be strictly preserved. While results of this project may be available for public inspection, any data so displayed will in no way be linked to any specific individual participating in this investigation.

Upon completion of the study, the data collected will be securely stored for 7 years in such a way that only the researchers involved in this investigation will be able to gain access to it. However, you are most welcome to request a copy of the results once you have completed your tests for this project, and we will be available to explain and interpret your specific data approximately 2 weeks following your final test.

What if participants have any questions?

If you have any questions about our project, either now or in the future, please feel free to contact:

Andrew Jones, Ph.D.
+44(0)1392 262886
a.m.jones@exeter.ac.uk

This project has been reviewed and approved by the Ethics Committee,
Sport and Health Sciences, College of Life and Environmental Sciences.

Appendix 5.

Exercise diary (used in all 3 studies)

To take part in this study, we need a record of your activity over a 14 day period

Please write down every exercise session, such as going to the gym, running, swimming etc. and the length of time taken

Day	Date	Activity	Length of time
1			
2			
3			
4			
5			
6			
7			
8			
9			

10			
11			
12			
13			
14			

Thank you!

Ann Ashworth

ann.ashworth@plymouth.ac.uk

07729 810765

Appendix 6.

Advice sheet for participants to avoid high nitrate vegetables.



SPORT AND HEALTH SCIENCES,
College of Life and Environmental Sciences

**St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom**

List of foods and drinks for participants to avoid:

Please do not eat/drink:

Beetroot

Rocket/mizuna

Lettuce (all types)

Kale

Watercress

Chinese leaf

Chard

Fennel

Endive

Processed meats (hot dog sausages,
ham, bacon, salami)

Vegetable juice

Beetroot juice

Cranberry juice

Perrier water

Instead you may have:

Tomatoes

Cucumber

Coleslaw

Carrot (cooked)

Cauliflower (white florets only -
cooked)

Peppers

Onions

Green beans

Cheese

Orange juice

Tap water

Squash

lemonade

If you agree to participate in this study you will be required to avoid eating the above listed foods for two weeks. If having read this list you feel you will be unable to avoid these foods due to dietary or any other reasons you may discontinue your involvement in this study immediately and we completely respect your right to do so.

Thank you.

Ann Ashworth

Appendix 7.

Food diary (used in all 3 studies)

Date:	Quantity: Tablespoons, bowl, number of slices	Food or drink: Give as many details as possible, for example, thick sliced bread from large wholemeal loaf
Breakfast		
Mid- morning		
Lunch		
Mid- afternoon		
Evening meal		
During evening		
Bedtime		
Notes		

Filling in your diary

Please keep a food diary every day for a week. Please record **everything** you eat and drink.

Please be honest and write everything down so I can get a true idea of your eating pattern. It might be helpful if you can take photos with your phone too.

Try to fill in your diary at the time of eating so keep it handy. If you try to do it at the end of the day, it will not be as accurate.

This diary will be treated as confidential information. All records will be stored appropriately to maintain confidentiality.

Please tell me about;

The quantity

Household measures used, such as tablespoons, teaspoons, cupful's

If you have a ready-made snack, there will be a weight on the packet, so write down the number of grams.

The type

As much information as possible, such as

Type of spread: for example, reduced fat, sunflower margarine

Type of milk: for example, semi-skimmed cow's milk

The method of cooking

Grilled, fried, roast, steamed, microwaved, stir fried

Remember to put in

Snacks between meals

Meals eaten out

Food/drink eaten whilst preparing meals

Sauces, pickles and salad dressings

Oil used in cooking

Thank you! ©Ann Ashworth MSc RD 2017

Appendix 8.

Recruitment poster study 2

Free vegetables for 2 weeks!

Are you female, healthy and aged 18 - 40 years?

If so, please read on....

You are invited to participate in a 5 week study into the effects of different vegetables on health. The study will include:

Measurement of blood pressure

4 blood samples

2 free vegetable boxes

Interested? Please e-mail Ann Ashworth, at the address below:

eea207@exeter.ac.uk

Appendix 9.

Ethical approval: 'Effects of vegetable consumption on blood pressure in women'

Email from Sally Discombe, Ethics Administrator, University of Exeter:

'I've located an entry in one of our spreadsheet records and, after consulting the Chairman of the Ethics Committee, I can let you have the details of that and we really hope this will be adequate for your needs.

The entry is as follows:

Ref: 2012/384 – *Effects of different types of vegetables on health* – Dr Andrew Jones, Ann Ashworth, Klaus Mitchell, Dr Anni Vanhatalo – approved on 20/7/2012'

Appendix 10.

Consent form study 2



SPORT AND HEALTH SCIENCES,
College of Life and Environmental Sciences

**St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom**

Effects of vegetable consumption on blood pressure in females

CONSENT FORM FOR PARTICIPANTS

I understand that:

My participation in the project is entirely voluntary

I am free to withdraw from the project at any time without any disadvantage.

The data collected will be coded (assigned a number to allow the identification of each subject) and stored securely in the principal investigator's filing cabinet. Following the date of collection, the data will be retained for a seven-year period. It will be destroyed following this time.

I will be required to provide a venous blood sample and understand the blood sampling procedure:

Yes, I understand the blood sampling procedure:	(please tick)
---	---------------

The results of the project may be published, but my anonymity will be preserved.

I have read and understand the Information Sheet concerning this project. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I agree to take part in this project.

..... (Signature of participant)..... (Date)

This study has been reviewed and approved by the Ethics Committee of Sport and Health Sciences, College of Life and Environmental Sciences.

Professor Andrew. M. Jones (A.M.Jones@exeter.ac.uk)

Appendix 11.

Information sheet study 2



SPORT AND HEALTH SCIENCES,
College of Life and Environmental Sciences

**St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom**

Effects of vegetable consumption on blood pressure in females

INFORMATION SHEET FOR PARTICIPANTS

Thank you for showing an interest in this study. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate, we thank you in advance for the time and effort you have decided to devote to our investigation. If you decide not to take part, there will be no disadvantage to you of any kind and we thank you for considering our request.

What is the aim of the study?

This study is being undertaken as part of a PhD study in Sport and Health Sciences. The aim of the current investigation is to determine the effects of dietary supplementation with different types of vegetables on blood pressure. A diet high in vegetables can reduce blood pressure, but it is not known which component of vegetables is responsible. This will provide an interesting model to see if eating certain vegetables has an effect on your blood pressure.

What types of participants are needed?

We seek 18-40 year old, healthy, physically active, non-smoking females free from any disease and not on any medications. Participants must be willing to eat commonly available vegetables.

What will participants be asked to do?

You will be asked to report to the laboratory at an agreed time in the Baring Court building in St Luke's Campus on 5 occasions over about 6 weeks. Each visit will take no longer than 15 minutes.

During the first visit to the laboratory;

the project will be explained and you will be asked to sign a consent form
your height and weight will be measured
you will be given food and exercise diaries to complete

During the following laboratory visits;

you will be given a short questionnaire to fill in
your blood pressure will be measured four times
a blood sample will be taken from your arm
your weight will be measured
you will be asked about your food and exercise diary

You will be then randomised into either the dietary intervention group or the control group.

If you are assigned to the control group, you will receive two boxes of vegetables at agreed dates after the study. You will be asked to attend the laboratory for measurements as above. You will be asked to keep to your normal diet throughout the study.

If you are assigned to the dietary intervention group, you will collect a box of vegetables on the second laboratory visit. You will be asked to eat these every day until the third laboratory visit 7 days later. Following this 7 day diet period, you will be asked to return to your normal diet for 21 days to allow the effects of the first diet to 'wash out'. You will then visit the laboratory for the fourth time where you will collect another box of vegetables. You will be asked to eat these every day until the fifth laboratory visit 7 days later.

If you are assigned to the dietary intervention group, you will be asked to eat your normal food alongside the vegetables provided. You may be asked to avoid green leafy vegetables, salad and beetroot unless contained in one of the boxes.

Please do not try to lose weight during this study.

If at any time you decide that you no longer want to take part in this project you will be allowed to withdraw without disadvantage to yourself of any kind.
--

Are there any side effects of the diets?

The vegetables which will be supplied to you have not been treated in any way. They are normal vegetables as sold to the general public. The portion sizes and amounts advised are in line with Department of Health recommendations to eat 'at least 5 portions of fruit and vegetables a day'.

What use will be made from the measurements taken?

The data collected will be used to establish cumulative scores across the group of subjects involved. These cumulative scores may be made available for public inspection in research journals and/or at seminars and conferences. In addition, individual data indicative of the typical response may also be presented. However, in all cases, anonymity will be strictly preserved. While results of this study may be available for public inspection, any data so displayed will in no way be linked to any specific individual participating in this investigation. Upon completion of the study, the data collected will be securely stored for 5 years in such a way that only the researchers involved in this investigation will be able to gain access to it. However, you are most welcome to request a copy of the results once you have completed your tests for this project, and we will be available to explain and interpret your specific data approximately 2 weeks following your final test.

What if participants have any questions?

If you have any questions about our project, either now or in the future, please feel free to contact:

Andrew Jones, Ph.D.
+44(0)1392 262886
a.m.jones@exeter.ac.uk

This project has been reviewed and approved by the Ethics Committee, Sport and Health Sciences, College of Life and Environmental Sciences.

Appendix 12.

Checklist study 2



SPORT AND HEALTH SCIENCES,
College of Life and Environmental Sciences

**St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom**

Effects of vegetable consumption on blood pressure in females

1) Have you consumed any of the following in the last 7 days?

Beetroot	Yes <input type="checkbox"/> No <input type="checkbox"/>
Celery	Yes <input type="checkbox"/> No <input type="checkbox"/>
Chinese leaf	Yes <input type="checkbox"/> No <input type="checkbox"/>
Cress	Yes <input type="checkbox"/> No <input type="checkbox"/>
Endive	Yes <input type="checkbox"/> No <input type="checkbox"/>
Fennel	Yes <input type="checkbox"/> No <input type="checkbox"/>
Lettuce (all types)	Yes <input type="checkbox"/> No <input type="checkbox"/>
Radish	Yes <input type="checkbox"/> No <input type="checkbox"/>
Rocket	Yes <input type="checkbox"/> No <input type="checkbox"/>
Spinach	Yes <input type="checkbox"/> No <input type="checkbox"/>
Swiss chard	Yes <input type="checkbox"/> No <input type="checkbox"/>
Watercress	Yes <input type="checkbox"/> No <input type="checkbox"/>
Vegetable juice	Yes <input type="checkbox"/> No <input type="checkbox"/>

If you answered yes to any of these, please provide further details (i.e. what, when and how much):

.....

.....

.....

2) Have you taken any medication in the last 7 days, including the contraceptive pill?
Yes ☐ No ☐

If you answered yes, please provide further details (i.e. what, when and dose):

.....

.....

3) Do your menstrual cycles last approximately 28 days? Yes ☐ No ☐

If no, please give an estimate.....

Please give date of first day of last period

Many thanks

Appendix 13.

Ethics application Study 3

Dear Dr Bescos

Application for Approval by Faculty Research Ethics Committee

Reference Number: 15/16-469

Application Title: The effect of dietary inorganic nitrate consumption on blood pressure and resting metabolic rate in vegetarians compared with omnivores

I am pleased to inform you that the Committee has granted approval to you to conduct this research.

Please note that this approval is for three years, after which you will be required to seek extension of existing approval.

Please note that should any MAJOR changes to your research design occur which effect the ethics of procedures involved you must inform the Committee. Please contact Sarah Jones (email sarah.c.jones@plymouth.ac.uk).

Yours sincerely

Professor Michael Sheppard, PhD, FAcSS

Chair, Research Ethics Committee -

Faculty of Health & Human Sciences and

Peninsula Schools of Medicine & Dentistry

Appendix 14.

Consent form for participants involved in research

CONSENT FORM FOR PARTICIPANTS INVOLVED IN RESEARCH

INFORMATION for PARTICIPANTS:

We would like to invite you to be a part of a study looking at the effect of dietary nitrate ingestion on blood pressure and metabolic rate in vegetarian and omnivore subjects.

The first aim of this study is to determine if vegetarians consume higher amounts of dietary inorganic nitrate and if this fact is related with higher levels of this anion in saliva and blood. The second aim is to investigate if the blockage of the nitrate pathway by the use of antibacterial mouthwash for 7 days alters blood pressure and the resting metabolic rate of vegetarians and omnivores.

Prior to your participation in this study it is required that you fill out a medical questionnaire. This information is needed to confirm that you are healthy and you do not have any metabolic disturbance or are taking any medication that could alter the results of this study.

Risk involved

The procedures involved in participating in this study are of low risk. Aseptic techniques will be used at all times to reduce the risk of infection during the blood collections.

CERTIFICATION BY SUBJECT

I, _____ (Full name)

of _____ (Address)

certify that I am at least 18 years old and that I am voluntarily giving my consent to participate in the study:

“The effect of dietary inorganic nitrate consumption on blood pressure and resting metabolic rate in vegetarians compared with omnivores” being conducted at the University of Plymouth by: Dr. Raul Bescos.

I certify that the objectives of the study, together with any risks and safeguards associated with the procedures listed hereunder to be carried out in the research, have been fully explained to me by Dr. Raul Bescos or Ms Ann Ashworth and that I freely consent to participation involving the below mentioned procedures:

Medical questionnaire
Dietary and physical activity record (14 days)
Resting Metabolic Rate
Blood Pressure
Blood sampling (plasma will be stored)
Saliva sampling (salivary cells will be stored and used for DNA analysis of bacteria)

I certify that I have had the opportunity to have any questions answered and that I understand that **I can withdraw from this study at any time and that this withdrawal will not jeopardise me in any way.**

I have been informed that the information I provide will be kept confidential.

Signed:

Date:

Any queries about your participation in this project may be directed to the researcher

Dr. Raul Bescos Ph: 01752 587 585 Mob: 07599 034710 Email:
raul.bescos@plymouth.ac.uk

If you have any queries or complaints about the way you have been treated, you may contact the Health & Human Science Research Ethics Committee of Plymouth University: Sarah C Jones, 01752 585339, hhsethics@plymouth.ac.uk

Appendix 15.

Study 3 Recruitment poster

Appendix 16.

Study 3 Information sheet



INFORMATION SHEET:

You are invited to participate in a research project entitled "*The effect of dietary inorganic nitrate consumption on blood pressure and resting metabolic rate in vegetarians compared with omnivores*" led by Dr. Raul Bescos and approved by the Plymouth University Human Research Ethics Committee.

Project explanation

Aim: Vegetarian diets are associated with lower risk of cardiovascular disease. Such healthy benefits can be associated with several factors, but it has been recently suggested that vegetarians eat more nitrate than omnivores and this fact might explain, at least partially, the effect of vegetarian diets in regulating blood pressure. The first aim of this study is to analyse the vegetable consumption of a group of vegetarians to estimate the consumption of nitrate compared with omnivores. Then, these data will be correlated with levels of nitrate and nitrite in blood and saliva in both populations to investigate the impact of diet in the circulatory levels of these nitrogen anions. A second aim of this study is to analyse the impact of vegetarian diets in the reducing-nitrate capacity of oral bacteria and its role in the control of blood pressure and energy expenditure under resting conditions.

What will I be asked to do?

Before the study you will be asked to attend a screening visit (1st visit) where you will fill out a medical questionnaire to determine your eligibility to participate. This will ask questions on issues such as alcohol and caffeine consumption, use of recreational drugs, use of medications, health issues and menstrual and reproductive history (for females only). If you meet the criteria, you will be invited to participate. You will be asked to sign a consent form. Your body weight and height will be recorded. You will be asked to fill out food and physical activity diaries for 7 days and you will be shown how to do this. You may accompany your records with photos of food and drinks consumed during this period of time to enhance the quality of the analysis. You will be given individual bottles of mouthwash to use twice a day every day for the next week.

On your 2nd visit (visit 2), 7 days later, you will arrive between 8am to 12pm at the Nutrition, Exercise and Health Laboratory (Room 206, 2nd Floor Smeaton Building) at Plymouth University under fasting conditions (water only, no tea or coffee for > 7 hours). You must avoid strenuous exercise for at least 24 hours before the visit. You will be asked to return the previous bottles of mouthwash. Your body weight and height will be recorded. Then, you will be placed on a couch for about 30 min to assess the resting metabolic rate (RMR) using indirect calorimetry. This is a method by which the type and rate of substrate

utilization, and energy metabolism are estimated in vivo starting from gas exchange measurements (carbon dioxide production and oxygen consumption during rest). The calorimeter has a gas collector that adapts to the subject and a system that measures the volume concentrations of carbon dioxide and oxygen. This will involve a plastic hood being placed over your head. You will be asked to breathe normally, lie still and relax during this time.

After that, blood pressure will be taken in triplicate over the next 5-10 minutes using an electronic sphygmomanometer (blood pressure cuff) according to established guidelines. After these measures, you will be taken to a different laboratory, where blood samples of 15 mL will be taken from the antecubital vein of your arm to analyse blood glucose, cholesterol, triglycerides, nitrate and nitrite levels. Additionally, a saliva sample (~ 5 mL) will also be collected to analyse nitrate and nitrite levels. Lastly, nitrate-reducing capacity of oral bacterial will be analysed. This is a simple and non-invasive test consisting of holding 10 ml of a solution containing nitrate in the oral cavity for 5 min, after which time the mouth rinse will be collected into a sterile tube to analyse salivary levels of nitrite and nitrate.

At the end of this session, you will be given another set of bottles of mouthwash in order to rinse the oral cavity with the solution twice daily for a week. During the same period of time, you will also be instructed to record and match as much as possible your previous dietary intake and physical activity records. To validate dietary intake and physical activity of both periods of study, you will be asked to complete dietary records (including photos if helpful) and physical activity diaries again. After 7 days of exposure to the different mouthwash, you will be required to report to the lab (visit 3) at the same time as on visit 2 (+/- 1 hour). The same protocol and parameters will be assessed again.

Important: You will be allowed to withdraw from the study at any time with no consequence for your person in any way. If you are a student at Plymouth University your studies will not be affected if you do not choose to participate in the study or withdraw from it once it has started.

What will I obtain from participating?

You will be provided with your own results and a summary of the main findings of this study. We may be able to provide some funding for travel expenses. We will provide a simple breakfast for you after all the tests have been carried out.

How will the information I give be used?

The information you give in the form of answering questions in the medical questionnaire will be simply used to assess if you are safe to complete the study. The answers you give will be kept confidential within the research team. The results of this study will be published in scientific journals and presented at conferences. All data will be presented anonymously; however complete confidentiality of data cannot be guaranteed. Confidential

data and consent forms, as well as physiological and biological information in a hard form (paper) obtained during the course of this study will be kept in a locked filing cabinet in the office space of Dr. Raul Bescos. Biological samples (blood and saliva) will be treated properly in the Nutrition, Exercise and Health Laboratory (Link Building). We will keep all these samples in a freezer at -80°C in the same laboratory. Samples will be analysed at the Plymouth University in due course. A computerized database will be created by Dr. Raul Bescos in order to keep the results of all parameters analysed in this study. This database will be kept in the Dr. Bescos' computer. Using external hard disks at least 2 copies of these data will be made every day after data collection.

What are the potential risks of participating in this project?

The procedures involved in participating in this study are of low risk.

Management of risks associated with Venepuncture and blood sampling: Aseptic techniques will be used at all times during the blood collections. Furthermore, the use of sterile, disposable vacutainers, syringes, swabs, etc., will markedly reduce the possibility of infection. The use of a qualified and experienced phlebotomist will reduce the likelihood of bruising as this is primarily caused by poor venepuncture technique. When the needle will be removed, direct pressure will be applied to the area to reduce the changes of bruising.

Who is conducting the study?

The chief investigator of this study is Dr. Raul Bescos. Other researchers involved in this study are Dr. Melanie Moore and Ann Ashworth (PhD candidate). The study will be carried out in the Nutrition, Exercise and Health Laboratory (Link Building) at the Plymouth University. This study has been approved by the Health & Human Science Research Ethics Committee of Plymouth University

Any queries about your participation in this project may be directed to the Chief Investigator or associate researchers listed below. If you have any queries or complaints about the way you have been treated, you may contact the Health & Human Science Research Ethics Committee of Plymouth University: Sarah C Jones, 01752 585339, hhsethics@plymouth.ac.uk.

Chief Investigator

Dr. Raul Bescos

Ph: 01752 587 585

Email: raul.bescos@plymouth.ac.uk

Researcher

Ann Ashworth

Mob: 07599 034710

Email: ann.ashworth@plymouth.ac.uk

3rd May 2016

Appendix 17.

Medical questionnaire

Medical Questionnaire

**PLEASE ANSWER THE FOLLOWING QUESTIONS COMPLETELY AND ACCURATELY.
ALL INFORMATION IS CONFIDENTIAL.**

General Questions

What is your date of birth? ____/____/____ Age _____

Are you currently or have you ever participated in a medical study? ☐ Yes ☐ No

If yes, please provide further information. If possible, please state the type of study, duration, approximate dates, were blood samples taken and if any drugs were administered:

Approximately how many years have you been vegetarian? _____

Please give your reasons for becoming a vegetarian.....

Nicotine

Do you currently smoke cigarettes (including casual)? ☐ Yes ☐ No

If yes, how many cigarettes do you smoke per day? _____

Do you currently use any chewing tobacco, cigars, nicotine patches or gum? ☐ Yes

Do you have any allergies? If so, please specify ☐ Yes ☐ No

Alcohol**Office
use only**

Please indicate the number of serves you regularly consume each week of:

Beer (pints/cans) _____ x 1 =

Lager (pints/cans) _____ x 1 =

Premium lager (pints/cans) _____ x 1 =

Spirit (measures) _____ x 1 =

Alcopops (bottles) _____ x 1 =

Pre mixed ready to drink (cans/bottles) _____ x 1 =

Wine (red/ white) (125 mls average glass) _____ x 1 =

In how many sittings do you consume these drinks? _____

How many times a month do you consume more than 5 serves of alcohol at a time?

What is the most number of drinks you consumed at one time during the last 3 months? _____

How many times in the last three months have you consumed these many drinks?

Caffeine

Please indicate the number of serves (250mls) you consume each day of:

Office use only

Instant Coffee (1 tspn)	_____	x 1 =
Roasted and ground coffee	_____	x 1 =
Decaffeinated coffee, all types	_____	x 1 =
Tea	_____	x 1 =
Soft drinks (such as Coke, Pepsi)	_____	x 1 =
Energy Drinks (such as Redbull)	_____	x 1 =

Medication and recreational drugs

Are you taking (or have you taken in the last 3 months) any prescription or non-prescription medications or recreational drugs? If yes, please list name/type, duration and dates of use.

☐ Yes

☐ No

Name/Type of Medication	Duration	Dates of Use

Are you currently taking any health food supplements or herbal remedies? If yes, please list name/type, duration and dates of use.

☐ Yes

☐ No

Name/Type of Medication	Duration	Dates of Use

Medication

If yes, please provide details on type, length of time taken, date last used and reason for use.

Have you ever taken, or are you currently taking medicines for anxiety, such as Benzodiazepines?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you taken antibiotics within the last 3 months?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking medications for the stomach, such as omeprazole or other proton pump inhibitor?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken a chronic pain reliever on a regular basis for more than 2 months? (e.g. Panadol, Panadeine, Ibuprofen, Aspirin)	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking anti-depressants (e.g. Prozac, Zoloft, Effexor)?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking anti-epileptic medication (e.g. Dilantin, Epilim)?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking anti-psychotic medication (e.g. Risperidone)?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever used, or are you currently using an asthma inhaler (e.g. Ventolin/Salbutamol)?	<input type="checkbox"/> Yes <input type="checkbox"/> No	

Have you ever taken, or are you currently taking medicines for blood pressure, such as Beta-blockers?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking topical (cream) or oral steroids (e.g. Prednisolone, Sigmacort, DHEA or Andro)?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking antihistamines with sleepiness/sedation listed as a possible side effect?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
Have you ever taken, or are you currently taking thyroid hormones or medications that affect thyroid hormones?	<input type="checkbox"/> Yes <input type="checkbox"/> No	
If male, have you taken, or are you currently taking sex hormones (e.g. testosterone, estrogen, progesterone)?	<input type="checkbox"/> Yes <input type="checkbox"/> No	

Health

Have you ever, or are you currently experiencing: (please tick)

High blood pressure?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
Diabetes, thyroid or other metabolic disorders?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
Surgery (major or minor)?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
Dental conditions such as gingivitis or periodontitis?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
Vision or hearing impairment?	<input type="checkbox"/> Yes	<input type="checkbox"/> No
Other eye disorders or diseases?	<input type="checkbox"/> Yes	<input type="checkbox"/> No

Are you currently, or have you ever been,
under the care of a psychiatrist, psychologist or
counsellor? If yes, please provide details: ☐ Yes ☐ No

Do you use mouthwash or tongue scrapes? ☐ Yes ☐ No

Can you provide the name of toothpaste you use and the frequency of daily tooth brushing?

Menstrual and Reproductive History (for FEMALES ONLY)

Are you currently pregnant or breast feeding? ☐ Yes ☐ No

Have you been pregnant within the last 6 months? ☐ Yes ☐ No

Are you currently taking oral contraceptives? ☐ Yes ☐ No

Do you have regular periods (every 28 days)? ☐ Yes ☐ No

Appendix 18.

Protocol vegetarian Study

Protocol Vegetarian Study

Prior to visit: Participant to be sent the Information Sheet and Medical Questionnaire. If no contraindications (age, smoking, medications etc.), invite to 1st visit.

1st Visit:

Researcher to check the medical questionnaire for any queries

Participant to sign and date the consent form

Researcher to explain to participants how to complete food and physical activity diaries (plus photos if possible)

Researcher to give participant:

Week one mouthwash

Toothpaste sample

7 day food diary (1 page per day)

EPIC FFQ

Physical activity diary

Researcher to remind participants before the next two lab visits:

To arrive in the fasted state (only drinking water allowed for at least 7 hours before the test. No food or caffeine containing drinks including coffee and tea).

To try to get > 7 hours sleep the previous night

To refrain from any strenuous exercise for at least 24 hours before each lab test

To bring their completed food and exercise diaries and empty mouthwash bottles

2nd Visit (Lab test)

*After 7 days of recording food/drinks and physical activity and using mouthwash twice daily.

Key points before participants arrive at the lab (30-60 minutes before):

Turn on the respirometer and to perform a calibration.

Check BP monitor.

Participants arrive at the Clinical lab (Room 206 Smeaton Building):

Anthropometrical measurements (body weight and height; BMI)

Record the temperature, atmospheric pressure and humidity of the lab.

Resting Metabolic Rate: The respiratory analyser (Jaeger Oxycon Pro, Germany) will be warmed up and calibrated before the first test according to the manufacturer instructions. Participants will lie down on a bed for 30 minutes and respiratory gases will be analysed using the Canopy.

Participants will be encouraged to relax during the test. They will not be allowed to play with smartphones, tablets or other devices or read books.

Any noise and conversation should be avoided by researchers into the lab during the test.

Very important: save all the data on the PC (Vegetarian Study folder) and on a USB before ending the test with name and date of test.

Blood Pressure: Will be assessed upon completing the RMR assessment using the Welch Allyn digital blood pressure monitor. Keep the participant on the bed and with the canopy. Three successive readings will be taken with a 1 minute rest between readings. All the readings will be noted on the data collection sheet.

Participant goes to the Nutrition, Exercise and Health lab (Link Building)

Before participants arrive:

Calibration of the pH meter.

Label the tubes with the code of each participant (Initials of first name and surname plus date of collection (e.g. AB0607) indicating the origin of the sample as plasma (PLA) or saliva (SAL).

Get a container with ice ready to keep the samples cold.

Participants arrive at the lab:

Blood collection: 2 tubes of 6 mL (1 lithium heparin + 1 serum separator) and immediately placed on ice.

Lithium Heparin tube (green top): For the analysis of nitrate/nitrite. Tubes should be centrifuged (4,000 rpm, 4°C, 10 min) quickly (<3 minutes). Then, separate the plasma using a plastic pipette in three different Eppendorf tubes **(Very important: all the tubes have to be labelled before use)**. Store both tubes in different freezers at -80°C.

Serum separator tube (golden top): For the analysis of cholesterol and lipids. Using a plastic pipette, take about 0.5 ml of whole blood and put it into an Eppendorf tube to measure blood glucose*** **(Very important: all the tubes have to be labelled before use)**. Then, invert the tube several times and keep the sample at room temperature. Seal the tube and double wrap in 2 specimen bags with the lab request form.

***Blood glucose will be measured using the YSI machine in the lab by the end of the day. **Very important: keep the Eppendorf tube containing whole blood in the container with ice**

Saliva collection: Via sterilised glass funnel, ask participant to produce ~4mls saliva into 1 Fisher tube of 15 mL for the analysis of saliva pH, nitrate/nitrite and microbiome.

- a. pH analysis (before centrifuging the sample)
- b. Centrifuge the tube (4,000 rpm, 4°C, 10 min)
- c. Separation: Put the liquid phase into 3 different **labelled** Eppendorf tubes. Store both tubes in different freezers at -80°C. These samples will be used to measure nitrate/nitrite.
- d. Take the pellet from the bottom of the tube and put it into another **labelled** Eppendorf tube. Store the tube at -80°C. It will be used to analyse the microbiome.

Assessment of the oral nitrate-reducing capacity: Participant to hold 10 ml of nitrate containing solution (NaNO_3) for 5 min in the oral cavity. Then, the mouth rinse should be collected into a sterile beaker, volume measured and centrifuged (4,000 rpm, 4°C, 10 min). The supernatant is collected in 3 different **labelled Eppendorf tubes**. Store tubes in different freezers at -80°C.

Collection of dietary and exercise records. Participant should receive a copy of all these records in order to match the dietary intake and physical activity over the second week of the study.

3rd visit Repeat method as above.

*After 7 days of recording food and drinks and physical activity and using mouthwash twice daily.

***Very important: To respect the same pre-conditions than in the visit 2 and to match diet and physical activity previously recorded as much as possible.

Appendix 19. Examples of vegetables and fruit consumed during 14 days low and high nitrate diet by one participant.

Food List – Low nitrate diet

Food Name	Quantity
Green pepper raw	672.0 g
Tomatoes, raw	800.0 g
Carrots, old, raw	560.0 g
Onions, raw	800.0 g
Garlic, raw	79.0 g
Bananas, weighed with skin	6.0 medium por (900.0g)
Cucumber, raw	776.0 g

Grams Total 4587.0 g

Food List - High nitrate diet

Food Name	Quantity
Rocket average	600.0 g
Leeks raw	616.0 g
Fennel raw	616.0 g
Lettuce average not iceberg	800.0 g
Broccoli purple sprouting raw	900.0 g
Satsumas, weighed with peel	14.0 medium (1400.0g)
Bananas	14.0 medium (1400.0g)

Grams Total 6332.0 g

Appendix 20.

Plasma nitrite and nitrate in men (mean \pm SD) measured at pre-supplementation baseline and after 14 days of low-nitrate and high-nitrate vegetables.

Plasma	Low-nitrate diet		High-nitrate diet	
	Baseline	Post supplementation	Baseline	Post supplementation
Nitrate (μ M)	30 \pm 17	27 \pm 12	30 \pm 20	129 \pm 87*
Nitrite (nM)	140 \pm 51	125 \pm 54	119 \pm 35	227 \pm 89**

*Interaction effect of dietary supplementation and time; * $P = 0.001$, ** $P < 0.001$.*

Appendix 21

Draft protocol to establish a reference database for assessing dietary nitrate intake.

A systematic review would be conducted to search major databases from 1982-2018.

Search terms would include 'nitrate', 'nitrite', 'vegetables' together with individual vegetable names and combinations of the listed terms. Reports from the UK Food Standards Agency archives and other relevant government agencies would also be searched. Data on nitrate content, sampling method, measurement method and publication would be entered into an appropriate database to determine mean (SD), median (IQR), and range for each vegetable.

This database would then be used to assess nitrate intake of individual participants recruited from an existing cohort such as the UK Biobank or Exeter 10,000 database. Ethical approval would be sought from the appropriate bodies. Baseline data such as weight, height, BMI, BP, blood glucose, liver function tests and lipid profiles would be collected if not already available.

Using this database, a validated dietary assessment tool should be developed to assess nitrate intake. Participants would complete a 24-hour diet recall and the results of this would be used to analyse nitrate content by importing data from the nitrate database into an existing nutritional analysis software programme such as Microdiet. The results of this 24-hour dietary recall would be compared to analysis of an FFQ which would be amended to assess vegetable intake over the previous year. Nitrate intake would be calculated by estimating daily intake of these vegetables and multiplying by the nitrate value from the nitrate database. Participants would provide plasma, saliva and urine for nitrate and nitrite analysis. In addition, a salivary DNA sample would be collected for analysis of the oral microbiome.

Statistical analysis would include comparison of nitrate intake to biomarkers of plasma, urine and salivary nitrite and nitrate to assess relationships between variables.

Appendix 22

Draft protocol to assess effects of high-nitrate vegetables and unsaturated fatty acids on cardiovascular health

Recent research suggests that there may be a combined effect of nitrate and unsaturated fatty acids, such as oleic acid, found in olive oil. Nitrate and unsaturated fats form nitrolipids (nitro-fatty acids) (Hughan *et al.*, 2017). These have been shown in animal studies to induce anti-inflammatory, cardio-protective mechanisms, reducing ischaemic and reperfusion injury in myocardial ischaemia (Rudolph *et al.*, 2010). It is possible that the formation of nitro-fatty acids may exert the protective effects observed in the Mediterranean diet, which includes both high-nitrate vegetables and olive oil.

Proposed hypothesis.

That a meal of high-nitrate vegetables and olive oil will significantly affect plasma and salivary nitrate, nitrite, salivary pH, oral nitrate-reducing capacity, SBP, DBP, MAP and aortic pulse wave velocity (aPWV) (or another available method to measure postprandial vascular function) in a population with existing prehypertension, compared to a high-nitrate vegetables and coconut oil, low-nitrate vegetables and olive oil and low-nitrate vegetables and coconut oil (Tables 5.2 & 5.3).

Table 5.2. Fatty acids in 100 ml olive oil and coconut oil.

Oil/100ml*	Saturated (g)	Monounsaturated (g)	Polyunsaturated (g)
Olive Oil	14.3	73.0	8.2
Coconut oil	86.5	6.0	1.5

*Source; McCance and Widdowson's *The Composition of Foods* (Food Standards Agency, 2014).

Table 5.3. Energy and fatty acid content of 40 g of olive oil and coconut oil.

Oil/40g*	Energy (kcal)	Saturated (g)	Monounsaturated (g)	Polyunsaturated (g)
Olive oil	360	5.7	29.2	3.3
Coconut oil	360	34.6	2.4	0.6

*Source; McCance and Widdowson's *The Composition of Foods* (Food Standards Agency, 2014).

Method

Feasibility study:

Supplementation similar to previous acute beetroot juice supplementation studies, i.e. give one meal containing high-nitrate vegetables (~200-300 mg nitrate) and olive oil (40 ml), to healthy participants > 55 years, male and female with prehypertension (i.e. SBP 120-139 mmHg), but not currently on anti-hypertensive medications, and measure effects on plasma and salivary nitrate, nitrite, SBP, DBP, MAP, and aPWV regularly up to 3 hours afterwards. Compare with isocaloric high-nitrate vegetables with coconut oil, low-nitrate vegetables with olive oil and low-nitrate vegetables with coconut oil. Exclusions include; obesity, diabetes, hyperlipidaemia, smokers, mouthwash users and recent use of antibiotics, pre-existing stomach disorders (e.g. taking proton-pump inhibitors (PPI's). This methodology would not reflect long term dietary change, but would establish:

- acceptability of methodology to participants by involving research participants in research design (via PenCLARC)
- ease of recruitment of this defined group, using the Exeter 10000 database or the UK BioBank

- control for other dietary components that may be involved in BP regulation (unsaturated fatty acids, polyphenol and sodium intake), difficult to assess in free living participants
- if changes in salivary and plasma nitrate, nitrite concentrations, aPWV and BP etc. are similar to those previously observed in studies using single doses of beetroot juice
- ease of direct assessment of nitrate content of vegetables, using HPLC analysis

Using data from the feasibility study, a power calculation would be carried out to determine the number of participants required to observe, for example, 4 mmHg reductions in SBP plus expected changes in aPWV.

Main study design:

Depending on the findings from the feasibility study, the main study design would be a randomised crossover trial which would randomize participants to either:

- a meal with high-nitrate vegetables plus olive oil
- a meal with high-nitrate vegetables plus coconut oil
- a meal with low-nitrate vegetables plus olive oil
- a meal with low-nitrate vegetables plus coconut oil

Outcomes: Before and at intervals up to 3 hours after each intervention, measure SBP, DBP, MAP, aPWV, plasma and salivary nitrate, nitrite, oral nitrate-reducing capacity, salivary pH, urinary nitrate, sodium, potassium and total polyphenols. Blood glucose, liver function tests and lipid screen to check metabolic health, if not already available. Duplicate analysis of vegetables eaten to confirm nitrate content via HPLC analysis. Nutritional analysis of meals to include macronutrients and micronutrients as well as sodium,

potassium and total polyphenol content. Assessment of oral microbiome by salivary DNA and relationship to baseline BP. Assessment of perceived stress levels by validated questionnaire and salivary cortisol and alcohol intake by questionnaire and appropriate liver function tests. Physical activity monitors and body composition scales used throughout study to monitor any changes in physical activity and body composition.

Statistical analysis: Repeated-measures ANOVA to assess differences in BP, plasma and salivary nitrate and nitrite, salivary pH, oral nitrate-reducing capacity, nutrient intake of meals, weight and activity changes during trial. Pearson's correlation coefficient to measure correlations between changes in variables.

Appendix 23

Plasma nitrate and nitrite after placebo and after antibacterial mouthwash in both vegetarians and omnivores (mean \pm SD).

	Vegetarians			Omnivores		
	Placebo	Mouthwash	<i>P</i>	Placebo	Mouthwash	<i>P</i>
Nitrate (μ M)	44.8 \pm 32.9	39.5 \pm 22.6	0.379	40.3 \pm 18.3	38.2 \pm 18.7	0.642
Nitrite (nM)	82.8 \pm 41.4	65.0 \pm 14.6	0.033	74.4 \pm 21.4	60.7 \pm 16.6	0.005

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