INORGANIC NITRATE METABOLISM WITHIN THE HUMAN BODY: DIETARY NITRATE SUPPLEMENTATION AND FACTORS INFLUENCING NO BIOAVAILABILITY

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

ABSTRACT

The use of nitrate (NO_3^-) as a dietary supplement to augment health and improve exercise performance has gained considerable attention in the last decade, with substantial evidence supporting its efficacy as a therapeutic and ergogenic aid. Importantly, the documented benefits have primarily been attributed to changes in nitric oxide (NO) bioavailability throughout the body. Pathways related to NO metabolism have been extensively studied. However, emerging evidence provides novel insights into how the efficacy of dietary NO_3^- supplementation may be contingent on additional components such as the oral microbiome and biological tissues (i.e. skin and skeletal muscle). Evidence supporting beneficial effects of dietary NO_3^- ingestion on indices of cardiovascular and metabolic health, as well as on the physiological responses to exercise is abundant. Nonetheless, certain clinical conditions in which the composition and environment within the mouth may be altered such as with oral disease, diabetes, pulmonary hypertension, obesity, cardiovascular disease (Koch et al. 2017), as well as the use of anti-bacterial mouthwash, may limit the body's ability to sequester NO_3^- from foodstuffs.

In an attempt to circumvent the role of the oral microbiome in NO_3^- metabolism, chapter 5 investigated whether using a NO_3^- containing skin lotion could be employed to alter circulating NO_3^- and NO_2^-, a marker of NO bioavailability. Results from this chapter illustrated that in humans, NO_3^- does not appear to traverse through skin to reach the circulation and is ineffective at changing circulating NO_3^- and NO_2^-. It is therefore unlikely to influence NO_3^- metabolism or evoke physiological responses.

Chapter 6 highlights the role of habitual NO_3^- ingestion (or lack thereof) on the microbial profile in the oral cavity. It was found that when human volunteers were deprived of dietary NO_3^- (30 – 50 mg NO_3^-), salivary [NO_3^-] and [NO_2^-] decreased when compared to the ingestion of a diet containing a standard (180 mg NO_3^-) or a high (1000 mg NO_3^-)

NO_3^{--} content. Furthermore, exploratory analyses demonstrated that the alterations in the prevalence (relative abundance of OTUs) of certain bacterial genera and species began to occur following as little as 7-days of dietary NO_3^- deprivation.

Chapters 7 and 8 investigated the effect of NO_3^- ingestion on the presence of NO_3^- in skeletal muscle. Chapter 7 demonstrated that, contrary to early rodent work, the elevation of NO_3^- in human skeletal muscle following dietary NO_3^- supplementation is short-lived. The profile of changes in skeletal muscle [NO_3^-] following the ingestion of NO_3^- closely reflects that seen in biological fluids such as plasma and saliva, with skeletal muscle [NO_3^-] initially increasing as early as 30 minutes, peaking between 1 and 3 hours and returning to baseline values at around 9 hours post NO_3^- ingestion. Chapter 8 is the first study to directly quantify the relative contribution of ingested NO_3^- to changes in [NO_3^-] in plasma, saliva, urine and skeletal muscle. One hour following the ingestion of a $K^{15}NO_3$ stable isotope tracer, labelled NO_3^- accounted for 68% of the total NO_3^- in skeletal muscle, and ~94%, 96% and 89% of total NO_3^- in plasma, saliva and urine, respectively.

The work that comprises this thesis evidences the importance of the oral microbiome and its NO_2^- generating properties by illustrating the ineffectiveness of alternative means of inducing change in circulating NO_3^- and NO_2^-. This, coupled with the findings that the oral microbiome is malleable following not only increased NO_3^- ingestion, but also the removal of NO_3^- from the diet, substantiates the importance of NO_3^- ingestion for NO metabolism. Furthermore, this thesis indicates that biological tissues are likely an integral component which influence NO-related processes and therefore should be considered when discussing the effect of NO_3^- ingestion. This is due to the findings that, following NO_3^- consumption, the circulation and distribution of NO_3^- is prompt and substantial increases of NO-related metabolites can be detected in biological fluids (i.e. saliva, whole blood, red blood cells, plasma) and tissues (i.e. skeletal muscle) simultaneously.

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Figure 3: Mean ± SD plasma/muscle ([NO_3^-] ratio (**Panel A**) and plasma/muscle [NO_2^-] ratio (**Panel B**) prior to (0-h) and at 1-h and 3-h following the ingestion of a $K^{15}NO_3$ supplement. a = significant difference compared to 0-h (P<0.05).

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Chapter 9.5: Figure 1: Time-course of change in circulation and skeletal muscle (and other biological tissues which are likely to be storage and active sites). How concentration gradients are involved in the movement of NO_3^- in the human body. The suggested relationship between circulation and biological tissues (skeletal muscle) in the movement of NO_3^- following ingestion. At rest, skeletal muscle NO_3^- concentrations ([NO_3^-], where '[ ]' indicates concentration) is greater than in circulation due to the endogenous generation of NO. Following the ingestion of NO_3^-, there is an initial shift in the concentration gradient whereby [NO_3^-] in circulation surpasses that in muscle. This enables NO_3^- to enter skeletal muscle from circulation. Both circulation and muscle [NO_3^-] peaks at about 3 h post-supplementation suggesting NO bioavailability is greatest during this period (optimal period, NO bioavailability at its peak: green). Between 3 – 6 h post NO_3^- ingestion,

the concentration gradient is likely to begin shifting, meaning that a [NO_3^-] decrease in circulation will lead to lower [NO_3^-] in circulation than skeletal muscle. This may in turn enable the movement of NO_3^- from skeletal muscle, thereby facilitating the sustained elevation of [NO_3^-] in circulation and not skeletal muscle. During this period, NO bioavailability is still likely to remain elevated and may therefore still be pertinent in altering physiological function (circulating [NO_3^-] remains elevated in circulation and skeletal muscle: yellow). Substantial quantities of NO_3^- are present in urine meaning that surplus ingested NO_3^- is excreted, with peaks in the expulsion of NO_3^- occurring between 6 – 9 h. Although circulating [NO_3^-] remains elevated for up to 24 h post ingestion, movement of NO_3^- from skeletal muscle to circulation may still be occurring before skeletal muscle [NO_3^-] returns to resting values by 9 h (little to no physiological relevance in NO bioavailability: amber).

N.B. The active transport of NO_3^- may also occur due to the presence of the NO_3^- transporter sialin and CLC-1. This would be characterised by the movement of NO_3^- against existing concentration gradients.

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Following its ingestion, NO_3^- swiftly enters the body with substantial increases in circulating NO_3^- being seen as early as 30 mins. Additionally, in processes facilitated by the oral microbiome, substantial quantities of NO_2^- also enters the body through the bacteria facilitated reduction of NO_3^- which ultimately leads to the formation of NO. The increased NO bioavailability following NO_3^- ingestion has been suggested to lead to ergogenic and therapeutic effects. However, the movement of NO_3^- from circulation to active or storage sites (i.e. skeletal muscle) has been reported in animals (Piknova et al. 2016; Gilliard et al. 2018)

and humans, with increases in muscle [NO_3^-] almost entirely linked to ingested NO_3^-. Interestingly, skeletal muscle possesses the necessary enzymatic machinery to generate NO from the reduction of NO_3^- to/ and NO_2^-. These findings make it reasonable to suggest that biological tissues such as skeletal muscle are also implicit in the therapeutic and ergogenic effects of NO_3^- supplementation.

Abbreviations

Nitric Oxide: NO

Nitric Oxide Synthase: NOS

Nitrate: NO_3^-

Nitrite: NO_2^-

Potassium Nitrate: KNO_3^-

Neuronal Constitutive NOS: nNOS, NOS1

Inducible or Calcium-independent NOS: iNOS, NOS2

Endothelial Constitutive NOS: eNOS, NOS3

Nicotinamide-adenine-dinucleotide phosphate: NADPH

Flavin adenine dicucleotide: FAD

Flavin mononucleotide: FMN

(6R-)5,6,7,8-tetrahydro-l-biopterin: BH_4

Iron protoporphyrin IX: haem

Oxygen: O_2

Calcium: Ca^{2+}

Adenosine Triphosphate: ATP

Phosphocreatine: PCr

Sodium Nitrate: $NaNO_3^-$

Gastrointestinal: GI

Ammonia: NH_3

Nitrous Oxide: N_2O

Xanthine Oxidoreductase: XOR

Aldehyde Oxidase: AO

Extensor Digtorum Longus: EDL

Tibialis Anterior: TA

Declaration

The material contained within this thesis is original work, conducted and written by Stefan Kadach. The following publications and communications are a direct consequence of this work.

Peer Reviewed Journal Articles

Kadach, S., Park, J.W., Stoyanov, Z., Black, M.I., Vanhatalo, A., Burnley, M., Walter, P.J., Cai, H., Schechter, A.N., Piknova, B. and Jones, A.M., 2023. 15N-labelled dietary nitrate supplementation increases human skeletal muscle nitrate concentration and improves muscle torque production. *Acta Physiologica*, p.e13924.

Kadach, S., Piknova, B., Black, M.I., Park, J.W., Wylie, L.J., Stoyanov, Z., Thomas, S.M., McMahon, N.F., Vanhatalo, A., Schechter, A.N. and Jones, A.M., 2022. Time course of human skeletal muscle nitrate and nitrite concentration changes following dietary nitrate ingestion. *Nitric Oxide*.

Conference Activity

Kadach, S., Black, M.I., Piknova, B., Park, J.W., Wylie, L.J., Stoyanov, Z., Vanhatalo, A., Schechter, A.N. and Jones, A.M., 2022. Pharmacokinetics of skeletal muscle nitrate concentration changes following dietary nitrate ingestion. *Medicine & Science in Sports & Exercise*. ACSM annual conference, San Diego, USA [Poster].

Kadach, S., Park, J.W., Stoyanov, Z., Black, M.I., Vanhatalo, A., Burnley, M., Walter, P.J., Cai, H., Schechter, A.N., Piknova, B. and Jones, A.M., 2023. 15N-labelled dietary nitrate supplementation increases human skeletal muscle nitrate concentration and improves muscle torque production. ECSS annual conference, Seville. Spain [Oral and Poster].

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CHAPTER 1: INTRODUCTION

The discovery of the gaseous molecule nitric oxide (NO) (also known as nitrogen monoxide) dates back to the 18th century and is credited to a chemist by the name of Joseph Priestly who published his findings in his work 'Observations on different kinds of air' (Priestly, 1772). However, following its identification, an extensive period of time elapsed and it was not until the 1970s and 1980s that the pharmacological and physiological relevance of NO was identified. A series of works subsequently led to NO being named "Molecule of the Year" in 1992, with the researchers leading several groups (Robert F. Furchgott, PhD; Louis J. Ignarro, PhD; and Ferid Murad, MD, PhD) being awarded the Nobel Prize for Physiology and Medicine in 1998 (SoRelle, 1998). Since its introduction into the scientific community, NO is now one of the most investigated molecules in physiological and medical research due to its versatility and the variety of roles it plays in the human body. Notably, the consequences that NO has on health-related processes include, but are not limited to, blood pressure maintenance (Bonilla Ocampo et al. 2018), platelet aggregation and function (Alonso et al. 2003), nerve transmission (Calabrese et al. 2007), host defence (Bogdan et al. 2001), pulmonary function (Ricciardolo et al. 2004), blood flow regulation (Bian et al. 2008) and metabolic control (Moncada et al. 2002).

Shortly following this early work which led to a link between NO and human physiological processes being identified, a number of investigations detailed that NO was generated within the body, endogenously, in a pathway termed the L-arginine – nitric oxide synthase (NOS) system. Within this pathway, the generation of NO leads to the NO-related metabolites nitrate (NO_3^-) and nitrite (NO_2^-) being formed, both of which were thought to be inert by-products of this process (Stuehr et al. 1985, Hibbs et al. 1987, Moncada et al. 1993). The physiological relevance of NO_3^- and NO_2^- was not detailed for some time, however it is now known that the reduction of these molecules enables various nitrogen oxides including NO to be produced *in*

vivo via the recycling of these molecules in processes involving numerous enzymes, proteins, and bacteria (Lundberg at el. 1994, Zweier et al. 1995; Weitzberg et al. 1998). The reduction of NO_3^- and NO_2^- to NO occurs through processes in a secondary pathway termed the 'NO_3^- - NO_2^- - NO pathway'. Notably, during instances of 'challenge' including during periods of low relative oxygen tension (hypoxia) and high local acidity (low pH) which attenuates the endogenous production of NO via the former pathway, the generation of NO via this secondary pathway is increased (Castello et al. 2006; Modin et al. 2001; Van Faassen et al. 2009, Lundberg et al. 2009). This demonstrates that it is the combination of these pathways which ensures sufficient NO generation, and as a result sustained NO bioavailability, in all circumstances which is central in supporting normal physiological function. A notable and distinct characteristic of the NO_3^- - NO_2^- - NO pathway is that the ingestion of food sources containing NO_3^- and NO_2^- can lead to the exogenous increase in these NO-related metabolites in the body. Examples of dietary sources particularly rich in NO_3^- include food types such as leafy green and some root vegetables (Lundberg at al. 2009; Ysart et al. 1999, Hord et al. 2009). As such, this pathway represents a valuable alternative method for ensuring NO bioavailability through the provision of these higher order nitrogen oxides, and therefore is integral in the maintenance of transient fluxes of NO formation within the human body (Lundberg et al. 2008, Weitzberg et al. 2010). Importantly, the relative influence of these pathways and their respective roles in sustaining NO bioavailability is complex. Factors such as diet, physical activity, disease/ clinical conditions as well as medication use (Lundberg et al. 2011) all lead to fluctuations of varying degrees in each pathways. This makes it exceedingly difficult to understand the contribution of these pathways, however it is clear that dietary NO_3^- ingestion represents a nutritional intervention which can be utilised to alter NO bioavailability and potentially NO bioactivity.

The popularity of research into the influence of dietary NO_3^- supplementation and its effects on NO bioavailability has led to a continued source of new and novel evidence regarding mechanisms and consequences of altering NO, via exogenous NO_3^-, and the importance of this in human physiological function. The involvement of NO_3^- supplementation on inherent NO-related processes such as vasodilation (Moncada et al. 1993), mitochondrial respiration (Brown et al. 1994), glucose and calcium homeostasis (Merry et al. 2010; Viner et al. 2000), skeletal muscle function and contractility (Stamler et al. 2001; Coggan et al. 2021), as well as fatigue development (Percival et al. 2010), suggests a range of therapeutic and ergogenic applications. Recently, there has been increased interest in research focussing on two specific components of NO bioavailability and homeostasis. The first being the oral microbiome, which is the initial rate-limiting factor in the metabolism of NO_3^- and NO_2^- (Duncan et al. 1995, Kapil et al. 2013). Specifically, this research demonstrates that not only the rate but also the magnitude of increased NO bioavailability from orally administered NO_3^- and NO_2^- sources appears to be contingent on the symbiotic relationship between the host and bacteria present in the oral cavity. Thereby being identified as significantly contributing to processes involved in altering cognitive function and cardiovascular health (Vanhatalo et al. 2021). The importance of the oral microbiota in the processing of dietary NO_3^- has been underlined in studies using antibacterial mouthwashes whereby the increase in plasma NO_2^- concentration (as well as subsequent alterations in cardiovascular related measures such as blood pressure) is blunted/ abolished (Govoni et al. 2008, Kapil et al. 2012, Petersson et al. 2009, McDonagh et al. 2015). However, recently it has been highlighted that the bacteria inhabiting the oral cavity display substantial plasticity with specific differences within- and between individuals, gender and habitual dietary NO_3^- exposure appearing to exert effects (Kapil et al. 2018, Vanhatalo et al. 2018, Burleigh et al. 2019, Rosier et al. 2020). These data warrant further

work in this area as these aforementioned factors influencing the oral microbiome are yet to be fully understood.

The secondary area of recent focus in dietary NO_3^- research is the storage and transport of NO_3^- and NO_2^- to biological tissues and organs. Specific organs including the liver (Li et al. 2001, Li et al. 2008) and heart (Omar et al. 2014, Pellegrino et al. 2010, Tota et al. 2010, McNulty et al. 2008, Sandvik et al. 2012) are significant contributors to the reduction of NO_2^- in addition to containing substantial quantities of NO_3^- and therefore may support the synthesis and distribution of NO. In the past decade, research has demonstrated the existence of an endogenous NO_3^- reservoir in skeletal muscle with concentrations surpassing concentrations found in the blood, liver and heart (Piknova et al. 2015). Moreover, the NO_3^- concentration within skeletal muscle has been demonstrated to increase following the provision of dietary NO_3^- in both rodents (Piknova et al. 2016, Gilliard et al. 2018) and humans (Nyakayiru et al. 2017; Wylie et al. 2019). This skeletal muscle NO_3^- reservoir appears to be a newly discovered addition to the factors which facilitate NO bioavailability, whereby NO homeostasis and metabolism may be supported via the storage and release of NO_3^- and NO_2^- from skeletal muscle (Gilliard et al. 2018, Wylie et al. 2019, Park et al. 2019). This may become important during instances in which systemic NO bioavailability may be compromised such as during periods of participation in exercise or periods of dietary NO_3^- deprivation. Emerging evidence indicates that skeletal muscle (at least in cell cultures) may possess the capacity to reduce NO_3^- to NO_2^- and NO (Srihirun et al. 2020). Consequently, not only does skeletal muscle appear to be an additional 'piece to the puzzle' in NO homeostasis via the uptake and subsequent release of NO_3^- and NO_2^-, but it may also possess the necessary 'machinery' to reduce these at an intracellular level to support local NO bioavailability and bioactivity.

CHAPTER 2: LITERATURE REVIEW

2.1 Nitric oxide generation via the Nitric Oxide Synthase (NOS) dependent pathway

NO production is an incessant process within the human body via an endogenous production pathway and is vital for the maintenance of normal physiological function. However, the work within this thesis will focus on the exogenous production of NO as this is a manner in which the generation of NO can be promoted via exogenous means (i.e. NO_3^- supplementation).

The messenger molecule, NO, is an extremely small (30 Da) and simple biosynthesis product in the human body, but relies on some of the most complex and largest (300 kDa) enzymes for its generation (Nathan et al. 1994; Wennmalm et al. 1994). Historically, the generation of NO was thought to solely be a product of an endogenous pathway termed the L-arginine pathway in which the amino acid L-arginine is utilised as a substrate to synthesize NO with L-citrulline, NO_3^- and nitrite NO_2^- as by-products (Moncada et al. 1993; Neilly et al. 1994) (Figure 1).

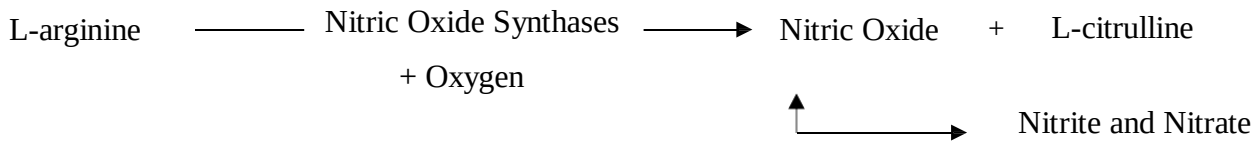

Figure 1: *Simplified schematic demonstrating the generation of nitric oxide in the L-arginine pathway. Nitric oxide synthases function as a catalyst in the process and L-citrulline, nitrate and nitrite are products of this reaction.*

Three distinct isoforms of NOS have been identified and are all products of different genes with diverging localisation throughout the human body. The common nomenclature for these are neuronal constitutive NOS (nNOS, NOS1), inducible or calcium-independent NOS (iNOS, NOS2), and endothelial constitutive NOS (eNOS, NOS3) (Bredt et al. 1994; Griffith et al. 1995; Alderton et al. 2001; Gaston et al. 2002; Förstermann et al. 2012). The varying isoforms of NOS catalyse the oxidation of the guanidino nitrogen of L-arginine to NO (Gaston et al. 2002). In the process of NO generation in this pathway, molecular oxygen (O_2) and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) are co-substrates, and flavin adenine dicucleotide (FAD), flavin mononucleotide (FMN), (6R-)5,6,7,8-tetrahydro-l-biopterin (BH_4) and iron protoporphyrin IX (haem) are cofactors for these isozymes (Crane et al. 1998; Kotsonis et al. 2000; Stuehr et al. 2001). The efficacy of the five-electron oxidation of L-arginine is dependent on the aforementioned cofactors and substrates (Alderton et al. 2001; Förstermann et al. 2012).

The half-life of NO within the circulation is less than 2 milliseconds (Crawford et al. 2006; Lundberg et al. 2011), whereas the more stable anions NO_3^- and NO_2^- have substantially longer half-lives of approximately 5-6 hours and 42-45 minutes respectively (Dejam et al. 2007; Pluta et al. 2011; Rix et al. 2015). Typically, within circulating blood and certain tissues such as vascular smooth muscle, NO_3^- is the predominant final product of endogenous NO oxidation (via the L-arginine – NOS pathway) and NO_3^- concentrations within these compartments are in the micromolar range which is two orders of magnitude greater than the nanomolar concentrations of NO_2^- (Moncada et al. 1993). The sustained provision of NO and therefore the maintenance of NO bioavailability is supported through the rapid oxidation of NO to these more stable products, NO_3^- and NO_2^- (Moncada. 1991; Rhodes et al. 1995). A process which is vital due to the highly reactive properties of NO. It is this rapid oxidation of NO in combination with the NOS tight regulation and short half-life of NO which has

guarantees the specificity of NO signalling (Maia et al. 2018). The oxidation of NO is facilitated by various factors including: oxy-hemoglobin and oxy-myoglobin leading to the generation of NO_3^- from NO (Doyle et al. 1981; Liu et al. 1998; Gow et al. 1999; Brunori et al. 2001; Huang et al. 2001; Gardner et al. 2006), as well as the generation of NO_2^- being facilitated by ceruplasmin, cytochrome c oxidase, and/ or dioxygen (Wink et al. 1993; Goldstein et al. 1995; Brunori et al. 2004; Liu et al. 2005; Shiva et al. 2006).

The apparent dependence on oxygen in this pathway to form NO means that an array of factors including alterations in local pH and oxygen tension (which if reduced attenuates the generation of NO) influence the extent of NO generation via both enzymatic and non-enzymatic means (Van Faassen et al. 2009; Lundberg et al. 2009). Instances of NOS dysfunction can also occur in a variety of circumstances in humans and is identifiable in aging where eNOS (NOS3) dysfunction is prevalent, as well as in certain metabolic and cardiovascular medical conditions in which NOS (dys)function is a central contributor (El Assar De La Fuente et al. 2012; Omar et al. 2016; Lundberg et al. 2018). It is evident that sole reliance on the endogenous production of NO could potentially lead to instances in which NO bioavailability may be compromised, a phenomenon which would be problematic to the maintenance of normal physiological processes.

2.2 Nitric oxide production via the Nitrate- Nitrite- Nitric Oxide pathway

The NOS-dependent generation of NO only explains part of the story of how NO bioavailability is maintained, with an alternative pathway termed the NO_3^- - NO_2^- - NO pathway forming a secondary element to this two-component system. This alternative pathway enables the NOS-derived by-products NO_3^-, and to a lesser extent NO_2^-, of endogenous NO generation to be utilised as a precursor and intermediate respectively of NO production (Lundberg et al. 1994). Importantly, this alternative pathway functions in a

synergistic manner with the aforementioned L-arginine pathway. This is achieved via the combination of the products of the endogenous pathway which, coupled with the non-enzymatic reduction of exogenously supplied NO_3^- (and NO_2^-), generates NO and other nitrogen oxides (Lundberg et al. 1994; Weitzberg et al. 1998; Zweier et al. 1999).

In contrast to the endogenous NO generation pathway, the NO_3^- - NO_2^- - NO pathway utilises NO_2^- as a substrate and through the one-electron reduction to NO does not necessitate the presence of NOS or multiple co-factors (Lundberg et al. 2009). However, it is notable that with the exception of extreme conditions including highly acidic incidences which may be present in the stomach (McKnight et al. 1997; Tsuchiya et al. 2005), urine (Lundberg et al. 1997), or ischemic tissue (Zweier et al. 1995; Dennis et al. 1991) the direct unsupported catalysation of NO_2^- is unremarkable (very slow) (Vanin et al. 2007). Therefore enzymatic facilitators/mediators of NO_2^- reduction are important to ensure sufficient NO_2^- to NO reduction in all circumstances. There are a number of cofactors which can be broadly categorised into heme-based nitrite reductases, molybdopterin- containing proteins and nitrite anhydrases which facilitate the reduction of NO_2^- to NO, particularly when oxygen tension and pH are low (Kim-Shapiro et al. 2014; DeMartino et al. 2019).

This pathway can be exogenously supplemented via the ingestion of dietary NO_3^- (and NO_2^-), the consumption of which leads to substantial increases in circulating concentrations of these ions (Lundberg et al. 2004; Bryan et al. 2015). The ability to augment circulating NO_3^- (and NO_2^-) via dietary supplementation is a potentially meaningful tool for inducing physiological effects typically associated with NO-like bioactivity. This thesis has utilised NO_3^- (and NO_2^-) supplementation as a means for increasing NO bioavailability in attempts to identify factors which may influence NO metabolism and physiological processes.

Inorganic NO_3^- and NO_2^- are naturally occurring anions which are a constituent part of all modern diets as a consequence of the variety of differing food sources they are present within

(Ysart et al. 1999; Lundberg et al. 2009). The predominant dietary sources of NO_3^- (and NO_2^-) for humans are from leafy green vegetables that are particularly rich in NO_3^-, i.e. beetroot, endive, fennel, lettuce, radish, rocket and spinach, typically share similar characteristics and together account for approximately 50-85% of the average daily intake (Gangolli et al. 1994; Van der Avoort et al. 2018). There are however further food sources which contain considerable quantities of NO_3^- such as some root vegetables (i.e. potatoes), fruits, as well as cured meats (Ysart et al. 1999; Hord et al. 2009; Jonvik et al. 2017). The ingestion of NO_3^- (and NO_2^-) can fluctuate considerably between individuals and populations, as highlighted by reports that NO_3^- intake approximates 31-185 mg/day in European countries and 40-100 mg/day in the United States (Gangolli et al., 1994; Hord et al. 2009). However, it should be noted that NO_3^- intake is likely to differ considerably from population-based estimates due to potential limitations and associated error in estimation through self-reported food frequency questionnaires. This is likely to be result of a distinct sparsity in the quantification of NO_3^- (and NO_2^-) in foods from differing countries, times of the year, cooking methods, and other agricultural related influences.

A plethora of evidence exists regarding the potential benefits that the ingestion of dietary NO_3^- may have (due to resulting increases in NO-like bioavailability/ bioactivity) (Lundberg et al. 2015; Jones et al. 2020). It can, however, be difficult to accurately quantify habitual nitrate ingestion and its potential effects on markers of health and physical performance (Shannon et al. 2021). Therefore, in the experimental chapters included in this work, a series of stringent dietary control periods were implemented. These were characterised by periods of abstaining from dietary sources rich in NO_3^- and NO_2^- while consuming a habitual diet, or the provision of controlled diets to account for variation that may result from differences in habitual dietary NO_3^- intake prior to the experimental procedures.

2.3 Therapeutic and Ergogenic Effects of Nitrate Supplementation

Administration of NO_3^-, specifically via convenient means such as dietary NO_3^- supplements in the form of beetroot juice and NO_3^- salts, has gained popularity due to the wide variety of beneficial effects that may be elicited as a consequence of the associated NO-like bioactivity (Lundberg et al. 2015).

There is growing evidence from cell culture, animal and human studies for beneficial effects of ingesting dietary NO_3^- on protection against cardiovascular and coronary heart disease via mechanisms such as altering blood pressure, platelet aggregation and attenuating ischemia reperfusion injury (Joshipura et al. 1999; Joshipura et al. 2001; Visioli et al. 2004; Vosioli et al. 2005; Trichopoulou et al. 2004; Gladwin et al. 2005). When inspecting the current literature base it is apparent that the ingestion of dietary NO_3^- has the potential to exert beneficial effects on cardiovascular health via the reduction of blood pressure, which can amount to 5 to 12 mmHg in systolic blood pressure and 5 to 6 mmHg in diastolic blood pressure in a variety of populations including both young and older adults, as well as individuals that are overweight and hypertensive (Larsen et al. 2006; Webb et al. 2008; Kapil et al. 2010; Vanhatalo et al. 2010; Bonilla Ocampo et al. 2018). Moreover, in addition to the aforementioned cardiovascular influences of NO_3^- ingestion, research reveals that supplementation may reduce the development of type II diabetes through reversing specific aspects of metabolic syndrome, augmenting pancreatic islet function (in murine and rodent models) as well as upregulating mitochondrial function (in humans) (Bazzano et al. 2008; Carter et al. 2010; Carlström et al. 2010; Nyström et al. 2012; Carlström et al. 2015). Dietary NO_3^- is a potential alternative method to typical pharmaceutical approaches and may potentially offer a practical alternative.

The administration of NO_3^- is of benefit in a variety of sports/ exercise modalities which include running, cycling and rowing exercise, whereby a variety of studies have presented

positive (improved performance) outcomes in both normoxia and hypoxia (See Review: Senefeld et al. 2020). These positive findings are not isolated to exercises/ sports of similar nature. For instance, the ingestion of NO_3^- with the efficacy of NO_3^- being evidenced over numerous exercise modalities such as "explosive" efforts lasting less than or 6 seconds (Tan et al. 2022) which may relate to weightlifting (San Juan et al. 2020) and team sport specific (Wylie et al. 2013; Thompson et al. 2015; Thompson et al. 2016; Jones et al. 2016; Baranauskas et al. 2020) scenarios but also NO_3^- leads to favourable endurance sports performance (Gao et al. 2022). This demonstrates that dietary NO_3^- ingestion may differ from other dietary aids in evoking positive consequences on exercise performance due to its mechanism of action being almost 'multi-layered' through the sheer number of physiological functions affected by NO_3^- ingestion. The mechanisms by which NO_3^- supplementation may enhance performance spans various factors including reducing the O_2 cost of sub-maximal exercise (Larsen et al. 2007; Bailey et al. 2009), augmenting muscle contractility via alterations in calcium (Ca^{2+}) handling and sensitivity (Hernández et al. 2012; Whitfield et al. 2016; Dominguez et al. 2018; Coggan et al. 2018), reducing adenosine triphosphate (ATP) utilisation and phosphocreatine (PCr) degradation (Bailey et al. 2010) as well as speeding PCr resynthesis kinetics (Vanhatalo et al. 2011; Vanhatalo et al. 2014).

It should however be noted that ergogenic effects appear to be limited in highly trained athletes (and perhaps females (Wickham et al. 2019)), (Jones. 2014; Jones. 2014b). There is a strong rationale forming for potential sex differences between males and females following ingestion of NO_3^- supplements (Wickham and Spriet. 2019; Kapil et al. 2018). It should however be considered that, like with any form of research, there are disparate findings in relation to the ergogenic influences of NO_3^- supplementation (McMahon et al. 2015; Jones et al. 2018), a phenomenon which has been attributed to a potential 'responder and non-responder' or 'high vs. low responder' (Wilkerson et al. 2012; Christensen et al. 2013;

Coggan et al. 2018). The work constituting this thesis will provide a new insight into potential factors involved in the metabolism of NO_3^- and therefore NO bioavailability, thus potentially going some way to providing explanations for why individuals may respond differently to NO_3^- supplementation.

2.4 Novel Approach to Influencing Circulating Nitrate: Skin

The limited research into alternative methodologies for augmenting NO via the exogenous provision of NO_3^- and NO_2^- is likely a product of the success of inducing favourable changes via the ingestion of NO_3^- and NO_2^- in the form of beetroot juice or NO_3^- salts (i.e. potassium nitrate (KNO_3) or sodium nitrate ($NaNO_3$)) (James et al. 2015; Jakubcik et al. 2021) . In spite of the abundance of evidence providing a rationale for consuming dietary NO_3^-, anecdotal evidence illustrates that there is continuing reluctance for ingesting NO_3^- due to gastrointestinal (GI) intolerability and unpalatability (Vitale et al. 2019). Therefore, in order to avoid these negative consequences, it would be desirable to explore novel approached for inducing similar consequences as the ingestion of NO_3^-. The skin could provide one such alternative, with emerging evidence demonstrating that skin tissue retains significant quantities of NO_3^- and NO_2^- (Villar et al. 2021).

The presence of NO_3^- and NO_2^- in skin was first demonstrated in 1996 (Weller et al. 1996). Interestingly, due to the acidic environment present on the skin surface the concentration of these ions was found to be significantly greater than that seen in circulating plasma (Weller et al. 1996). Although further research is required for understanding the influence of the NO metabolites retained in skin on physiological function, it appears that skin NO_3^- and NO_2^- may potentially contribute to body NO bioavailability (Liu et al. 2014). NO_3^- is the predominant nitrogen oxide present in skin tissue with concentrations approximating 82.4 µmol l^{-1}, with NO_2^- concentrations at 5.1 µmol l^{-1}, and is primarily located in the dermis and

epidermis (Paunel et al. 2005; Mowbray et al. 2009). Villar and colleagues (2021) demonstrated that not only muscle, but also skin may potentially act as a reservoir for NO_3^- and NO_2^- with the estimated recovery of intravenous injected ^{15}N-nitrate and ^{15}N-nitrite being greater in skin but still considerable for both of these tissue types (Skin: ^{15}N-nitrate recovery = 22.8%, ^{15}N-nitrite recovery = 26.9 %; Muscle: ^{15}N-nitrate recovery = 4.5 %, ^{15}N-nitrite recovery = 12.9 %). However, it should be noted that this study was performed in sheep and the estimated values in tissues were made 60 minutes post supplementation so further work is required to determine the extent of NO_3^- and NO_2^- retention in skin and whether the same phenomenon occurs in humans.

Notably, the below section focusses on and describes the importance of the enterosalivary pathway in altering NO bioavailability following the ingestion of NO_3^-. The unequivocal reliance on the enterosalivary pathway and thereby the oral microbiome provides a reason for exploring alternative methods as the potential to bypass would be potentially important in situations where the oral microbiome may be compromised or changes (i.e. in aging: Vanhatalo et al. 2018; Ahmed et al. 2021, mouthwash use: Lundberg and Govoni 2004; Govoni et al. 2008; Kapil et al. 2015, McDonagh et al. 2015). Hereby providing further rationale for the exploration of alternative methods to elicit equivalent effects on NO bioavailability through changes in circulating NO_3^- and NO_2^-.

2.5 Role of the Enterosalivary Pathway in the formation of Nitric Oxide

2.5.1 Oral Cavity: The oral cavity and its constituents are central in the uptake and reduction of NO_3^- and NO_2^- from dietary sources, and ultimately is considered a rate-limiting step in processes influencing NO bioavailability (Duncan et al. 1995; Kapil et al. 2013).

Following its ingestion from dietary sources, up to 25% NO_3^- enters the enterosalivary circulation via the salivary glands in processes facilitated by sialin, an active transporter of NO_3^- found tissues that aids transport in both saliva and blood (Qin et al. 2012), prior to being concentrated by up to 20-fold in saliva (Govoni et al. 2008; Lundberg et al. 2008). Anaerobic facultative bacteria present on the dorsal face of the tongue facilitate the reduction of NO_3^- to NO_2^- within the saliva which once swallowed, enters circulation (Spiegelhalder et al. 1976; Lundberg et al. 2004; Lundberg et al. 2011). These commensal bacteria reduce NO_3^- by using this as a terminal electron acceptor for ATP synthesis, and can lead to NO_2^- formation (which can be vasoactive in certain conditions; i.e. low pH and oxygen tension) which can be further reduced to generate NO (Dejam et al. 2004). Although, much research focusses on the NO_2^- generating capacity of the microbial profile of the mouth, it is important to understand the nuanced role which supports both the generation and depletion of NO_2^- as both obligate and facultative bacteria reside in the mouth (Li et al. 1997; Koch et al. 2015). Three distinct bacterial NO_3^- to NO_2^- reducing pathways exist and lead to alterations in NO bioavailability through the formation of NO or the depletion of NO_2^- to form ammonia (NH_3) leading to reduced NO bioavailability (Sparacino-Watkins et al. 2014; Koch et al. 2015). Firstly, a NO_3^- / NO_2^- movement pathway allows the movement of these NO related ions through the plasma membrane through a NO_3^- / NO_2^- transporter family coded by the gene nark (Goh et al. 2022). Respiratory denitrification is a NO_3^- - NO_2^- pathway which leads to the formation of NO and therefore availability of NO through the systematic reduction of NO_3^- to NO_2^-. The reduction of NO_3^- to NO_2^- is enabled through the nitrate reductase Nar which is coded by several genes (NarB, narG, narZ, nxrA, narY, nxrB, narI, and narV), and the reduction of NO_2^- requires the nitrite reductase Nir (coded by the genes nirK and nirS) (Goh et al. 2022). Finally, dissimilation and assimilation pathways in which the generation of ammonia from NO_3^- leads to the depletion of NO_3^- and NO_2^-, NO availability may be reduced as a result. In these

dissimilatory and assimilatory NO_2^- reduction pathways, the reduction of NO_3^- to NO_2^- occurs and involved the NO_3^- reductases Nap (coded by the genes napA and napB) and Nas (coded by the genes (nasA and nasB) respectively. Following the formation of NO_2^-, NH_3 is produced by enzyme proteins Nrf (coded by the genes nrfA and nrfB) in dissimilation and Nir (coded by the genes nirA, nirB and nirD) in assimilation (Goh et al. 2022).

The central role exerted by bacteria within the oral cavity has been illustrated in studies that utilised antiseptic mouthwash (Lundberg and Govoni 2004; Govoni et al. 2008; Kapil et al. 2015, McDonagh et al. 2015), germ-free mice (Petersson et al. 2015), or manipulating saliva deglutition (Lundberg and Govoni 2004; Webb et al. 2008), to show that the expected increases in plasma and saliva NO_2^- following the ingestion of dietary inorganic NO_3^- are abolished or significantly blunted. The synergistic relationship between the microbial communities in the mouth as well as other communities in the host (i.e. gut) appears to be a fundamental rate-limiting step in generating favourable effects on markers of cardiac and pulmonary vascular health (Koch et al. 2017; Blekkenhorst et al. 2018).

The oral cavity has the second most diverse microbial community in the human body, in which 50-100 billion bacteria from more than 700 prokaryotic taxa reside (Kilian et al. 2016). The oral microbiota is comprised primarily of approximately 200 species (spp) in the phylum Firmicutes (incl. *Streptococcus, Veillonella, Granulicatella, Gemella spp*.) and Proteobacteria (incl. *Neisseria, Haemophilus spp.*) which account for approximately 80% the bacteria present in the oral cavity, and further contributions from Bacteroidetes, Actinobacteria and Fusobacteria account for a further 15% and therefore totalling upwards of 95% of the bacteria present in the mouth (Zaura et al. 2009; Dewhirst et al. 2010; Segata et al. 2012; Ahn et al. 2014). These bacteria include numerous anaerobic facultative and obligate anaerobes with NO_3^- reductase genes that facilitate the denitrification of NO_3^- to nitrogen gas (N_2) through NO and nitrous oxide (N_2O) generation (Li et al. 1997), with specific microbes

present in the deep clefts of the dorsal face of the tongue potentiating the reduction of NO_3^- to NO_2^- in saliva (Lundberg et al. 2004; Doel et al. 2005; Hyde et al. 2014).

Emerging evidence indicates that the concurrent ingestion of NO_3^- in the habitual diet with the addition of NO_3^- supplements results in an increase of Proteobacteria (i.e. Neisseria (*genus*)) (+225%) and a reduction in Bacteroidetes (i.e. Prevotella (*genus*)) (-46%) in the oral microbiome (Vanhatalo et al. 2018; González-Soltero et al. 2020). Moreover, there appears to be a preferential phenotype for bacteria present in the mouth. It has been suggested that high levels of *Rothia* and *Neisseria* and low levels of *Prevotella* and *Veillonella* genera are correlated to enhanced plasma $[NO_2^-]$ in response to NO_3^- ingestion (Vanhatalo et al. 2018). Vanhatalo and colleagues (2018) elucidated that in response to chronic NO_3^- supplementation, individuals who have a greater proportion of *Prevotella melaninogenica* and *Campylobacter concisus* at baseline were less responsive. Conversely, greater abundances of *Fusobacterium nucleatum* subspecies and Actinomycetales at baseline were correlated with greater augmentation of $[NO_2^-]$ in plasma and a greater reduction in blood pressure following a 10-day NO_3^- supplementation period. In partial agreement, Burleigh and associates (2018) reported that several oral microbial species such as *Prevotella melaninogenica, Veillonella parvula* and *Rothia mucilaginosa* were positively correlated to increases in salivary $[NO_2^-]$. These authors also demonstrated that NO_3^- reduction in the mouth influences the pharmacokinetic profile of salivary NO_2^- which is slowed in individuals with a lower abundance of NO_3^- reducing bacteria following the acute ingestion of NO_3^-. Furthermore, higher relative abundances of these microbial species did not lead to additional increases in circulating NO biomarkers (NO_2^- concentrations in plasma) or changes in markers or cardiovascular health (blood pressure) (Burleigh et al. 2018). Therefore, it appears that focussing on individuals or populations which the oral microbiome may be compromised (i.e. lower abundances of NO_2^- producing bacteria in the mouth) is of greater importance than

those with sufficient microbial profiles. It is however important to note that these bacterial species were selected due to their NO_3^- reducing capacity and not the production of NO_2^-. If the product of bacterial NO_3^- reduction is NH_3 and not NO_2^- or NO, this would not increase systemic NO availability.

Notably, data from Vanhatalo and colleagues (2018) suggest that a consequence of manipulating the NO_2^- producing oral microbiota through increased dietary NO_3^-, may be more meaningful in elderly populations through increasing circulating NO_2^- (in plasma) and reducing blood pressure. This illustrates the influence of diet in combination with bacterial profiles in the mouth and how these may impact indices of NO homeostasis and vascular health (at least in older individuals). This highlights that although the microbiome may be seen as a rate-limiting factor in the metabolism of dietary NO_3^-, additional factors must be considered when attempting to gain an oversight of factors modulating the NO_3^- and NO_2^- in systemic circulation.

Synthesis of the above evidence regarding the role of the oral microbiome on NO metabolism indicates that the bacteria residing within the mouth may rate-limit the reduction of NO_3^- to NO_2^- (Blekkenhorst et al. 2018). There is emerging evidence focussed on several confounding variables and how the microbial profile within the mouth varies as a consequence of gender and age (Kapil et al. 2018; Ahmed et al. 2021) as well as being malleable through dietary exposure to NO_3^- (Vanhatalo et al. 2018). There is, however, a distinct lack of investigation into the influence of habitual NO_3^- intake and how alterations in this (i.e. low vs. high NO_3^- ingestion) may influence the metabolism of NO.

At present, the gap in the literature and evident fluctuations in habitual NO_3^- ingestion, not only in periods of high-NO_3^- but also during periods of low-NO_3^- ingestion, makes it difficult to fully interpret how the diet interacts with and potentially changes the malleable oral

microbiome. Investigation into this may provide valuable information which may partially explain variations in the responsiveness to NO_3^- supplementation and subsequent impacts on physiological systems.

2.5.2 NO Synthesis: Gastrointestinal Tract/ Stomach: Following the ingestion of NO_3^- and NO_2^-, the NO-related metabolites are rapidly absorbed from the gastrointestinal tract into the bloodstream where it is integrates with the existing endogenously synthesised NO_3^- and NO_2^- (Dejam et al. 2004, Lundberg et al. 2004). Upon coming into contact with the acidic gastric environment, NO_2^- is rapidly protonated to form nitrous acid which decomposes further to NO and various nitrogen oxides (Benjamin et al. 1994; Lundberg et al. 1994). The relative complexity regarding the chemistry of acidified NO_2^- makes it difficult to quantify the amount of NO produced from this reaction (Weitzberg et al. 1998). The quantity of NO generated within the gastrointestinal tract is a multifaceted process and is not only contingent on the acidity of the stomach and the concentration of NO_2^- but also factors such as the presence of additional reducing agents which may be co-ingested with and are present to varying degrees in the diet (vitamins, thiocyanate, ascorbic acid, and polyphenols) (Bjorne et al 2005, Gago et al. 2007). The high quantities of NO generated from the acidified NO_2^- in the stomach are in the range of 10-100 ppm, which is several orders greater than that required for expected vasodilatory properties (Benjamin et al. 1994; Lundberg et al. 1994; Duncan et al. 1995). The process of gastric NO generation has been suggested to contribute to primary host defence of swallowed pathogens, a result of the relative concentration of NO yielded from these reactions being toxic to numerous micro-organisms and entero-pathogens (Lundberg et al. 2011). Notably, it is the addition of NO_2^- and other products such as reactive nitrogen intermediates resulting from the production of NO (in combination with NO) in the acidic environment present in the stomach which leads to the eradication of entero-pathogens *Escherichia coli* and *Candida albicans* species (Benjamin et al. 1994) and inhibition of

growth of *Salmonella, Shigella,* and *Helicobacter pylori* (Dykhuizen et al. 1996; Duncan et al. 1997). NO_2^- has been shown to exert a variety of effects which are integral in maintaining gastric integrity via antibacterial properties and increasing gastric blood flow and mucus production (Bjorne et al. 2004; Petersson et al. 2007), as well as providing protective properties in models of gastric injury (ulceration) (Jansson et al. 2007; Miyoshi et al. 2003; Larauche et al. 2003; Larauche et al. 2003b). In addition to the aforementioned physiological roles for gastric NO and other reactive nitrogen oxides/ intermediates metabolised from salivary-derived NO_2^-, the majority of NO_2^- is not involved in the gastric conversion within this passage and enters the systemic circulation (Lundberg et al. 2004).

Similar to the oral cavity, the concept of a host- microbe symbiotic relationship also exists in the gut. Approximately 70% of the microorganisms which constitute the dynamic ecosystems inhabiting the varying surfaces and compartments of the human body are present in the gastrointestinal tract (Sekirov et al. 2010). The human gut microbiome is comprised of up to 1000 different bacterial species which are involved in various processes including the harvesting of otherwise indigestible nutrients, being involved in cell renewal, and modulating host defence via innate and adaptive immune responses (Turnbaugh et al. 2007). The microbial communities present have been associated with the maintenance of health via the contribution to metabolic, defensive and tropic function (Alonso et al. 2013). Isolated strains of bacteria present within the stomach such as *Escherichia coli* and lactobacilli (Ji et al. 1988; Ji et al. 1989) have been demonstrated to be involved in the generation of NO. This phenomenon was further substantiated in a study illustrating that Lactobacilli and bifidobacterial generate a substantial proportion of NO from nitrite, in contrast to *Escherichia coli, Bacteroides thetaiotaomicron* and *Clostridium difficile,* which did not significantly contribute to NO generation from either NO_3^- or NO_2^- (Sobko et al. 2005). However, some studies have failed to observe a relationship between dietary NO_3^- supplementation and gut

microbiome composition (Conley et al. 2017; Rocha et al. 2019). At present there is very limited evidence available in rodents, and even less in humans, relating to the gut microbiome and NO bioavailability. Thus, it is currently not clearly understood whether the ingestion of dietary nitrate induces alterations in the gut microbiome (Gonzalez-Soltero et al. 2020) and further work is needed to bridge the current gap in the literature.

2.5.3 Nitrate Excretion: An extensive amount, encompassing approximately 60-75% of endogenously and exogenously derived NO_3^-, is excreted in urine via the kidneys within 48 hours of its ingestion (Wagner et al. 1985; Hobbs et al. 2002). Additionally, although large quantities of NO_3^- are excreted, there is an active uptake process whereby up to 25% of NO_3^- is actively reabsorbed and concentrated by up to 10-fold in saliva from plasma and re-secreted into the upper intestinal tract (Duncan et al. 1995; Spiegelhalder et al. 1976; Tannenbaum et al. 1978) (Figure 2).

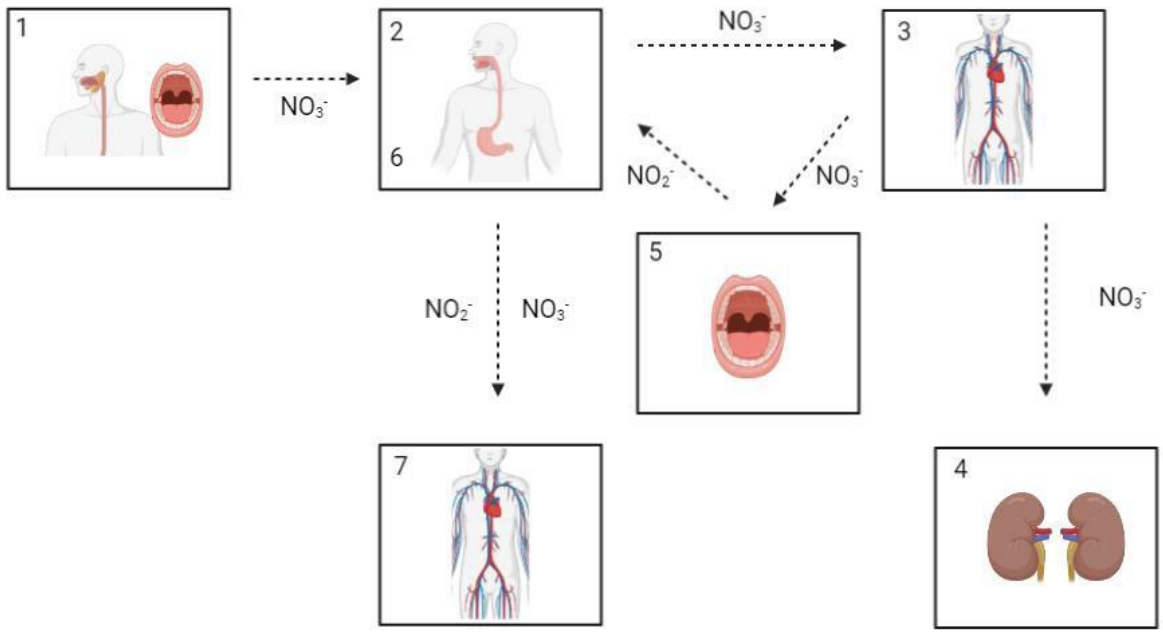

Figure 2: *Illustration of the metabolic fate of nitrate (NO_3^-). Once ingested (1), the initial absorption of NO_3^- occurs in the gastrointestinal tract (2) and subsequently enters systemic circulation (3). Up to 70% of NO_3^- is excreted (4) by the kidneys, but approximately 25% of*

NO_3^- enters the enterosalivary pathway where bacteria within the mouth generate nitrite (NO_2^-) (5). The concentrated NO_2^- in saliva is then swallowed and enters the stomach, where nitric oxide (NO) and other reactive nitrogen species are generated (6). NO_3^- in the swallowed saliva that was not reduced also re-enters the systemic circulation via the small intestine alongside the remaining NO_2^- that did not form NO (7).

2.6 Skeletal Muscle Nitrate Metabolism and Storage

Until 2015, it was generally considered that NO metabolites were transported through the body in blood to intended active sites but could also be recycled or potentially stored at specific organs which primarily spanned certain internal organs including the heart and liver (Rodriguez et al. 2002; Bryan et al. 2003; Carlström et al. 2010). This was enabled due to processes regulated by xanthine oxidase (XO) and aldehyde oxidase (AO), especially in the heart and liver, having been demonstrated to contribute to the generation of NO from NO_2^- via specific NO_3^-/ NO_2^- to NO reductase activity to a greater extent than that seen in the blood (Li et al. 2003; Li et al. 2008). However, more recently there is a growing body of literature suggesting that NO_3^- and NO_2^- may be transported to various other biological tissues that were previously unidentified as retaining NO_3^- (and NO_2^-).

2.6.1 Murine Models*:* Piknova and colleagues (2015) were the first to evidence the existence of a basal NO_3^- and NO_2^- reservoir in skeletal muscle in rodents, where significant quantities of these metabolites are held (approximately 3-fold greater than that seen in circulation and 17-fold higher than liver). Although the expression of nNOS in skeletal muscle was previously described (Nakane et al. 1993), no link had been made between the skeletal muscle and whole body NO homeostasis. It has been suggested that the transport of NO_3^- and NO_2^- from skeletal muscle to circulating blood and vice versa occurs via passive diffusion due to the existence of the large gradient in concentrations (Piknova et al. 2016; Park et al.

2019). Nevertheless, the transport of NO_3^- and NO_2^- may also be facilitated via an active transport chain which is supported by the NO_3^- transporter sialin, a protein which is also present in skeletal muscle (Piknova et al. 2016; Park et al. 2019). The presence of xanthine oxidoreductase (XOR) (*in part responsible for this*) (and aldehyde oxidase (AO)) in skeletal muscle means that active NO_3^- to NO_2^- and NO reductase activity within muscle can occur (Piknova et al. 2016). Park and associates (2019) demonstrated the existence of compensatory mechanisms whereby XOR and sialin were both upregulated in engineered myoglobin deficient mice. The development of the compensatory mechanisms suggests that maintenance of NO_3^- levels within muscle is important for preservation of NO homeostasis (Park et al. 2019). This illustrates, at least in rodents, that NO_3^- levels within skeletal muscle may support NO metabolism throughout the body. The importance of exogenously derived NO_3^- and NO_2^- is not negated by the existence of the substantial endogenous pool of NO_3^- and therefore further work is required to investigate the influence of ingesting NO_3^- on skeletal muscle NO_3^- concentrations as this work remains in its infancy in human models.

Notably, evidence indicates that NO_3^- concentration in skeletal muscle is subject to bidirectional alterations via chronic manipulation of the provision of inorganic NO_3^-, meaning that this store can be augmented by 1.3-fold or depleted by 0.4-fold of the original value with dietary NO_3^- supplementation or restriction, respectively (Gilliard et al. 2018). The authors suggested that when nitrate is absent in the diet, the skeletal muscle store appears to contribute extensively in NO metabolism (Gilliard et al. 2018). These findings illustrate the importance of NO_3^- derivation from dietary means for the sustenance of NO_3^- levels within skeletal muscle. To this end, nutritional or dietary history is likely to substantially influence fluxes in skeletal muscle NO_3^-. High NO_3^- diets induce alterations in NO_3^- reductases and transporters, i.e. XOR, NOS1 and sialin, all of which increase following 21 d supplementation (Park et al. 2021). This likely causes augmented NO_3^- to NO_2^- reduction;

however upon the termination of these diets, there was continued reduction in muscle NO_3^- content (Park et al. 2021). It may be speculated that this may be due to sustained increases in NO_3^- - related reductase and transporter activity/ expression. Hereby, dietary interventions may lead to changes in these NO_3^- related reductases and transporters but this may only occur over sustained periods, with any changes being sustained for a time following the cessation of NO_3^- supplementation.

A novel finding from Park and colleagues (2021) was disproportionate storage of NO_3^- across muscle groups, likely due to the fibre type content of the muscle sample collected (Long et al. 2020). In this study, the NO_3^- and NO_2^- in the soleus muscles of rodents which is primarily comprised of type I muscle fibres (slow-twitch) contained 3.4- and 1.8- fold higher concentrations respectively than the vastus lateralis. This finding was substantiated by Park et al. (2021) who described a 1.4-fold increase in NO_3^- and 1.2-fold increase in NO_2^- in the soleus when compared to the extensor digitorum longus which is primarily comprise of type II muscle fibres (EDL). Interestingly, these findings are in contrast to the hypothesis that NO_3^- is of greater benefit for improving the performance of type II muscle fibres (Jones et al. 2016; Jones et al. 2018). The study of skeletal muscle NO_3^- and NO_2^- storage is in its infancy with the majority of the existing information base stemming from murine or rodent work meaning caution must be taken in the interpretation of findings.

2.6.2 Human Trials: The presence of NO_3^- in skeletal muscle has since been reported in humans (NO_3^-: 226 ± 213 nmol.g^{-1}, NO_2^-: 5.7 ± 7.4 nmol.g^{-1}) with this being significantly greater than plasma (NO_3^-: plasma 54 ± 27 nmol.g^{-1}, NO_2^-: 0.2 ± 0.2 nmol.g^{-1}) (Wylie et al. 2019). Skeletal muscle NO_3^- concentrations have also been quantified across both young and older age ranges (Young; 21 ± 1 yr, n = 10. Old; 75 ± 1 yr, n = 10) as well as in individuals with type II diabetes mellitus (Type II diabetes; 72 ± 1 yr, n = 17), showing that muscle was greater than plasma in all age groups and young individuals had elevated NO_3^- levels

compared to older counterparts under basal conditions (Nyakayiru et al. 2017). Similar to rodent models, the ingestion of inorganic NO_3^- induces profound increases in skeletal muscle NO_3^- content with an approximate 5-fold increases in skeletal muscle NO_3^- (226 ± 213 to 1139 ± 894 nmol.g^{-1}) and a 3-fold increase in muscle NO_2^- (5.7 ± 7.4 to 14.2 ± 21.4 nmol.g^{-1}, *P*>0.05) (Wylie et al. 2019). The elevation of skeletal muscle NO_3^- has been tracked over a 7-hour period in type II diabetes mellitus patients and it was found to be significantly increased for all time-points, with its peak occurring at 75 ± 22 minutes at a concentrations of 217 ± 24 nmol.g^{-1} (Nyakayiru et al. 2017). Synthesis of the information in this section emphasises that there is emerging evidence confirming the existence of NO_3^- and NO_2^- in skeletal muscle under basal condition in humans, and that this indeed can be elevated via the ingestion of dietary NO_3^-. However, with only two investigations focussing on this new topic in humans, there is little data concerning the rate- and duration- of change in NO-related metabolites in skeletal muscle tissue. Moreover, to date we do not understand how the fluxes in NO_3^- and NO_2^- in muscle may relate to other biological compartments within the body which is vital for interpreting potential implications for NO-related processes in the human body.

When coupled with information generated from rodent/ murine models, it is becoming evident that the maintenance of NO_3^- levels in muscle is a complex phenomenon in which there are still unknown factors, however it can be speculated that multiple processes are integrated in preserving the intracellular concentrations of NO_3^- in muscle. However, with the limited data from only two studies in humans, it remains difficult to draw conclusions regarding the influence of skeletal muscle NO_3^- and NO_2^- under basal conditions and following the ingestion of inorganic NO_3^- on physiological processes. The initial animal model studies that yielded novel information on the existence of NO_3^- retention in skeletal muscle paved the way for human experimental studies confirming the existence of a skeletal muscle NO_3^- reservoir which is receptive to change following the ingestion of dietary NO_3^-

(Nyakayiru et al. 2017; Wylie et al. 2019). The retention of NO_3^- in skeletal muscle tissue is of particular interest in humans as, following the early work in rodents, it has been postulated that skeletal muscle NO_3^- may be integral in modulating transient fluxes in NO bioavailability and therefore central in NO homeostasis (Piknova et al. 2016; Wylie et al. 2019; Park et al. 2019). This emerging evidence is providing new insights into factors which may contribute to the maintenance of NO bioavailability in humans. However, there is presently limited evidence confirming the role and relevance of skeletal muscle NO_3^- for this purpose in humans. Prior to being able to understand how the retention of NO_3^- in skeletal muscle influences certain outcome variables correlated to exercise- or health-related parameters, knowledge of the pharmacokinetic profile of NO_3^- in skeletal muscle and how this relates to fluxes in NO_3^- in other biological compartments is required. Moreover, the relative contribution of endogenous and exogenous NO_3^- and NO_2^- from skeletal muscle tissue on NO homeostasis remains unclear (Figure 3).

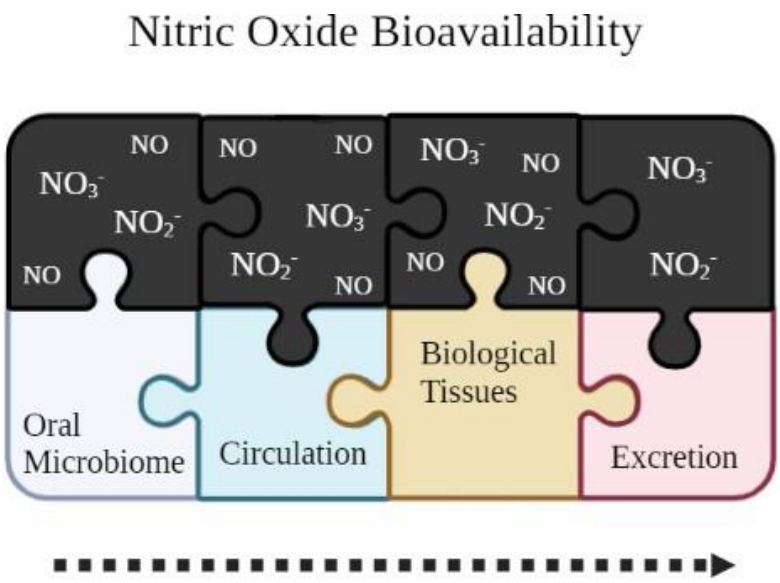

Figure 3: Are biological tissues the missing part of the NO bioavailability puzzle? *Historically, many of the associated effects of ingesting NO_3^- have been attributed to increased circulating NO_3^-. Emerging evidence suggests that biological tissues such as*

skeletal muscle may also be innately involved in the sustenance and maintenance of NO within the human body.

2.7 Novel Contributions of this Thesis

The literature review highlights the complex nature of NO metabolism and the numerous factors which contribute to the maintenance of NO bioavailability in humans. When examining the current literature, there are two largely under examined factors which are likely to contribute significantly and be central to NO homeostasis in humans: the oral microbiome and the retention of NO_3^- in skeletal muscle tissue (Jones et al. 2020).

CHAPTER 3: AIMS AND OBJECTIVES

The aims of this work were three-fold and all tie into the optimisation of NO_3^- supplementation and the effect this may have on NO bioavailability by measuring fluxes in NO-related metabolites in varying biological tissue and fluids in the human body. Firstly, we aimed to address whether the application of a NO_3^- containing lotion can provide a novel alternative for altering circulating NO bioavailability. Secondly, we aimed to explore the influence of variations in habitual dietary NO_3^- and how this may influence known contributory factors for NO bioavailability such as the oral microbiome. Finally, and the crux of this thesis was focussed on ascertaining the effects of dietary NO_3^- ingestion on the newly-discovered skeletal muscle NO_3^- and NO_2^- concentrations. An objective which was achieved through generating a pharmacokinetic profile of skeletal muscle in response to acute NO_3^- supplementation and then quantifying the relative contribution of exogenous vs. endogenous NO_3^- in skeletal muscle.

The following research objectives will be addressed:

1.) **To ascertain whether circulating [NO_3^-] and [NO_2^-] concentrations can be altered via transdermal application of a NO_3^- containing lotion.**
    - Does the transdermal application of a NO_3^- containing lotion influence circulating [NO_3^-] and [NO_2^-] in blood plasma?

2.) **To identify the influence of alterations in habitual NO_3^- ingestion on the oral microbiome**.
    - What are the effects of habitually ingesting varying quantities of NO_3^- on the oral and gut microbiome? Specifically, how does NO_3^- deprivation influence the oral microbiota?

3.) **To investigate the pharmacokinetic response of skeletal muscle [NO_3^-] and [NO_2^-] following the ingestion of an acute bolus of NO_3^-.**

- What is the influence of an acute bolus of potassium NO_3^- (~12.8 mmol, ~800 mg NO_3^-) on skeletal muscle NO_3^- and NO_2^- concentrations over the course of a 24-hour period?
- How do these compare with other biological compartments which are typically measured in dietary NO_3^- research, including saliva, blood and urine?

4.) **To determine the metabolic fate of 15N-labelled NO_3^- in young ostensibly healthy individuals.**

- What is the relative contribution of exogenously supplied NO_3^- compared to endogenously generated NO_3^- in skeletal muscle tissue?
- What is the metabolic fate of dietary NO_3^- in varying biological compartments including skeletal muscle tissue, blood, saliva and urine following the ingestion of a 15N- labelled NO_3^- tracer?

CHAPTER 4: GENERAL METHODS

Overview

Data collection for the experimental chapters in this thesis involved approximately 175 laboratory visits for participants that completed all trials. All study visits were performed in a temperature regulated laboratory with an ambient temperature of 18 to 22°C at the University of Exeter, St Luke's Campus. Prior to data collection, the University of Exeter, School of Sport and Health Sciences Research Ethics Committee approved all study procedures.

Health and Safety

The University of Exeter's Health and Safety procedure were adhered to, and great care was taken to ensure that informed consent, human tissue act (HTA) and risk assessment procedures were followed.

Participants

The participants that volunteered for the research studies in this thesis were recruited from the University of Exeter student and staff population, and the local community. Following provision of a written information document containing the protocols and procedures involved in the corresponding experimental chapters, informed consent was taken prior to the initiation of experimental test processes. All participants were ostensibly healthy, did not smoke, were free from cardiovascular or metabolic conditions, did not regularly use dietary supplements or aids, did not use antibacterial mouthwash, and were not on medication that would influence the findings of the investigations. For each experimental visit, participants arrived in a rested and fasted state. For experimental chapters 2, 3 and 4 participants arrived at the laboratory having followed an individualised diet that controlled for nitrate intake whilst ensuring that daily calorie intake was matched between visits and that the diet fulfilled the recommended macro- and micro-nutrient intake (See: *Dietary Manipulation* for further

details). Participants arrived for all visits well hydrated following a 24-hour abstinence from strenuous exercise and alcohol intake. In the experimental chapters that required repeated visits to the laboratory, participants arrived at the laboratory at the same time of day (± 1 hour).

Study procedures

The Control and Manipulation of Dietary NO_3^-

To enhance the validity and reliability of study observations, the adequate control and manipulation of dietary nitrate was of great importance for all experimental chapters (Experimental chapter 1 to 3. In each experimental chapter, each participant was firstly asked to record their habitual diet using a 4-day self-report food diary. The daily habitual NO_3^- intake of the diet was determined using a comprehensive NO_3^- database that provided the NO_3^- and NO_2^- concentrations in food and drink products from published investigations. The total daily calorie intake and the macro- and micro-nutrient composition of the diet was also determined as part of the database by using normative values for the varying food types. Subsequently, for experimental chapters 2-4, participants followed a dietary NO_3^- intervention for which NO_3^- intake was manipulated (either low-, 15 – 35 mg; normal-, ~180 mg, or high- ~1000 mg), whilst ensuring that the total daily calorie intake was not different to their habitual diet, and that the diet consisted of approximately 50% carbohydrates, 20% protein, 30% fat and the recommended daily intakes for micronutrients. During chapter 1, Participants were provided with a list of high NO_3^- containing foods and were instructed not to consume these in the 24 hours preceding the visit.

Venous Blood Collection and Sample Preparation

For Experimental Chapters 1, 3 and 4 venous blood samples were obtained from a vein in the antecubital fossa, via cannulation or venepuncture, for the quantification of [NO_3^-] and [NO_2^-]

in whole blood, red blood cells, and blood plasma. For these measurements, a tourniquet was firstly applied to the top of the arm at a site proximal to the antecubital fossa. Subsequently, a vein in the antecubital fossa was identified via palpation and the skin was disinfected using an alcohol swab (70% isopropyl alcohol).

Either a 20-gauge cannula (Insyte-W^{TM}; Becton-Dickinson, Madrid, Spain) or venepuncture needle (BD Safety-Lok Blood Collection Set; Becton-Dickson, UK) was inserted into the vein. Venous blood was drawn into a 6 mL vacutainer pre-treated with lithium-heparin (LH) (Becton Dickinson, NJ). The tourniquet was removed prior to the collection of all samples as occlusion during the collection of blood samples has been shown to influence plasma [NO_2^-] concentrations (Liddle et al. 2020). The blood sample was divided into separate aliquots for subsequent analyses. Whole blood samples were processed by extracting (800 µL per micro-centrifuge tube) from a vacutainer and combining with 200 µL of a pre-prepared NO_2^- preservation solution in micro-centrifuge tubes. These tubes were thoroughly vortexed (Piknova et al. 2016b) and immediately placed in liquid nitrogen. Simultaneously (within ~30 seconds of collection), whole blood samples were centrifuged at 3300 g and 4°C for 7 minutes (Adapted from Piknova et al. 2016b). Subsequently, plasma was extracted and aliquoted into micro-centrifuge tubes before being immediately frozen in liquid nitrogen. For the red blood cell samples, 900 µL was aliquoted and added to 100 µL of the NO_2^- preservation solution prior to being placed in liquid nitrogen. The NO_2^- preservation solution consisted of 890.9 mM potassium ferricyanide ($K_3Fe(CN)_6$), 118.13 mM N-Ethylmaleimide (NEM), NP-40 (octyl phenoxylpolyethoxylethanol) added in a 1:9 ratio (v/v, NP-40/solution), and 4.5 mL deionised water (dH2O) (Piknova et al. 2011).

Muscle Biopsy Procedure and Sampling

The percutaneous Bergström needle technique modified for manual vacuum (Bergström et al. 1975) was used to collect muscle tissue samples from participants. Trained and experienced members of staff performed this procedure. The initial process involved in this procedure was to shave a local area of the quadricep (vastus lateralis) with a disposable razor and to sterilise the skin using an iodine-based solution (Videne Antiseptic Solution, 10% w/w Cutaneous Solution, Iodinated Povidone). A local anaesthetic was then applied to the region through the injection of ~2-3 mL (Lidocaine, 2% Lidocaine Hydrochloride) using a syringe and needle. Once the local anaesthetic had taken effect, an incision spanning 4-5 mm was made through the skin, subcutaneous tissue (dermis and adipose), and fascia. The muscle biopsy needle configuration was inserted into the muscle via the incision, and while suction was applied a muscle sample was collected and immediately taken to a pre-prepared area which had been sterilised prior to the procedure. The muscle sample, typically between 20 and 200 mg, was removed from the needle and blotted on sterile non-woven swabs. This blotting process was performed as quickly and efficiently as possible (< 30 s) ensuring that as much blood was removed from the tissue as possible. Following blotting, the sample was immediately placed in liquid nitrogen to be snap frozen and subsequently transferred into a cryotube for storage in -80°C freezers until subsequent analysis. Each incision was usable for 2 muscle sampling periods before being sealed using 3 steri-strips and a waterproof plaster being applied to the site. Post biopsy treatment recommendations were provided verbally and a written document detailing all relevant information was given to participants. All muscle tissue samples that were collected over the course of the experimental chapters comprising this thesis were stored in accordance with the guidelines set by the Human Tissue Act (2004). Muscle biopsy samples were collected in experimental chapters 1 and 3.

Urinary Nitrate and Nitrite

Urinary [NO_3^-] and [NO_2^-] was measured in experimental chapters 1 and 3. Urine was collected in small containers (Kartell^{TM}; Milan, Italy) at selected collection periods. Males were asked to urinate into the toilet and then mid flow collect a sample in the collection container. In contrast, female participants were given a collection pan which was inserted into the toilet and urine was transferred from this to the collection container. The sample was taken immediately, aliquoted into separate micro-centrifuge tubes, and snap frozen in liquid nitrogen for subsequent analysis of [NO_3^-] and [NO_2^-].

Salivary Nitrate and Nitrite

Saliva sampling was undertaken in experimental chapters 1, 2 and 4. This entailed the collection of saliva at selected time-points in processes which required participants to stimulate saliva production (e.g., moving tongue around mouth, chewing on tongue, thinking of food, yawning) for 2 minutes and expelling the saliva into a 30 mL tube (Thermo Scientific^{TM} Sterilin^{TM}; Massachusetts, USA). Upon conclusion of the 2-minute period, the sample was taken from the participant, extracted into separate micro-centrifuge tubes and placed in liquid nitrogen until storage at -80°C.

Analysis: Nitrate and Nitrite Concentrations in Biological Samples

[NO_3^-] and [NO_2^-] was determined using ozone-based gas-phase chemiluminescence in a variety of biological sample types including muscle, blood, saliva and urine (Piknova et al. 2011; Piknova et al. 2016). Ozone-based chemiluminescence is widely regarded as the most accurate and sensitive technique available for measuring NO and its metabolites (Tsikas et al. 2005). The general concept of this technique focuses on the luminescent nature of specific chemical reactions, something that is true for NO metabolites which are cleaved from parent compounds or reduced back to radical form by utilising varying chemical reagents (Pinder et

al. 2008). Differing chemical reagents can be utilised to induce reductions of various NO metabolites (such as NO_3^-, NO_2^-, RSNO, N-nitrosamine (RNNO), and S-nitrosothiols) to NOx which is typically associated with either NO_3^- or NO_2^-. Determination of [NO_3^-] and [NO_2^-] in select biological samples such as plasma required the samples to be deproteinised through the addition of ethanol/ methanol, whereby the sample was combined with ice-cold ethanol/ methanol, vortexed, and subsequently centrifuged at 11,000 g for 5 minutes. Saliva and urine samples were diluted using deionised water but underwent the same vortex and centrifugation processes. The supernatant was then extracted and injected into the Sievers gas-phase chemiluminescence NO analyser (Sievers 280i Nitric Oxide Analyser, GE Analytical Instruments, Boulder, CO, USA). Muscle tissue preparation differed slightly from the aforementioned processes and was in line with Park and colleagues' (2021) publication. The analysis of muscle tissue consisted of the preliminary weighing (subject to analysis; typically ~25 to 50 mg) which was subsequently combined with a NO_2^- preservation solution ($K_3Fe(CN)_6$, *N*-ethylmaleimide, water, Nonidet P-40) and homogenised via bead homogenisation as defined previously (Wylie et al. 2019; Park et al. 2021b).

For the quantification of [NO_2^-] in the included biological samples, the supernatant of the samples were injected into a gas-sealed purge vessel which was heated to 35°C and contained 10 mL glacial acetic acid, 4 mL sodium iodide (NaI: 4% w/v) and 100 µL antifoam agent. Experimental chapter 2, 3 and 4 utilised this method for the analysis of plasma, saliva and urine NO_2^-. In experimental chapter 3 and 4 the tri-iodide method was utilised as this was consistent to that which is performed by our collaborators. The tri-iodide solution consisted of 301 mM potassium iodide (KI) together with 138 mM iodine (I_2) solution in water, which was then mixed with glacial acetic acid in 2:7 ratio (v/v solution/acid) on a magnetic stirrer for ~ 30 min until all crystals were dissolved. Alternatively, [NO_3^-] was quantified by injecting the supernatant of samples into a gas-tight purge vessel heated to 95°C containing

10 mL 0.1M vanadium chloride (VCl_3, 0.8 w/v) and 100 µL antifoam agent, which induces release and reduction of NO_3^-. In the processes involved in the determination of [NO_2^-] and [NO_3^-], an additional trap containing 15 mL sodium hydroxide (NaOH) was attached to the purge vessel and analyser to prevent acid vapours from entering the analyser.

A calibration curve was generated for both the NO_2^- and NO_3^- analyses by injecting varying quantities of a known concentration of either sodium nitrite ($NaNO_2^-$) or sodium nitrate ($NaNO_3^-$). The calibration curve concentrations spanned the concentrations that were measured for the respective ions. The generated calibration curves were used to calculate the concentration of the biological samples that were analysed, with a 'blank' sample also being injected which was subsequently subtracted to ensure the measured concentrations were indicative of that in the sample. Internal standards were periodically injected to ascertain the accuracy and reproducibility of the analysis.

EXPERIMENTAL CHAPTER 1: THE EFFECT OF A SKIN LOTION CONTAINING NITRATE ON PLASMA NITRITE AND NITRATE CONCENTRATIONS: A PILOT STUDY

ABSTRACT

Introduction: The efficacy of ingesting dietary nitrate (NO_3^-) to increase nitric oxide (NO) bioavailability and ultimately benefit sporting performance and health is well established. There is anecdotal evidence to suggest that the typical form of NO_3^- supplements, i.e. beetroot juice, are unpalatable and may cause gastrointestinal distress. Therefore, investigating novel alternatives which can provide similar effects as ingestion of NO_3^- is desirable.

Methods: We tested the effectiveness of a NO_3^- containing skin lotion in changing circulating NO_3^- and NO_2^- concentrations. In a randomized cross-over study, 10 healthy young males applied a NO_3^- lotion (NIT) containing 2.18 g $NaNO_3$ or a placebo lotion. Blood samples were collected from an intravenous cannula inserted in the antecubital fossa prior to the application of the lotion and then at 15 mins, 30 mins, 1 h, 2 h, 3 h and 4 h post. Plasma [NO_3^-] and nitrite ([NO_2^-]) was determined using ozone-based chemiluminescence.

Results: Neither plasma [NO_3^-] nor [NO_2^-] were significantly altered by the application of either NIT or PLA lotions.

Conclusion: For the first time, we demonstrate that dermal application of a NO_3^- containing lotion does not induce alterations in circulating [NO_3^-] and [NO_2^-].

INTRODUCTION

Nitric oxide (NO) is an omnipotent gaseous signalling molecule produced endogenously within the body via the conversion of the amino acid L-arginine to L-citrulline and NO (Moncada et al. 1993; Ignarro et al. 2002). The process of oxidation from L-arginine to these products is catalysed by Nitric Oxide Synthases (NOS) (Förstermann et al. 2006; Förstermann et al. 2012). In this endogenous generation of NO, the NO-related metabolites nitrate (NO_3^-) and nitrite (NO_2^-) are formed, both of which are substantially more stable than NO which rapidly oxidises in varying biological tissues and fluids throughout the body (Moncada et al. 1991; Rhodes et al. 1995).

The existence of a diverging, complimentary pathway the importance of which is exaggerated in certain instances (i.e. reduction in pH and oxygen tension) has since been discovered and is termed the NO_3^- - NO_2^- - NO pathway (Lundberg et al. 1994; Modin et al. 2001; Stuehr et al. 2004; Castello et al. 2006). Importantly, this alternative pathway enables the reduction of NO_3^- and NO_2^- to the form of NO and works synergistically (via potential crosstalk and interactions) with the NOS-dependent pathway (Lundberg et al. 2008; Lundberg et al. 2010; Carlström et al. 2015). The maintenance of NO levels within the body requires both pathways. Importantly, the generation of NO via the NO_3^- - NO_2^- - NO pathway can be exogenously augmented through the ingestion of NO_3^- and NO_2^- which are naturally formed in varying foods and drinks. This provides a vital avenue to facilitate NO bioavailability and bioactivity within the human body (DeMartino et al. 2019; Jones et al. 2020).

Research into dietary NO_3^- supplementation has gained popularity in the last two decades numerous reported positive influences on cardiovascular, metabolic and neurological function (Weitzberg et al. 1998; Zweier et al. 1999; Lundberg et al. 2018; McDonagh et al. 2019; Jones et al. 2020). Notably, these positive influences reflect alterations in the formation NO

via the reduction of the NO-related metabolites NO_3^- and NO_2^- (Jones et al. 2020). Although the positive effects of ingesting dietary NO_3^- on ergogenic and therapeutic outcomes are well established, anecdotal evidence suggests that some individuals are reluctant to consume NO_3^- containing supplements. NO_3^- supplements are typically in the form of beetroot juice but there are reports that these may be unpalatable or because these may cause gastrointestinal distress (Vitale et al. 2019). Therefore, attempts to induce equivalent alterations in NO-bioavailability via alterations in circulating NO_3^- and NO_2^- are a reasonable endeavour. It is possible to speculate that the skin may offer one such alternatives to the "typical" ingestion of NO_3 for increasing circulating NO-related metabolite fluxes.

The skin is the largest organ of the human body, with an extensive surface area which can account for approximately 10 to 15% body mass and is composed of three main layers; the epidermis, the dermis and subcutaneous tissue/ sub-dermal layers or hypodermis (Kanitakis, 2002; Kolarsick et al. 2011). Notably, NO-related metabolites including nitrosothiols, NO_3^- and NO_2^- are present throughout the varying compartments of human skin tissue, and in some instances, concentrations of these NO related metabolites are greater than in circulation (Weller et al. 1996; Paunel et al. 2005; Mowbray et al. 2009). Furthermore, bacteria present in the normal skin flora, coupled with the slightly acidic conditions present on the skin surface, facilitate the reduction of NO_3^- to NO_2^- and NO (Weller. 1997). The presence of NO_3^- and NO_2^- on the skin may be linked to the presence of NO on the skin and therefore involved in local NO metabolism, however it is not currently known whether this may translate to systemic alterations in NO and NO-related metabolite fluxes within the human body.

The relevance of these NO-related metabolites in the skin may in fact be of physiological importance as other biological tissues (i.e. skeletal muscle) throughout the human body are innately linked with maintaining systemic NO via the capacity to retain and "release" NO_3^-

and NO_2^- (Wylie et al. 2019; Piknova et al. 2022; Kadach et al. 2022). Furthermore, recent research in an animal model (sheep) has shown that following the infusion of a ^{15}N NO_3^- tracer, substantial quantities of up to 22.8% and 26.9% of intravenously infused NO_3^- and NO_2^- respectively is stored in skin tissue (these quantities of which exceeded those seen in other biological tissues and compartments) (Villar et al. 2021). Based on these findings, it is reasonable to speculate that, similar to skeletal muscle, skin tissue may also be linked to sustaining NO bioavailability. This is due to the intravenously infused ^{15}N NO_3^- and ^{15}N NO_2^- potentially being transported from circulation to skin which may, in turn, suggest that transient movement of these NO-related metabolites and may therefore modulate physiological processes (Liu et al. 2014; Muggeridge et al. 2015).

Although, as aforementioned the presence of NO-related metabolites have been identified in human skin, it is not currently known whether skin NO_3^- concentration can be manipulated in humans. The purpose of the present study, therefore, was to determine whether the transdermal application of a NO_3^- containing lotion may influence systemic NO_3^- concentrations ($[NO_3^-]$ where [ ] denote concentration). We hypothesised that plasma $[NO_3^-]$ would be elevated following the topical application of a NO_3^- - containing lotion, with a peak $[NO_3^-]$ achieved 1-3 h post application.

METHODS

Participants

Ten ostensibly healthy, recreationally active males (Age: 27 ± 2 years, Height: 1.79 ± 0.06 m, Mass: 79 ± 9 kg, BMI: 25 ± 2) volunteered to participate in this study. The participants provided written consent following explanation of the possible risks and benefits of taking part in the study. Participants were not taking any medication or nutritional supplements and did not suffer from or have a history of respiratory, cardiovascular, metabolic or

musculoskeletal disease. Additionally, individuals that participated were non-smokers and did not regularly use antibacterial mouthwash. Approval for all the procedures included in this investigation was granted by the Sport and Health Sciences Ethical Committee at the University of Exeter, and the study was conducted in accordance with the *Declaration of Helsinki*.

Experimental Design and Protocol

Participants visited the laboratory on two separate occasions. Participants were instructed to abstain from consuming NO_3^- rich foods and to replicate food intake on for the 24 h preceding each of the experimental visit. A list of NO_3^- rich foods was provided and participants were requested to consume the same evening meal on the day prior to the two experimental visits. Following an overnight fast, participants were required to attend the laboratory in a rested state and having removed hair from the lotion application site by shaving the area. Participants arrived at the laboratory at 07:30 am (± 2 hours) with the time of arrival consistent for each individual on both test days (Figure 1). Upon arrival, height and body mass measurements were taken using a stadiometer (Seca stadiometer SEC-225; Seca, Hamburg, Germany) and digital weighing scales (Seca digital column scale SEC-170; Seca, Hamburg, Germany). An intravenous cannula (Insyte-W; Becton-Dickinson, Franklin Lakes, NJ, USA) was inserted into a vein in the antecubital fossa while the participants were in a supine position. Following the collection of the baseline venous blood samples, 10 g of either nitrate containing lotion/ active (NIT) (20 g in total; 2.18 g NO_3^-) or placebo (PLA) (20 g; negligible NO_3^-) lotion was applied to each leg. Participants applied the lotion onto both legs (quadriceps) ensuring that maximal surface area was covered, and a timer was immediately started for subsequent blood sample collections. Venous blood samples were subsequently taken at 0.25, 0.5, 1-, 2-, 3- and 4-hours post-lotion application (Figure 1). Participants were then required to attend the laboratory on a second occasion in which the above processes

were replicated with the alternative lotion (i.e., NIT or PLA).

Placebo (PLA) and Nitrate containing lotion (NIT): 20 g (10 g per leg) of either PLA or NIT lotion was applied to the frontal portion of the thigh. Both lotions contained the same ingredients: water, isopropyl palmitate, lecithin, poloxamer 407, cetyl alcohol, propylene glycol, denatured alcohol, benzyl alcohol, polyglyceryl-4 laurate, sodium hydroxide, sodium lauryl sulfate, sorbic acid, fragrance. The NIT lotion varied to the PLA due to the addition of NO_3^- in the form of $NaNO_3^-$ in the amount of 0.109 g per g of lotion, this equated to an approximate serving of 2.18 g NO_3^- being administered in this study.

Sample Collection and Processing: 7 venous blood samples were collected during the experimental visits (see Figure 1). Blood samples were drawn into lithium-heparin vacutainers (7.5 mL, Becton-Dickinson) and immediately underwent centrifugation at 3300 g for 7 minutes at 4°C.The plasma supernatant was extracted and aliquoted into 1.5 mL microcentrifuge tubes and then placed in liquid nitrogen to be snap frozen, prior to being stored at -80°C in preparation for subsequent quantification of NO_3^- and NO_2^- concentrations.

Measurements

Plasma Nitrate and Nitrite: Quantification of NO_3^- and NO_2^- was performed using ozone-based gas-phase chemiluminescence (Piknova et al. 2016). Samples were defrosted in batches to reduce the potential alterations in NO_2^- and NO_3^- that may occur with time. Following an initial thawing period, samples were deproteinised with cold ethanol and centrifuged for 10 minutes at 10,000 g at 4°C. The supernatant was immediately collected and injected into the nitric oxide analyser (NOA, Sievers, Model 280 NO analyzer, Boulder, CO), which used nitrogen as the carrier gas for determination of NO. Determination of NO_2^- was performed using acetic acid and sodium iodide, whereas NO_3^-analysis entailed a vanadium(III) chloride (VCl_3) chemiluminescence assay.

Statistical Analysis: Two-way repeated measures ANOVAs were performed to determine

differences between plasma NO_3^- and NO_2^- between conditions and across time. Statistical significance was accepted at $P \leq 0.05$. All data was expressed as mean ± standard deviation. Statistical analyses were performed using the IBM Statistical Package for Social Scientists (SPSS v.28, SPSS Inc., Chicago, IL, USA).

RESULTS

Plasma Nitrate: At baseline, there was no difference in plasma [NO_3^-] between conditions (52.4 ± 31.4 nmol.g^{-1} vs. 47.6 ± 25.6 nmol.g^{-1}; $P > 0.05$). No changes were observed in [NO_3^-] following the application of the NIT lotion at any time-point ($P > 0.05$), furthermore no changes were observed following the application of the PLA lotion in [NO_3^-] ($P > 0.05$). There was no difference between the NIT and PLA condition over the varying time-points ($P > 0.05$). (**Figure 2A**; $P > 0.05$).

Plasma Nitrite: Plasma [NO_2^-] were not different at prior to the application of either NIT or PLA lotion ($P > 0.05$). Additionally, plasma [NO_2^-] remained unchanged between conditions ($P > 0.05$). There was a reduction in plasma [NO_2^-] over the course of the visit in both groups (**Figure 2B**; $P < 0.05$).

DISCUSSION

Dietary NO_3^- has long been used for purposes of enhancing indices of health and exercise performance through increasing NO bioavailability. To our knowledge, this is the first study to examine the effects of a NO_3^- containing lotion and whether this can be an efficacious alternative for altering circulating levels of NO_3^- and NO_2^- as opposed to the conventional ingestion of NO_3. This would provide an important insight into whether NO_3^- and NO_2^- can be altered in the absence of the enterosalivary pathway which is central in NO metabolism (Govoni et al. 2008; Kapil et al. 2013). There is sparse data relating to the potential role of

the skin in NO_3^- metabolism and storage, however data available provides promising evidence. Therefore we hypothesised that the transdermal application of a NO_3^- containing lotion would elevate plasma NO_3^- and NO_2^-. In contrast to the original hypothesis, the present study demonstrates that a NO_3^- containing transdermal lotion does not alter resting plasma [NO_3^-] and [NO_2^-] over a 4-hour period. Therefore, the percutaneous application of NO_3^- via a lotion does not necessarily lead to alterations in circulating NO_3^- and NO_2^- and therefore NO bioavailability under the specific conditions of the present study.

Human skin plays a complex, multifactorial role in physiological processes whereby its complex multi-layered composition provides an integral protective barrier against foreign materials such as chemicals and microbes while also preventing the excessive loss of moisture and endogenous products (Kanitakis, 2002; Segre, 2006). However, it can also be pertinent method of delivering specific drugs and solutes into systemic circulation via topical administration (Brown et al. 2006; Singh et al. 2011). The skin is an organ composed of multiple layers and structures, but these layers and structures do not necessarily comprise function *per se.* It is understood that these multiple layers and structures may be associated with a diverse microbiome of commensal, symbiotic and pathogenic bacteria that contribute to both health and disease (Roth et al. 1988; Grice et al. 2008; Byrd et al. 2018). Importantly, human skin contains NO related metabolites such as NO_3^- and NO_2^- (and nitrosothiols) at greater concentrations than those seen in circulation (Weller et al. 1996). In the skin, these NO-isoforms alter exposure to UVA irradiation and sunlight and are linked with the promotion of the non-enzymatic formation of NO (Paunel et al. 2005; Mowbray et al. 2009). The presence of NO metabolites associated NO generation in the skin has been suggested to be integrated in processes involved in inflammation, antimicrobial defence and wound healing (Carls-Grierson et al. 2004). The presence of these NO-related metabolites may suggest a local requirement for NO. Therefore, the existence of a potential active transport or

reduction chain of NO_3^- and NO_2^- may enable the movement of NO metabolites from skin to circulation and *vice versa*. The present investigation provides no evidence to support the transportation of NO_3^- (and NO_2^-) from the lotion to plasma. However, whether there was a greater availability of NO_3^- and NO_2^- due to enzymatic and bacterial reduction to NO in skin is yet to be investigated. A factor which may be pertinent to how the skin is involved in biological processes.

NO can be directly generated via the metabolism of bacteria present on the skin surface (Weller et al. 1996). The skin microbiome includes commensal bacteria which possess NO_3^- reductase properties including *S.epidemidis* and *Staphylococcis aureus* as well as bacteria that can further reduce NO_2^- to NO such as *Nitrosomonas spp*. (Weller et al. 1996). The cell-specific isoforms of NOS enzymes are present throughout the human body; however, there are profound differences in the expression, distribution, regulation and function of isoforms (Förstermann et al, 1994; Griffith and Stuehr, 1995). Notably, these NOS isoforms are expressed throughout the various constituents of the skin (Bruch-Gerharz et al. 1998; Cals-Grierson et al. 2004). It may be speculated that the generation of NO to varying degrees may be linked with the skin's unique adaptability that through varying distinct expression patterns and mechanisms of regulation (Bruch-Gerharz et al. 1998; Cals-Grierson et al. 2004). The nature of the present study did not allow us to determine whether the NO_3^- and NO_2^- delivered by the transdermal lotion impacted local NO metabolism on the skin surface or throughout its varying layers via bacterial reduction. A finding which could translate to meaningful consequences on the aforementioned role that the skin plays in human health.

Exposure to UVA irradiation has been shown to influence blood pressure via vasodilation and this is likely due to the release of NO from storage sites facilitated by the more stable isoforms NO_3^- and NO_2^- located in the epidermis (Liu et al. 2014). When UVA irradiation is coupled with the ingestion of NO_3^-, it has been demonstrated to augment cycle time-trial

performance (Muggeridge et al. 2015). Interestingly, this change was only evident when UVA irradiation and NO_3^- was combined, with no changes occurring when these two conditions (UVA irradiation and NO_3^-) were isolated (Muggeridge et al. 2015). This is a likely consequence of photodecomposition which occurs at the skin meaning that (RSNO and) NO_2^- can be reduced to form NO at the skin (Paunel et al. 2005; Liddle et al. 2022). When exposed to UVA, greater quantities of NO_3^- and NO_2^- are released into circulation from the skin (Liddle et al. 2022). This suggests that UVA may act as a catalyst *per se*, which when coupled with alterations in local oxygen tension or acidity (typically seen during exercise), may be required to mobilise the NO related ions from skin tissue. For the entirety of the experimental visits, participants rested indoors (i.e. without sunlight exposure). This may have led to limited reduction or transport of NO_3^- or NO_2^- present on the skin following application of the lotion as the above illustrates the potential for a catalyst to facilitate this. However, it should be noted that in this pilot study our intention was to explore the effectiveness of a NO_3^- containing skin lotion on altering circulating NO-metabolite concentrations. Therefore investigating whether certain circumstances stimulate NO (or NO_3^- / NO_2^- mobilisation) in skin (i.e. sunlight exposure or exercise) and thereby altering plasma NO_3^- and NO_2^- was outside the scope of the present study.

The site of lotion application may also be a factor influencing the efficacy of NO_3^- lotion application. The uniformity of expression and localisation of NO_3^-, NO_2^- and RSNO is not currently fully known in the context of varying biological tissues and organs in humans, by this we do not understand whether these may vary. There is likely regional variation in percutaneous penetration rates in humans with locations including the scrotum, forehead and intertriginous axilla (armpit) having the greatest absorption rates (Maibach at el. 1971). If similar to how localisation and "storage" of NO_3^- alters between muscles, i.e. certain muscles display higher NO_3^- (soleus, extensor digitorum longus and tibialis anterior) influxes than

others (gluteus and gastrocnemius) (Long et al. 2020; Park et al. 2021), the site of lotion application may be an important factor when determining its effectiveness.

At present, we cannot discount the possibility that the lotion may in fact have increased local NO_3^- in skin similar to that seen via the infusion of NO_3^- by Villar and colleagues (2021) however this may not have entered circulation. The reduction of NO_3^- and NO_2^- throughout biological systems has been attributed to reductases associated with NO_3^- metabolism such as sialin, xanthine oxidoreductase (XOR), aldehyde oxidase (AO), nitric oxide synthases (eNOS and iNOS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Notably, these proteins are present in the skin (Shimizu et al. 1997; Miyamoto et al. 2011), skeletal muscle (Wylie et al. 2019; Park et al. 2019) and organs (incl. liver and eye) (Piknova et al. 2015; Park et al. 2020). It is possible to speculated that due to the presence of these reductases in the skin, the NO_3^- delivered via the transdermal application of NO_3^- may have led to local increases in NO_3^- and/or NO_2^- reduction and therefore may elicit augmented NO bioavailability without entering the circulation. Furthermore, the sialic acid / H^+ cotransporter, sialin, has been described as functioning as a unique transporter of NO_3^- through its role in the uptake of NO_3^- from salivary glands and at the plasma membrane (Qin et al. 2012). It is notable that the expression of sialin has also been identified in a numerous biological tissues including the central nervous system (Morse et al. 2005), lysosomes and cytoplasmic membranes in salivary samples (Jin et al. 2013), muscle tissue (Park et al. 2019; Wylie et al. 2019) and skin fibroblasts (Qin et al. 2012). However, although expressed within these tissues and organs, we currently do not know whether the activity of sialin would facilitate the movement of NO_3^- and/ or NO_2^- across varying biological compartments such as from the skin to circulation. Therefore, without a substantial concentration gradient which would enable the passive diffusion of NO_3^- and/ or NO_2^- the rationale for using a NO_3^- containing lotion becomes flawed.

Experimental Considerations

A limitation of this investigation is the absence of strictly controlled dietary NO_3^- and NO_2^- intake prior to the experimental visits; rather, we advised participants to abstain from ingesting high NO_3^- and NO_2^- food sources and to consume similar foods prior to each experimental visit. It is however, worth noting that although an experimental consideration, plasma [NO_3^-] and [NO_2^-] were similar at baseline. The effect of time on plasma [NO_2^-] that was present in this study may be expected due to the duration of time that participants were fasting during the study prior to and during the experimental visits. This suggestion is supported by the findings of a previous investigation which showed that, depending on the dose of NO_3^- ingested, NO_2^- levels can remain elevated for up to 24 hours post supplement ingestion (Wylie et al. 2013; James et al. 2015).

Conclusion

The primary finding of the present investigation is that the transdermal application of a NO_3^- containing lotion did not alter basal circulating [NO_3^-] or [NO_2^-] over a 4-hour period. Therefore, the application of a transdermal lotion may not be a viable method for inducing changes in systemic NO bioavailability in the specific and controlled circumstances which constituted the experimental methods in this study. Further research is warranted to determine whether the use of this NO_3^- containing lotion would be efficacious when combined with a stimulus such as UV irradiation or exercise.

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Appendix: Figure Captions

Figure 1: Schematic representation of the experimental visit and timings of the blood samples. '↓' illustrate the venous blood sample collection from the cannula inserted into the antecubital fossa throughout the course of the visit.

Figure 2: A: Plasma nitrate concentration prior to and following the application of a nitrate containing lotion (NIT) or placebo lotion (PLA). **B:** Plasma nitrite concentrations in the diverging conditions over the course of a 4-hour period following the application of the transdermal lotions. Closed squares (■) with a solid line indicate the NIT condition and open squares with a dashed line represents the PLA condition (□) at the varying time-points. '#' illustrates a significant main effect by time in plasma nitrite concentrations.

Figures 1:

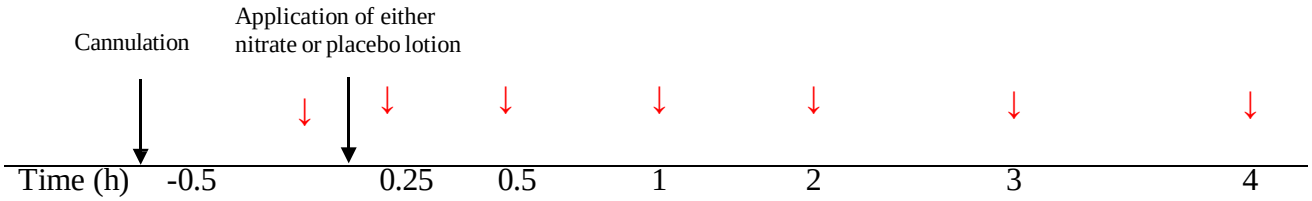
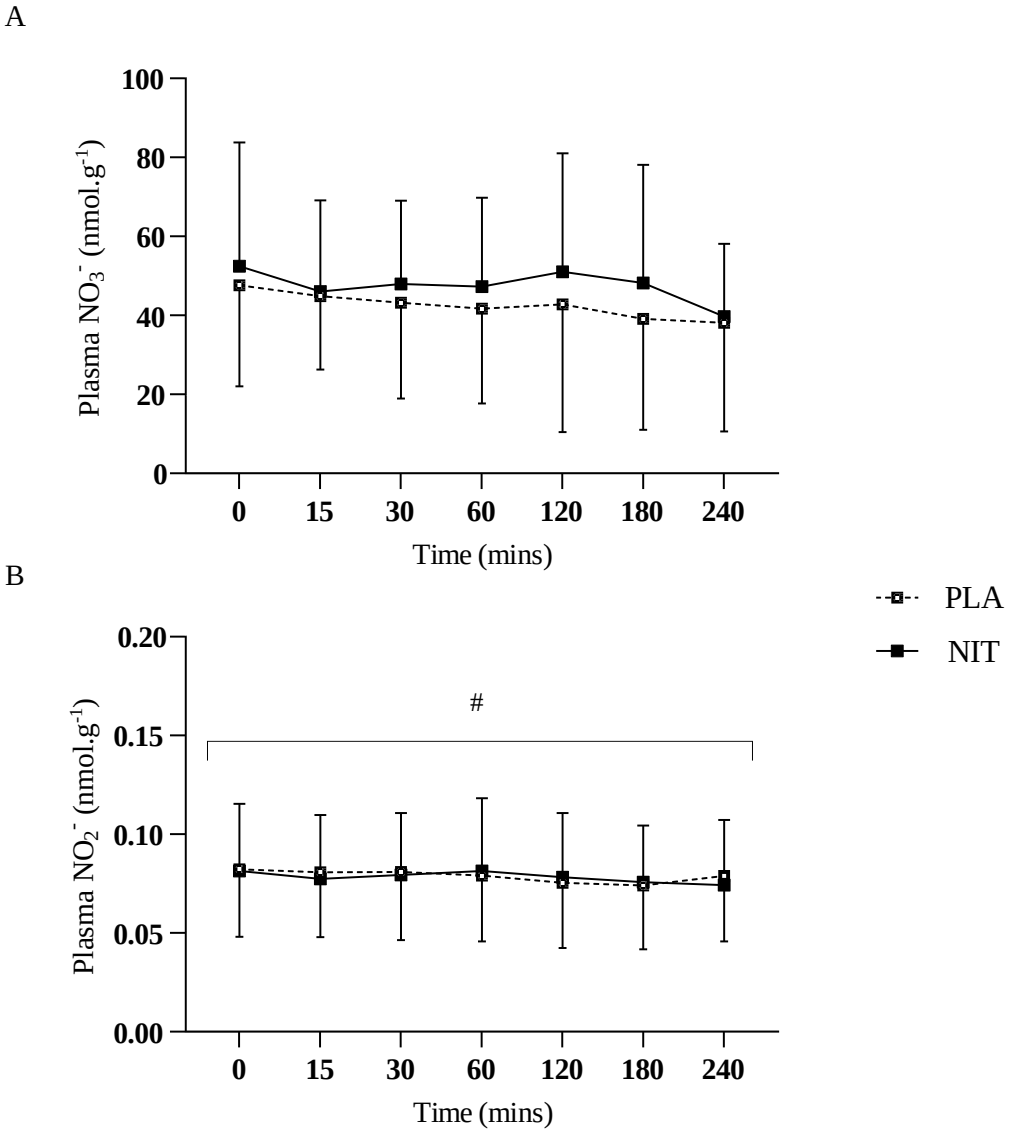


Figure 2:



EXPERIMENTAL CHAPTER 2: THE INFLUENCE OF DIETARY NITRATE INGESTION AND DEPRIVATION ON SALIVARY NITRATE AND NITRITE CONCENTRATIONS AND THE ORAL MICROBIOME

ABSTRACT

Purpose: The purpose of this study was to examine to the effect of NO_3^- deprivation on salivary NO-related biomarkers (NO_3^- and NO_2^-) and the relative abundance of microbial communities in the mouth.

Methods: In a randomized, cross-over design, 11 healthy individuals completed two dietary conditions. Both conditions consisted of an initial 3-day standard (STD) diet containing 180 mg NO_3^- and a final 3-day high (HIGH) NO_3^- diet containing approximately 1000 mg NO_3^-. These diets were interspersed with either a 7-day NO_3^- deprived diet containing 30 – 50 mg NO_3^- (LOW) or a 7-day STD diet. Saliva samples were collected and salivary [NO_3^-] and [NO_2^-] was measured using ozone based chemiluminescence. Exploratory analysis of the oral microbiome was performed on a sub-set of individuals using oral swabs and sequenced using 16S rRNA gene analysis to determine whether the microbial profile of the mouth changed following NO_3^- deprivation.

Results: No difference was observed in NO_3^- or NO_2^- during the 3-day STD diet periods or 3-day HIGH diets between conditions. Following the ingestion of a 7-day NO_3^- depleted diet (LOW), both saliva [NO_3^-] and [NO_2^-] was reduced compared to a 7-day STD diet. Exploratory analyses of the oral microbiome demonstrate differences in Candidatus Saccharibacteria (phyla) and Genus Candidatus Nanosynbacter (genus) and Candidatus Nanosynbacter Lyticus (species) during 7-day NO_3^- compared to a 7-day STD NO_3^- diet.

Conclusion: NO_3^- deprivation has profound effects on salivary NO_3^- and NO_2^-, with concentrations being significantly lower than those seen following the ingestion of a standard or high NO_3^- containing diet. Exploratory analyses indicate that certain bacterial phyla and

genera which constitute the oral microbiome may change during instances of NO_3^- deprivation.

INTRODUCTION

Inorganic nitrate (NO_3^-) and nitrite (NO_2^-) are naturally occurring anions which constitute a large variety of diets and are found in numerous foods types including root and leafy green vegetables (Ysart et al. 1999; Lundberg et al. 2009). Beneficial effects of dietary NO_3^- ingestion span a variety of physiological and health related parameters and are a consequence of increases in Nitric Oxide (NO) bioavailability and bioactivity (Lundberg et al. 2015; Jones et al. 2020). The NO_3^- - NO_2^- - NO pathway entails the *in vivo* recycling and reduction of NO_3^- and NO_2^- to ultimately form NO (Lundberg et al. 1994; Weitzberg et al. 1998; Zweier et al. 1999). Importantly, the reduction and subsequent uptake of NO_3^- and NO_2^- from dietary sources is modulated by the oral microbiome, which is central in the maintenance of NO bioavailability and ultimately cardiovascular health (Kapil et al. 2013; Bondono et al. 2015; Blekkenhorst et al. 2018). The initial reduction of NO_3^- to NO_2^- following the ingestion of NO_3^- containing foods occurs within the oral cavity in processes facilitated by facultative or obligate bacteria, both types of which have NO_3^- reductase capabilities (Li et al. 1997; Lundberg et al. 2004; Doel et al. 2005; Hyde et al. 2014). Importantly, NO_2^- can be generated or depleted via three bacterial NO_3^- – NO_2^- reduction pathways. In these pathways, certain bacteria are involved in the reduction of NO_3^- to NO_2^- in a process called Respiratory Denitrification (RD) and the generation of NO_2^- from NO_3^- in Assimilatory / Dissimilatory NO_3^- Reduction to Ammonia (ANR / DNRA) (Koch et al. 2015; Sparacino-Watkins et al. 2014; Goh et al. 2022). However, it should be noted that not all these pathways result in the increase of NO bioavailability as the NO_2^- can also form ammonia (Goh et al. 2022). When the bacterium in the mouth is eradicated through the use of antibacterial mouthwash, the expected increases in plasma and salivary NO_2^- concentrations, as well as the associated decreases in blood pressure, are abolished (Govoni et al. 2008; Kapil et al. 2013; McDonagh et al. 2015). This demonstrates the importance for the conservation of oral bacterial

communities. Indeed, Burleigh et al. (2018) found that changes in salivary NO_2^-, an index of NO_3^- reduction in the mouth, was attenuated following NO_3^- ingestion in individuals with fewer NO_3^- reducing bacteria. The oral microbiome is extremely adaptive with the delicate symbiotic relationship between host and microbiota being influenced by factors such as age, gender and diet (Kapil et al. 2018; Sato-Suzuki et al. 2021; Ahmed et al. 2021; Rosier et al. 2022). Furthermore, the malleability of the microbiome in response to the addition of NO_3^- has also been confirmed by Vanhatalo and colleagues (2018), who used the NO_3^- supplementation to induce a shift to a more preferential NO_2^- generating microbial profile. This was achieved through prolonged ingestion of NO_3^-, and led to increases in the relative abundance of Rothia and Neisseria and decreasing Prevotella and Veillonella when compared to a placebo condition. However, it is not known whether the oral microbiota are altered by a low NO_3^- ingestion, and how this might influence NO bioavailability.

The aim of this study was therefore, to investigate how consuming diets characterized by standard (STD), NO_3^- deprivation (LOW) and high NO_3^- (HIGH) quantities influence salivary NO_3^- and NO_2^- concentrations. Furthermore, due to the lack of information regarding the modulation of the host to microbiome relationship and how the removal of NO_3^- from the diet may influence this, we aim to perform exploratory analyses on the microbiota of the mouth to assess whether these change with altered NO_3^- ingestion. We hypothesize that NO_3^- deprivation will decrease the relative abundances of Rothia and Neisseria and increase the relative abundances of Prevotella and Veillonella.

METHODS

Ethical Approval: Authorisation to conduct this study was granted by the University of Exeter Sport and Health Sciences Ethics Committee and the study protocol was in accordance with the Declaration of Helsinki and World Medical Association. Following the provision of a comprehensive information document and oral explanation detailing the study and experimental processes included, prospective subjects signed an informed consent document and were enrolled into the study.

Participants: Eleven (10 males, mean ± SD: age 22 ± 2 y; body mass 74.2 ± 8.7 kg; height 1.77 ± 0.07 m; 1 female: age 21 y; body mass 80.5 kg; height 1.69 m) ostensibly healthy individuals volunteered to participate in this study. Inclusion criteria specified that participants were absent of having a history of medical conditions, were not tobacco smokers, use mouth wash or consume dietary supplements.

Experimental Design: Participants were enrolled in the study for a minimum of 37 days in which they were exposed to 2 conditions, each containing 3 diets. Condition 1 (DEP) was STD, LOW, HIGH and condition 2 (NORM) was STD, STD, HIGH. All food ingested by participants was provided. The study was a repeated measures, cross-over design meaning that participants were randomly allocated to either the DEP or NORM condition and following a wash-out period, completed the second condition meaning that all participants completed both conditions. A 3-day 'STD' NO_3^- diet containing ~180 mg NO_3^- was ingested as the initial period in both conditions and acted as baseline measurement periods. These periods were followed by either an identical 'STD' diet in the NORM condition or a 'LOW' diet in the DEP condition which contained approximately 30 mg NO_3^- per day. Finally, both conditions contained a 'HIGH' NO_3^- diet in which participants consumed ~1000 mg nitrate per day. A minimum of 10 days separated the two conditions as a wash-out period (Figure 1).

Saliva Sample Collection: Saliva samples were collected for determination of [NO_3^-] and

[NO_2^-]. To do this, participants actively stimulating saliva production for a 2-minute period and then dispensed the accumulated saliva into a collection (30 mL universal) tube. These samples were aliquoted into separate micro centrifuge tubes and immediately snap frozen in liquid nitrogen prior to being stored at -80°C in preparation of analysis.

Quantification of Nitrate and Nitrite Concentrations: Ozone-based gas-phase chemiluminescence was utilised to determine nitrate and nitrite concentrations (Pinder et al. 2008; Piknova et al 2011) in the saliva samples. These samples were centrifuged at 11,000g for 5 minutes and then diluted with deionised water prior to being injected into a Sievers gas-phase chemiluminescence NO analyser (Sievers 280i Nitric Oxide Analyser, GE Analytical Instruments, Boulder, CO, USA) for determination of [NO_3^-] and [NO_2^-].

Oral Bacterial Sequencing: Oral commensal bacteria samples were collected at the beginning of the experimental visits by swabbing the tongue dorsum, subsequently placed in eppendorfs containing 600 μL cell lysis solution (Qiagen, Germantown, MD) and then placed into a -80°C freezer until analyses was performed. 16S rRNA genes were sequenced to identify bacteria present in the oral microbiome in an identical manner to those described in Vanahatalo et al. (2018). Genomic DNA was isolated from tongue swabs using Gentra Puregene Buccal Cell Kit (Qiagen, Germantown, MD). The concentration of double-stranded DNA was subsequently fluorometrically determined (Qubit 3.0 high-sensitivity fluorescence detection, ThermoFisher Scientific, Waltham, MA). NEXTflex 16 S V1-V3 Amplicon-Seq Kit (Bioo Scientific, Austin, USA) was used as library preparation. The 16S V1-V3 rDNA region was subsequently amplified with 5 ng of dsDNA and exposed to 8 varying thermal cycles (30s at 98°C, 30s at 60°C, and 30s at 72°C) with primers A and B. Identification of individual samples occurred by PCR with indexing primers containing Illumina flow cell binding sites and preceded a AMPure® XP bead cleanup (Becton Dickinson, Franklin Lakes, NJ). Using v3 MiSeq reagents, samples were sequenced using

paired-end 300 base pair MiSeq Illumina platform (Illumina, San Diego, CA).The nucleotide sequence data for each sample was then trimmed in FASTQ format using Trim-Galore! (Krueger F. Trim-Galore!, accessible at http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Low-quality cases from the 3' read ends are removed for quality trimming purposes. Following this, adapter sequences were removed from the 3' end (the first 13 base pairs). Once the trimming was completed Trim-Galore! paired-end validation eliminated short sequences. The abundance and taxonomy of bacteria was subsequently assigned using Kraken standard build which utilises the genomes in Refseq and NCBI information (Kraken manual, accessible at: http://ccb.jhu.edu/ software/kraken/MANUAL.html#kraken-databases). Finally, classification and processing of the paired read sequences occurred by the Kraken Taxonomic Sequence Classification System (Wood et al. 2014).

Statistical Analysis: The statistical analyses were performed using The Statistical Package for Social Scientists (SPSS Version 28, SPSS Inc., Chicago, IL, USA). Two-way repeated measures ANOVAs were used to determine differences in saliva [NO_3^-] and [NO_2^-] between and within conditions using all the time-points (NORM: STD, STD, HIGH / DEP: STD, LOW, HIGH). In the instance of a main effects and interaction effects, data was further analysed using the least significant difference (LSD) post hoc test. Significance was accepted at an alpha level of $P < 0.05$. Saliva [NO_3^-] and [NO_2^-] (n = 11) was expressed as mean ± std deviation (SD). Paired samples t-tests were performed to analyse differences in phyla, genus and species of bacteria in the mouth. A series of exploratory analyses were performed using paired samples t-tests to identify differences in the relative abundance of OTUs at a bacterial phyla, genus and species level.

RESULTS

Saliva NO_3^- (Figure 2A): There was an effect of time on saliva [NO_3^-] meaning that there was a difference between the 3-day STD, 7-day DEP / STD and 3-day HIGH diets ($P < 0.001$). There was no difference between the DEP and NORM conditions ($P > 0.05$, $P = 0.140$). However, there was no interaction effect between time and condition was observed ($P > 0.05$, $P = 0.143$). There was no difference between condition in saliva [NO_3^-] at either baseline (following the initial 3-day STD diets; ($P = 0.130$)) or following the 3-day HIGH diets ($P > 0.05$, $P = 0.338$). Saliva [NO_3^-] was lower in the DEP condition following the 7-day LOW diet compared to the 7-day STD diet in the NORM condition ($P < 0.001$). In the DEP condition, saliva [NO_3^-] was lower in both STD and LOW than HIGH ($P < 0.001$), with LOW also lowering saliva [NO_3^-] compared to the STD diet ($P < 0.001$). In the NORM condition, no difference in saliva [NO_3^-] was evident between the 3-day and 7-day STD diets ($P > 0.05$, $P = 0.651$) but both STD diets led to decreased saliva [NO_3^-] when compared to HIGH ($P < 0.001$).

Saliva NO_2^- (Figure 2B): There was an effect of diet on saliva [NO_2^-] meaning that there was a difference between the 3-day STD, 7-day DEP / STD and 3-day HIGH diets ($P < 0.001$). There was however, no difference in saliva [NO_2^-] between DEP and NORM ($P = 0.314$). However, there was no interaction effect between time and condition was observed ($P > 0.05$, $P = 0.105$). There was no difference between the DEP and NORM conditions at following the initial 3-day STD or 3-day HIGH dietary periods ($P = 0.819$ and $P = 0.380$, respectively). Saliva [NO_2^-] was lower following 7-day LOW diet in the DEP condition compared to the 7-day STD diet in the NORM condition ($P < 0.001$). In the DEP condition, saliva [NO_2^-] was higher following the initial 3-day STD diet compared to the 7-day LOW ($P = 0.005$) but lower than the HIGH diet ($P = 0.010$). The 7-day LOW diet was also lower than the 3-day HIGH diet ($P = 0.002$). In the NORM condition, there was no difference between the 3-day

STD diet and the 7-day STD diet (P = 0.483). Both the 3-day and 7-day STD diets were lower than the HIGH diet (P = 0.004 and P = 0.012, respectively).

Exploratory Analysis: Oral Microbiome (Figure 3 and 4)

Between condition: There was no difference in the relative abundance of OTUs at a phylum, genus or species level between DEP and NORM following the initial 3-day STD diet ingestion (P > 0.05). Candidatus Saccharibacteria was lower following the 7-day LOW diet in the DEP condition when compared to the 7-day STD diet in NORM (P = 0.034, n = 5), and also Proteobacteria tended to be lower in the DEP condition following the 7-day diet (P = 0.53, n = 5). At a genus level, Candidatus Nanosynbacter was lower following the 7-day intervention in the DEP arm compared to NORM (P = 0.036, n = 5). At a species level, the relative abundance of OTUs of Candidatus Nanosynbacter Lyticus was lower following the 7-day DEP diet compared to the STD diet in the NORM condition (P = 0.036, n = 5).

DISCUSSION

When habitual NO_3^- ingestion is increased through the addition of NO_3^- supplements (concentrated beetroot juice), significant alterations are noted in the oral microbiome (Vanhatalo et al. 2018; González-Soltero et al. 2020). The relevance of which is described by Vanhatalo and colleagues (2018), who illustrate that manipulating the NO_2^- producing oral microbiota which was a consequence of increased dietary NO_3^-, may in fact lead to meaningful implications on circulating NO_2^- (in plasma) and therefore impact indices of NO homeostasis and vascular health. This highlights that the integrity of the symbiotic relationship between the host and microbiota is imperative in, not only the sustenance of NO metabolism, but also illustrates the importance of the presence of NO_3^- in the diet. Importantly, the maintenance of this symbiotic relationship is ultimately achieved and upheld by the malleability of the oral microbiome. The current paper provides novel insights into

changes in the oral microbiome following the removal of NO_3^- from the diet. We demonstrate that the removal of NO_3^- from the diet decreases saliva [NO_3^-] and [NO_2^-]. We also confirm the malleability of the oral microbiome by demonstrating that the removal of NO_3^- from the diet leads to significant change in certain bacterial phyla and genera also. The findings of this experimental chapter are in line with Rosier and colleagues (2020), who concluded that the introduction of NO_3^- to oral communities leads to a rapid and functional alteration in the bacterial profiles (*in vitro*) which may be of benefit to human health. NO_3^- intake is therefore an important determinant of the composition and function of the oral microbiome and its associated role in health-related outcomes.

Alterations in saliva [NO_3^-] and [NO_2^-]

The presence and ingestion of NO_3^- within food substances provides an important opportunity for the human body to complement an endogenous NO generating pathway and therefore is considered a therapeutic and ergogenic aid (McDonagh et al. 2019). The environment exclusive to the oral cavity and thereby symbiotic relationship between host and bacteria represents an integral link facilitating the reduction of NO_3^- from dietary sources to NO_2^- which ultimately influences NO fluxes within the human body (Jones et al. 2020). Once ingested, NO_3^- is rapidly absorbed into circulation from the stomach and small intestine, and is subsequently concentrated by the salivary glands leading to substantial increases of NO-related anions (NO_3^- and NO_2^-) (Benjamin et al. 1999; Duncan et al. 1995; Pannala et al. 2003). Moreover, salivary NO_3^- and NO_2^- following the ingestion of NO_3^- is dose-dependent (Duncan et al. 1995; Pannala et al. 2003) and is influenced by the type of food stuff ingested (McDonagh et al. 2018). We demonstrate that following a 3-day standardised diet (STD), as well as a diet containing less than 50 mg NO_3^- for 7-days (LOW) leads to a marked reduction in salivary [NO_3^-] and [NO_2^-] when compared to a HIGH NO_3^- containing diet. This reduction may have implications on health as NO_3^- within the mouth (in saliva) is linked to protecting

against gastric damage, may be linked with inflammatory processes, and is protects from pH shifts in the mouth which is key in the physiology, ecology and pathogenicity of oral biofilms in the mouth (Burne et al. 2000; Duncan et al. 1997; Jansson et al. 2007; Thompson et al. 2007; Qu et al. 2016).

Alterations in Oral Microbiome Bacteria

A number of bacterial genera which populate the oral cavity have been described as possessing characteristics involved with NO metabolism via possessing NO_3^- reducing properties, with Veillonella, Actinomyces, Rothia, Staphylococcus (Doel et al. 2005), Prevotella, Neisseria and Haemophilus (Hyde et al. 2014) all being implicated in NO generating processes (via NO_2^- production) to varying degrees (Rosier et al. 2020). Interestingly, Vanhatalo et al. (2018) characterised what constitutes a favourable microbial profile in terms of NO_2^- producing bacteria and suggest that high abundances of Prevotella and Veillonella are likely detrimental for NO metabolism, while high abundances of Rothia and Neisseria likely positively influenced NO bioavailability. These authors found that following a 10-day NO_3^- supplementation (2 x 70mL beetroot juice shots: 12.8 mmol NO_3^- per day) period, the relative abundance of the NO_3^- reducers Neisseria and Rothia increased, while the obligate anaerobes Prevotella and Veillonella decreased compared to a placebo condition, which would be indicative of a microbiome that would be positive for NO generation. In corroboration, the work of Burleigh and colleagues (2019), who used an 8-day NO_3^- supplementation regimen (2 x 70mL beetroot juice shots: 12.8 mmol NO_3^- per day) and observed reductions in Prevotella, Streptococcus, Actinomyces, and increases in the relative abundance of Neisseria. However, changes in the aforementioned bacterial constituents of the mouth were not observed in the present study.

At the inception of the present study, we anticipated that substantial changes in the bacterial profile of the mouth would occur in response to changes in habitual NO_3^- ingestion. Indeed,

this is the first study to demonstrate that at a phylum, genus and species level, the bacterial composition of the mouth alters in response to not only the provision of but also the removal of dietary NO_3^-. A novel finding of the present study is that the removal of dietary NO_3^- for 7-days, leads to the reduction in the relative abundance of OTUs of Candidatus Saccharibacteria when compared to that seen following a 7-day diet containing ~180 mg NO_3^-. This was mirrored at a genus level, in which Candidatus Nanosynbacter Lyticus, a constituent of the candidatus saccharibacteria phylum was lower following the 7-day LOW diet in the DEP condition compared to the STD diet in the NORM. The oral microbiome is comprised of microbes which form complex and highly structured, multi-species communities termed biofilms, which are intricate in nature and contain polysaccharides, proteins, DNA, and lipids which form an extracellular matrix (Bowen et al. 2018). These biofilms ensure the persistence of microbiota within the oral cavity by protecting against salivary flow, oral hygiene interventions, and clearance by the immune system (Sanz et al. 2017). Notably, bacteria from the Saccharibacteria phylum have been suggested to be beneficial to host bacteria through the promotion of biofilm formation (Bedree et al. 2018). These have also been linked with influencing oral microbial ecology by modulating the structure hierarchy and functionality of the host's bacterial physiology, inhibition of the host's bacterial growth dynamics and affecting the relative abundance of the host via direct virulent killing of bacterium (Bor et al. 2019). This is achieved through Saccharibacteria representing a negative epiparasite which induces increased stress responses, reduced cellular division and growth rates as well as cell lysis under nutrient starvation (Bor et al. 2016; He et al. 2015).

Experimental Considerations

This study is a pilot study with the oral microbiome analysis containing a reduced number of participants. We utilized the maximum number of cases for each analysis of the oral

microbiome, therefore as illustrated in the results section, the n = alters depending on the comparison. This was due to technical error and therefore caution must be taken when interpreting results from a sample of this size. This study was primarily designed to investigate the chronic effects of altering dietary NO_3^- consumption on salivary NO_3^- and NO_2^-. However, without directly measuring NO_3^- reduction / NO_2^- production, which was not possible in the present study, it is not possible to determine a causal relationship or effect between the outcome of altering salivary NO_3^- and NO_2^-. Furthermore, it should be noted that in this experimental work, we employed a 2x 13-day total duration of the dietary interventions, with only 7-days of NO_3^- deprivation and 3-days of high NO_3^- consumption. Although there is some indication that the duration of these interventions may be sufficient a further consideration when interpreting these findings is the duration of dietary manipulation.

CONCLUSION

The present investigation indicates that the ingestion of variable quantities of NO_3^- may modulate salivary NO_3^- and NO_2^- with the deprivation of NO_3^- from the diet abolishing both NO-related metabolites following as little as 7-days. It is reasonable to speculate that this may influence NO bioavailability through the body, however we did not measure this in the current study. We illustrate that the highly sensitive bacterial communities within the oral cavity are susceptible to change through the removal of NO_3^- from the diet, specifically changes in Candidatus Saccharibacteria (phylum) and Candidatus Nanosynbacter Lyticus (genus). Moreover, when exploring changes in bacterial profiles, we highlight a surprising consistency in the oral microbiome whereby repeating a 3-day STD diets (not consecutively) leads to comparable microbial profiles in the relative abundance of OTUs of bacterial phylum, genus and species.

The present study therefore warrants further work to investigate how the complex symbiosis

between habitual diets and how altering dietary preferences may influence health over prolonged periods through consequences on NO bioavailability. Furthermore, we describe the profound influence of the removal of NO_3^- from the diet on salivary [NO_3^-] and [NO_2^-]. Unfortunately, we are unable to ascertain the consequence of the reported changes in salivary [NO_3^-] and [NO_2^-] and this should therefore also be explored further.

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Figure Legends:

Figure 1. Experimental Design Overview: Dietary control throughout the study was strictly controlled with food and drink being supplied for all periods. ‘A’ denote a 3-day STD diet in which individuals consumed ~180 mg nitrate per day. ‘B’ indicates a further 7-day STD diet containing ~180 mg nitrate per day. ‘C’ depict the HIGH diet in which dietary nitrate consumption equated to ~1000 mg nitrate per day. ‘D’ illustrates a 7-day LOW diet in which individuals consumed ~30 mg nitrate per day. A minimum of 10 days separated the two 13-day experimental conditions as a washout period. Red arrows denote the test days which took place on the first day of the subsequent condition.

Figure 2: A: Saliva [NO_3^-] B: Saliva [NO_2^-] following the ingestion of diets containing varying quantities of NO_3. The initial 3-day periods both consisted of STD diets containing 180 mg NO_3, 7-day periods were either a LOW diet containing 15 – 30 mg NO_3 in closed bars or STD diet in open bars. The final 3-day HIGH diets contained ~1000 mg. Differences ($P < 0.05$) between visits but within condition are displayed with "*", "$" depicts differences between condition.

Figure 3: Changes in the relative abundance of oral microbiome at a phyla level in response to the varying dietary interventions.

Figure 4: Changes in the relative abundance of oral microbiome at a genus level in response to the varying dietary interventions.

Figure 1:

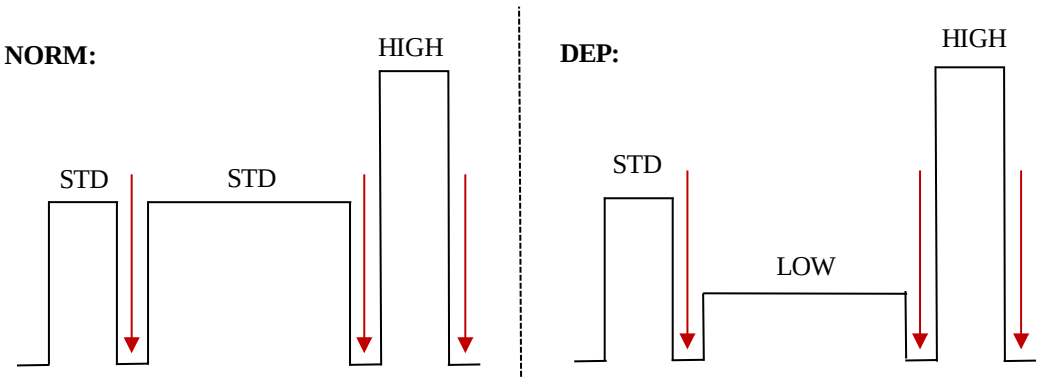


Figure 2:

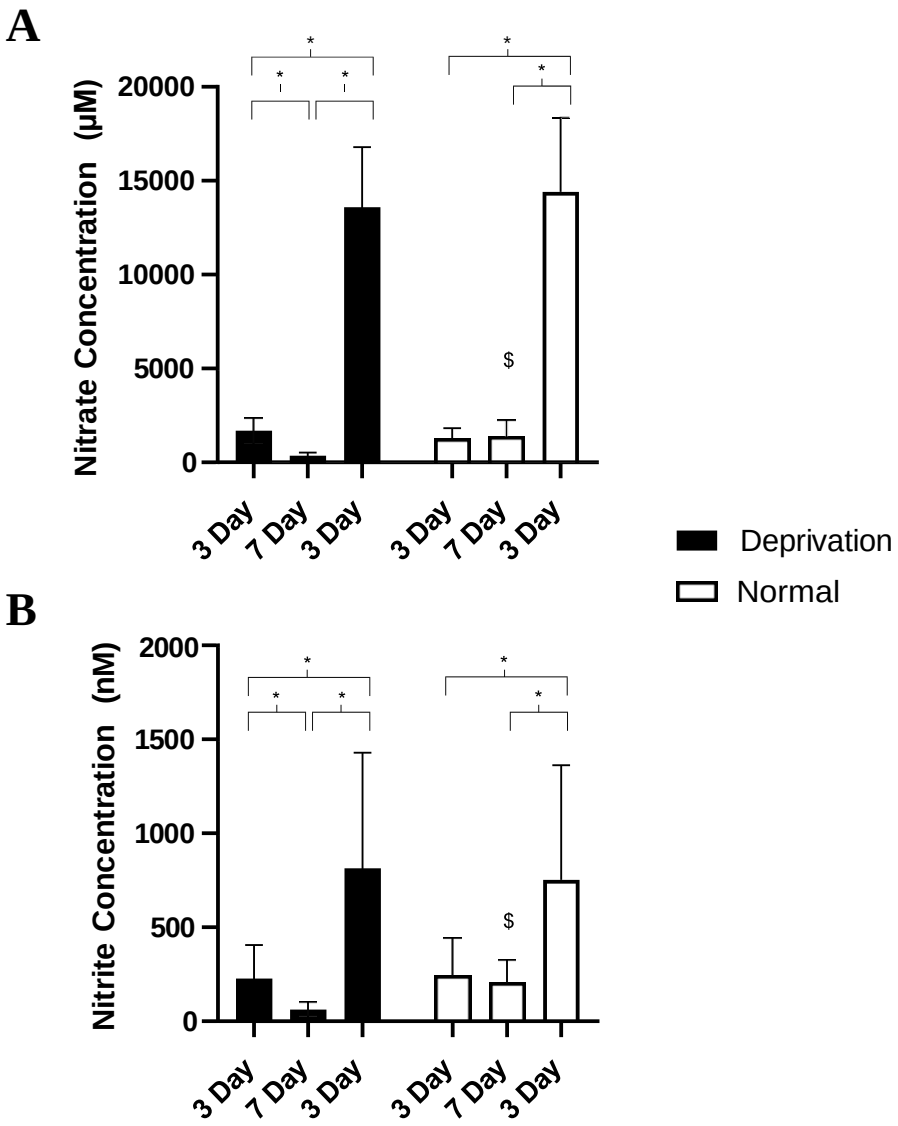


Figure 3:

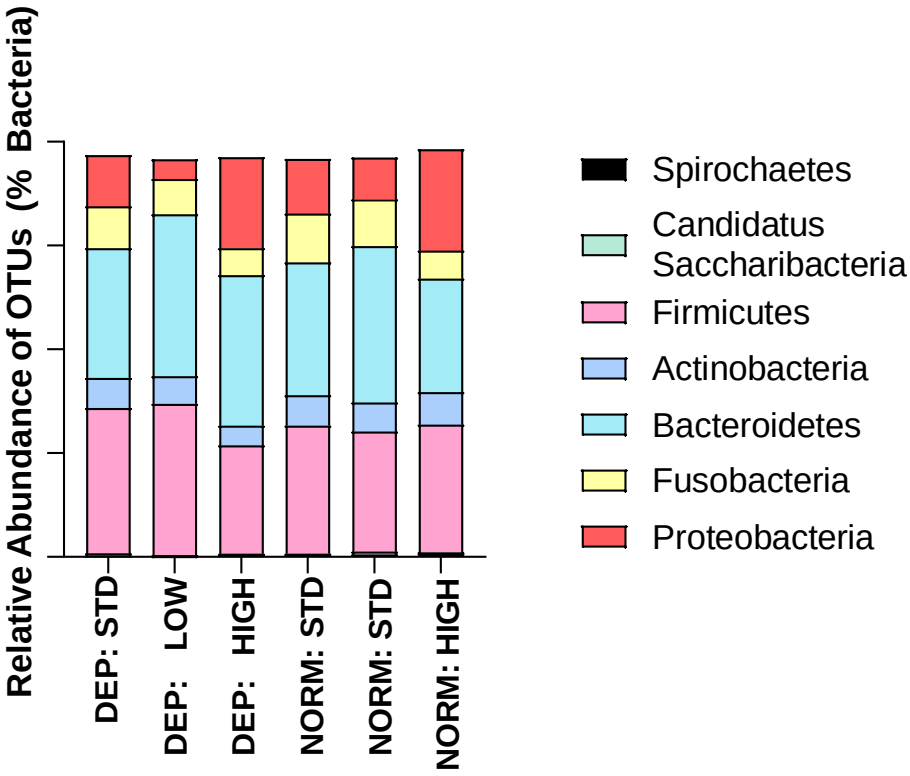
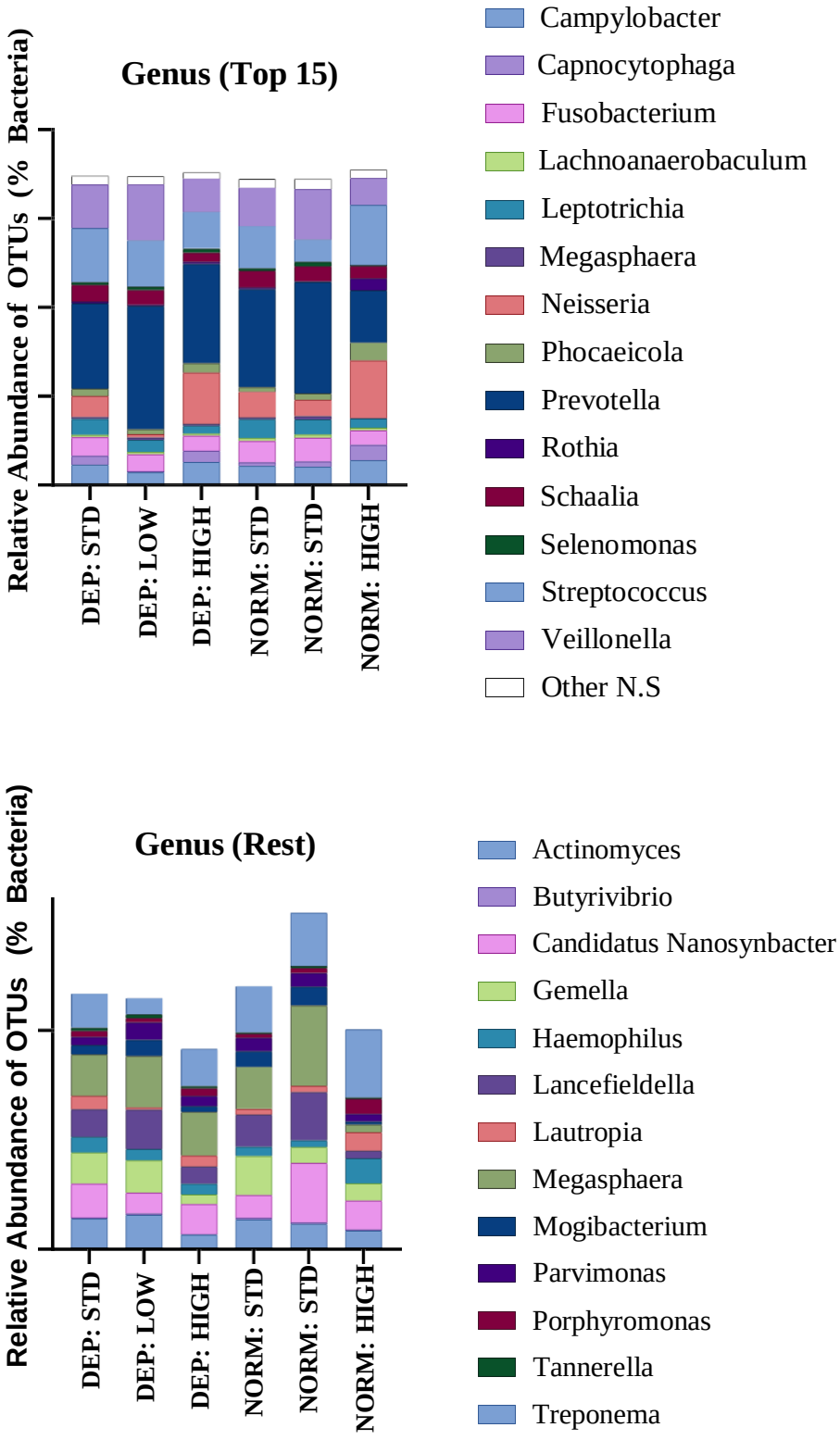

Relative Abundance of OTUs (% Bacteria)
DEP: STD
DEP: LOW
DEP: HIGH
NORM: STD
NORM: STD
NORM: HIGH
Spirochaetes
Candidatus Saccharibacteria
Firmicutes
Actinobacteria
Bacteroidetes
Fusobacteria
Proteobacteria

Figure 4:


Genus (Top 15)
Relative Abundance of OTUs (% Bacteria)
DEP: STD
DEP: LOW
DEP: HIGH
NORM: STD
NORM: STD
NORM: HIGH
Campylobacter
Capnocytophaga
Fusobacterium
Lachnoanaerobaculum
Leptotrichia
Megasphaera
Neisseria
Phocaeicola
Prevotella
Rothia
Schaalia
Selenomonas
Streptococcus
Veillonella
Other N.S
Genus (Rest)
Relative Abundance of OTUs (% Bacteria)
DEP: STD
DEP: LOW
DEP: HIGH
NORM: STD
NORM: STD
NORM: HIGH
Actinomyces
Butyrivibrio
Candidatus Nanosynbacter
Gemella
Haemophilus
Lancefieldella
Lautropia
Megasphaera
Mogibacterium
Parvimonas
Porphyromonas
Tannerella
Treponema

EXPERIMENTAL CHAPTER 3:

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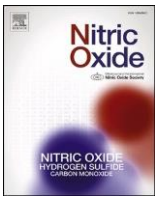

Time course of human skeletal muscle nitrate and nitrite concentration changes following dietary nitrate ingestion

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Skeletal muscle

ABSTRACT

Dietary nitrate (NO_3^-) ingestion can be beneficial for health and exercise performance. Recently, based on animal and limited human studies, a skeletal muscle NO_3^- reservoir has been suggested to be important in whole body nitric oxide (NO) homeostasis. The purpose of this study was to determine the time course of changes in human skeletal muscle NO_3^- concentration ($[NO_3^-]$) following the ingestion of dietary NO_3^-. Sixteen participants were allocated to either an experimental group (NIT: n = 11) which consumed a bolus of ~1300 mg (12.8 mmol) potassium nitrate (KNO_3), or a placebo group (PLA: n = 5) which consumed a bolus of potassium chloride (KCl). Biological samples (muscle (*vastus lateralis*), blood, saliva and urine) were collected shortly before NIT or PLA ingestion and at intervals over the course of the subsequent 24 h. At baseline, no differences were observed for muscle $[NO_3^-]$ and $[NO_2^-]$ between NIT and PLA ($P > 0.05$). In PLA, there were no changes in muscle $[NO_3^-]$ or $[NO_2^-]$ over time. In NIT, muscle $[NO_3^-]$ was significantly elevated above baseline (54 ± 29 nmol/g) at 0.5 h, reached a peak at 3 h (181 ± 128 nmol/g), and was not different to baseline from 9 h onwards ($P > 0.05$). Muscle $[NO_2^-]$ did not change significantly over time. Following ingestion of a bolus of dietary NO_3^-, skeletal muscle $[NO_3^-]$ increases rapidly, reaches a peak at ~3 h and subsequently declines towards baseline values. Following dietary NO_3^- ingestion, human m. *vastus lateralis* $[NO_3^-]$ expressed a slightly delayed pharmacokinetic profile compared to plasma $[NO_3^-]$.

1. Introduction

Pharmacokinetics studies are important for determining the fate of administered substances or drugs, and can be broken down into several factors: absorption, distribution, metabolism and excretion [1]. In human physiology, knowledge of the time course of the uptake of an ingested substance into a target tissue, such as blood or muscle, is imperative in optimising the therapeutic or ergogenic effect of that substance, while also minimising the occurrence of potential adverse events.

Nitrate (NO_3^-) and nitrite (NO_2^-) are metabolites related to the production of the signalling molecule, nitric oxide (NO), which is essential in the maintenance of normal physiological function, including the regulation of blood pressure [2,3]. The ingestion of inorganic NO_3^- in NO_3^--rich food and drink sources augments NO bioavailability and may have important physiological effects [4,5]. Several studies have described dynamic changes in $[NO_3^-]$ and $[NO_2^-]$ in biological tissues such as saliva [6–8], plasma [9,10 for review see Ref. [11]], and urine [12–14] following dietary NO_3^- ingestion or supplementation.

Piknova et al. [15,16] reported that *gluteus maximus* muscle $[NO_3^-]$ was higher than that of blood and other organs in rodents. The existence of higher $[NO_3^-]$ in *vastus lateralis* muscle, compared to plasma, and its elevation following dietary NO_3^- ingestion, has recently been confirmed in humans [17,18]. It is possible that this relatively high muscle $[NO_3^-]$ has functional significance [19]. Skeletal muscle possesses the enzymatic machinery required for the reduction of NO_3^- and NO_2^- to NO (i.e., xanthine oxidoreductase, aldehyde oxidase, sulphite oxidase [16]); and it is possible that skeletal muscle serves as a NO_3^- 'reservoir' that

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might be drawn upon when access to dietary NO_3^- is restricted [5,15]. However, the time course over which muscle [NO_3^-] changes following acute dietary NO_3^- ingestion, and how this relates temporally and quantitatively to changes in [NO_3^-] in other biological compartments (saliva, blood and urine), has yet to be fully investigated.

The purpose of the present study was therefore to describe, for the first time, the pharmacokinetic profile of human skeletal muscle [NO_3^-] and [NO_2^-] over 24 h following bolus dietary NO_3^- ingestion. We hypothesised that skeletal muscle [NO_3^-] and [NO_2^-] would follow a similar profile to that described previously for plasma [9,10], with a peak value reached 1–3 h post NO_3^- ingestion and a subsequent fall back to the pre-ingestion baseline by 24 h.

2. Methods

The local Research Ethics Committee (Sport and Health Sciences, University of EXeter) approved this study, which was conducted in accordance with the principles of the Declaration of Helsinki. Participants provided written informed consent prior to commencement of the study after the experimental procedures and associated benefits and risks associated with participation had been explained.

3. Participants

Participants were ostensibly healthy and free of cardiovascular, respiratory, metabolic or musculoskeletal diseases or disorders. EXclusion criteria included use of dietary supplements, blood pressure medication, tobacco smoking, vegan or vegetarian diet, and use of antibacterial mouthwash. Sixteen individuals were enrolled and successfully completed the study (Table 1).

4. Experimental design

Participants were allocated into one of two groups in which either a placebo (PLA; n=5 males) or active (NIT; n 11, 9 males) dietary supplement was ingested. We employed a parallel group, rather than crossover, experimental design to reduce the number of muscle biopsies required per participant, recognising that no changes in muscle [NO_3^-] or [NO_2^-] were expected with PLA. After adhering to a prescribed low-NO_3^- diet (~20–40 mg NO_3^-/day; ~0.004–0.005 mmol/kg) for 24 h, participants attended the laboratory on three separate occasions within the following 24-h period during which all food and drink was provided. Fig. 1 illustrates the experimental procedures and the times at which biological tissue samples were collected. On the day of the experimental visits, participants arrived in a rested and fasted state at 07:00 a.m. Participants were asked a series of questions related to adherence to the prescribed diet, body mass measurement was completed and the initial urine sample was collected. After a 40-min period of rest, a cannula was inserted into a vein in the antecubital fossa and the initial blood samples were collected. Preparations for the muscle biopsies were completed and the first muscle tissue sample was collected. Following this, a low-NO_3^- breakfast was provided and, at 09:00 a.m., 140 mL of a drink containing either 12.8 mmol (~1300 mg) potassium nitrate (KNO_3) (NIT) or 12.8 mmol potassium chloride, providing an equivalent dose of potassium but negligible NO_3^- (PLA), was ingested. All ensuing measurements and sample collections corresponded to the time of supplement ingestion and occurred at 0.5, 1, 2, 3, 9 and 24 h following ingestion of the supplement (Fig. 1).

Table 1
Characteristics of participants and groups that completed the study.

Group	Sex	Age (y)	Height (m)	Body Mass (kg)
Active (NIT)	Male (n = 9)	24 ± 4	1.79 ± 0.07	81.3 ± 17
	Female (n = 2)	22 ± 3	1.67 ± 0.04	57.6 ± 6.2
	Total (n = 11)	23 ± 4	1.77 ± 0.08	76.5 ± 17.3
Placebo (PLA)	Male (n = 5)	25 ± 6	1.78 ± 0.06	73.1 ± 6.8

5. Measurements

A total of seven muscle biopsies were collected from the m. *vastus lateralis* muscle using the percutaneous Bergström needle technique modified for manual vacuum [20]. Immediately upon collection, the samples were blotted using sterile gauze to remove blood and any visible adipose tissue. The sample was then placed in liquid nitrogen prior to being stored at −80 °C until subsequent analysis.

Venous cannulation (20 g Insyte-WTM cannula; Becton Dickinson, Madrid, Spain) and venepuncture (BD Safety-Lok Blood Collection Set; Becton Dickson, UK) were used to collect blood samples from a vein in the antecubital fossa to determine [NO_3^-] and [NO_2^-] in whole blood, red blood cells, and plasma. Blood was drawn into 6 mL vacutainers pre-treated with lithium-heparin (Becton Dickinson, NJ). 800 μL of the whole blood sample was extracted and inserted into a 1.5 mL Eppendorf containing 200 μL of a NO_2^- preservation solution and thoroughly vortexed prior to freezing in liquid nitrogen. Simultaneously two further vacutainers were centrifuged within 30 s of collection at 3300 g for 7 min at 4 °C. Following centrifugation, plasma was aliquoted into Eppendorfs and placed in liquid nitrogen. 900 μL of red blood cells remaining in the Eppendorf were subsequently extracted and combined with 100 μL of the NO_2^- preservation solution in separate tubes, vortexed, and placed in liquid nitrogen. The NO_2^- preservation solution consisted of 890.9 mM potassium ferricyanide ($K_3Fe(CN)_6$), 118.13 mM *N*-Ethylmaleimide (NEM), NP-40 (octyl phenoXylpolyethoXylethanol) added in a 1:9 ratio (v/v, NP-40/solution), and 4.5 mL deionised water (dH2O) [15].

Participants collected a sample of urine in a separate container (Kartell™; Milan, Italy) and this was aliquoted into Eppendorfs and immediately frozen in liquid nitrogen for subsequent NO_3^- and NO_2^- analysis.

Saliva sampling occurred at the same time points (Fig. 1) as muscle biopsies and entailed participants collecting their saliva in a 30 mL tube (Thermo Scientific™ Sterilin™; Massachusetts, USA) over a 2-min period. The samples were aliquoted into Eppendorfs and placed in liquid nitrogen before being stored in a —80 °C freezer.

NO_3^- and NO_2^- concentration measurements in all biological samples was performed using ozone-based gas-phase chemiluminescence [21,22]. The initial step for determination of NO_3^- and NO_2^- in blood samples was the addition of ice-cold methanol to deproteinize samples which then underwent centrifugation at 11,000 g for 5 min. Urine and saliva samples were diluted with deionised water and were centrifuged similarly to plasma samples. The supernatant was extracted and used in the [NO_3^-] and [NO_2^-] quantification by a Sievers gas-phase chemiluminescence NO analyser (Sievers 280i Nitric Oxide Analyser, GE Analytical Instruments, Boulder, CO, USA). The analysis of muscle NO_3^- and NO_2^- content entailed the preliminary weighing of 15–20 mg muscle which was subsequently mixed with a NO_2^- preservation solution ($K_3Fe(CN)_6$, *N*-ethylmaleimide, water, Nonidet P-40) and homogenized using a bead homogenizer (see Ref. [22]).

6. Statistical analysis

Statistical analyses were performed using the IBM Statistical Package for Social Scientists (SPSS Version 27, SPSS Inc., Chicago, IL, USA) statistical software. Two-way repeated measures ANOVAs were used to determine differences in NO_3^- and NO_2^- concentrations in plasma, red blood cells, whole blood, saliva, urine and muscle across time (0, 0.5, 1, 2, 3, 9 and 24 h post supplement) and between groups (PLA and NIT). Significant main and interaction effects were analysed further and, where appropriate, least significant difference (LSD) post hoc tests were applied to identify the point at which significant differences occurred. Pearson product moment correlation coefficients were used to evaluate the significance of relationships between changes in plasma and changes

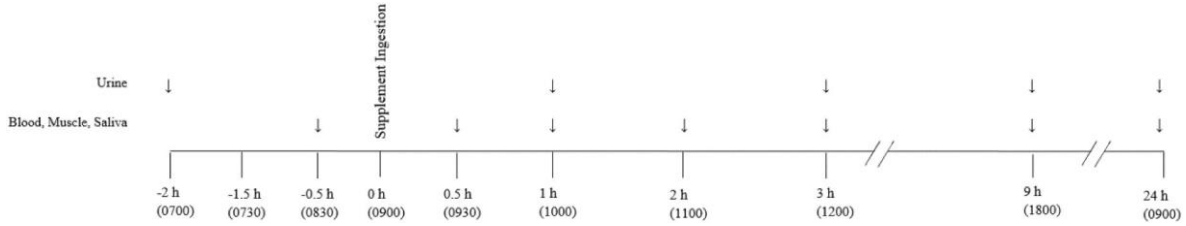

Fig. 1. Schematic of experimental procedures and timings of measurement and sample collection. The '↓' refer to the time-points at which each measurement was taken and the closed circle '●' illustrates the ingestion of a single bolus of either a potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo(PLA) supplement.

in muscle [NO_3^-] and [NO_2^-]. Statistical significance was accepted at $P < 0.05$. The results are presented as mean ± standard deviation (SD).

7. Results

7.1. Baseline muscle, blood, saliva, and urinary [NO_3^-] and [NO_2^-]

At baseline, prior to the ingestion of any supplement, no differences were observed for muscle [NO_3^-] and [NO_2^-] between NIT (NO_3^-, 54 ± 29 nmol/g; NO_2^-, 2.4 ± 0.9 nmol/g) and PLA (NO_3^- 56 ± 11 nmol/g; NO_2^-, 2.5 ± 0.6 nmol/g) groups (both $P > 0.05$; Fig. 2). Similarly, no between-group differences were observed in [NO_3^-] and [NO_2^-] for saliva (Fig. 3), plasma or whole blood (Fig. 4), or urine (Fig. 5), (all $P > 0.05$). Red blood cell [NO_3^-] was greater ($P < 0.05$) for the PLA (14 ± 6 nmol/g) compared to the NIT (7 ± 4 nmol/g) group, but no differences ($P > 0.05$) were observed in red blood cell [NO_2^-] (NIT, 0.08 ± 0.05 nmol/g; PLA, 0.08 ± 0.03 nmol/g), (Fig. 4).

When considered across both NIT and PLA groups (n = 16), no

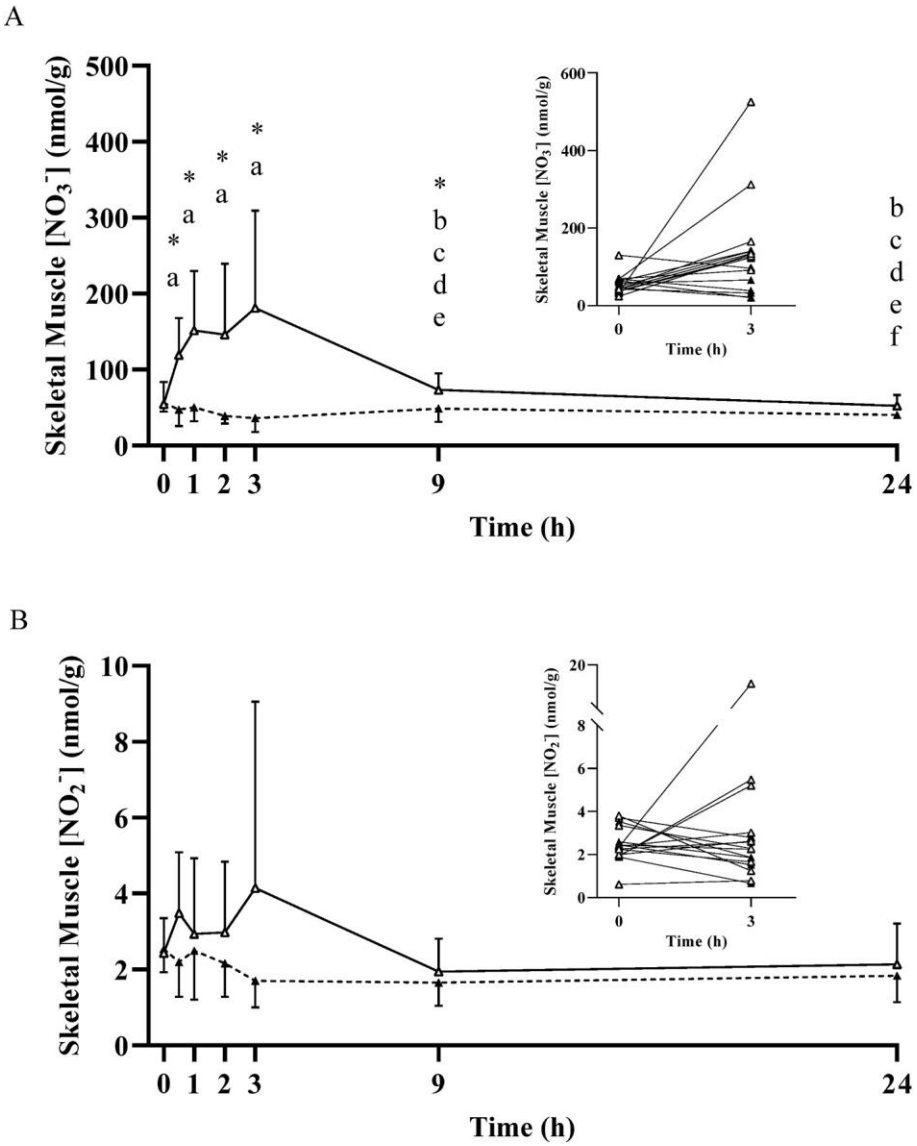

Fig. 2. Mean ± SD skeletal muscle nitrate concentration ([NO_3^-]; **Panel A**) and nitrite concentration (NO_2^-; **Panel B**) prior to and following the ingestion of either a potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo (PLA) supplement over a 24-h period. No significant ($P>0.05$) differences were observed in the PLA group over time. Significant differences ($P < 0.05$) between groups are shown with '*'. In the NIT group, 'a' = significant difference when compared to baseline (0 h), 'b' = significant difference when compared to 0.5 h, 'c' = significant difference when compared to 1 h, 'd' = significant difference when compared to 2 h, 'e' = significant differences when compared to 3 h, and 'f' = significant difference when compared to 9 h following NO_3^- ingestion. Open triangles (△) represent the NIT group and closed triangles (▲) represent the PLA group. See text for further information. To exemplify the inter-individual variability in response, the inset figures show the individual participant responses between 0 and 3 h.

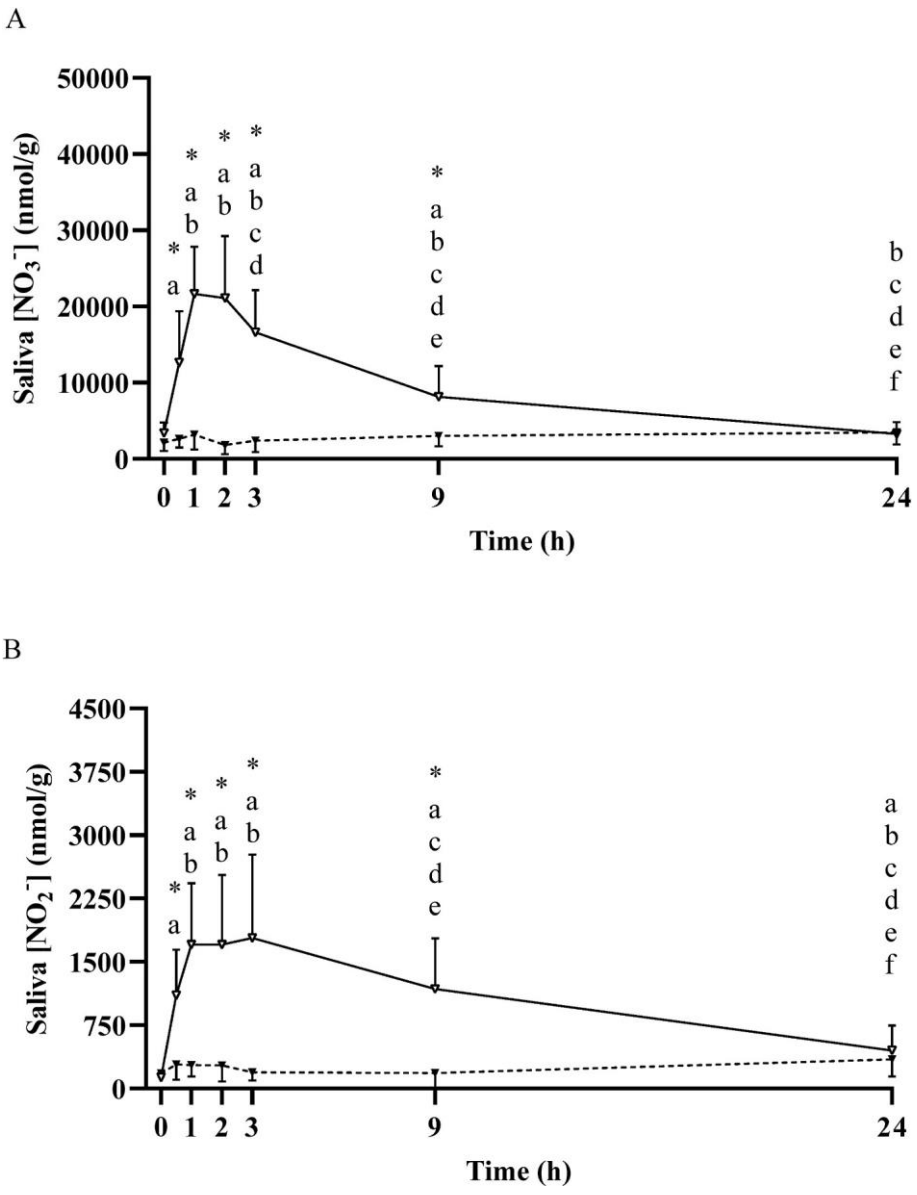

Fig. 3. Mean ± SD salivary nitrate concentration ([NO_3^-]; **Panel A**) and nitrite concentration ([NO_2^-]; **Panel B**) prior to and following the ingestion of either a potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo (PLA) supplement over a 24-h period. No significant ($P>0.05$) differences were observed in the PLA group over time. Significant differences ($P < 0.05$) between groups are shown with '*'. In the NIT group; 'a' = significant difference when compared to baseline (0 h), 'b' = significant difference when compared to 0.5 h, 'c' = significant difference when compared to 1 h, 'd' = significant difference when compared to 2 h, 'e' = significant difference when compared to 3 h, and 'f' = significant difference when compared to 9 h following NO_3^- ingestion. Open triangles (△) represent the NIT group and closed triangles (▲) represent the PLA group. See text for further information.

differences ($P > 0.05$) were observed between baseline skeletal muscle [NO_3^-] (55 ± 25 nmol/g) and plasma [NO_3^-] (63 ± 38 nmol/g), but muscle [NO_2^-] (2.5 ± 0.8 nmol/g) was ~12-fold greater than plasma [NO_2^-] (0.2 ± 0.1 nmol/g) ($P < 0.05$). Plasma [NO_3^-] (63 ± 38 nmol/g; n = 16) was ~2.5-fold greater than whole blood [NO_3^-] (25 ± 15 nmol/g, $P < 0.01$), and ~7-fold greater than red blood cell [NO_3^-] (9 ± 6 nmol/g, $P < 0.001$). In contrast, plasma [NO_2^-] (0.2 ± 0.1 nmol/g) was not different from whole blood [NO_2^-], (0.3 ± 0.2 nmol/g, $P = 0.06$), but both were greater than red blood cell [NO_2^-] (0.1 ± 0.04 nmol/g, both $P < 0.001$).

7.2. Effect of dietary NO_3^- ingestion on muscle, blood, saliva, and urinary [NO_3^-] and [NO_2^-]

No changes in muscle, blood, saliva or urinary [NO_3^-] or [NO_2^-] were observed across time in the PLA group (all $P > 0.05$; Figs. 2–5). In contrast, following NO_3^- ingestion, [NO_3^-] and/or [NO_2^-] changed with time in all tissues assessed. Muscle [NO_3^-] and [NO_2^-] values during the 24 h measurement period are presented in Fig. 2. Muscle [NO_3^-] was significantly greater than baseline at 0.5 h (119 ± 49 nmol/g), increased ~3-fold (181 ± 128 nmol/g) to its peak at 3 h, and was not different to baseline from 9 h ($P > 0.05$) onwards. No significant changes in muscle [NO_2^-] were observed (all $P > 0.05$).

Plasma [NO_3^-] and [NO_2^-] across the 24 h period are presented in Fig. 4. Plasma [NO_3^-] was significantly greater than baseline at 0.5 h (351 ± 126 nmol/g), increased ~8-fold (514 ± 122 nmol/g) to its peak at 2 h, remained elevated at 9 h (230 ± 42 nmol/g, $P < 0.05$), and was not different from baseline at 24 h (88 ± 16 nmol/g, $P > 0.05$). Plasma [NO_2^-] was greater than baseline at 1 h (0.3 ± 0.1 nmol/g, $P < 0.05$), attained its peak at 3 h (0.6 ± 0.3 nmol/g), and was not different from baseline at 24 h (0.2 ± 0.1 nmol/g, $P > 0.05$). Saliva [NO_3^-] was significantly increased above baseline from 0.5 h to 9 h (all $P < 0.05$; Fig. 3). Both whole blood [NO_3^-] and [NO_2^-], red blood cell [NO_3^-] and saliva [NO_2^-] were elevated above baseline across the 24 h period (all $P < 0.05$; Figs. 3 and 4). No significant increases were observed in red blood cell [NO_2^-], although there was a tendency towards an increase above baseline at 0.5 h ($P = 0.09$) and 2 h ($P = 0.05$). Urinary [NO_3^-] was increased above baseline from 1 h to 9 h (all $P < 0.05$), and urinary [NO_2^-] tended to be higher at 1 h ($P = 0.08$), was greater than baseline at 3 h and 9 h (both $P < 0.05$), and was not different from baseline at 24 h ($P > 0.05$; Fig. 5).

The ratios between plasma and muscle [NO_3^-] and [NO_2^-] across

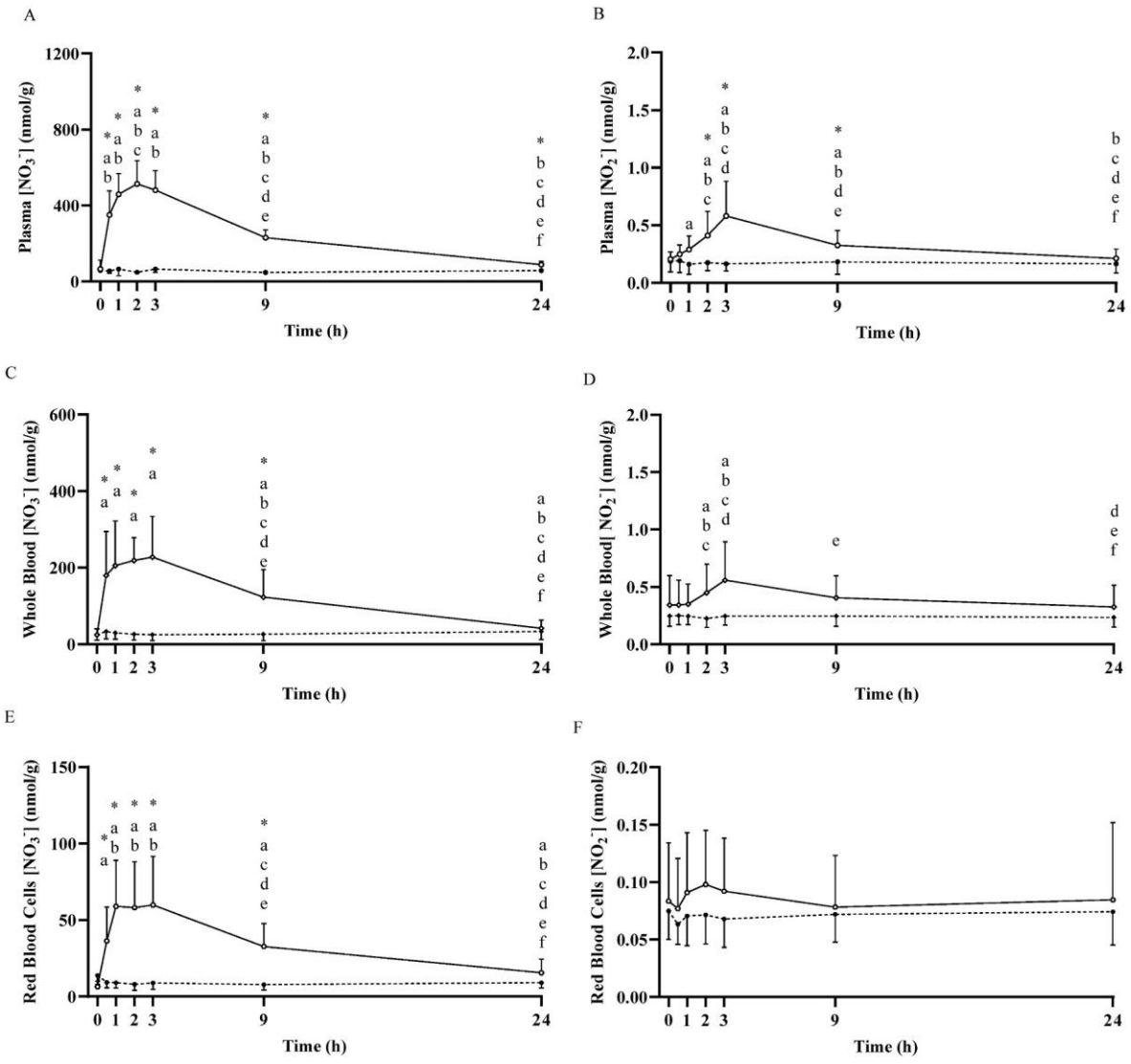

Fig. 4. Mean ± SD plasma nitrate concentration ([NO_3^-]; **Panel A**) and nitrite concentration (NO_2^-; **Panel B**), whole blood [NO_3^-] (**Panel C**) and [NO_2^-] (**Panel D**), and red blood cell [NO_3^-] (**Panel E**) and [NO_2^-] (**Panel F**) prior to and following the ingestion of either a potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo (PLA) supplement over a 24-h period. No significant ($P>0.05$) differences were observed in the PLA group over time. Significant differences ($P < 0.05$) between groups are shown with '*'. In the NIT group, 'a' = significant differences when compared to baseline (0 h), 'b' = significant difference when compared to 0.5 h, 'c' = significant difference when compared to 1 h, 'd' = significant difference when compared to 2 h, 'e' = significant difference when compared to 3 h, and 'f' = significant difference when compared to 9 h following NO_3^- ingestion. Open triangles (△) represent the NIT group and closed triangles (▲) represent the PLA group. See text for further information.

time are presented in Fig. 6. At baseline, the plasma/muscle [NO_3^-] ratio was close to unity, indicating no clear muscle-to-blood or blood-to-muscle gradient, whereas the plasma/muscle [NO_2^-] ratio was ~0.1, indicating that [NO_2^-] is substantially higher in muscle than blood. No changes were observed in the plasma/muscle [NO_3^-] ratio in PLA ($P >$ 0.05). In NIT, following supplementation, the plasma/muscle [NO_3^-] ratio was significantly greater than baseline (1.3 ± 0.7) at 0.5 h (3.3 ± 1.3), attaining its peak at 2 h (4.6 ± 2.4), and returning to baseline at 24 h ($P > 0.05$). No changes were identified in the plasma/muscle [NO_2^-] ratio in PLA ($P > 0.05$). In NIT, the plasma/muscle [NO_2^-] ratio was significantly elevated at 2 h (0.2 ±0.1 a.u.), reached its peak at 3 h (0.3 ±0.2 a.u.) and remained elevated at 24 h (0.1 0.1 a.u.), compared to baseline (all $P < 0.05$).

There was no significant correlation between plasma and muscle [NO_3^-] at baseline (r = 0.13; $P > 0.05$). However, the percentage change in plasma [NO_3^-] was significantly correlated with the percentage change in muscle [NO_3^-] at 0.5, 1, 2, and 9 h following NO_3^- ingestion (r = 0.52–0.70; $P < 0.05$). Similarly, there was no significant correlation between plasma and muscle [NO_2^-] at baseline (r = 0.48; $P > 0.05$) but the percentage change in plasma [NO_2^-] was significantly correlated with the percentage change in muscle [NO_2^-] at 1, 2, 3 and 9 h following NO_3^- ingestion (r = 0.58–0.78; $P < 0.05$).

The changes in muscle, plasma, saliva, and urinary [NO_3^-] and [NO_2^-] across time relative to their respective baseline are presented in Fig. 7. At 1 h post NO_3^- ingestion, the change in [NO_3^-] was significantly greater for plasma (+815 ± 426%) compared to muscle (+225 ± 153%, $P < 0.001$) and urine (+400 ± 386%, $P < 0.001$), but was not different from saliva (+686 ± 455%, $P > 0.05$). At 3 h, urinary [NO_3^-] had increased further from baseline (+618 ± 469%) and was not different from plasma (+799 ± 397%), saliva (+492 ± 327%) or muscle (+328 414%) (all $P > 0.05$). At 9 h, plasma [NO_3^-] remained elevated above baseline (+341 185%) ($P < 0.05$) and was significantly higher than muscle (+68 ±96%), but was not different from saliva (214 245%) and urine (+624 598%) (both $P > 0.05$). At 24 h, there was no difference in the relative change from baseline in any of the variables except for between plasma and muscle ($P < 0.05$). The change in saliva

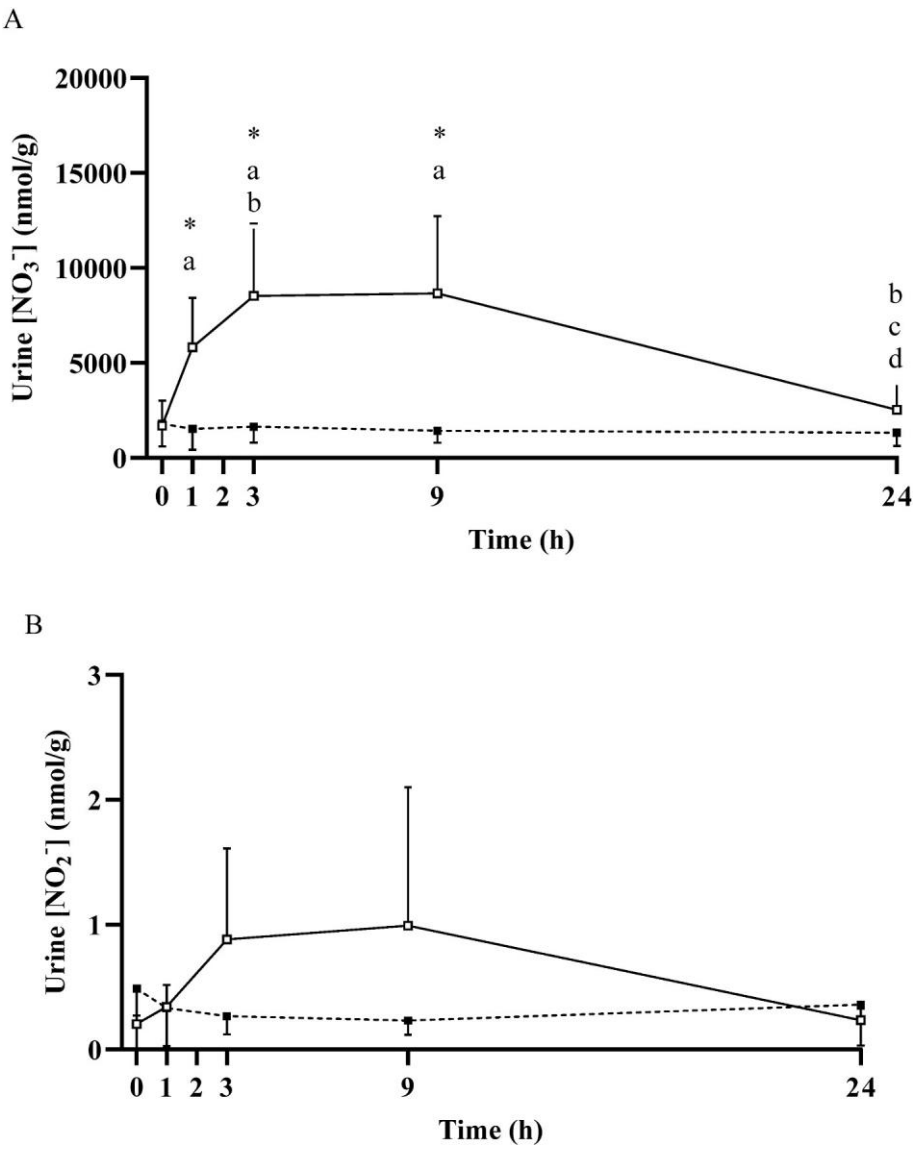

Fig. 5. Mean ± SD urinary nitrate concentration ([NO_3^-]; **Panel A**) and nitrite concentration (NO_2^-; **Panel B**) prior to and following the ingestion of either a potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo (PLA) supplement over a 24-h period. No significant ($P>0.05$) differences were observed in the PLA group over time. Significant differences ($P < 0.05$) between groups are shown with '*'. In the NIT group, 'a' = significant differences when compared to baseline (0 h), 'b' = significant difference when compared to 1 h, 'c' = significant difference when compared to 3 h, 'd' = significant difference when compared to 9 h following NO_3^- ingestion. Open triangles (△) represent the NIT group and closed triangles (▲) represent the PLA group. See text for further information.

[NO_2^-] was greater than for all other samples across all measurement time points ($P < 0.05$). Both plasma and muscle [NO_2^-] were greater than baseline 1–3 h following NO_3^- ingestion, and there were no differences between these tissues at any time (all $P > 0.05$). Urinary [NO_2^-] was greater than baseline at 3 and 9 h post supplementation ($P < 0.05$), and the magnitude of change was greater than observed for both muscle and plasma.

8. Discussion

The effects of dietary NO_3^- supplementation on physiological responses (e.g., blood pressure and exercise performance) are complex and highly variable between individuals [11,23]. The efficacy of NO_3^- supplementation is often evaluated in relation to the magnitude of elevation in plasma [NO_3^-] and [NO_2^-]. However, NO_3^- metabolism is a dynamic process involving the transport of NO_3^- and NO_2^- between biological compartments, and a more complete understanding of the biological fate of the ingested NO_3^-, and its functional consequences, requires concurrent consideration of NO_3^- and NO_2^- pharmacokinetics in numerous tissues (e.g., saliva, blood, skeletal muscle, urine). It has been reported that rodent muscle has a relatively high [NO_3^-] [15,16], and that this NO_3^- reservoir is highly sensitive to the NO_3^- content of the diet [24]. However, further studies in rodents showed non-uniform distribution of NO_3^- over different skeletal muscle groups [25,26], which complicates the originally formulated simple hypothesis that declared skeletal muscle to be a whole body NO_3^- reservoir [15]. The primary purpose of the present investigation was to characterize, for the first time, the pharmacokinetic profile of human *vastus lateralis* muscle [NO_3^-] and [NO_2^-] following bolus ingestion of dietary NO_3^-. Consistent with our hypothesis, we found that skeletal muscle [NO_3^-] was significantly increased above baseline at 0.5 h, reached its peak at ~3 h, remained elevated for ~9 h and then returned to baseline 24 h after NO_3^- ingestion. In contrast, muscle [NO_2^-] was not significantly elevated following NO_3^- ingestion.

8.1. Time course of nitrate elevation in biological tissues

With the exception of urine, the pharmacokinetic profiles of NO_3^- elevation in the biological tissues we sampled were broadly similar, with a rapid elevation leading to peak concentration at 1–3 h, followed by a slower decline thereafter, and a return to baseline at 24 h following NO_3^- ingestion. These profiles, which are illustrated as a percentage change from baseline in Fig. 7, indicate that the time-to-peak concentration occurs in saliva at ~1 h, in plasma at ~2 h, in muscle at ~3 h,

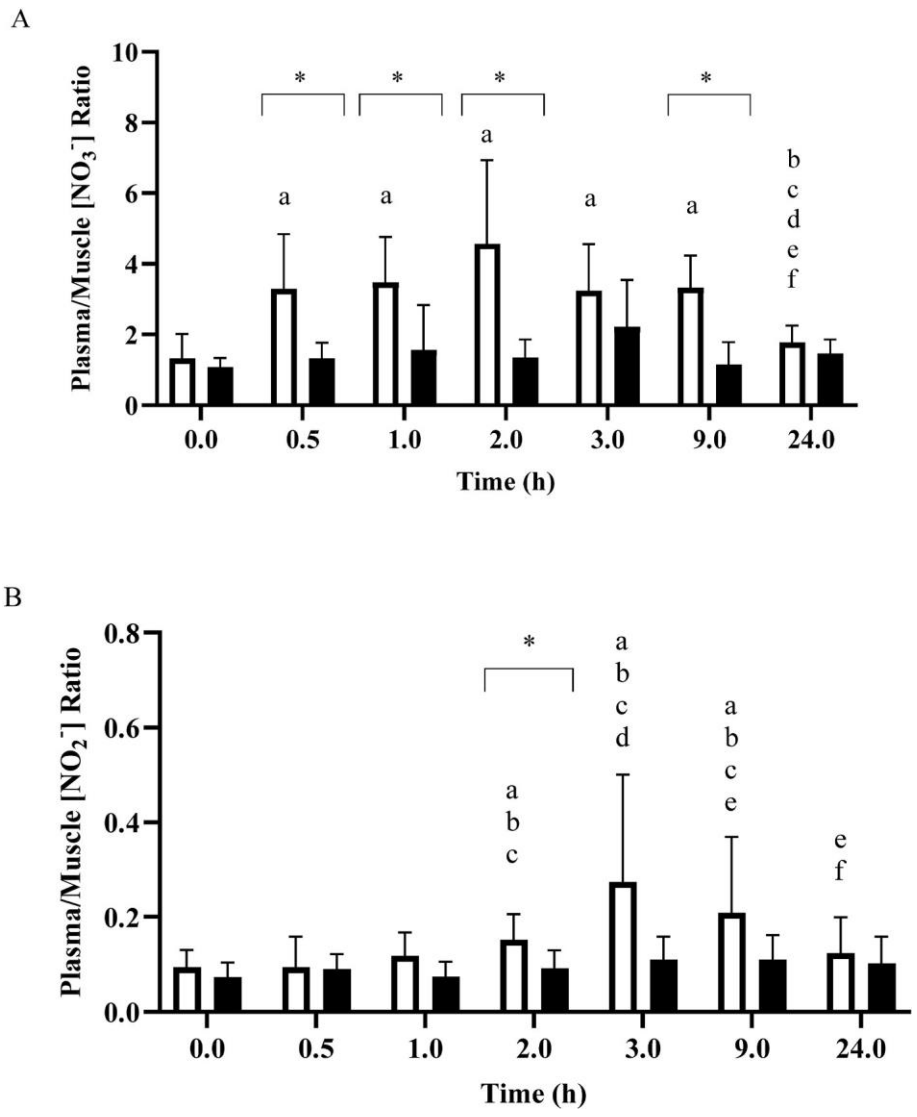

Fig. 6. Mean ± SD plasma/muscle nitrate concentration ($[NO_3^-]$) ratio (**Panel A**) and plasma/muscle nitrite concentration ($[NO_2^-]$) ratio (**Panel B**) prior to and for the 24-h following the ingestion of either a potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo (PLA) supplement. Significant differences ($P < 0.05$) between the NIT and PLA group are illustrated with '*'. For NIT, 'a' = significant difference when compared to 0 h, 'b' = significant difference when compared to 0.5 h, 'c' = significant difference when compared to 1 h, 'd' = significant difference when compared to 2 h, 'e' = significant difference when compared to 3 h, and 'f' = significant difference when compared to 9 h following NO_3^- ingestion. Open symbols represents the NIT group and closed symbols represent the PLA group.

and in urine between 3 and 9 h.

Our results for saliva, plasma and urine are consistent with previous reports [10,11,13,14,27,28]. However, a novel finding of the present study is the rapid uptake of NO_3^- into skeletal muscle, which was evident at 0.5 h following the ingestion of an acute bolus of KNO_3. This highlights the speed with which NO_3^- is absorbed from the intestine into the bloodstream, transported to other tissues, and sequestered from the circulation into storage sites including muscle [19]. This process may be facilitated via the actions of sialin, a protein involved in the active transport of NO_3^- [29], which has been demonstrated to be present in skeletal muscle [18,30]. The change in muscle $[NO_3^-]$ was correlated with the change in plasma $[NO_3^-]$, consistent with the existence of blood to muscle NO_3^- exchange following NO_3^- ingestion.

Two previous studies have measured $[NO_3^-]$ in human skeletal muscle following dietary NO_3^- ingestion but with more limited temporal resolution. Nyakayiru et al. [17] reported that *vastus lateralis* muscle $[NO_3^-]$ was significantly elevated in type II diabetes mellitus patents at 2, 4, and 7 h, following the ingestion of ~12 mmol $NaNO_3^-$. Similarly, Wylie et al. [18], reported that *vastus lateralis* muscle $[NO_3^-]$ was significantly increased at 2 h post ingestion of beetroot juice containing 12.8 mmol NO_3^-. In the present study, muscle $[NO_3^-]$ approximated 40–50 nmol/g at baseline and was elevated to a peak of ~180 nmol/g following NO_3^- ingestion (Fig. 2). These values are similar to those reported by Nyakayiru et al. [17] but considerably lower than those reported by Wylie et al. [18]. It is notable that in all three studies, the influence of dietary NO_3^- ingestion on muscle $[NO_3^-]$ is qualitatively similar (i.e., a 4–5 fold increase in $[NO_3^-]$) despite the considerable differences in absolute concentrations reported between laboratories, which may be related in part to factors such as diet, age and physical activity levels. The measurement of $[NO_3^-]$ in muscle is both relatively new and technically challenging, and experimental techniques continue to evolve [26].

To our knowledge, this is the first study in any species to determine the time course of $[NO_3^-]$ changes in plasma, red blood cells and whole blood following dietary NO_3^- ingestion. Interestingly, the time-to-peak $[NO_3^-]$ in red blood cells and whole blood occurred at 3 h, which was slightly later than in plasma and may suggest a slight lag in the entry of NO_3^- into the red cell from the plasma. However, it should be noted that although the absolute increase and time-to-peak $[NO_3^-]$ differed in plasma, red blood cells and whole blood, the values were not significantly different between 1 and 3 h following NO_3^- ingestion (Fig. 4).

The time course of changes in urine $[NO_3^-]$ differed from the pattern observed in the other compartments, reflecting delayed dynamics for the excretion of NO_3^-. The results indicate that urine $[NO_3^-]$ was significantly increased 1 h post NO_3^- ingestion and remained elevated for 9 h before returning to baseline at 24 h. These results are consistent with previous literature [12–14,28]. It has been reported that up to 75% of ingested NO_3^- is ultimately expelled in the urine [31]. The sustained

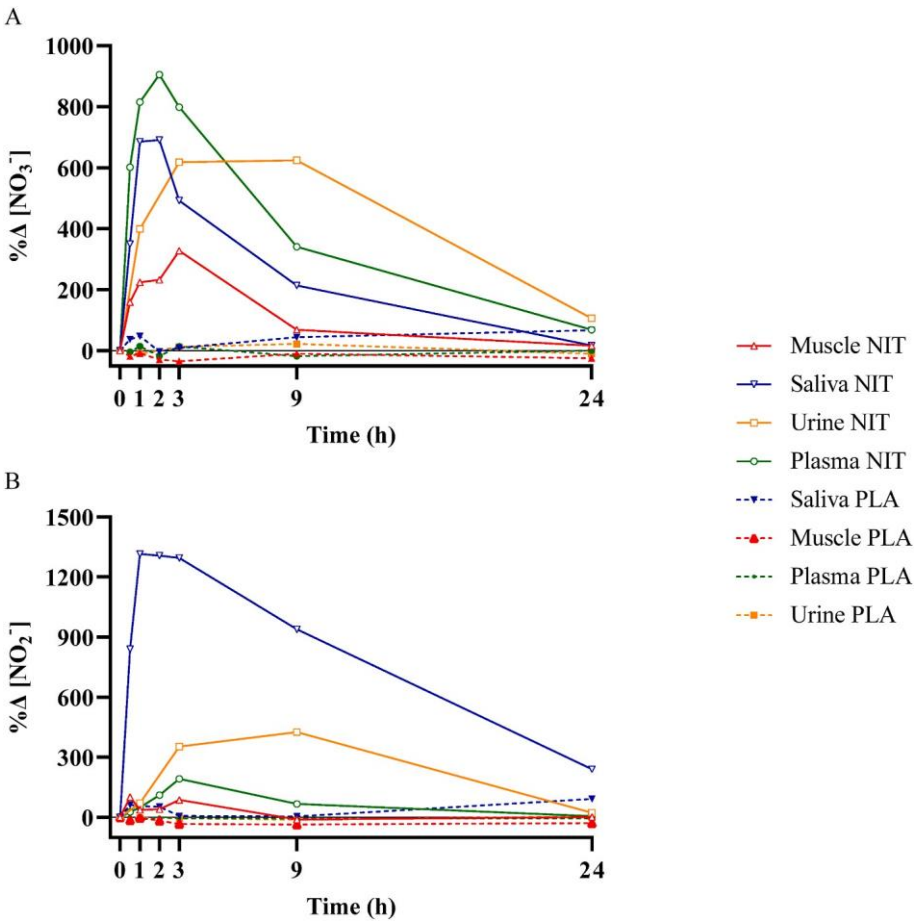

Fig. 7. The relative change compared to baseline (%Δ) in nitrate concentration ([NO_3^-]; **Panel A**) and nitrite concentration (NO_2^-; **Panel B**) in different biological compartments for 24-h following the acute ingestion of a bolus of potassium nitrate (NIT: 12.8 mmol, ~1300 mg KNO_3) or placebo (PLA). For clarity of visualisation, only group mean responses are shown.

elevation of urinary [NO_3^-] indicates that the body is still expelling significant quantities of NO_3^- 9 h following NO_3^- ingestion and is consistent with the earlier decrease in [NO_3^-] in blood and muscle.

8.2. Time course of nitrite elevation in biological tissues

NO_2^- is an important ion in NO metabolism, being both the product of NO oxidation and the precursor to NO formation depending on the prevailing physiological milieu [32,33]. Following dietary NO_3^- ingestion, salivary [NO_2^-] was significantly increased at 0.5 h, reached peak values between 1 and 3 h, and remained elevated above baseline for 24 h. Salivary [NO_3^-] and [NO_2^-] changes displayed similar temporal dynamics. In contrast, the change in plasma [NO_2^-], including time-to-peak concentration, was delayed compared to changes in plasma [NO_3^-], in accordance with previous reports [9–11,14]. This sequence of events is consistent with the existence of an enterosalivary pathway by which ingested NO_3^- that enters the bloodstream is concentrated in the salivary glands and reduced to NO_2^- via the action of anaerobic bacteria in the oral cavity [34]. This NO_2^- is subsequently swallowed, contributing to an increased circulating blood [NO_2^-], and may in turn be reduced to NO under certain physiological conditions such as tissue hypoxia [35,36]. Urinary [NO_2^-] evidenced a different time course to that of other biological compartments, being significantly increased at 3 h and 9 h before returning to baseline at 24 h following NO_3^- ingestion.

There was no significant increase in skeletal muscle [NO_2^-] at any time point following NO_3^- ingestion, although appreciable measurement and inter-individual variability may have obscured the existence of a true difference, at least at 3 h (see Fig. 2B). Similarly, Wylie et al. [18] found that muscle [NO_2^-] was elevated 3-fold by NO_3^- ingestion but this increase was also non-significant. Nyakayiru et al. [17], due to their extraction protocol, were unable to detect NO_2^- in their muscle samples. Overall, it appears that dietary NO_3^- ingestion does not consistently or appreciably increase muscle [NO_2^-] in humans. This is in contrast to rodents in which dietary NO_3^- supplementation significantly elevated both muscle [NO_3^-] and [NO_2^-] [24]. Muscle NO_3^- and NO_2^- is likely only reduced to NO 'on demand' and thus differences in the degree of activity between species may be important (i.e., the participants in our study were mainly confined to bed rest whereas the rats in earlier studies were able to move freely around their cages). It is also possible that there is a species-related difference in the activity of xanthine oxidoreductase (the main native mammalian nitrate reductase, and also nitrite reductase) in rodents and humans. It is worth noting that there are challenges to the sensitive measurement of small concentrations of NO_2^- in the relatively small muscle biopsy samples that can be harvested in human volunteers compared to the whole muscles which are excised and analysed in rodent studies [26]. However, the possibility of genuine inter-species difference in muscle [NO_2^-] should be also considered and further explored [19].

8.3. Plasma to muscle ratios for nitrate and nitrite

The plasma/muscle [NO_3^-] ratio at baseline was approximately 1, indicating a similar concentration in these two tissues. This contrasts with the two previous studies in humans, both of which reported a higher baseline [NO_3^-] in *vastus lateralis* muscle compared to plasma [17,18], and also with several investigations in rat *gluteus maximus* [15,

16]. Following dietary NO_3^- ingestion, the plasma/muscle $[NO_3^-]$ ratio widened, indicating a relatively greater increase in plasma compared to muscle $[NO_3^-]$, results which are also consistent with previous studies regardless of the muscle used [17,18,24]. Our results for the plasma/-muscle $[NO_2^-]$ ratio are, however, consistent with Wylie et al. [18], with both studies showing higher muscle compared to plasma $[NO_2^-]$ both at baseline and following NO_3^- ingestion, despite a greater relative change in plasma $[NO_2^-]$. It is possible that this difference has functional significance: skeletal muscle possesses the enzymatic machinery required for nitrite reduction to NO (i.e., xanthine oxidoreductase, aldehyde oxidase, sulphite oxidase [16]), and the presence of high NO_2^- availability may therefore be functionally more important than high NO_3^-.

It has been speculated that skeletal muscle serves as a NO_3^- store or reservoir which can be drawn on to support NO metabolism in other regions of the body, perhaps especially in situations when dietary NO_3^- intake is limited or the need for NO is suddenly increased [5,15,19]. This hypothesis was formulated on the basis of reports that skeletal muscle $[NO_3^-]$ is appreciable higher than plasma $[NO_3^-]$ such that there is a muscle-to-blood gradient for NO_3^- [15,18] and that muscle $[NO_3^-]$ not only increases following dietary NO_3^- supplementation but also falls when dietary NO_3^- is restricted [24,25]. The results of the present study challenge the original formulation of this proposal by showing that human *vastus lateralis* muscle $[NO_3^-]$ was not higher than plasma $[NO_3^-]$ either at baseline or following dietary NO_3^- supplementation. Moreover, there was no evidence in the present study that NO_3^- is stored for any longer in human muscle than in blood, with both tissues returning to baseline $[NO_3^-]$ values at 24 h following dietary NO_3^- ingestion.

It is important to note that recent studies indicate substantial differences in $[NO_3^-]$ between different skeletal muscles in the rat with the *gluteus maximus* having the highest concentration [19]. In the present study with human volunteers, we only obtained a muscle biopsy from the *vastus lateralis* and it is not known whether differences in $[NO_3^-]$ exist between different muscles in humans. This apparent paradox between earlier animal studies and human studies highlights the hazards of inter-species comparisons when formulating hypotheses. However, it is not inconceivable that the preferred storage forms for NO differ between species, owing perhaps to differences in the capacity for NO_3^- and/or NO_2^- reduction, and/or that, compared to rodents, humans rely relatively more on nitrite, *S*-nitrosothiols or some other mediator for NO production. In this respect, in humans, a muscle-to-blood NO_2^- gradient might potentially serve a similar role to the muscle-to-blood NO_3^- gradient previously identified in rodents [16]. The hypothesis that human skeletal muscle serves as a whole-body NO_3^- reservoir requires further study.

8.4. Experimental considerations and implications

This study was designed to ascertain the time course, including the time-to-peak concentration, of skeletal muscle $[NO_3^-]$ and $[NO_2^-]$ following the acute ingestion of 12.8 mmol NO_3^- and to relate it to the time course of $[NO_3^-]$ and $[NO_2^-]$ in other biological compartments (saliva, blood (including plasma, red cells and whole blood), and urine). One strength of this study was that the diet and physical activity of the participants was carefully controlled. For the 24 h preceding the experiment and during the experiment, participants were provided with a low NO_3^- (~30 mg) diet, which was designed using a custom-made database (Nick McMahon, personal communication). This enabled better isolation of the specific physiological response to the acute ingestion of NO_3^- during the experiment as well as attenuating variability between participants at baseline. Participants remained in bed (either in a supine or seated position) for the majority of the initial 9 h sample collection period, with restrictions placed on the amount of movement individuals were allowed to complete. This meant that it was possible to remove the potential unknown consequence of movement on the pharmacokinetic response to NO_3^- ingestion. It should be considered, however, that these restrictions on diet and physical activity may not reflect the typical 'real world' situation, in particular with regard to NO_3^- to NO_2^- conversion.

In the present study, participants received an acute bolus of KNO_3 when previously we have administered NO_3^- via beetroot juice [18]. It is possible that tissue NO_3^- uptake might differ between these different sources with the other bioactive components in beetroot juice facilitating a greater retention of NO_3^- and greater conversion of NO_3^- to NO_2^- [11,37].

One limitation to the present study was the number of muscle biopsies donated by the participants, which we restricted to seven. While this provided good granularity in describing the time course of muscle NO_3^- loading and retention, especially in the first 3 h following NO_3^- ingestion, the lack of measurements made between 3 h and 9 h means that we cannot exclude the possibility that peak muscle $[NO_3^-]$ and $[NO_2^-]$ was reached at a later time than 3 h. The requirement to limit the number of muscle biopsies also meant that we were unable to explore the dose-response relationship between the quantity of NO_3^- ingested and the pharmacokinetics of tissue concentration changes. The focus of our study was on muscle $[NO_3^-]$ and $[NO_2^-]$ pharmacokinetic profiles following acute bolus ingestion of NO_3^-, and the influence of chronic NO_3^- supplementation (i.e. daily NO_3^- ingestion) on muscle NO_3^- and NO_2^- retention, and plasma/muscle $[NO_3^-]$ and $[NO_2^-]$ ratios, remains to be determined.

We harvested muscle tissue via needle biopsy from the m. *vastus lateralis* because this is a relatively safe and convenient procedure in humans. However, it is possible that, as for rodents [19], there are differences in $[NO_3^-]$ and $[NO_2^-]$ between human muscles, possibly as a consequence of differences in the predominant muscle fiber type. At rest, blood flow is higher to rat skeletal muscles that are comprised predominantly of type I fibers (slow-twitch; e.g., *soleus*) compared to type II fibers (fast-twitch; e.g. *biceps femoris*) [38] and differences in blood flow and NO_3^- delivery might be expected to impact muscle NO_3^- uptake. Differences in the properties of type I and type II muscle, including with regard to O_2 delivery and the propensity for oxidative metabolism and fatigue development, have also been suggested to be relevant to the efficacy of NO_3^- supplementation for enhancing exercise performance [39,40]. The human *vastus lateralis* is a mixed muscle containing ~42% type I fibers [41] but there are substantial differences in muscle fiber typology and metabolic properties across other human muscles that might potentially impact NO_3^- storage. At the present time, it is not possible to discern whether differences between the results of the present study in humans and previous studies in rodents reflect genuine inter-species differences or methodological factors including the characteristics of the muscles sampled.

It is presently unknown whether the potential ergogenic effects of dietary NO_3^- supplementation are more strongly related to increased muscle or blood $[NO_3^-]$ and $[NO_2^-]$. However, the results of this study indicate that peak $[NO_3^-]$ and $[NO_2^-]$ in both compartments are reached at ~3 h following NO_3^- ingestion. This suggests high nitrate flow between blood and muscle compartments and enables estimation of the time at which peak muscle $[NO_3^-]$ is reached following NO_3^- ingestion. Assuming that ergogenic effects are more likely to arise when NO bioavailability is at its highest, the present results support the current recommendation to ingest NO_3^- approximately 3 h prior to the performance of exercise [5].

In summary, the present study provides the first comprehensive description of changes in skeletal muscle $[NO_3^-]$ and $[NO_2^-]$ following acute dietary NO_3^- ingestion. We show that muscle $[NO_3^-]$ rises rapidly (within 0.5 h) and reaches a peak at ~3 h before declining to the initial baseline within 24 h. The dynamics of muscle NO_3^- loading are slightly slower than saliva and blood, and faster than urine. While muscle $[NO_3^-]$ was significantly elevated following NO_3^- ingestion, the rise in muscle $[NO_2^-]$ was not statistically significant, in part due to appreciable measurement variability. Unlike some previous studies, we did

not find that muscle [NO_3^-] was higher than blood [NO_3^-] at baseline or at any other time point following NO_3^- ingestion, challenging the previously formulated hypothesis that human skeletal muscle serves as a NO_3^- reservoir, at least in the case of m. *vastus lateralis*. However, [NO_2^-] was appreciably higher in muscle than blood, raising the possibility that, in humans, a muscle NO_2^- 'store' and/or a muscle-to-blood NO_2^- gradient may have functional significance.

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EXPERIMENTAL CHAPTER 4: THE METABOLIC FATE OF DIETARY NITRATE SUPPLEMENTATION: THE INFLUENCE OF ACUTE DIETARY NITRATE SUPPLEMENTATION AND ITS RELATIVE CONTRIBUTION TO SKELETAL MUSCLE NITRATE AND NITRITE AT REST.

From:

^{15}N-LABELLED DIETARY NITRATE SUPPLEMENTATION INCREASES HUMAN SKELETAL MUSCLE NITRATE CONCENTRATION AND IMPROVES MUSCLE TORQUE PRODUCTION

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ABSTRACT

Introduction: Dietary nitrate (NO_3^-) supplementation increases nitric oxide (NO) bioavailability and can enhance exercise performance and health. Once ingested, concentrations of NO_3^- increase in biological fluids such as saliva, blood, and urine. However, there are also transient fluxes in NO_3^- within biological tissues with skeletal muscle recently being implicated as being central in NO homeostasis. These compartments all are integrated in processes sustaining NO availability through the human body. However, to date, no research has evidenced the contribution of exogenously supplied NO_3^- compared to that produced endogenously within the body. We therefore investigated the distribution and metabolic fate of ingested NO_3^- at rest with a specific focus on skeletal muscle.

Methods: Ten healthy volunteers consumed 12.8 mmol ^{15}N-labelled potassium nitrate ($K^{15}NO_3$; NIT). Muscle biopsies were taken at baseline, at 1-h and 3-h post-supplement ingestion. Muscle, plasma, saliva and urine samples were analysed using chemiluminescence to determine absolute [NO_3^-] and [NO_2^-], and by mass spectrometry to determine the proportion of NO_3^- and NO_2^- that was ^{15}N-labelled.

Results: Following NIT, muscle [NO_3^-] (but not [NO_2^-]) was elevated at both 1-h (from ~35 to 147 nmol.g^{-1}, P<0.001) and 3-h, with almost all of the increase being ^{15}N-labelled.

Conclusion: This study shows, for the first time, that skeletal muscle rapidly takes up dietary NO_3^- and that the elevated muscle [NO_3^-] is almost entirely consequent to exogenous NO_3^-.

INTRODUCTION

The signalling molecule, nitric oxide (NO), is essential for the maintenance of normal physiological function (Moncada et al. 1991; Lundberg et al. 2008). The highly reactive nature and relatively short half-life of NO means that sustained provision of this molecule may be compromised if it is not continually synthesised. Following its production from L-arginine in a reaction catalysed by the nitric oxide synthase (NOS) enzymes, NO may be oxidized to form the more stable metabolites, nitrite (NO_2^-) and nitrate (NO_3^-). NO_2^- and NO_3^- are now considered to be storage forms of NO since they can be also reduced under appropriate physiological conditions (i.e., low PO_2) to form NO non-enzymatically (Piknova et al. 2022). Tissue and whole body NO homeostasis is regulated via the synergistic relationship between these complementary oxidation (L-arginine-NOS-NO) and reduction (NO_3^- - NO_2^- - NO) pathways.

Following dietary NO_3^- ingestion or supplementation, several studies have described elevations in [NO_3^-] and [NO_2^-] in biological tissues such as saliva (Spiegelhalder et al. 1976; McDonagh et al. 2018; Capper et al. 2022), plasma (Webb et al. 2008; Wylie et al. 2013; Jonvik et al. 2016), urine (Bartholomew and Hill, 1984; Pannala et al. 2003; McDonagh et al. 2018) and, most recently, skeletal muscle (Piknova et al. 2015; Piknova et al. 2016; Nyakayiru et al. 2017; Wylie et al. 2019; Kadach et al. 2022). The augmentation of body NO bioavailability following dietary NO_3^- ingestion may have important physiological and therapeutic effects (DeMartino et al. 2018; Jones et al. 2021).

At any given time, skeletal muscle [NO_3^-] reflects the balance between metabolism of NO_3^- into other nitrogen-containing species, oxidation of NO produced via NOS into NO_3^-, and NO_3^- and NO_2^- exchange between muscle and blood with the latter facilitated by sialin (Qin et al. 2012) and chloride channels (Srihirun et al. 2020). While muscle [NO_3^-] has been

shown to be elevated following dietary NO_3^- supplementation (Piknova et al. 2015; Nyakayiru et al. 2017; Wylie et al. 2019; Kadach et al. 2022), the extent to which this results from direct accretion of the exogenously-supplied NO_3^-, via uptake from the circulation, is uncertain. Determining the proportional contribution of exogenous NO_3^- and endogenously-generated NO_3^- to total [NO_3^-] in muscle as well as other tissues, following dietary NO_3^- supplementation, would provide important insight into the distribution of ingested NO_3^-. Recent observations that muscle [NO_3^-] is higher than blood [NO_3^-] in both rodents (*gluteus maximus;* Piknova et al. 2015; 2016) and humans (*vastus lateralis*; Nyakayiru et al. 2017; Wylie et al. 2019) has led to speculation that this relatively high muscle [NO_3^-] may have functional significance (Piknova et al. 2022). It has been suggested that skeletal muscle serves as a NO_3^- 'reservoir' that might be drawn upon, via the circulation, to enhance NO bioavailability in other tissues when access to dietary NO_3^- is restricted (Piknova et al. 2015; Gilliard et al. 2018; Jones et al. 2021). Moreover, skeletal muscle possesses the enzymatic machinery required for the reduction of NO_3^- and NO_2^- to NO (i.e., xanthine oxidoreductase (XO), aldehyde oxidase (AO), mitochondrial amidoxime-reducing component (MARC); Piknova et al. 2016; Maia and Moura, 2018). It is therefore possible that local muscular NO_3^- and/or NO_2^- stores are important in muscle function, including the regulation of contractile activity, blood flow distribution and mitochondrial respiration, particularly during exercise when lower PO_2 and pH may favour non-enzymatic NO production (Piknova et al. 2022).

The purpose of the present investigation was to employ a stable isotope tracer ($K^{15}NO_3$) to determine the distribution and metabolic fate of ingested dietary NO_3^- at rest. To provide insight into the relative contribution of exogenously-supplied vs endogenously-generated NO_3^- on [NO_3^-] and [NO_2^-] in skeletal muscle as well as other tissues, we measured the

absolute concentrations of NO_3^- and NO_2^- using chemiluminescence and the percentage of ^{15}N labelled NO_3^- and NO_2^- by ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS). We hypothesised that there would be significant increases in skeletal muscle [NO_3^-] following the ingestion of a $K^{15}NO_3$ tracer, with this increase being consequent to the accretion of the labelled NO_3^- onto an unchanged basal [NO_3^-].

METHODS

This study was approved by the Sport and Health Sciences Ethics Committee (University of Exeter) in line with the principles of the Declaration of Helsinki. Once the associated risks and benefits of the investigation were thoroughly explained, all participants that enrolled in the study provided written consent before taking part in any experimental procedures.

Participants

Inclusion criteria were ostensibly healthy men and women, free of cardiovascular, respiratory, metabolic and musculoskeletal disorders, or having any contraindication to maximal exercise. Exclusion criteria included use of antibacterial mouthwash or tongue scrapers, dietary supplements, blood pressure medication, and tobacco smoking. Ten healthy males (age: 23 ± 4 years, height: 1.80 ± 0.07 m, body mass: 87.7 ± 8.5 kg, BMI: 26.4 ± 1.0 kg/m^2) volunteered for the study.

Experimental Design

Participants ingested a $K^{15}NO_3^-$ tracer (NIT group: 12.8 mmol, ~1300 mg NO_3^-; 1g/L, 99 % ^{15}N, CK Isotopes, Desford, UK). To minimise the number of muscle biopsies and because we have previously shown that no changes occur in skeletal muscle [NO_3^-] following the ingestion of PLA (Wylie et al. 2019; Kadach et al. 2022), a PLA condition was not included.

To control for potential variations in the participants' habitual diets, a 3-day dietary control period preceded the experimental visits. These consisted of an initial two day period in which participants abstained from consuming foods with high concentrations of NO_3^- and NO_2^- and a final day during which participants were provided with a controlled diet containing ~25-30 mg NO_3^-. The experimental visits were separated by a minimum of 7 days and a maximum of 10 days.

On each experimental visit, participants arrived at the laboratory in a rested and fasted state at 07:30 a.m. Upon arrival, participants were asked a series of questions regarding their adherence to the prescribed diet. An initial urine sample was collected after which participants were seated on a bed and were requested to refrain from excessive movement for the remainder of the sample collection period. A saliva sample was then collected, and an intravenous cannula was inserted into the antecubital fossa and a blood sample was collected. Preparations for the muscle biopsies were completed after the initial blood samples had been processed and the initial muscle tissue sample was collected. Following the biopsy, a low NO_3^- breakfast (two slices of toast with 10 g butter) was provided at ~08:50 a.m., and at 09:00 a.m. the $K^{15}NO_3^-$ tracer was ingested in the form of a 140 mL drink. The drink was created on the morning of the visit by dissolving 1.31 g of the $K^{15}NO_3$ tracer (NIT, 12.8 mmol, ~1300 mg NO_3^-) in 140 mL deionised water. The container was vigorously shaken to ensure that the powder had fully dissolved before the participant ingested the drink. All subsequent biological samples were collected in relation to the supplement ingestion time. The collection time of the muscle tissue extraction was at 1-h and 3-h post-supplement ingestion, with saliva and blood collected before, and urine collected after, the muscle sampling was completed.

Sample Collection

Muscle tissue samples were collected from the *vastus lateralis* muscle using a modified percutaneous Bergström needle procedure adapted for manual vacuum (Bergström, 1975). These samples were collected at rest and at 1-h and 3-h post supplement ingestion. A venous cannula (20 G Insyte-WTM cannula; Becton Dickinson, Madrid, Spain) inserted in the antecubital fossa was used to draw blood samples for the determination of plasma [NO_3^-] and [NO_2^-]. These samples were collected in lithium heparin vacutainers (Becton Dickinson, NJ) and centrifuged at 3300 g for 7 min at 4°C. The extracted plasma was then placed in liquid nitrogen before being stored in a -80°C freezer. Two-min saliva collection periods were employed to enable participants to generate sufficient saliva before expelling it into a 30-mL universal tube (Thermo Scientific^{TM} Sterilin^{TM}; Massachusetts, USA). The saliva was then aliquoted and placed in liquid nitrogen before being stored at -80°C. Urine samples were collected in separate containers (Kartell^{TM}; Milan, Italy) and aliquoted into micro-centrifuge tubes for storage.

Measurement of total [NO_3^-] and [NO_2^-] in biological samples

Ozone-based gas-phase chemiluminescence was utilised to quantify [NO_3^-] and [NO_2^-] in the biological samples collected during the study using helium as the carrier gas (Sievers 280i Nitric Oxide Analyser, GE Analytical Instruments, Boulder, CO, USA). The initial step for sample processing prior to injection into the NO analyser was to add methanol to the plasma, urine and saliva samples (1:2 ratio by volume). These were thoroughly vortexed, left to incubate at room temperature for 30 min and subsequently centrifuged at 4°C and 11,000 g for 5 min. The supernatant was then collected and injected into the analyser configuration. Vanadium chloride or tri-iodide solution was used for nitrate or nitrite analysis, respectively. Muscle samples were weighed and processed to ensure consistency between the sample sizes

(~40-60 mg), a NO_2^- preservation solution was added ($K_3Fe(CN)_6$, *N*-ethylmaleimide, water, Nonidet P-40), and then the samples underwent a series of homogenisation steps using a bead homogeniser (Bertin Minilys, Bertin Instruments, France). Muscle [NO_3^-] and [NO_2^-] was then determined using the methods described by Park et al. (2021b). For all tissues, part of the supernatant was processed for UPLC-MS/MS analysis (see below) and the rest was used for NOA.

Determination of $^{15}NO_3^-$ or $^{15}NO_2^-$ proportion by UPLC-MS/MS

To measure NO_3^- content by UPLC-MS/MS, NO_3^- in all samples were first reduced to NO_2^- enzymatically by bacterial nitrate reductase from Aspergillus niger (N7265, Sigma-Aldrich, St. Louis, MO, USA) as previously described (Chao et al. 2016) with some modification. Briefly, the sample (20 µl, or 2 µl for urine) was mixed with nitrate reductase (0.1 U/ml) and NADPH (100 µM) and incubated for 2 h at room temperature. Then NO_2^- in samples were derivatized with 2,3-diaminonaphthalene (DAN, D2757, Sigma-Aldrich, 5 mM) for 30 min at 37°C to yield 2,3-naphthotriazole (NAT). NaOH (58 mM) was added to terminate the reaction. For measuring NO_2^- content only, samples (50 µl) were directly subjected to DAN derivatization.

High-performance liquid chromatography (HPLC) grade solvents and LC-MS modifiers were purchased from Sigma-Aldrich (St. Louis, MO, USA). Detection and quantification were achieved by ultra-performance liquid chromatography - tandem mass spectrometry (UPLC-MS/MS) utilizing a Thermo Scientific Vanquish UPLC with a Thermo Scientific Altis triple quadrupole mass spectrometer, heated electrospray ionization (HESI-II) in positive ion mode (3500V). 50 µl of sample was mixed with 200 µl of acetonitrile (ACN), vortexed for 5 min and then centrifuged at 4°C and 17,000 g for 15 min. The supernatant was transferred to an LC-MS vial for analysis. Injection volume was 1 µl. A Waters Cortecs T3 column, 2.1 x 100

mm, 1.6 µm column was maintained at 35°C. Solvent A: H_2O with 0.1% formic acid (FA) and Solvent B: ACN with 0.1% FA. The flow rate was 250 µl/min, the gradient was 25% B at 0 min for 0.25 min, increasing to 65% B at 5 min, further increased to 90% B at 5.5 min, remained at 90% B until 7.5 min, and then decreased to 25% B at 8 min. The total running time was 10 min. Samples were analysed in triplicate. Quantitation of ^{14}NAT and ^{15}NAT were based on multiple reaction monitoring (MRM) transitions *m/z*, 170.062 → 115.042 and 171.062 → 115.042, respectively. The result was based on the percentage ratio of ^{15}NAT/(^{14}NAT + ^{15}NAT).

Statistical Analysis

The Statistical Package for Social Scientists (SPSS Version 28, SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the data. Two-way repeated measures ANOVAs were used to determine differences in [NO_3^-] and [NO_2^-] in muscle, plasma, saliva and urine across time (pre-exercise and post-exercise) and condition (PLA and NIT). A separate one-way repeated measures ANOVA was also run for the NIT condition for the exercise component of the study which included a control and experimental leg. Where appropriate, significant main and interaction effects were analysed further using least significant difference (LSD) post hoc tests. Relationships between variables were evaluated using Pearson's product moment correlation coefficients. The alpha level to denote statistical significance was $P<0.05$. All results are expressed as mean ± standard deviation (SD).

RESULTS

Distribution of Ingested NO_3^- at Rest: Skeletal Muscle [NO_3^-] and [NO_2^-]

Total skeletal muscle [NO_3^-] was elevated above baseline at 1-h (from 35 ± 9 to 147 ± 71 nmol.g^{-1}, $P<0.001$) before falling slightly but remaining elevated compared to baseline at 3-h

following NO_3^- ingestion (105 ± 41 nmol.g^{-1}, P<0.001), (**Figure 2A**). ^{15}N labelled muscle [NO_3^-] was elevated above baseline at both 1-h (100 ± 63 nmol.g^{-1}, P<0.001) and 3-h (50 ± 25 nmol.g^{-1}, P<0.001), with the value at 1-h being higher than that at 3-h (P<0.05). Endogenous (i.e., unlabelled) muscle [NO_3^-] did not increase above baseline at 1-h (47 ± 16 nmol.g^{-1}) but was higher than baseline at 3-h (55 ± 21 nmol.g^{-1}, P<0.05). There was no change in total muscle [NO_2^-] following NO_3^- ingestion (**Figure 2B**). ^{15}N labelled muscle [NO_2^-**]** was greater than baseline at 1-h (0.12 ± 0.14 nmol.g^{-1}, P<0.05), and tended to be higher at 3-h (0.40 ± 0.54 nmol.g^{-1}, P=0.05) whereas unlabelled muscle [NO_2^-] was unchanged.

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

Total plasma [NO_3^-] was elevated above baseline at both 1-h and 3-h (from 29 ± 6 to 451 ± 46 and 431 ± 48 nmol.g^{-1}, respectively, P<0.001) following NO_3^- ingestion, with no difference between 1-h and 3-h (**Figure 2C**). ^{15}N labelled plasma [NO_3^-] was increased above baseline at 1-h (423 ± 45 nmol.g^{-1}; P<0.001) and 3-h (405 ± 45 nmol.g^{-1}; P<0.001). Unlabelled plasma [NO_3^-] was not different from baseline at 1-h (from 28 ± 6 to 28 ± 5 nmol.g^{-1}) but was lower than baseline at 3-h (27 ± 6 nmol.g^{-1}; P<0.05). Total plasma [NO_2^-] was greater than baseline at 1-h (from 0.13 ± 0.02 to 0.29 ± 0.07 nmol.g^{-1}; P<0.001) and 3-h (0.47 ± 0.14 nmol.g^{-1}; P<0.001) and the value at 3-h was greater than that at 1-h (P<0.01), (**Figure 2D**). ^{15}N labelled [NO_2^-] was greater than baseline at 1-h (0.08 ± 0.04 nmol.g^{-1}, P<0.001) and 3-h (0.21 ± 0.10 nmol.g^{-1}; P<0.001) and the value at 3-h was greater than that at 1-h (P<0.05). Unlabelled plasma [NO_2^-] was also elevated above baseline at 1-h (from 0.12 ± 0.02 to 0.20 ± 0.03 nmol.g^{-1}; P<0.001) and 3-h (0.26 ± 0.05 nmol.g^{-1}; P<0.001), and the value at 3-h was greater than that at 1-h (P<0.05).

Salivary [NO_3^-] and [NO_2^-]

Total salivary [NO_3^-] was increased above baseline at both 1-h and 3-h following NO_3^- ingestion (Baseline: 194 ± 182; 1-h: 14364 ± 5782; 3-h: 16536 ± 8300 nmol.g^{-1}, both P<0.001) and there was no difference between 1-h and 3-h (**Figure 2E**). Both ^{15}N labelled and unlabelled salivary [NO_3^-] were greater than baseline at 1-h and 3-h. Total salivary [NO_2^-] was increased above baseline at both 1-h and 3-h (Baseline: 229 ± 213; 1-h: 2530 ± 1775; 3-h: 3108 ± 1460 nmol.g^{-1}; both P<0.001), (**Figure 2F**). ^{15}N labelled salivary [NO_2^-] was greater than baseline at both 1-h (P<0.05) and 3-h (P<0.001) but there was no change in unlabelled salivary [NO_2^-] following NO_3^- ingestion.

Urinary [NO_3^-] and [NO_2^-]

Total urinary [NO_3^-] was greater than baseline at 1-h (from 739 ± 225 to 2567 ± 1360 nmol.g^{-1}, P<0.05) and 3-h (2466 ± 1070 nmol.g^{-1}; P<0.001) following NO_3^- ingestion, and there was no difference between 1-h and 3-h (**Figure 2G**). ^{15}N labelled urinary [NO_3^-] was greater than baseline at 1-h and 3-h (P<0.001) whereas unlabelled urinary [NO_3^-] was greater at baseline compared to 1-h (P<0.05) and 3-h (P<0.001). Total urinary [NO_2^-] was not different from baseline at either 1-h or 3-h (**Figure 2H**). ^{15}N labelled urinary [NO_2^-] was greater than baseline at 1-h (P<0.001) and 3-h (P<0.05). In contrast, unlabelled urinary [NO_2^-] was lower than baseline at 1-h and 3-h (P<0.05).

Plasma/Muscle [NO_3^-] and Plasma/Muscle [NO_2^-] Ratios

At baseline, muscle [NO_3^-] was greater than plasma [NO_3^-] (ratio of 0.8; plasma: 28 ± 6; muscle: 35 ± 9 nmol.g^{-1}; P<0.05). Following NO_3^- ingestion, the ratio increased to 3.6 at 1-h (plasma: 451 ± 46; muscle: 160 ± 63 nmol.g^{-1}; P<0.001) and 4.4 at 3-h (plasma: 431 ± 56; muscle: 111 ± 39 nmol.g^{-1}; P<0.001). The plasma/muscle [NO_3^-] ratio was greater than baseline at both 1-h (P<0.05) and 3-h (P<0.001) (**Figure 3A**). At baseline, plasma [NO_2^-] was lower than muscle [NO_2^-] (ratio of 0.18; plasma: 0.13 ± 0.02; muscle: 0.91 ± 0.51 nmol.g^{-1};

P<0.05). Following NO_3^- ingestion, the ratio was 0.45 at 1-h (plasma: 0.29 ± 0.07; muscle: 0.69 ± 0.23 nmol.g^{-1}, *P*<0.001) and 0.63 at 3-h (plasma: 0.48 ± 0.14; muscle: 0.94 ± 0.48 nmol.g^{-1}; *P*<0.05). The plasma/muscle $[NO_2^-]$ ratio was greater than baseline at both 1-h and 3-h (*P*<0.01), (**Figure 3B**).

DISCUSSION

We used a stable isotope tracer ($K^{15}NO_3$) to determine the distribution of ingested dietary NO_3^- at rest and its metabolic fate during exercise. The principal original findings of the present study confirmed our hypotheses by showing that: 1) labelled dietary NO_3^- accumulates in skeletal muscle within 1-h of its ingestion, elevating total muscle $[NO_3^-]$ in the absence of changes in basal (unlabelled) $[NO_3^-]$.

Distribution of Ingested NO_3^- at Rest

Several previous studies have shown that tissue and body fluid (e.g., skeletal muscle, plasma, saliva, urine) $[NO_3^-]$ and/or $[NO_2^-]$ are increased following dietary NO_3^- supplementation (Webb et al. 2008; Wylie et al. 2013; Piknova et al. 2016; Nyakayiru et al. 2017; McDonagh et al. 2018; Wylie et al. 2019; Kadach et al. 2022) and it has been assumed that it is the ingested NO_3^- that is directly responsible for this increase. However, NO_3^- is also derived from NOS activity and it is important to clearly distinguish whether dietary NO_3^-, *per se*, is responsible for the observed increases in tissue $[NO_3^-]$ and $[NO_2^-]$. In the present study we employed a stable isotope tracer ($K^{15}NO_3$) to determine the distribution of ingested dietary NO_3^-. To achieve this, we used standard chemiluminescence to measure the absolute $[NO_3^-]$ and $[NO_2^-]$ in skeletal muscle, plasma, saliva and urine, and we determined the percentage of $^{15}NO_3^-$ and $^{15}NO_2^-$ in those same tissues and bodily fluids using UPLC-MS/MS. Our results confirm that elevations in tissue and body fluid $[NO_3^-]$ and $[NO_2^-]$ following dietary NO_3^-

supplementation are almost exclusively consequent to the introduction of exogenous NO_3^- to the body.

The profiles of changes in [NO_3^-] above baseline following dietary NO_3^- supplementation were broadly similar in muscle, plasma, saliva and urine, with rapid dynamics (i.e., a significant increase within 1-h of NO_3^- ingestion) and with [NO_3^-] remaining elevated at 3-h. These findings are consistent with earlier reports (Pannala et al. 2003; Wylie et al. 2013; Burleigh et al. 2018; McDonagh et al. 2018; Capper et al. 2022; Kadach et al. 2022). Interestingly, at 1-h, ^{15}N labelled NO_3^- accounted for ~94%, 96% and 89% of the total NO_3^- present in plasma, saliva and urine, respectively, but only ~68% of the total NO_3^- in muscle. This is consistent with earlier reports of a relatively smaller change in muscle [NO_3^-] compared to plasma [NO_3^-] following NO_3^- ingestion (Wylie et al. 2019; Kadach et al. 2022) and may suggest the existence of a barrier to the entry of NO_3^- into muscle such as saturation of the sialin transporter or chloride channels (Qin et al. 2012; Wylie et al. 2019; Srihirun et al. 2020).

Consistent with our previous study (Kadach et al. 2022), there were significant increases in total salivary [NO_2^-] and total plasma [NO_2^-] but no change in total muscle [NO_2^-] or total urinary [NO_2^-] at 1-h or 3-h following NO_3^- ingestion. The temporal profiles of salivary and plasma [NO_2^-] we observed are consistent with our understanding of the nitrate-nitrite-NO pathway (Lundberg et al. 2008) in which ingested NO_3^- enters the enterosalivary circulation, is concentrated by the salivary gland and is then reduced to NO_2^- by the oral microbiota before being swallowed and entering the bloodstream, resulting in increased plasma [NO_2^-]. We found that the increased total salivary [NO_2^-] was entirely due to increased ^{15}N labelled [NO_2^-] whereas the increased total plasma [NO_2^-] resulted from increases in both labelled and unlabelled [NO_2^-]. The lack of change in total muscle [NO_2^-] following NO_3^- ingestion is

consistent with our previous work (Wylie et al. 2019; Kadach et al. 2022). It is noteworthy that these results in humans are in contrast to rodent studies in which muscle [NO_2^-] is significantly increased by NO_3^- ingestion (Gilliard et al. 2018). Possible explanations for these inter-species differences were discussed previously (Kadach et al. 2022) but might also include differences between bolus ingestion (present study) and continuous feeding of NO_3^- via drinking water (Gilliard et al. 2018). Despite the lack of change in total muscle [NO_2^-] in the present study, there was a significant increase in ^{15}N labelled [NO_2^-] at 1-h post NO_3^- ingestion, which might be due either to local NO_3^- reduction or absorption from the circulation.

An intriguing finding in the present study was that total muscle [NO_3^-] fell by ~29% from 1-h to 3-h. This is in contrast to our previous study in which muscle [NO_3^-] was unchanged over the same time period following the same acute NO_3^- ingestion protocol (Kadach et al. 2022). Moreover, we found that the ^{15}N labelled fraction of the total muscle [NO_3^-] fell from 100 to 50 nmol.g^{-1}, mirrored by a non-significant rise in labelled muscle [NO_2^-], whereas the unlabelled fraction of the total muscle [NO_3^-] increased from 34 to 47 nmol.g^{-1}. These changes over time suggest continued 'processing' of the ingested NO_3^- within muscle which might include reduction to NO_2^- and other nitrogen-containing metabolites, and potentially cross-talk with NOS-mediated NO generation. However, interpretation of these data, along with the finding of an increased unlabelled plasma [NO_2^-] following ^{15}N labelled NO_3^- ingestion, is complicated by the possibility of continued movement of labelled and unlabelled NO_3^- and NO_2^- between the muscle and bloodstream, or *vice versa*, which we were unable to quantify using the present experimental model.

At baseline, skeletal muscle [NO_3^-] was greater than plasma [NO_3^-] with the plasma/muscle [NO_3^-] ratio being ~0.8. This is in agreement with previous findings in rodents (Piknova et al.

2015; Piknova et al. 2016) and humans (Nyakayiru et al. 2017; Wylie et al. 2019; cf. Kadach et al. 2022). It has been suggested that this NO_3^- concentration gradient between skeletal muscle and plasma enables passive diffusion of NO_3^- from muscle to support NO requirements in other tissues or organs especially when demand is high or dietary NO_3^- intake is restricted (Piknova et al. 2015; Gilliard et al. 2018; Jones et al. 2021; Piknova et al. 2022). Following NO_3^- ingestion, the plasma/muscle [NO_3^-] ratio increased to 3.6 and 4.4 at 1-h and 3-h, respectively, reversing the muscle-to-plasma NO_3^- concentration gradient that was evident at baseline. Immediately following NO_3^- ingestion, it is possible that the negative plasma-to-muscle NO_3^- gradient mandates that muscle NO_3^- accretion depends on active NO_3^- transport via sialin and/or the chloride channels, whereas when the plasma-to-muscle NO_3^- gradient later becomes positive, muscle NO_3^- uptake from blood may also occur via diffusion. We confirmed a previous report (Kadach et al. 2022) that, in humans, muscle [NO_2^-] is considerably greater both in the basal state (plasma/muscle [NO_2^-] ratio of 0.18) and following NO_3^- ingestion (plasma/muscle [NO_2^-] ratio of 0.63 at 3-h). This substantial muscle-to-plasma NO_2^- concentration gradient in humans differs from that reported in rodents (Piknova et al. 2015; Piknova et al. 2016) and, while NO_3^- is considered more stable and therefore better suited to a role as an NO storage molecule (Piknova et al. 2022), it is possible that NO_2^- is favoured for this purpose in humans (Kadach et al. 2022).

In conclusion, we used a stable isotope tracer ($K^{15}NO_3$) to show that acute inorganic NO_3^- ingestion increases plasma, salivary and urinary [NO_3^-] and that the ingested NO_3^- is also taken up rapidly by skeletal muscle.

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Figure Legends

Figure 2: Mean ± SD skeletal muscle [NO_3^-] (**Panel A**) and [NO_2^-] (**Panel B**), plasma [NO_3^-] (**Panel C**) and [NO_2^-] (**Panel D**), salivary [NO_3^-] (**Panel E**) and [NO_2^-] (**Panel F**), and urinary [NO_3^-] (**Panel G**) and [NO_2^-] (**Panel H**), prior to (0-h) and at 1-h and 3-h following the ingestion of $K^{15}NO_3$ (12.8 mmol NO_3^-; ~1300 mg). The height of the bars represent the total concentrations and the proportion of unlabelled and ^{15}N labelled NO_3^- and NO_2^- is shown in light blue and cerise, respectively. a = significant difference compared to 0-h ($P<0.05$); b = significant difference between 1-h and 3-h ($P<0.05$); black, blue and cerise letters refer to comparisons between total, unlabelled and ^{15}N labelled data, respectively.

Figure 3: Mean ± SD plasma/muscle ([NO_3^-] ratio (**Panel A**) and plasma/muscle [NO_2^-] ratio (**Panel B**) prior to (0-h) and at 1-h and 3-h following the ingestion of a $K^{15}NO_3$ supplement. a = significant difference compared to 0-h ($P<0.05$).

Figure 2.

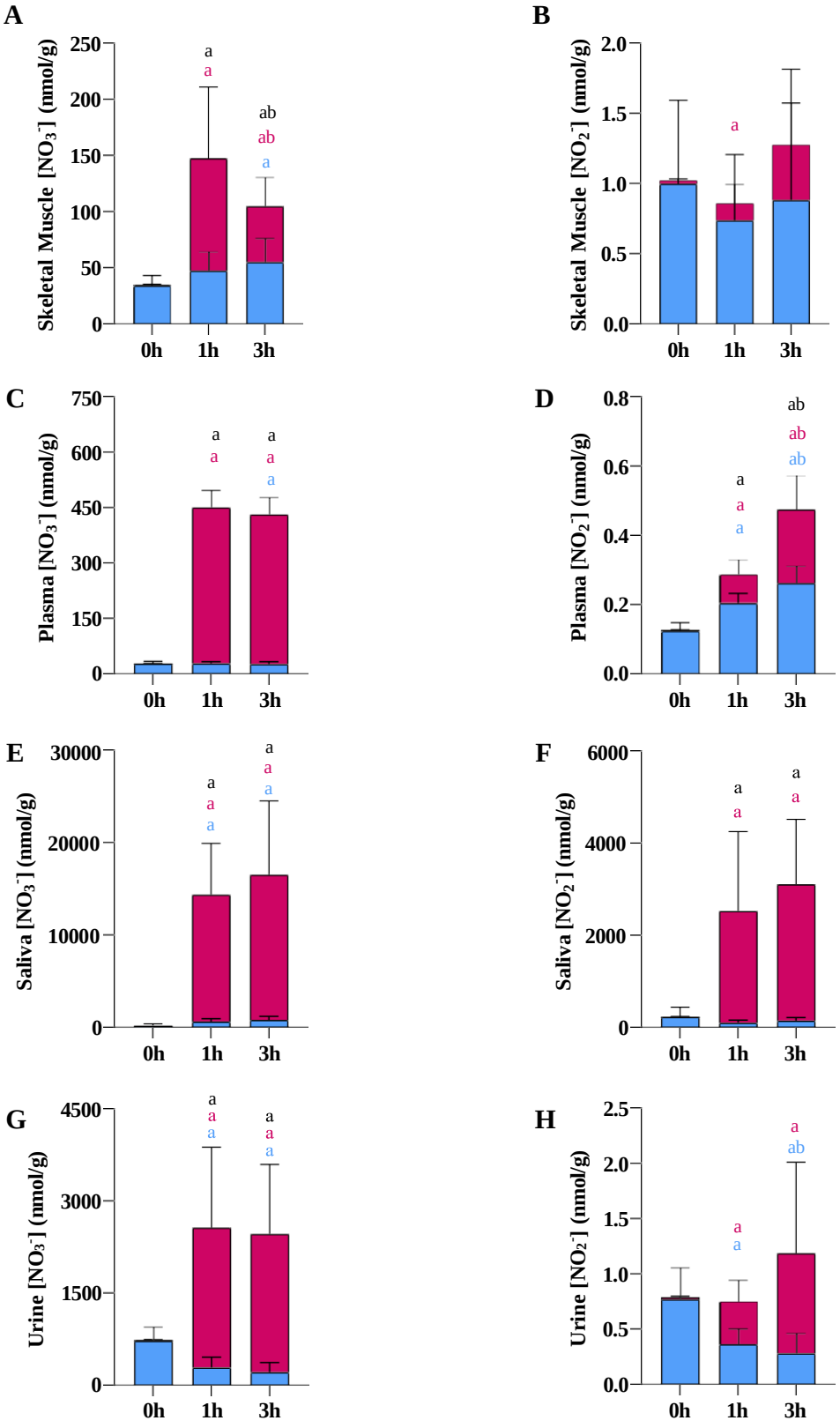
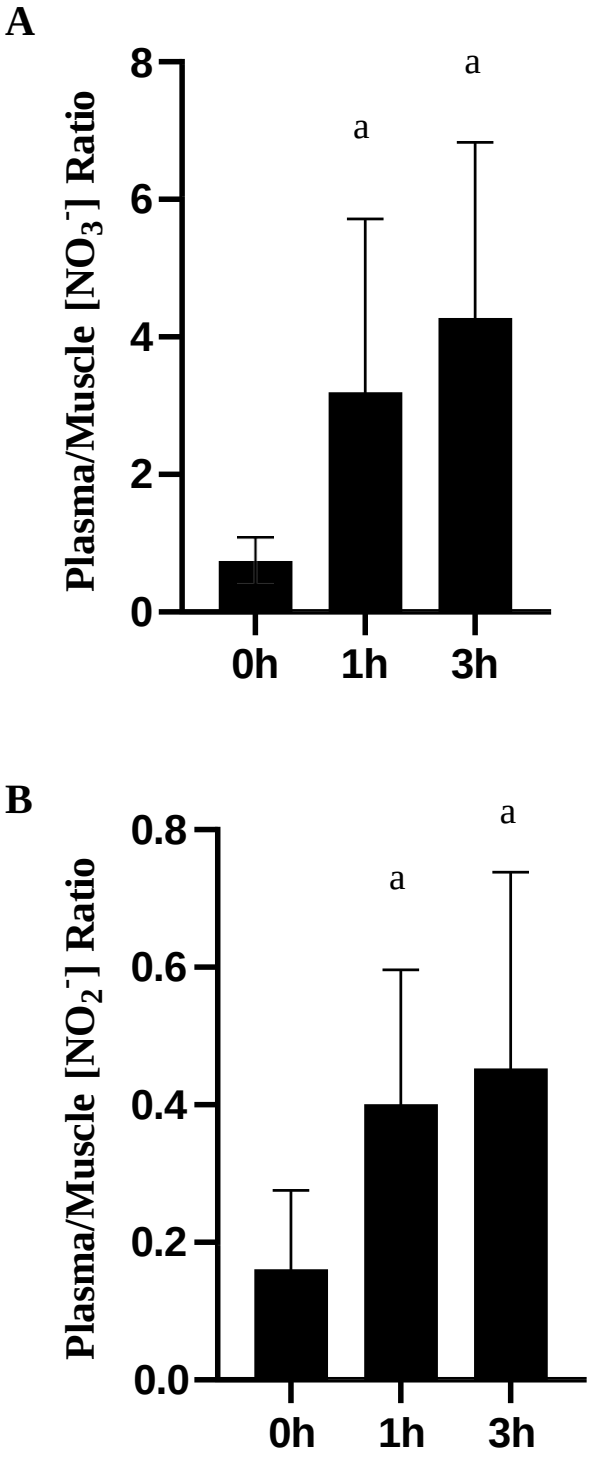

A
Skeletal Muscle [NO3-] (nmol/g)
250
200
150
100
50
0
a
a
ab
ab
a
0h
1h
3h
B
Skeletal Muscle [NO2-] (nmol/g)
2.0
1.5
1.0
0.5
0.0
a
0h
1h
3h
C
Plasma [NO3-] (nmol/g)
750
600
450
300
150
0
a
a
a
a
0h
1h
3h
D
Plasma [NO2-] (nmol/g)
0.8
0.6
0.4
0.2
0.0
ab
ab
ab
a
a
a
0h
1h
3h
E
Saliva [NO3-] (nmol/g)
30000
20000
10000
0
a
a
a
a
a
a
0h
1h
3h
F
Saliva [NO2-] (nmol/g)
6000
4000
2000
0
a
a
a
a
0h
1h
3h
G
Urine [NO3-] (nmol/g)
4500
3000
1500
0
a
a
a
a
a
a
0h
1h
3h
H
Urine [NO2-] (nmol/g)
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
a
a
0h
1h
3h

Figure 3.


A
Plasma/Muscle [NO3-] Ratio
8
6
4
2
0
a
a
0h
1h
3h
B
Plasma/Muscle [NO2-] Ratio
0.8
0.6
0.4
0.2
0.0
a
a
0h
1h
3h

CHAPTER 9: GENERAL DISCUSSION

The presence of NO_3^- within the diet provides an opportunity for endogenous NO generation to be supplemented. This results in greater [NO_3^-] and [NO_2^-] within the body, therefore altering NO homeostasis (Lundberg et al. 2015; Jones et al. 2020). The mouth is the gateway to the human body and possesses the characteristic of containing an active microbiome. This is comprised of bacteria central to NO_3^- metabolism as these have an inherent reductase capacity which is essential in NO generation (Li et al. 1997; Govoni et al. 2008; Kapil et al. 2013; McDonagh et al. 2015). Interestingly, the addition of NO_3^- to a normal diet elicits favourable alterations in the relative abundance of certain bacteria which are integral in the reduction of NO_3^- to NO_2^- in the mouth (Vanhatalo et al. 2018; Burleigh et al. 2018). One goal of the present thesis was to investigate whether it was possible to by-pass the oral microbiome by using a novel NO_3^- containing skin lotion on circulating [NO_3^-] as this may provide a practical alternative for individuals that possess a compromised oral microbial profile (i.e. in certain clinical conditions) or who do not wish to ingest dietary NO_3^- supplements. The delicate nature of the relationship between microbiome and host is easily altered by factors such as increased NO_3^- ingestion (Vanhatalo et al. 2018; Rosier et al. 2020). However, with significant variance in habitual dietary NO_3^- intake occurring in differing populations, a notion which is entirely feasible in today's society, it is also important to consider how the absence of NO_3^- may influence the microbial profile of the mouth. This was therefore a question which we also aimed to address.

Piknova and colleagues (2015) discovered that, in rodents, skeletal muscle contained substantial quantities of NO_3^- consequent to the endogenous generation of NO via NOS. Since then, several studies from the laboratory led by Prof. Alan Schechter at the US National Institutes of Health, Bethesda, have advanced understanding of skeletal muscle NO_3^- and

NO_2^- storage and metabolism, including how the concentrations of these NO-related metabolites can be increased following dietary NO_3^- ingestion (Piknova et al. 2016; Gilliard et al. 2018; Park et al. 2021). Importantly, NO is continuously produced by skeletal muscle at rest and during exercise (Balon et al. 1994) and is likely central in modulating signalling cascades and proteins which regulate contractile function, metabolism and perfusion (Stamler et al. 2001). Recently, it has been proposed that these effects might be facilitated by the presence of NO_3^- and NO_2^- in skeletal muscle (Jones et al. 2020; Piknova et al. 2021). However, studies investigating NO_3^- and NO_2^- in skeletal muscle in humans are sparse. The recent emergence of data demonstrating the importance of skeletal muscle as a storage site of NO_3^- in rodents, and its subsequent role in NO homeostatic processes and the sustenance of NO bioavailability warrants further work to be carried out in humans. A key aim of this thesis was therefore to investigate the tole of skeletal muscle in NO_3^- storage and metabolism in humans.

9.1. Research Questions Addressed

The principal focus of this thesis was the importance of exogenously supplied NO_3^- for NO bioavailability and bioactivity. Specifically, we aimed to identify whether a novel transdermal NO_3^- lotion could influence systemic [NO_3^-] and therefore be a viable alternative to dietary supplementation regimens. Moreover, as the oral microbiome is a rate-limiting factor which influences the magnitude of increase in NO_3^- following its consumption, we addressed whether variations in dietary NO_3^- intake influences the oral microbiota.

However, a key focus was on the investigation of dietary NO_3^- on NO bioavailability by measuring transient fluxes in NO-related metabolites throughout the body but with an emphasis on skeletal muscle.

The specific research questions addressed were:

1.) Does the application of a NO_3^- containing lotion to the skin influence [NO_3^-] and [NO_2^-] in blood plasma?

2.) What are the effects of habitually ingesting varying quantities of NO_3^- on saliva [NO_3^-] and [NO_2^-] as well as the oral microbiome? Specifically, how does NO_3^- deprivation influence the oral microbiota?

3.) What is the influence of an acute bolus of potassium NO_3^- (~12.8 mmol, ~800 mg NO_3^-) on skeletal muscle [NO_3^-] and [NO_2^-] over the course of a 24-hour period?

4.) How much of the increase in NO_3^- within skeletal muscle tissue is accounted for by ingested (exogenous) compared to endogenously produced NO_3^-?

9.2. Summary of Main Findings

1.) The application of a NO_3^- containing skin lotion does not induce changes in circulating [NO_3^-] or [NO_2^-].

2.) During periods of NO_3^- deprivation, saliva [NO_3^-] and [NO_2^-] is substantially decreased when compared to both a standard and high NO_3^- containing diet. Also, exploratory analysis indicate changes in Candidatus Saccharibacteria during NO_3^- deprivation.

3.) NO_3^- enters skeletal muscle within 30 minutes of the acute ingestion of KNO_3, reaches its peak with 1-3 hours and returns to baseline 9 hours post-supplementation. This profile closely resembled that seen in plasma and saliva. No changes were observed in skeletal muscle NO_2^-.

4.) Increases in skeletal muscle [NO_3^-] at both 1 and 3 hours following NO_3^- ingestion is almost exclusively a result of the exogenously supplied NO_3^-, with minimal alterations in endogenous proportion of NO_3^- within skeletal muscle tissue.

9.3. Novel Alternative Method of Altering NO Bioavailability

In Experimental Chapter 1, it was demonstrated for the first time, that the application of a NO_3^- containing lotion on the skin above the quadriceps does not induce changes in NO_3^- and NO_2^- within blood at rest. Interestingly, this finding was made in spite of the apparent characteristic of skin to retain substantial quantities of NO_3^- and NO_2^- (Paunel et al. 2005; Mowbray et al. 2009), and with NO_3^- and NO_2^- being found in sweat (Weller et al. 1996). The pilot data which comprise Experimental Chapter 1 was exploratory and therefore several further questions arose from this investigation. According to prior research, skin may contain the necessary machinery to transport and reduce NO_3^-: the skin contains NOS enzymes (Förstermann et al. 1994; Griffith et al. 1995) which are central in the endogenous production of NO, as well as skin fibroblasts containing sialin which acts as a unique transporter of NO_3^- (Qin et al. 2012). These were, however, not measured in our pilot study. Moreover, Villar and colleagues (2021) demonstrated that NO_3^- and NO_2^- were increased in skin following the infusion of NO_3^- which would suggest that there is at least uni-directional 'movement' (via active transport or passive diffusion) of NO_3^- and NO_2^-. Accordingly, the skin as an alternative medium for increasing systemic NO_3^- is unlikely; however, further work is warranted to confirm whether potential alterations in local NO_3^- may have functional implications for health or performance.

9.4. The Oral Microbiome Changes in Response to Dietary Nitrate Deprivation

The exploratory nature of the analysis of the oral microbiome has yielded interesting findings which demonstrate that the deprivation of NO_3^- may lead to alterations of the microbial profile within the mouth, and is the first study to do so. These findings indicate that the oral microbiome responds, not only to the ingestion of dietary NO_3^- (Rosier et al. 2020; Vanhatalo

et al. 2018; Burleigh et al. 2018; González-Soltero et al. 2020), but to changes in the quantity of NO_3^- ingested. Furthering our understanding that by reducing NO_3^- intake from 180 mg to 30-50 mg, substantial changes manifest in the environment in the mouth. Although not investigated in the experimental chapter, it is reasonable to speculate that such alterations may have relevance in factors such as the NO_2^- generating properties of the mouth, oral health and NO homeostasis.

Experimental Chapter 2 contributes to the literature base by being the first study to describe the influence of a NO_3^- deprived diet on salivary [NO_3^-] and [NO_2^-] as well as the oral microbiome. We describe that the continued ingestion of a NO_3^- deprived diet will likely lead to undesirable changes in the microbial profile of the mouth, with changes in both phyla and genera being more pronounced in the absence of NO_3^- compared to a standard quantity of NO_3^-. Although we were unable to ascertain whether these changes would lead to significant effects on biological or physiological processes, we did confirm a marked decrease in salivary [NO_3^-] and [NO^-]. If sustained for prolonged periods, it is possible that the changes we observed may be detrimental to NO homeostasis and therefore parameters of cardiovascular health (Vanhatalo et al. 2021; Morou-Bermúdez et al. 2022). A further observation in Experimental Chapter 2 was the absence of a NO_3^- "overshoot" or super-compensation effect when transitioning from a LOW diet to a HIGH diet compared to STD to HIGH, a phenomenon which was reported in rodents (Gilliard et al. 2018). The existence of a super-compensation effect could have potentially informed periodization of NO_3^- supplementation regimens during training or in preparation for sports competition but may also have been applicable in instances such as prior to hospitalization. However, a limitation of Experimental Chapter 2 is that samples were collected 3-days post initiation of the HIGH diet and therefore we do not know whether a different response to increasing NO_3- occurred acutely (i.e. within this 3-day period). Nonetheless, a positive finding in Experimental

Chapter 2 was that following both HIGH diets, irrespective of intervention (being preceded by LOW or STD), substantial and comparable increases in saliva [NO_3^-] and [NO_2^-] occurred.

9.5. The Time-course of Change in Skeletal Muscle [NO_3^-] and [NO_2^-]

9.5.1. Resting skeletal muscle NO_3^- and NO_2^- concentrations in humans

It has been proposed that skeletal muscle serves as a NO_3^- store/ reservoir and thereby may facilitate processes involved in NO metabolism (Piknova et al. 2015; Park et al. 2019). The existence of substantial quantities of NO_3^- within muscle, greater than those seen in blood, has been reported and this has led to the suggestion that a concentration gradient may exist between these compartments (Piknova et al. 2015; Wylie et al. 2019). The notion that skeletal muscle possesses NO-related transporters coupled with this concentration gradient could hypothetically allow NO_3^- to be actively sequestered or to passively diffuse from muscle to blood and *vice versa* (Jones et al. 2021; Piknova et al. 2022). It is possible to speculate that, due to shifts in a proverbial pendulum of [NO_3^-] within the body (in varying compartments of i.e. circulation and biological tissues), movement of NO_3^- may change because of instances of challenge such as during dietary NO_3^- restriction or during abrupt increases in the requirement for NO. However, to date there is no evidence to support this notion in humans. The work presented in this thesis challenges suggestions that skeletal muscle acts as a storage site of NO_3^-. Findings from Experimental Chapter 3 indicate that there were no differences between skeletal muscle [NO_3^-] and plasma [NO_3^-] at rest. Nevertheless, Experimental Chapter 4 was in agreement with earlier work and suggests that the potential for skeletal muscle to act as a reservoir for NO_3^- is potentially related to factors such as habitual NO_3^- intake. Chapters 3 and 4 contribute to current knowledge by demonstrating that skeletal muscle [NO_2^-] appear to be consistently greater than that in blood and therefore this raises the possibility of functional

significance of these NO_2^- 'stores' due to the muscle to blood gradient (Figure 4).

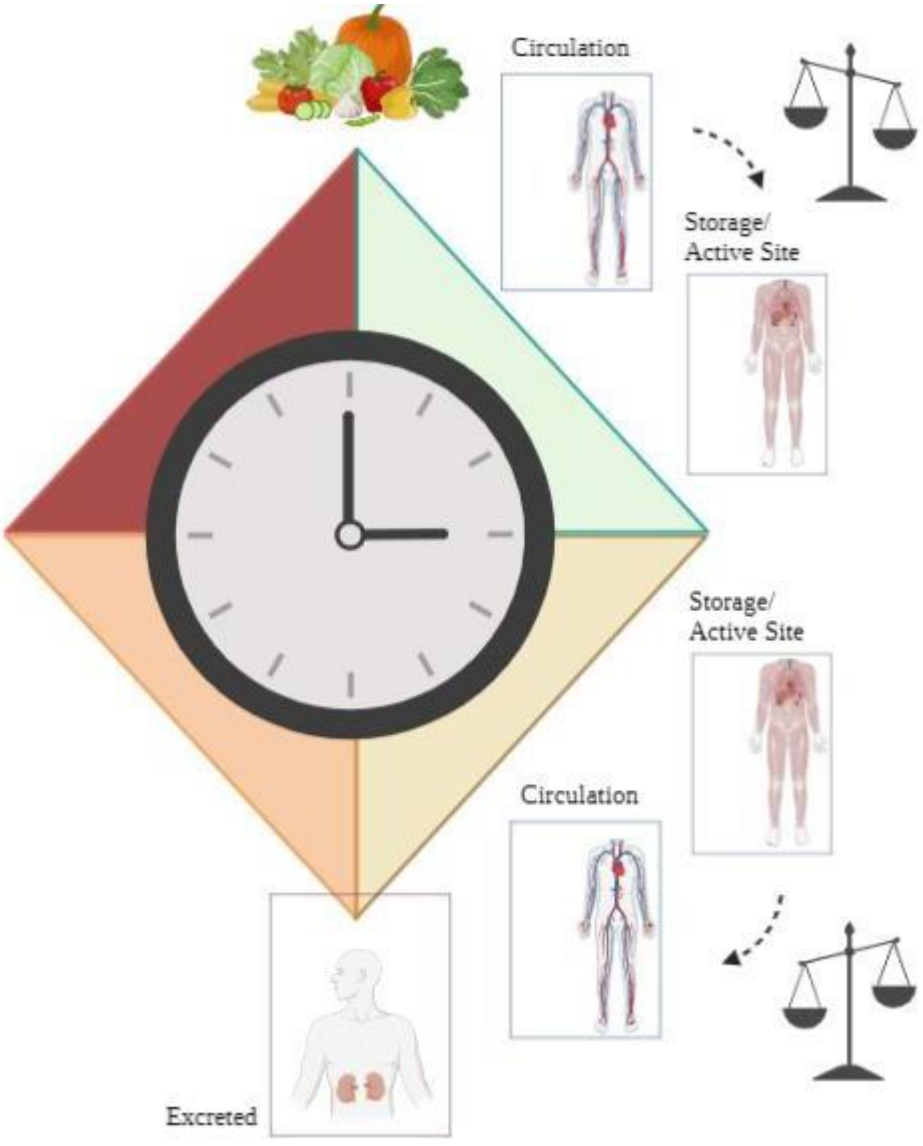


Figure 1: Time-course of change in circulation and skeletal muscle (and other biological tissues which are likely to be storage and active sites). How concentration gradients are involved in the movement of NO_3^- in the human body. *The suggested relationship between circulation and biological tissues (skeletal muscle) in the movement of NO_3^- following ingestion. At rest, skeletal muscle NO_3^- concentrations ($[NO_3^-]$, where '[ ]' indicates*

concentration) is greater than in circulation due to the endogenous generation of NO. Following the ingestion of NO_3^-, there is an initial shift in the concentration gradient whereby [NO_3^-] in circulation surpasses that in muscle. This enables NO_3^- to enter skeletal muscle from circulation. Both circulation and muscle [NO_3^-] peaks at about 3 h post-supplementation suggesting NO bioavailability is greatest during this period (optimal period, NO bioavailability at its peak: green). Between 3 – 6 h post NO_3^- ingestion, the concentration gradient is likely to begin shifting, meaning that a [NO_3^-] decrease in circulation will lead to lower [NO_3^-] in circulation than skeletal muscle. This may in turn enable the movement of NO_3^- from skeletal muscle, thereby facilitating the sustained elevation of [NO_3^-] in circulation and not skeletal muscle. During this period, NO bioavailability is still likely to remain elevated and may therefore still be pertinent in altering physiological function (circulating [NO_3^-] remains elevated in circulation and skeletal muscle: yellow). Substantial quantities of NO_3^- are present in urine meaning that surplus ingested NO_3^- is excreted, with peaks in the expulsion of NO_3^- occurring between 6 – 9 h. Although circulating [NO_3^-] remains elevated for up to 24 h post ingestion, movement of NO_3^- from skeletal muscle to circulation may still be occurring before skeletal muscle [NO_3^-] returns to resting values by 9 h (little to no physiological relevance in NO bioavailability: amber).

N.B. The active transport of NO_3^- may also occur due to the presence of the NO_3^- transporter sialin and CLC-1. This would be characterised by the movement of NO_3^- against existing concentration gradients.

What is evident from Experimental Chapters 3 and 4 is that skeletal muscle retains considerable quantities of both NO_3^- and NO_2^- which are the product of endogenous NO generation. It may be hypothesized that these concentrations represent an essential buffer

which may ensure the sustenance of NO and therefore 'normal' physiological function. This is supported when considering the distinct lack of change during instances of 'challenge' such as dietary NO_3^- deprivation and during exercise (when skeletal muscle [NO_3^-] is not elevated prior to the initiation of exercise (Wylie et al. 2019)).

In the absence of prior elevation caused by the ingestion of NO_3^-, skeletal muscle [NO_3^-] does not alter during exercise (Wylie et al. 2019). This may indicate that a critical threshold may exist whereby a stimulus in the form of surplus or an abundance of NO_3^- within skeletal muscle may be required to induce the correct physiological conditions to allow the movement of NO_3^- from biological tissues. A consequence of shifting the concentration gradient between biological compartments.

9.5.2 The pharmacokinetics of skeletal muscle [NO_3^-] following the ingestion of dietary NO_3^-.

At the inception of this thesis, two studies had examined skeletal muscle [NO_3^-] in humans (Nyakayiru et al. 2017; Wylie et al. 2019). These studies both confirmed that dietary NO_3^- consumption leads to increases in skeletal muscle [NO_3^-], with the work of Wylie and colleagues (2019) reporting increases in [NO_2^-] for the first time. Nonetheless it was clear that further work was required to identify the time-course of change in skeletal muscle [NO_3^-] and [NO_2^-] following the acute ingestion of dietary NO_3^-. Experimental Chapter 3 sought to add to the literature base by investigating the rate, duration and magnitude of change in skeletal muscle [NO_3^-] and [NO_2^-] following the consumption of KNO_3 in healthy adults. We hypothesised that skeletal muscle [NO_3^-] and [NO_2^-] would follow a similar profile to that described previously for plasma, with a peak value reached 1–3 h post NO_3^- ingestion and a subsequent fall back to the pre-ingestion baseline by 24 h. A novel finding of Experimental Chapter 3 was that skeletal muscle [NO_3^-] increased above baseline as early as 30 mins

following the acute ingestion of KNO_3, persisted for 3 h, and then returned to baseline after 9 h. As discussed in Experimental Chapter 3, a novel finding of this thesis was the rapidity of the rate of increase in skeletal muscle [NO_3^-]. The change in skeletal muscle [NO_3^-] was correlated with plasma [NO_3^-], demonstrating the existence of a cross-compartment exchange, with the speed of NO_3^- absorption from dietary ingestion to the intestine, bloodstream and ultimately storage sites (i.e. skeletal muscle) (Piknova et al. 2022) being somewhat faster than expected.

Importantly, Experimental Chapter 3 was the first study in healthy adults to demonstrate that the profile of increase (although at lower concentrations), at least for the initial 3 h, mimicked changes in saliva and blood [NO_3^-]. Importantly, we characterised that the time-to-peak in [NO_3^-] within saliva (~1 h), plasma (~2 h) and muscle (~3 h), which was in line with previous research, with urine being an exception with the peak occurring between 3 h and 9 h (Wylie et al. 2013; James et al. 2015; Pannala et al. 2003, McDonagh et al. 2018; Burleigh et al. 2018; Capper et al. 2021). However, understanding the time-course of change in skeletal muscle [NO_3^-] is complicated by data presented in Experimental Chapter 4. In Experimental Chapter 4 it was found that total skeletal muscle [NO_3^-] was increased above baseline at both 1 h and 3 h post-ingestion, but a reduction was observed at 3 h compared to 1 h. The reduced temporal resolution of measurement in Experimental Chapter 4 would lead to the conclusion that skeletal muscle [NO_3^-] peaks at 1 h post and therefore would be in line with that seen in saliva. Unfortunately, the reason for these reported differences is not clear at present and further work is required to determine inter-individual differences in response to ingesting dietary NO_3^-. Nonetheless, this highlights an overarching theme in this thesis in relation to the almost instantaneous changes in biological tissue (skeletal muscle) [NO_3^-] which mimic alterations in biological fluids in humans.

9.5.3 Plasma to muscle ratios for nitrate and nitrite prior to and following NO_3^- ingestion.

It has previously been speculated that the existence of a [NO_3^-] gradient enables the passive diffusion of NO_3^- to sustain NO bioavailability (and potentially bioactivity) in tissues or organs when demand is high, i.e. during instances of NO_3^- deprivation (Piknova et al. 2015; Gilliard et al. 2018; Jones et al. 2021; Piknova et al. 2022). Notably, experimental chapter 3 and 4 contributed to knowledge by characterising the plasma to muscle ratio of both NO_3^- and NO_2^- at rest and following the acute intake of NO_3^-. Experimental chapter 3 described a ratio of ~1 in plasma to muscle [NO_3^-] meaning that concentrations in both compartments were similar. This was in contrast to the results of Experimental Chapter 4 which found a plasma/ muscle ratio of ~0.8. Experimental Chapter 4 builds on data from rodents (Piknova et al. 2015; Piknova et al. 2016) and humans (Nyakayiru et al. 2017; Wylie et al. 2019). This therefore illustrates that, at rest, skeletal muscle [NO_3^-] tends to be greater than circulating [NO_3^-] in plasma. This suggests that NO_3^- accretion within muscle (from circulation) is contingent on the active transport of NO_3^- via sialin and/or the chloride channels. In contrast, the ingestion of NO_3^- induces a shift in the plasma/ muscle [NO_3^-] ratio meaning this becomes positive and therefore passive diffusion may be the primary mechanism by which muscle [NO_3^-] uptake occurs. Indeed, the plasma/ muscle [NO_3^-] ratio widened following the acute ingestion of dietary NO_3^- in experimental chapter 3, to 3.5 and 3.2 at 1 h and 3 h, respectively. Moreover, the gradient between plasma and muscle [NO_3^-] was reversed in experimental chapter 4, to 3.6 at 1 h and 4.4 at 3 h indicating that plasma [NO_3^-] was greater than skeletal muscle [NO_3^-]. These results highlight that the generation of NO_3^- and NO_2^- via endogenous NO production is substantial in biological tissues with concentrations of both being equivalent or greater than that in circulation at rest in humans. It is reasonable to speculate

that under normal conditions, muscle may act as a reservoir of NO_3^- and via passive diffusion, NO_3^- may enter into circulation. This would not necessitate the presence of transporters (i.e. sialin or chloride channels) but would ensure the provision of sufficient NO during instances of 'challenge' that would necessitate a sudden increase in NO requirements (Piknova et al. 2015; Jones et al. 2021; Piknova et al. 2022). The work in Experimental Chapters 3 and 4 build on the only study that was successful in measuring NO_2^- in skeletal muscle in humans (Wylie et al. 2019). Herein we present data to support that, in spite of a larger relative changes in plasma [NO_2^-], there was a consistent negative ratio between plasma and muscle meaning that [NO_2^-] was greater in skeletal muscle than plasma following acute NO_3^- supplementation. This is in contrast with results from rodent work (Piknova et al. 2015; Piknova et al. 2016).

This data illustrates the importance of NO_3^- in the maintenance of NO availability in the body due to its greater relative stability when compared to NO_2^-. NO_3^- may be the form in which NO-related metabolites can move throughout the body. Moreover, the magnitude of change in [NO_3^-] within biological tissues following supplementation and how the ratio between muscle and plasma changed may reflect the requirement for the transfer of NO metabolites throughout the body. These effects may occur to prevent 'extreme' alterations in NO fluxes throughout the body in response to situations such as NO_3^- deprivation or during exercise. This would explain why skeletal muscle may not necessarily be a "storage pool" for NO_3^- but instead a site for NO_3^- to be temporarily deposited. NO_2^-, on the other hand, is more bioactive and may be more functionally important than NO_3^- within biological tissues. The stability (lack of change) in the gradient of NO_2^- suggests that the presence of this is somewhat important in NO generation locally and therefore any transport from this site (muscle) would require its active transport. This may be facilitated via the enzymatic machinery that muscle

possesses (i.e. xanthine oxidoreductase, aldehyde oxidase, sulphite oxidase (Piknova et al. 2016; Wylie et al. 2019)). These findings reveal the potential importance of the direction of the plasma to muscle concentration gradient in allowing the relocation of NO_3^- and NO_2^- in biological tissues both at rest and following the ingestion of NO_3^- rich food sources.

9.6. The Relative Contribution of Exogenous Nitrate vs. Endogenous Nitrate in Skeletal Muscle

9.6.1. The metabolic fate and relative contribution of dietary NO_3^- ingestion on biological tissue and fluid [NO_3^-].

Experimental Chapter 3 describes changes in skeletal muscle [NO_3^-] and [NO_2^-] (with greater resolution through the number of time-points), while drawing comparisons to other biological compartments such as blood, urine and saliva. This is an important first step in describing the distribution of NO_3^- and NO_2^- in the body following NO_3^- ingestion. However, using the approach taken in Chapter 3, it is not possible to determine with certainty whether the concentration changes in these compartments is consequent solely to the ingested NO_3^- or whether changes in endogenously produced NO_3^- and NO_2^- had some effect. Therefore, in experimental chapter 4, a potassium nitrate stable isotope tracer ($^{15}KNO_3$) was used and muscle, blood plasma, urine and saliva samples were collected to quantify the metabolic fate and relative contribution of the ingested NO_3^-.

Experimental chapter 4 highlights that, NO_3^- from ingested sources swiftly enters skeletal muscle and leads to increases in [NO_3^-] to levels which eclipse existing quantities of NO_3^- present from endogenous NO production. The profile of change in NO_3^- is predominantly the result of exogenously derived sources rather than its endogenous counterpart. This is an important finding as previously it may have been speculated that NO_3^- generated from the L-

arginine pathway may comprise a significant proportion of [NO_3^-] within skeletal muscle (Piknova et al. 2015; Piknova et al. 2016). Moreover, these changes in skeletal muscle [NO_3^-] coincide with changes in biological fluids, with ingested NO_3^- also being the principle factor which evokes increases in [NO_3^-] within plasma, saliva and urine.

Notably, within skeletal muscle, the exogenous (^{15}N labelled) portion accounted for ~68% at 1 h post-ingestion which was substantially lower than that seen in plasma (~94%), saliva (~96%) and urine (89%). At 3 h following supplementation, the percentage attributed to the ingested NO_3^- fell to 48% in skeletal muscle compared to 94%, 95% and 91% in plasma, saliva and urine, respectively. We noted a slight reduction in skeletal muscle [NO_3^-] at 3 h compared to 1 h which was in contrast with the findings of experimental chapter 3. This data is also in contrast to work in rodents, where although not isolated to exogenous vs. endogenous [NO_3^-], changes in skeletal muscle [NO_3^-] are equivalent or greater in terms of magnitude of response between muscle and other biological compartments (Piknova et al. 2015; Piknova et al. 2016; Park et al. 2021). A possible explanation for the divergent responses seen in humans compared to rodents in the mode of supplementation selected. The investigations in this thesis entailed the ingestion of a single bolus of KNO_3 or $^{15}KNO_3$, meaning that we are studying responses to an acute increase in [NO_3^-] throughout the body. In contrast, the rodent work entails regular, intermittent NO_3^- ingestion via chow or drinking water. Therefore, the attenuated NO_3^- 'uptake' in skeletal muscle seen in our work may be consequent to the existence of a limitation in humans whereby diffusion or sialin / chloride channel transporters may become saturated (Qin et al. 2012; Wylie et al. 2019; Srihirun et al. 2020). However, without investigating the influence of smaller, more regular patterns of NO_3 ingestion, we are unable to determine whether these pathways are becoming saturated or whether this response is typical of the dose provided. A limitation of the specific approach

utilised in Experimental Chapter 4, i.e. the use of an orally ingested isotope tracer, was the inability to determine whether the observed alterations in endogenous (i.e., unlabelled) [NO_3^-] in skeletal muscle at 3 h was the result of augmented endogenous pathway generation of NO or uptake of NO_3^- from the circulation. An important experimental consideration in the work included in this thesis is the selection of KNO_3 as the supplement that was provided in Experimental Chapter 3 and 4. KNO_3 was used as this enabled the same equimolar dose to be supplied to participants however the ingestion of differing sources of NO_3^- may lead to subtle but significant changes in the pharmacokinetics of NO_3^- and NO_2^- (James et al. 2015; McIlvenna et al. 2017).

9.6.2. The metabolic fate and relative contribution of dietary NO_3^- ingestion to tissue and fluid [NO_2^-].

NO_2^- is both the product of NO oxidation and the precursor to NO formation depending on the prevailing pathway for NO generation and physiological circumstance (Dejam et al. 2004; Gladwin et al. 2005). Additionally, due to its greater relative bioactivity compared to NO_3^- and its interactions with other NO metabolites through only requiring a one-electron reduction to form NO, NO_2^- is physiologically important and should be considered a storage pool for NO in biological fluids (Helms et al. 2017).

Experimental chapter 3 details the temporal changes in salivary and plasma [NO_2^-] following the ingestion of dietary NO_3^-, something which has previously been described (Webb et al. 2008; Wylie et al. 2013; James et al. 2015; McDonagh et al. 2018; Capper et al. 2022). Salivary [NO_2^-] increased as early as 0.5 h and reached its peak between 1 h and 3 h following the ingestion of KNO_3, and remained elevated up to 24 h. Relative to saliva, there was a delay in [NO_2^-] increases in plasma, with an elevation at 1 h, a peak at 3 h, and

sustained elevation for up to 9 h post supplementation. These data illustrate that although considered to be more bioactive, [NO_2^-] increases rapidly and remains elevated for substantial periods of time following the ingestion of a single bolus of NO_3^-. The ingestion of NO_3^- and the expected role of the enterosalivary pathway supports these findings through the sequence of events outlined in the literature summary through which NO_3^- enters the body, is concentrated in salivary glands and enters the blood stream whereafter NO_2^- may be reduced to NO (Cosby et al. 2003; Kapil et al. 2010). Importantly, Experimental Chapter 3 is only the second study to quantify changes in human skeletal muscle [NO_2^-] following the ingestion of NO_3^- and the first study to describe temporal changes in skeletal muscle tissue. In contrast to work carried out in rodents, the study conducted by Wylie and colleagues (2019) found no change in skeletal muscle [NO_2^-] 2 h following the ingestion of NO_3^- in the form of beetroot juice (~12.8 mmol). Experimental Chapter 3 subsequently confirmed the lack of change in [NO_2^-] in skeletal muscle tissue not only at 2 h post supplementation but also over 6 other time-points over a 24 h period. Experimental Chapter 4 corroborated the findings of Experimental Chapter 3, with no change in total muscle [NO_2^-] at either 1 h or 3 h following the ingestion of $^{15}KNO_3$. There is now an emerging body of evidence suggesting that [NO_2^-] in skeletal muscle remains stable even when a bolus of NO_3^- in varying forms is ingested. The use of the $^{15}KNO_3$ tracer in experimental chapter 4 enabled investigation of possible effects of ingested ^{15}N-Labelled NO_3^- on skeletal muscle [NO_2^-]. We evidence that ^{15}N-labelled muscle [NO_2^-] increased at 1 h post ingestion, an occurrence which may be explained by potential local NO_3^- reduction or the absorption from blood.

Based on this work, it is possible to speculate that the stability of [NO_2^-] in skeletal muscle even in the presence of supplementation, or challenge (i.e. exercise (Wylie et al. 2019)), may be indicative of the importance of the presence of NO_2^- and ultimately the maintenance of NO

bioavailability or bioactivity within biological tissues. Therefore, skeletal muscle [NO_2^-] may be integral for the preservation of normal function of skeletal muscle, a finding which needs to be examined further in the future. Interpretation of muscle data from the study which contained a wider dataset which was collected as part of Chapter 6 would support this hypothesis. In a study involving NO_3^- deprivation for 7 days, we saw no change in skeletal muscle [NO_2^-] compared to a 7 day normal diet. This indicates that skeletal muscle appears to retain NO_2^-, even in the absence of NO_3^- in the diet, presumably to support NO bioactivity. Additionally, when participants ingested a 7 day 'normal' diet containing 180 mg NO_3^-, there was a slight reduction in [NO_2^-] compared to baseline. This may support the above proposed theory, as sufficient NO_3^- is being delivered via the diet. It is therefore reasonable to speculate that the reduction of NO_3^- to NO_2^- and in turn NO would be adequate to support physiological function and NO homeostasis under these circumstances.

9.7. Skeletal muscle is not a storage site of NO_3^- and is instead a short-term reservoir which temporarily retains NO_3^-

As with most research, rodent models have been imperative in informing the initial experimental designs of studies in humans because they can often provide a good indication of phenomena which may occur in human physiological processes. Indeed, this has been the case for research into skeletal muscle NO_3^- and NO_2^- which has demonstrated the presence of these NO-related metabolites in skeletal muscle following the ingestion of NO_3^- in rodents (Piknova et al. 2015; Piknova et al. 2016). At this early stage of research in humans and in contrast to work performed in rodents (Piknova et al. 2015; Piknova et al. 2016), it appears that skeletal muscle is not a storage site *per se* in which NO_3^- is retained for prolonged periods. Experimental chapter 3 demonstrated that skeletal muscle [NO_3^-] returned to baseline

24 h following KNO_3 supplementation and skeletal muscle [NO_3^-] had begun to fall at 3 h when compared to 1 h post $^{15}KNO_3$ ingestion in Experimental Chapter 4. As alluded to above, a possible explanation for the potential differences which may have led to this prolonged elevation in skeletal muscle [NO_3^-] being identified in rodents is the difference in the manner in which NO_3^- is ingested between species. In all the human trials which have investigated the influence of dietary NO_3^- ingestion on skeletal muscle [NO_3^-] (and [NO_2^-]), acute supplementation regimens have been employed which fundamentally differs from the approach taken in rodent work which either uses a NO_3^- rich chow or the addition of NO_3^- in the drinking water (Nyakayiru et al. 2017; Wylie et al. 2019; Kadach et al. 2022). Interestingly, in contrast to the early rodent work showing substantial retention of [NO_3^-] in skeletal muscle and in line with the work completed as part of this thesis, recently Park and colleagues (2021) noted a rapid return to control concentrations in gluteus muscle 12 h following the cessation of NO_3^- administration. In this work, prior to cessation of NO_3^- ingestion, the rodents ingested NO_3^- for 5 days following which gluteus [NO_3^-] increased 2.4-fold compared to baseline (Park et al. 2021). However, the work in humans presented in this thesis indicates similar magnitudes of change with the rodent work whereby experimental chapter 3 and 4 demonstrated a ~3 fold and ~4 fold increase from baseline to the peak in [NO_3^-] at 3 h and 1 h post-acute supplementation, respectively. The work in rodents has only employed chronic supplementation regimes with skeletal muscle [NO_3^-] data thereafter. It is conceivable that the effects observed in humans may in fact also occur acutely in rodents. In this case, rodents and humans may in fact be remarkably similar when it comes to skeletal muscle [NO_3^-], with NO_3^- not being "stored" unless ingested systematically throughout a day. Therefore, if the findings from rodent work are translatable to humans, we may need to revise chronic supplementation regimes to ensure that dietary NO_3^- ingestion is evenly distributed

over the course of 24 h periods to ensure that elevated skeletal muscle [NO_3^-] is maintained over the course of supplementation regimes and thereby optimises effects on health or exercise performance-related parameters (Figure 5).

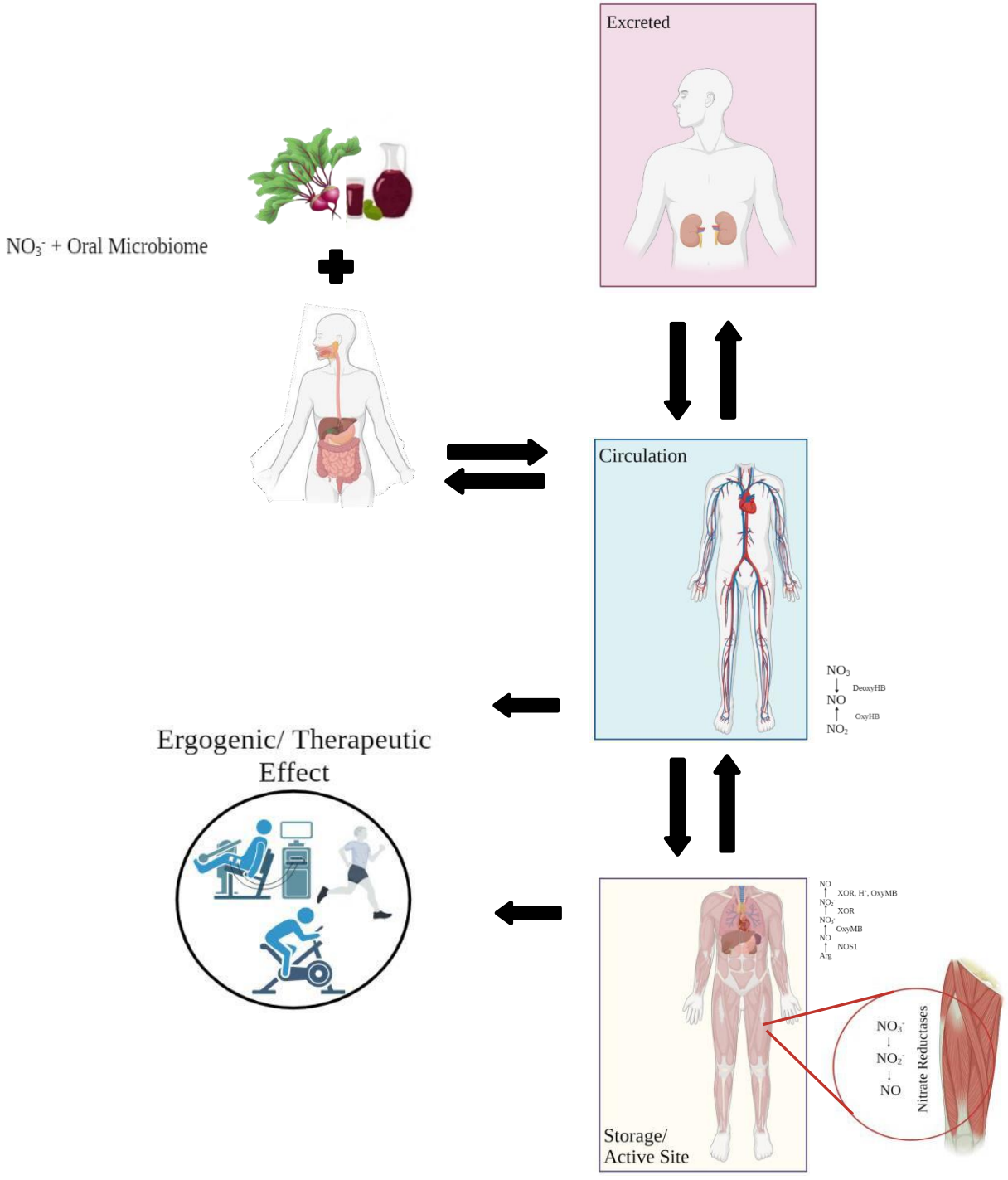


Figure 2: How NO_3^- in circulation and skeletal muscle (biological tissues) exert influences on NO bioavailability, ultimately leading to ergogenic and therapeutic effects.

Following its ingestion, NO_3^- swiftly enters the body with substantial increases in circulating NO_3^- being seen as early as 30 mins. Additionally, in processes facilitated by the oral microbiome, substantial quantities of NO_2^- also enters the body through the bacteria facilitated reduction of NO_3^- which ultimately leads to the formation of NO. The increased NO bioavailability following NO_3^- ingestion has been suggested to lead to ergogenic and therapeutic effects. However, the movement of NO_3^- from circulation to active or storage sites (i.e. skeletal muscle) has been reported in animals (Piknova et al. 2016; Gilliard et al. 2018) and humans, with increases in muscle [NO_3^-] almost entirely linked to ingested NO_3^-. Interestingly, skeletal muscle possesses the necessary enzymatic machinery to generate NO from the reduction of NO_3^- to/ and NO_2^-. These findings make it reasonable to suggest that biological tissues such as skeletal muscle are also implicit in the therapeutic and ergogenic effects of NO_3^- supplementation.

9.8. The role of fibre type composition on the retention of skeletal muscle NO_3^- and NO_2^-

In 2016, Jones and colleagues proposed that a possible reason for the variability in exercise performance related responses (i.e. muscle efficiency and exercise economy) following NO_3^- supplementation may in part be related muscle fibre type composition. These authors suggested that, although there is reasonable homogeneity between Type I (slow-twitch) and Type II (fast-twitch) fibres, there may be distinct differences in terms of myofibrillar protein content, calcium handling, as well as mitochondrial and capillary density. These differences are likely to translate into Type II muscle fibres relying more on nonoxidative pathways

compared to Type I fibres for ATP resynthesis (Bottinelli et al. 2000). It is anticipated that the greater reliance on nonoxidative processes might mean that Type II muscle fibres would be more susceptible to dietary NO_3^- ingestion. This could be explained by the role that local acidity and oxygen tension have on the production of NO via the NO_3^- - NO_2^- - NO pathway. Effects on muscle oxygenation and pH may be exaggerated during instances of 'challenge' (such as exercise) within Type II muscle fibres, resulting in the promotion of NO generation via the reduction of NO_2^- within these muscle fibres. It is understood that skeletal muscle possesses the "machinery" for NO_3^- generation, a notion supported by particularly high expression of nNOS particularly in Type II muscle fibres (Kobzik et al 1994), as well as myoglobin and XOR which are all integral in processes involved in NO metabolism (Jones et al. 2020; Jones et al. 2022). It is not known whether the expression/ activity of these reductases are different between human skeletal muscles due to fibre type differences and therefore further work is required in this area. Nonetheless, at the inception of this thesis, it was anticipated that Type II muscle fibres may retain greater quantities of NO_3^- and NO_2^- than Type I muscle fibres as this is the site at which significant NO generation via the reduction of NO_3^- and NO_2^- may occur.

Although not investigated in humans to date, there is emerging evidence from rodents suggesting that there may be distinct differences in the retention of NO_3^- and NO_2^- in skeletal muscle comprised of differing muscle fibre types. One investigation by Long et al. (2020) utilised two different muscle groups, the soleus and vastus lateralis, which represent two extremes of the spectrum as these muscles are composed of approximately 97% fast-twitch and 87% slow-twitch, respectively (see table below) (Armstrong et al. 1984). The other study that has published findings in this area used 4 different muscle groups, the fibre type

composition of which are presented in the below table (**Table 1)**: soleus, extensor digitorum longus (EDL), tibialis anterior (TA) and gluteus (Park et al. 2021).

	Myosin heavy chain isoform			
Muscle (N = 6)	Type I (%)	Type IIa (%)	Type IIx (%)	Type IIb (%)
Vastus lateralis	1.24 ± 0.80	12.36 ± 1.81	21.82 ± 0.71	64.58 ± 2.37
Tibialis anterior	0.43 ± 0.43	10.13 ± 0.64	19.69 ± 0.65	69.75 ± 0.90
Extensor digitorum longus	0.00 ± 0.00	10.52 ± 0.40	25.08 ± 1.40	64.40 ± 1.33
Gastrocnemius	5.71 ± 0.33	9.80 ± 1.07	19.01 ± 0.59	62.52 ± 1.52
Soleus	80.19 ± 4.10	19.81 ± 4.10	0.00 ± 0.00	0.00 ± 0.00
Gluteus (Glut medius)	0.00 ± 0.00	5.22 ± 0.62	19.01 ± 0.59	75.77 ± 0.13

Table 1: Myosin heavy chain isoform percentages in rat hind limb muscle. Table adapted from Eng et al. 2008. Values are means ± standard error of mean.

[NO_2^-]: At rest, [NO_2^-] differed between skeletal muscle with differing fibre type compositions, as follows: gluteus (0.28 ± 0.05 nmol/g tissue) contained the least [NO_2^-], whereas EDL (0.38 ± 0.04 nmol/g tissue) and soleus (0.44 ± 0.06 nmol/g tissue) had the greatest [NO_2^-] (Park et al. 2021). The gastrocnemius and tibialis anterior (TA) were also measured in this study, with the TA (predominantly type II (refer to above table for precise fibre type breakdown)) containing 0.31 ± 0.04 nmol/g [NO_2^-] and the gastrocnemius containing 0.26 ± 0.04 nmol/g [NO_2^-]. In agreement with this, the predominantly slow-twitch muscle, soleus, contained 1.8 fold greater [NO_2^-] than the vastus lateralis in the work by Long and colleagues (2020). Where the rodent data may contrast to that in humans is that this study

found increases of 2.9 fold, 2.5 fold and 3.3 fold in gluteus, EDL and soleus muscle [NO_2^-] following five days of NO_3^- supplementation. **[NO_3^-]**: Interestingly, [NO_3^-] in varying muscle groups does not appear to follow the same trend as [NO_2^-] with fibre type composition not appearing to be related to [NO_3^-] at baseline. According to the work by Park and colleagues (2021), the gluteus muscle contained the greatest [NO_3^-] at baseline (43.4 ± 14.6 nmol/g tissue), with significantly higher concentrations than both the soleus (22.8 ± 4 nmol/g tissue) and EDL (14.4 ± 5.2 nmol/g tissue) with differences of 1.9- and 2.6- fold, respectively. The TA contained the least NO_3^- with concentrations of 12.1 ± 3.5 nmol/g tissue being reported and [NO_3^-] in the gastrocnemius (18.8 ± 9.2 nmol/g tissue) falling between the gluteus and TA. However, in contrast, Long et al (2020) suggested that type I muscle may in fact contain higher quantities of [NO_3^-] at baseline. The reason for these differences in baseline [NO_3^-] is not clear at present. Following NO_3^- supplementation, there was an increase in [NO_3^-] in the different skeletal muscles (gluteus: 2.4- fold, EDL: 4.3- fold, soleus: 3.3- fold increases) (Park et al. 2021), demonstrating that irrespective of fibre type composition there is a substantial elevation in [NO_3^-] in skeletal muscle following the ingestion of NO_3. These observations suggest that there may be underlying characteristics between muscles groups (fibre type may be one such factor, especially in the case of [NO_2^-] storage) which may contribute to explaining differences in the retention and storage of [NO_3^-] and [NO_2^-] between muscle groups. An additional consideration when attempting to identify possible patterns and links between skeletal muscle characteristics and properties which may be related to NO_3^- and/or NO_2^- retention is the role the muscle plays in physiological processes. The above section demonstrates diversity in [NO_3^-] and [NO_2^-] in rodent skeletal muscle with the presence of a muscle to blood gradient for NO_3^- in several instances, i.e. gluteus, EDL, soleus and gastrocnemius. It is conceivable that only certain muscles possess the characteristics to

be NO_3^- reservoirs and thereby supply the body with NO_3^- while others may simply retain small quantities to support fundamental processes involved in the maintenance of local muscular function. Further work is therefore warranted in humans to examine which characteristics of muscle may be important in NO_3^- and NO_2^- retention.

Indeed, recent work completed as part of the larger study that experimental chapter 4 was a part of may support the concept that muscle fibre type may be associated with NO-metabolite related movement, utilisation or retention. It is currently understood that skeletal muscle possesses the ability to generate NO via an intracellular reduction pathway, thereby having intrinsic capacity to reduce NO_3^- to NO_2^- and NO (Srihirun et al. 2020; Piknova et al. 2022) and therefore may be central in the preservation of NO bioactivity during muscular work. Although we did not include fibre type composition measurement in the study, the apparent reduction of NO_3^- during exercise coupled with the improvement in force production during the early stages of exercise in which Type II muscle fibres would have been preferentially recruited may support the notion that Type II muscle fibres may utilise NO_3^- to support NO provision and bioactivity during muscle contractions whereas Type I fibres may retain NO_3^- as a temporary storage site. This hypothesis is consistent with a greater capacity of Type I fibres to rely more on the oxidative production of NO.

Caution must be taken when extrapolating from rodent work and inferring the same may occur in humans. This is especially true when considering fibre type composition with factors such as differences between species, anatomical location and gender potentially being influential (Pellegrino et al. 2003; Schiaffino et al. 2011; Haizlip et al. 2015). To date, evidence in humans in relation to NO_3^- and NO_2^- in skeletal muscle has been generated using the *vastus lateralis* muscle group in the quadriceps. While there are benefits to using such a heterogeneous muscle, which is well balanced in terms of fibre type composition, it is more

difficult to interrogate the influence of factors such as muscle fibre type on NO_3^- and NO_2^- retention. In humans the *vastus lateralis* is typically composed of approximately 50% Type I fibres and 50% Type II fibres (Staron et al. 2000). However, factors such as training status, aging and clinical conditions can all induce alterations in this fibre type distribution (Lexell et al. 1986; Gosker et al. 2007; Haizlip et al. 2015; Methenitis et al. 2016). Understanding the role of fibre type in the retention and utilisation of NO_3^- and NO_2^- may yield important information concerning how differences in muscle characteristics are related to NO homeostasis.

9.9. Future Directions:

Is the storage of NO_3^- in skeletal muscle contingent on anatomical location or muscle fibre type composition?

When synthesizing the points discussed in Chapter 9.8, it is possible to speculate that the retention of NO_3^- may vary between differing skeletal muscle sites and types (smooth, cardiac, skeletal). However, work in humans in relation to skeletal muscle NO_3^- retention has solely been performed using the *m. vastus lateralis*. A recent study illustrates the potential importance of muscle NO_3^- in muscle contraction (Kadach et al. 2023). We speculate that this reduction in $[NO_3^-]$ observed is likely to occur during the early phase of a maximal exercise protocol where improved force production occurred and where type II fibres may be most active. Our current understanding of how ingesting NO_3^- modulates NO-metabolite related fluxes in the human body both at rest and during exercise does not account for a factor such as skeletal muscle fibre type composition. Therefore, investigating the potential innate connection between the composition of skeletal muscle and its influence on NO bioavailability/ bioactivity, and how this may impact the magnitude and duration of change in NO_3^- and NO_2^- could provide novel insights into optimizing NO_3^- ingestion.

Acute dietary NO_3^- ingestion and its influence on skeletal muscle [NO_3^-]: What is the dose-response relationship between exogenous NO_3^- and skeletal muscle NO_3^- retention?

The dose-response relationship changes with differences in NO_3^- ingestion, with greater amounts of NO_3^- consumed eliciting more marked increases in circulating NO_3^- and NO_2^- (Wylie et al. 2013; James et al. 2015). This is consistent in both young and older adults and it is apparent that the ingestion of both beetroot and KNO_3 can effectively increase NO_3^- and NO_2^- concentrations within the human body (Capper et al. 2022). The incremental increases in bioavailable NO_3^- and NO_2^- following its ingestion has primarily been investigated in biological fluids such as plasma, saliva and urine (Wylie et al. 2013; Capper at el. 2022). Relatively few studies have assessed skeletal muscle [NO_3^-] and [NO_2^-] (Nyakayiru et al. 2017; Wylie et al. 2019; Kadach et al. 2022) and the dose-response relationship with dietary NO_3^- intake is not known. Therefore, it is important to further investigate whether, similar to plasma and saliva, skeletal muscle NO_3^- and NO_2^- incrementally increase and any potential implications of this on muscle or exercise performance.

Chronic dietary NO_3^- ingestion and its influence on skeletal muscle [NO_3^-]: Does skeletal muscle possess storage capabilities or is it a short-term active site?

To date, no papers have been published in humans exploring the long-term (more than 7 days) effects of supplementing with NO_3^- and the influence this may have on skeletal muscle [NO_3^-] and [NO_2^-]. The early reports from rodent work revealing substantial quantities of [NO_3^-] and [NO_2] in muscle (Piknova et al. 2015; Piknova et al. 2016) perhaps led to the expectation that human skeletal muscle would preserve [NO_3^-] and [NO_2^-] for prolonged periods and thereby act as a storage reservoir once these NO-related metabolites enter the

tissue. Experimental chapters 3 and 4 demonstrate that increases in [NO_3^-] from exogenous sources are short lived, with peaks in skeletal muscle [NO_3^-] occurring within 1-3 h post-ingestion, the exogenous portion decreasing at 3 h, and returning to baseline at 9 h post supplementation.

The early indication of whether skeletal muscle can act as a reservoir for prolonged NO_3^- does not appear to corroborate early suggestions. In a dietary NO_3^- deprivation study, we showed that following a 3-day high NO_3^- diet (~1000 mg), skeletal muscle [NO_3^-] increases compared to a diet containing standard (~180 mg) or low (<50 mg) NO_3^-. However, when compared to other investigations by our lab (Kadach et al. 2022; Kadach et al. 2023), total [NO_3^-] was comparable between the 3-day supplementation and the acute ingestion of NO_3^-. Further investigating the capacity of skeletal muscle to chronically retain NO_3^- would provide important insights into the role that NO_3^- ingestion and skeletal muscle tissue plays in the sustained provision of NO during periods of NO_3^- abundance. This could in turn inform supplementation regimens as our recent paper demonstrates the importance of skeletal muscle NO_3^- through describing correlations between exercise performance and muscle [NO_3^-] (Kadach et al. 2023). An important consideration with such a study would be the dose, timing and frequency of supplement ingestion. Therefore, in order to achieve an additive effect of supplementation, it may be pertinent to supplement before skeletal muscle [NO_3^-] returns to baseline which would be within 9 hours of the initial bolus.

Are humans in a constant state of NO_3^- "deprivation"?

Adopting a slightly different school of thought may provide interesting an important new perspective into the underlying factors of why NO_3^- research is yielding such favourable findings. It is irrefutable that NO_3^- ingestion causes increases in circulating NO-related

metabolite fluxes in both biological fluids and tissues as well as induces change in the human oral microbiome when ingested over chronic periods. An interesting notion to be considered is that many modern diets may contain inadequate quantities of NO_3^- and NO_2^-. It may be speculated that as a species, humans may have adopted a deficient/ substandard diet which may in fact be prohibiting the achievement of NO homeostasis. The absence of key constituents of the diet, such as NO_3^-, could be altering fundamental biological functions, processes and relationships (i.e host and microbiome) which would influence NO-metabolism. This concept may be supported by evidence that adhering to vegetarian and Mediterranean diets are beneficial for cardiovascular and metabolic health (Hord et al. 2011; Capurso et al. 2014; Yokoyama et al. 2014; Omar et al. 2015; Shannon et al. 2018) which is likely due to greater relative consumption of NO_3^- containing food sources (typically vegetables and fruits) (Lundberg et al. 2004; Koch et al. 2017; Hord et al. 2009). Experimental chapter 2 supports this theory as only subtle differences in the oral microbiome were observed between an intervention in which NO_3^- was removed from the diet compared to a "standard" NO_3^- containing diet. However, this chapter shows that in some instances and when compared to a "standard" diet, the deprivation of NO_3^- did exacerbate differences in oral microbial genera and species when compared to a diet with high NO_3^- concentrations. An occurrence which although not measured in the experimental chapter, may be linked to the body's capacity to process NO_3^- from food sources. Therefore, this is an area which requires further research to determine the point at which changes, not only in the oral microbiome but also potentially the body's capacity to retain NO_3^-, start occurring in humans thus this would inform how much NO_3^- should be present within diets.

CHAPTER 10. CONCLUSION

The importance of sustained NO bioavailability and its role in maintaining and supporting physiological function is clear, with the oral microbiome and skeletal muscle likely central in this. Dietary NO_3^- can be an effective avenue for supplementing endogenously generated levels of NO_3^- and NO_2^- within the human body. However, human research into the roles of the oral microbiome and skeletal muscle in NO homeostasis is still in its infancy and further work is required to investigate how these are influenced by dietary NO_3^- ingestion.

Experimental Chapter 1 provides an interesting addition to the current evidence base and demonstrates that the application of a NO_3^- lotion to the skin is ineffective at inducing changes in circulating NO-metabolite concentrations. Nonetheless, questions remain regarding the importance of the skin and its role in NO homeostasis. For example, in some research, the combination of NO_3^- supplementation and exposure to UVA irradiation and exercise, indicated that skin may be involved in NO metabolism. Because the enterosalivary pathway may be seen as a limiting factor in NO_3^- metabolism, it is important to examine alternative methods of augmenting NO-bioavailability. Therefore, although the NO_3^- containing lotion did not induce change in NO-related metabolite fluxes in circulation, it is nonetheless an important contribution in attempting to find viable alternatives that may by-pass the oral cavity. Experimental Chapter 1 dovetails nicely into Experimental Chapter 2 as we demonstrate the importance of the oral microbiome in sustaining NO bioavailability.

When considering NO fluxes within the human body and the pathways that are integral in this process, the initial "uptake" of NO_3^- from dietary sources is facilitated by an underlying relationship between host and the oral microbiome which is pivotal in NO metabolism. At the inception of this thesis, emerging work indicated that the oral microbiome changes when

dietary NO_3^- consumption increases. However, as dietary NO_3^- ingestion substantially differs between individuals and populations, we designed Experimental Chapter 2 to investigate how standard (based on European standards), low and high NO_3^- diets modulate the oral microbiota. The results of Experimental Chapter 2 support the potential for dietary NO_3^- to act as a prebiotic for inducing a "shift" to a more favourable oral microbial profile when compared to a "standard diet". This may in turn may be integral in supporting therapeutic effects of NO_3^- ingestion through eliciting favourable changes to bacteria associated with oral and cardiovascular health as well as NO metabolism. Furthermore, for the first time, we provide evidence that the removal of NO_3^- from the diet may in some cases exaggerate (compared to a standard diet) differences in the microbiota within the mouth compared to a high NO_3^- diet.

Once in the body, there are changes in the concentrations of NO_3^- and NO_2^- over time in the saliva and plasma as well as increased expulsion in urine. To achieve sustained NO bioavailability, the body likely draws on biological tissues which contain considerable quantities of these NO related metabolites. Skeletal muscle has been highlighted as one such tissue which, under normal conditions, contains levels of NO_3^- and NO_2^- which exceed those seen in circulation and which increase following the ingestion of NO_3^-. Nonetheless, studies to date had not comprehensively investigated the pharmacokinetic profile of change of skeletal muscle in response to the ingestion of NO_3^- and how this may interact with other biological compartments (i.e. saliva, plasma and urine). Experimental Chapters 3 and 4 make important original contributions in this growing area, with both studies indicating that NO_3^- and NO_2^- within skeletal muscle are likely central in NO metabolism. Therefore, these studies provide a better understanding of the effects of NO_3^- ingestion on biological tissues and how

these impact the maintenance of NO bioavailability and bioactivity. Experimental Chapter 4 provides a foundation for future work investigating NO_3^- and NO_2^- in skeletal muscle by describing the time-course of change in both of these NO-related metabolites, and provides additional information on how these alterations compare to biological fluids (i.e. saliva, plasma, urine) following the ingestion of ^{15}N-labelled KNO_3. The results illustrate the rapidity of increase and subsequent short-lived duration of elevated [NO_3^-]. In combination with the lack of change in [NO_2^-] within skeletal muscle, these results challenge the notion that skeletal muscle serves as a NO storage site, at least when NO_3^- is ingested as a 'bolus'. Experimental chapter 4 elucidates that the accretion of NO_3^- in skeletal muscle is almost exclusively consequent to ingested NO_3^-. An observation made in this chapter is that unlabelled/ endogenous NO_3^- also increases in skeletal muscle at 3 h following the ingestion of NO_3^-, and was accompanied with increases in NO_3^- in plasma at 3 h, saliva and urine at both 1 h and 3 h. Shifts in plasma to muscle concentration ratios/ gradients found in Experimental Chapters 3 and 4 illustrate the importance of alterations in concentration gradient**s** in response to exogenous NO_3^- provision. This appears to facilitate the movement of NO_3^- between compartments (i.e from circulation to muscle) within the human body rather than, or more likely in combination with, the initially implicated active NO_3^- transporters which may be more relevant under non-fed conditions. Based on the combined findings of the experimental work included in this thesis, it is evident that further research is warranted investigating factors influencing the movement of NO_3^- and NO_2^- between biological fluids (i.e. blood) and tissues (i.e. muscle) and how this influences physiological function.

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