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EFFECT OF NITRATE SUPPLEMENTATION ON *PORPHYROMONAS GINGIVALIS* ABUNDANCE IN THE ORAL MICROBIAL COMMUNITY

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THE EFFECT OF NITRATE SUPPLEMENTATION ON *Porphyromonas gingivalis* ABUNDANCE IN THE ORAL MICROBIAL COMMUNITY

by

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ABSTRACT

Alzheimer's disease, the most common type of dementia, is characterized by a gradual decline in cognitive function. As a neurodegenerative disease, Alzheimer's is distinguished by neuronal loss and atrophy in regions of the brain responsible for learning and memory. Despite many hypotheses to explain the potential causes of Alzheimer's disease, its pathogenesis remains poorly understood. Although risk factors such as aging and poor cardiovascular health have been historically well-established in Alzheimer's disease pathology, researchers are continually exploring new hypotheses to explain its onset and progression. *Porphyromonas gingivalis*, a gram-negative bacterium that is the main etiological agent in periodontal disease, has recently been attributed to Alzheimer's disease pathogenesis. Researchers suggest that *P. gingivalis* can travel from the oral cavity into the brain, where it may contribute to the development of Alzheimer's disease. As a result, treating *P. gingivalis* colonization in the oral cavity could potentially offer protection against Alzheimer's disease. One proposed treatment is a dietary nitrate supplement, which may reduce the abundance of *P. gingivalis* in the oral cavity and improve overall vascular health. In this study, we administered a 10-day potassium-nitrate supplement of 4.84 mmol nitrate per day to ten healthy participants and assessed changes in oral microbiome composition and vascular health before and after the intervention. Participants attended the laboratory twice: once before and once after the 10-day nitrate supplementation. At each lab visit, we took oral samples from saliva, hard tissue (floss), and soft tissue (tongue swab) to evaluate the oral microbiome composition. Additionally, we took measurements of endothelial function and blood pressure as markers of vascular health. We assessed the composition of the oral microbiome, specifically the relative abundance of *Porphyromonas*, using 16S rRNA gene sequencing. Blood pressure was measured using photoplethysmography in the finger and endothelial function was analyzed using a flow-mediated dilation test. Paired t-tests were used to analyze changes in the abundance of *Porphyromonas*, blood pressure, and flow-mediated dilation responses from pre to post nitrate supplementation. We hypothesized there would be a decrease in the relative abundance of *Porphyromonas* in the oral cavity, a decrease in blood pressure, and an improvement in FMD response post supplementation. We found that there were no clear changes in bacterial composition and no significant shifts in the abundance of *Porphyromonas* from pre to post nitrate supplementation from the different sample sites. However, we did find that the oral microbiome associated with each sample site was closely related among individuals, and that the oral

microbiome composition remained relatively stable over time. Furthermore, we found the highest relative abundance of *Porphyromonas* in the subgingival sulcus (i.e., the floss sample type) as compared to other areas of the oral cavity. As for vascular health, there were no significant changes in blood pressure or flow-mediated dilation response. Although we did not detect significant changes in oral microbiome composition or markers of vascular health after a short-term nitrate supplementation, we did detect differences in the prevalence of *Porphyromonas* in the oral cavity. The methodology and results presented here lay a foundation for future work and have the potential to be developed into a larger scale study. If future studies see improvements post supplementation in both oral microbiome composition and vascular health, the novel use of a dietary nitrate supplement could be administered to people suffering from cognitive impairment, with the hope of potentially providing protection against Alzheimer's disease.

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1 INTRODUCTION

Alzheimer's disease (AD), the most common form of dementia, is characterized by a gradual decline in cognitive function (Kametani and Hasegawa 2018). As the seventh leading cause of death in Canada, AD is a devastating disease that affects more than 40 million people worldwide (Statistics Canada 2022; Santos et al. 2017; d'Errico and Meyer-Luehmann 2020). AD is a neurodegenerative disease characterized by neuronal loss and brain atrophy, typically in the hippocampus and entorhinal cortex regions of the brain that are responsible for learning and memory (Raji 2009; Anand and Dhikav 2012). Due to its high prevalence, AD has been closely studied for decades; however, its pathogenesis remains poorly understood and with no treatments in place to effectively prevent or cure the disease (Santos et al. 2017). Although the etiology of AD is not clearly characterized, risk factors contributing to the development of the disease have been well-established, including aging, genetic predisposition, immune dysfunction, and poor cardiovascular health (Armstrong 2013). However, recent research indicates that oral health may also play an integral role in the development AD (Ide et al. 2016; Dominy et al. 2019).

For many years, researchers have distinguished AD by two abnormal structures formed and dispersed in the brain, known as amyloid-ß (Aß) plaques and neurofibrillary tangles (NFTs) (Glenner and Wong 1984; Grundke-Iqbal et al. 1986). Aß plaques are comprised of extracellular Aß peptide accumulations, while NFTs are aggregates of hyperphosphorylated tau proteins (d'Errico and Meyer-Luehmann 2020). Researchers suggest accumulations of these structures directly damage and destroy synapses in regions of the brain responsible for memory and cognition (d'Errico and Meyer-Luehmann 2020). Aß plaques and NFTs are considered the hallmarks of AD as their involvement either alone or together is suggested to contribute to AD pathology (Vogel et al. 2021).

Aß is a proteolytic product of the glycoprotein amyloid precursor protein (APP), and regulates neuron growth, synaptic functions, neuroprotection, as well as nervous system development (Chen et al. 2017; d'Errico and Meyer-Luehmann 2020). APP is a transmembrane protein expressed in various brain tissues and neuronal synapses and is cleaved sequentially by β- and γ-secretases, which are membrane-bound proteases (Murphy and Levine 2010; Chen et al. 2017). The cleavage of APP by γ-secretase is imprecise and therefore results in Aß fragments of varied sizes, most often being either 40 ($A\beta_{40}$) or 42 ($A\beta_{42}$) amino acids in length (Murphy and

LeVine 2010). $A\beta_{40}$ is the most abundant fragment form in healthy brains, while overproduction of $A\beta_{42}$ in relation to $A\beta_{40}$ has been linked to AD pathology (Findeis 2007).

Once Aß monomers are formed in the brain, they are typically released into the extracellular space where they undergo proteolytic degradation or transportation out of the brain (Chen et al. 2017). However, in AD, Aβ aggregates into soluble oligomers that can spread throughout the brain and drive synaptic dysfunction, or into larger insoluble amyloid fibrils that can further aggregate into amyloid plaques, with the majority being driven by $A\beta_{42}$ (d'Errico and Meyer-Luehmann 2020; Chen et al. 2017). Aβ plaque formation plays a role in AD pathogenesis by inducing neuronal loss, synaptic dysfunction, and disrupting neural connectivity (d'Errico and Meyer-Luehmann 2020; Chen et al. 2017; Murphy and Levine 2010). Although the mechanism that drives Aß deposition in AD remains unclear, early onset AD may be triggered by mutations in the *APP* gene to increase the production of $A\beta_{42}$, or mutations in the *PSEN1/2* gene which plays a role in the cleavage of APP (Chen et al. 2017). In contrast, late onset AD may result from an abnormal propensity of Aß to aggregate into plaques or may be due to the role of Aß as an antimicrobial peptide (Kumar et al. 2017).

Antimicrobial peptides are a class of innate immune defense molecules that serve to protect the host from microbial invasion (Soscia et al. 2010; Kumar et al. 2017; Gosztyla et al. 2018). The role of Aß as an antimicrobial peptide in the brain is to function as a first line of defense against invading pathogens, specifically by binding and intercepting microbes before entering neurons, reducing microbial adhesion to the host cell, and even directly killing bacteria (Kumar et al. 2017; Brothers et al. 2018). However, despite the protective role of Aß as an antimicrobial peptide, it may become dysregulated in response to microbial invasion and lead to host cell toxicity, inflammation, and degenerative pathologies, like those seen in AD (Kumar et al. 2017). Thus, the large deposition of Aß plaques in AD patients may result from over reactivity of the brain's innate immune system in response to invading microbes (Kumar et al. 2017).

In addition to Aß plaques, NFTs may also play a central role in AD. NFTs are composed of insoluble filaments of the microtubule-associated phosphoprotein tau, which is a protein responsible for microtubule assembly and stability (Kametani and Hasegawa 2018). In AD, tau becomes abnormally hyperphosphorylated, allowing microtubules to disassemble and free the tau protein, which then aggregate into NFTs in the neuronal cytosol (d'Errico and Meyer-Luehmann 2020; Medeiros et al. 2011). Once NFTs are formed, they inhibit the microtubule network and

normal neuronal signaling, thus potentially compromising neuronal viability (d'Errico and Meyer-Luehmann 2020). Healthy cells may also internalize aggregates of tau, or these aggregates may be transferred through synaptic contacts between neurons, which results in the rapid spread of NFTs in healthy brain areas (d'Errico and Meyer-Luehmann 2020).

Despite substantial evidence supporting the amyloid and tau hypotheses as drivers of AD, researchers have recently highlighted other mechanisms that may play integral roles in AD etiology. There is evidence suggesting cardiovascular health and more recently, oral health also contribute to AD pathogenesis (de Bruijn and Ikram 2014; Kelleher and Soiza 2013; Ide et al. 2016). Cardiovascular diseases including stroke, coronary heart disease, and heart failure, as well its risk factors, such as arterial stiffness and endothelial dysfunction, significantly increase the risk of developing AD (de Bruijn and Ikram 2014; Stephan et al. 2017).

Arterial stiffness has been linked to neural inflammation, synaptic injury, NFTs, and an increase in pulsative pressure that damages the brain microvascular system, which can all contribute to AD (Moore et al. 2021; de Bruijn and Ikram 2014). Similarly, endothelial dysfunction may also play a role in the development of AD (Kelleher and Soiza 2013). The endothelium is a monolayer of endothelial cells that lines the lumen of all blood vessels, including the blood-brain barrier (Krüger-Genge et al. 2019). This layer of cells controls the degree of vascular relaxation and constriction, regulates the exchange of molecules between the bloodstream and tissues, and maintains the integrity of blood vessels (Krüger-Genge et al. 2019; Deanfield et al. 2007). Healthy endothelial function is largely dictated by the availability of nitric oxide (NO) in the body, which is a molecule acquired both endogenously and exogenously, and acts as a potent vasodilator. In turn, endothelial dysfunction, which is the impaired functioning of the lining of blood vessels, is characterized by a decreased availability of NO, in which blood vessels are unable to properly dilate in response to an adequate stimulus (Hughes et al. 2013). In turn, the integrity of the endothelial layer may become increasingly compromised and the blood-brain barrier can become permeable to toxic molecules that contribute to AD progression (Dede et al. 2007; Kelleher and Soiza 2013; Lyros et al. 2014).

While the relationship between cardiovascular health and AD is relatively well-established, how oral health contributes to AD pathology is not as well-characterized. Recently, several studies have found periodontal disease to be closely linked to the development of AD (Ide et al. 2016; Dominy et al. 2019; Beydoun et al. 2020). Periodontal disease, also known as gum disease, is a

gum infection that damages the tissues surrounding the teeth, often because of poor oral hygiene, smoking, or genetic predisposition (Díaz-Zúñiga et al. 2020; Tettamanti et al. 2017). The disease is caused by the imbalance or dysbiosis of the commensal oral bacteria that leads to inflammation of the gingiva (gums), the breakdown of connective tissue, and the formation of periodontal pockets (Kinane et al. 2017; Leira et al. 2017). Notably, periodontal pockets, which are spaces around the teeth underneath the gum line, create an environment that favours the proliferation of bacteria, especially of gram-negative genera (Kamer et al. 2008).

One such bacterium is *Porphyromonas gingivalis*, a gram-negative anaerobic bacterium that is the main etiological agent in periodontal disease (Kamer et al. 2008; Dominy et al. 2019; Díaz-Zúñiga et al. 2020). *P. gingivalis* typically exists in a healthy oral cavity in a commensal relationship with the human host; however, these bacteria can quickly shift into a disease state if they accumulate at high numbers in the subgingival dental plaque (Lamont and Jenkinson 1998; Guo et al. 2010). *P. gingivalis* produces a variety of virulence factors including fimbriae and proteolytic enzymes, which likely mediate its toxicity in the host (Lamont and Jenkinson 1998; Guo et al. 2010). Of its proteolytic enzymes, gingipains, which are cysteine proteases, are involved in 85% of *P. gingivalis* tissue destruction, therefore playing an integral role in its pathology (Guo et al. 2010; Imamura 2003). Gingipains enable a tight adherence to sites in the oral cavity, allowing *P. gingivalis* to exploit the host for sources of heme and iron, sustain chronic inflammation in the host, and induce apoptosis in gingival epithelial cells (Shi et al. 1999; Guo et al. 2010; Stathopoulou et al. 2009; Sheets et al. 2005). In *P. gingivalis* pathology, gingipains play a vital role in exploiting host signalling pathways to invade the cell and degrade extracellular protein matrices, as well as acting to manipulate the cytokine network to avoid host defence (Guo et al. 2010).

The home of *P. gingivalis* within periodontal pockets also enables it to gain easy access to the circulatory system (Guo et al. 2010). *P. gingivalis* can travel out of the oral cavity by manipulating monocytes for transport to the circulatory system, or by processes of bacteremia, in which bacteria enter the bloodstream from gingival bleeding during teeth brushing and by chewing food in periodontally compromised teeth (Kanagasingam et al. 2020; Singhrao et al. 2015; Dominy et al. 2019). Once in the circulatory system, *P. gingivalis* can invade blood-brain barrier endothelial cells, which may have become permeable as a result of endothelial dysfunction

(Singhrao and Harding 2020). *P. gingivalis* invasion may also occur through olfactory or trigeminal cranial nerve infection that travels to the brain (Dominy et al. 2019).

In the brain, *P. gingivalis* spreads slowly from neuron to neuron, causing increased infection over time, and contributing to AD by several mechanisms (Dominy et al. 2019). Given the putative role of Aß as an antimicrobial peptide, $Aß_{42}$ has been shown to accumulate in the brain and form amyloid plaques in response to influxes of *P. gingivalis* (Battersby et al. 2016; Dominy et al. 2019). Furthermore, gingipains produced by *P. gingivalis*, can cleave tau proteins in the brain that may contribute to NFT formation and degenerate neurons (Dominy et al. 2019; Díaz-Zúñiga et al. 2020). Researchers also suggest that the inflammatory products produced in periodontal disease induce further Aß and tau protein production in brain tissue of patients with AD (Kamer et al. 2008; Ishida et al. 2017).

The new-found link between *P. gingivalis* and AD has sparked efforts to try to treat *P. gingivalis* associated AD. Dominy et al. (2019) have been instrumental in the field in developing a drug to inhibit *P. gingivalis* proliferation. In recognition that antibiotics do not offer reliable protection from *P. gingivalis* invasion, the researchers developed small molecule gingipain inhibitors as treatment (Dominy et al. 2019). Given gingipains are necessary for *P. gingivalis* host colonization, nutrient acquisition, and inactivation of host defenses, blocking gingipains in the brain has the potential to inhibit *P. gingivalis* infection (Dominy et al. 2019). Dominy et al. (2019) demonstrated that gingipain inhibitors can reduce the load of *P. gingivalis* in the brain, block gingipain-caused neurodegeneration, decrease the $Aß_{42}$ host response, and reduce overall neuroinflammation. These researchers suggest that gingipain inhibitors block the bacteria's ability to acquire heme and generate peptide nutrients, both of which are essential for energy production and growth (Dominy et al. 2019).

Alongside gingipain inhibitors, an additional intervention worth assessing is the administration of a dietary nitrate supplement. Vanhatalo et al. (2018) have demonstrated that nitrate supplements can alter the salivary microbiome community composition, notably with a decline in the abundance of Bacteroidetes, the phylum to which *P. gingivalis* belongs. Rosier et al. (2020) recently found that in vitro nitrate supplementation significantly reduces *P. gingivalis* abundance in saliva samples. These two studies suggest that a nitrate supplement could create an oral environment that would be unfavourable for *P. gingivalis* proliferation and thus may potentially protect against AD development and progression.

Dietary nitrate supplements may also improve systemic vascular health. A nitrate supplement increases the production of NO, a potent vasodilator, in the body and thus has the potential to improve overall physiological health by both lowering blood pressure and improving endothelial function. NO is naturally produced by two pathways in the body. The first is the endogenous L-arginine/NO-synthase pathway, and the second is the enterosalivary nitrate-nitrite-NO pathway. In the endogenous L-arginine/NO-synthase pathway, NO is synthesized from the amino acid L-arginine by endothelial NO synthases. However, NO can also be derived from nitrate in the diet, such as from vegetables or water (Koch et al. 2017; Tribble et al. 2019). This process is termed the enterosalivary nitrate-nitrite-NO pathway and provides the body with an alternate source of NO (Tribble et al. 2019).

The enterosalivary nitrate-nitrite-NO pathway relies on the reduction of dietary nitrate to nitrite by nitrate-reducing oral bacteria, in which these bacteria utilize nitrate as an alternative electron acceptor in anaerobic respiration (Koch et al. 2017; Tribble et al. 2019; Sundqvist et al. 2020; Alzahrani et al. 2021). Approximately 60-70% of consumed nitrate is released in the urine, while the remaining 25% is reabsorbed from the plasma and concentrated into the saliva to be used in the enterosalivary pathway (Hobbs et al. 2013). Upon the conversion of nitrate to nitrite by oral bacteria, roughly 80% of nitrites are swallowed and are protonated to form nitrous acid (HNO_2) which spontaneously decomposes into NO as facilitated by acids in the stomach (Alzahrani et al. 2021; Lundberg et al. 2008; Hobbs et al. 2013). The human body lacks nitrate reductase abilities and thus rely on the presence of oral bacteria to supplement NO and to regulate proper vasodilation in the body (Tribble et al. 2019).

It is important to consider that the production of NO from the endogenous L-arginine/NO-synthase pathway can often become compromised, such as in cardiovascular diseases, and the reduced availability of NO in the body limits the ability of the arteries to properly dilate or maintain endothelial layer integrity (Kelleher and Soiza 2013; Velmurugan et al. 2016). The consumption of nitrate, such as from a nitrate supplementation, may be able to restore adequate NO concentrations to ensure healthy endothelial function, decrease blood pressure, and maintain endothelial layer integrity, especially in the blood brain barrier (Dejam et al. 2004; Vanhalato et al. 2018; Rosier et al. 2020). In turn, these effects may be able to slow the entry of bacteria, such as *P. gingivalis*, into the brain and thus potentially protect against the development of AD.

In the present study, we assessed the effects of a dietary nitrate supplementation on the oral microbiome and overall vascular health in a healthy study population. We hypothesized that a dietary nitrate supplement will reduce the abundance of *P. gingivalis*, a microbe linked to AD pathogenesis, in the oral microbiome, as well as improve secondary vascular outcomes tied to cardiovascular disease. To test this hypothesis, we administered a 10-day dietary nitrate supplement, in the form of potassium nitrate, to ten healthy participants. We assessed changes in the oral microbiome, specifically the abundance of *Porphyromonas* genus, using 16S rRNA gene sequencing, as well as assessed changes in secondary vascular outcomes of blood pressure and endothelial function as surrogate markers of cardiovascular disease risk.

2 MATERIALS AND METHODS

2.1 Ethical approval

Ethical approval was granted by the Thompson Rivers University Research Ethics Board and Biosafety approval was granted by the Thompson Rivers University Biosafety Committee. All participants gave their written and informed consent, both after the experimental procedure and risks had been explained to them and prior to the start of testing.

2.2 Study participants

Ten healthy participants including 7 males and 3 females between 20-49 years of age were recruited to participate in the study. Mean participant age was 30.8 ± 10.5 years, height was 177.5 ± 7.6 cm, and weight was 73.8 ± 10.6 kg. Recruitment was of Thompson Rivers University professors and students, as to limit contact with the public due to the COVID-19 pandemic at the time of testing. Participants were screened prior to testing to ensure they met inclusion criteria. All participants had their initial vaccination for COVID-19 (> 2 weeks prior to testing), no blood or platelet/bleeding disorders, and no known cardiac diseases and/or cardiovascular risk factors. None of the participants were hypertensive, smokers, diabetics, or had other known metabolic diseases. The participants were not taking medication or being treated for any disease, did not experience aversion to the sight of blood and/or needles, and were over 19 years of age and under 60 years of age.

Preceding testing, participants were instructed to arrive at the laboratory rested, having refrained from strenuous physical activity, alcohol, marijuana, or non-prescription drug ingestion

24 hours prior to sampling. They were instructed not to cycle or run to and from the laboratory on sampling days, nor partake in any physically demanding work in the hours after the sampling day. Participants were asked to avoid caffeine on the day of sampling before their testing, eating within four hours prior to sampling, and blood donations eight weeks prior to, and eight weeks after participation. Additional participant exclusions included using antimicrobial or antiseptic tooth paste 48 hours prior to testing, mouthwash 48 hours prior to test, and flossing on the morning of test days, following criteria used in the NIH Human Microbiome Project (2010).

2.3 Experimental design

Participant testing consisted of blood pressure and ultrasonographic measurements, blood extraction, and oral bacterial sampling. Upon arrival at the lab, participants were placed in a supine position in a dimly lit, temperature controlled (21-24°C) room and were instrumented with a non-invasive continuous blood pressure monitor. Once an adequate blood pressure signal was recording, the lights were turned off and the participant was left in a supine position for ten minutes to ensure baseline sampling was taken at rest, with minimal sympathetic nervous system activation.

Once the rest period was complete, participants underwent a flow mediated dilation (FMD) protocol to measure endothelial dependent dilation of the brachial artery using Doppler ultrasound as described below. Following the FMD, 6 mL of blood was drawn from an antecubital vein for later analysis of blood nitrite-nitrate concentrations. Oral samples of saliva, floss, and tongue swab were then taken for oral microbiome analysis. At the end of sampling, participants were given twenty 400 mg potassium nitrate oral supplements, containing 2.42 mmol of nitrate each, to be taken twice a day for the 10 days preceding their post testing. The total intake of nitrate each day was therefore 4.84 mmol. The concentration of nitrate was chosen as per previous work by Wylie et al. (2013) and the WHO suggestion that 4.2 mmol of nitrate in a 70 kg adult is an acceptable daily intake (Hobbs et al. 2013). The duration of 10 days was chosen as per the work of Vanhatalo et al. (2018). Male participants returned two weeks after their pre-testing for post testing, while females returned 28 days after their pre-testing to control for potential influences of the female menstrual cycle. In both groups, the nitrate supplements were taken in the final 10 days preceding post supplement testing. Upon return for post testing, the same procedures described above were repeated (Figure 1).

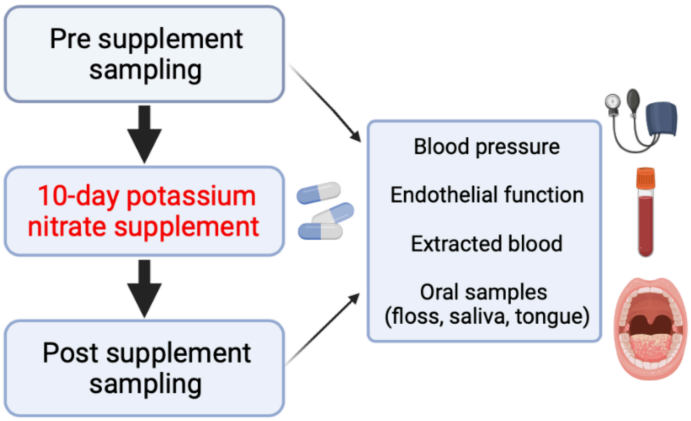


Figure 1. Experimental procedure of pre and post nitrate testing.

2.4 Vascular assessments

2.4.1 Blood pressure

Participants were instrumented with a non-invasive continuous blood pressure monitor that output an analog continuous waveform to a BIOPAC data acquisition system (Biopac MP160, Biopac Systems, California, USA). The CNAP monitor consisted of finger cuffs placed around the left index and middle fingers, one of which was monitored continuously using photoplethysmography. Blood pressure was recorded during the initial 10-minute rest period and through the duration of the FMD protocol. Each participant's resting blood pressure was determined by averaging their values over 300 seconds midway through the rest period.

2.4.2 Endothelial function

Endothelial function was assessed using a brachial artery flow mediated dilation (FMD) protocol that measures an endothelium-dependant dilation after a period of occlusion downstream of the assessed artery. A pneumatic cuff around their right forearm was connected to a pressure monitor and an inflation bulb. Doppler ultrasound was used to image a longitudinal section of the right brachial artery, with clearly defined intima-media borders (Epiq 5G, Philips Health Care, Canada). Prior to proceeding with the FMD protocol, a 30 second video was recorded of the right brachial artery at rest. To carry out the FMD protocol, the cuff around the right forearm was inflated to 220 mmHg and held for 5 minutes to inhibit blood flow into or out of tissues beyond the cuff (i.e., the right forearm and hand). A 3-minute video of the right brachial artery was taken, starting 30 seconds before the cuff was deflated (at 4:30 minutes of the 5-minute protocol) and

extended two and a half minutes after the cuff was deflated to capture the vessel's response to blood flow re-establishment.

Changes in FMD response were analyzed and compared between the pre and post nitrate supplementation conditions. First, end diastolic diameter (in millimeters) was averaged between 20 and 30 cardiac cycles during rest (FMD_{min}), and was compared to the average of the three maximum end diastolic diameters upon cuff deflation (FMD_{max}). Subsequently, calculations were carried out to determine the absolute and relative difference between FMD_{min} and FMD_{max}. The absolute difference was calculated using the equation $FMD_{max} - FMD_{min}$, while the relative difference was calculated using the equation $[(FMD_{max} - FMD_{min}) / FMD_{min}] \times 100\%$. These values were calculated for both the pre and post supplementation conditions. Baseline end diastolic diameters at rest were recorded both pre and post supplementation, as this value may have changed upon oral administration of the nitrate supplement and needed to be controlled for during the analysis.

2.4.3 Blood plasma nitrate and nitrite

To assess blood nitrite-nitrate levels, 6 mL of venous blood was drawn from an antecubital vein. The blood was centrifuged at 400 relative centrifugal force (rcf) and 21 °C for 8 minutes. Subsequently, 1 mL of plasma was separated, flash frozen in liquid nitrogen, and stored at -80°C for later determination of nitrate and nitrite concentration using a Griess Reaction modified from Romitelli et al. (2007) and Ghasemi et al. (2007).

2.5 Oral sampling

Following blood collection, three oral samples were collected for microbial analysis, as per methodology outlined in the NIH Human Microbiome Project – Core Microbiome Sampling Protocol (2010) and altered where necessary.

2.5.1 Saliva

To obtain unstimulated saliva, participants allowed saliva to build up in their mouth for 1 minute and drooled into a 15 mL centrifuge tube. In total, 1 mL of saliva was required, and so participants underwent this process numerous times to ensure sufficient sample was obtained. Subsequently, 200 µL of saliva was separated and placed into a 2 mL screw top tube containing 1 g sterile ceramic beads and 600 µL Buffer RLT Plus (lysis buffer), with the latter being obtained from an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). The remaining 800 µL of saliva was centrifuged at 3500 rcf and 21 °C for 10 minutes to pellet. The supernatant was discarded, and

600 µL Buffer RLT Plus was added to the pellet biomass. Both the saliva and centrifuged saliva samples were flash frozen in liquid nitrogen and stored at -80 °C until future DNA extraction.

2.5.2 Soft tissue tongue swab

Samples of soft tissue tongue swabs were obtained following saliva sampling. Participants rinsed their mouth with water to wash away any saliva that was coating their tongue, which ensured the tongue swab represented the tongue microbiota, and not the salivary microbiota. A sterile cotton tipped swab was used to swab 1 cm^2 of the center of the tongue for 5 to 10 seconds. The swab was then trimmed and placed in a 2 mL screw cap tube containing 1 g sterile ceramic beads and 600 µL Buffer RLT Plus. The tube was flash frozen in liquid nitrogen and stored at -80 °C until future DNA extraction.

2.5.3 Hard tissue floss

The third and last oral sample taken was a hard tissue gum line sample obtained by flossing. Participants were instructed to floss four teeth, each twice, with sterile disposable floss picks for a total of eight floss samples. The teeth flossed were between each canine and first premolar in the top right, bottom right, top left, and bottom left areas of the mouth. The floss of each pick was removed with a sterile razor and all eight floss samples were placed together in a 2 mL screw top tube containing 1 g sterile ceramic beads and 600 µL Buffer RLT Plus. If participants bled during flossing, the floss was discarded, and they were instructed to floss between the first and second premolar of the same area instead (e.g., top right). The tube was then flash frozen in liquid nitrogen and stored at -80 °C until future DNA extraction.

2.6 DNA sequencing and analysis

2.6.1 DNA extraction

Oral samples from both pre and post nitrate supplementation (saliva, centrifuged saliva, tongue swab, and floss) of the 10 participants were isolated using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Samples were frozen in Buffer RLT Plus and were stored in tubes containing 1 g of sterile silica beads, except for the centrifuged saliva samples that were transferred into bead containing tubes upon thaw. Once all samples were thawed, 6 µL of beta-mercaptoethanol (BME) was added to each sample, samples were homogenized by bead beating (vortexing) for 5 minutes and centrifuged at 8000 rpm for 30 seconds following the methodology outlined by Moen et al. (2016). From then onwards, manufacturer's instructions were followed. Once the DNA had been extracted, it was quantified on a Qubit 2.0 Fluorometer (Invitrogen,

Carlsbad, CA, USA) using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts, USA).

2.6.2 Amplification

Polymerase chain reaction (PCR) of the V4 hypervariable region in the bacterial 16S rRNA gene was carried out on each of the 80 extracted DNA samples. A 20 µL reaction mixture was prepared in a microcentrifuge tube containing a final concentration of 1X GoTaq Green Master Mix (Promega, Madison, USA), 0.05 µM forward primer (341F 5′TACGGGAGGCAGCAG), 0.05 µM reverse primer (806R 5′GGACTACVSGGGTATCTAAT), and 2 µL isolated DNA from each sample. In place of the 2 µL DNA sample, 2 µL of bacterial genomic DNA was used as a positive control and 2 µL of PCR-grade water as a negative control. Each 20 µL reaction mixture was transferred to a well in a 96-well PCR plate and PCR was completed in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Massachusetts, USA). PCR thermocycling conditions included an initial denaturation for 4 minutes at 95°C; 25 cycles of denaturation for 30 seconds at 95°C, primer annealing for 45 seconds at 53.4°C and 2 minutes at 72°C; and a final extension for 5 minutes at 72°C. PCR amplification was assessed in all samples and controls by running 5 µL of product on a 1% agarose gel prepared in TAE buffer, and product sizes were determined by comparison to 500 ng of 1 kb Plus Ladder run on the same gel. The gel was run at 80 volts for 30 minutes and visualized on a UV transilluminator.

The amplicons were purified prior to a second round of PCR by mixing 18 µL of agencourt AMPure XP (Beckman-Coulter, Brea, USA) beads with 10 µL of PCR product in a 96-well plate. After five minutes, the PCR plate was placed on a DynaMag-96 side magnetic PCR plate (Thermo Fisher Scientific, Massachusetts, USA) for two minutes to allow the beads now containing the DNA to migrate to the magnet. The reaction solution was discarded, and the beads were washed twice with 200 µL 70% ethanol for 30 seconds. After the second wash and all ethanol had been removed, the beads were left to dry for five minutes. The 96-well plate was taken off the DynaMag-96 side and 20 µL TE buffer was added to the wells and mixed to elute the DNA. The mixture was left to sit for three minutes and then transferred to the DynaMag-96 side for an additional minute to let the beads separate from the solution. The beads were discarded, and the solution was transferred to a new plate and stored at -20 °C. The purified amplicons were quantified with the on the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts, USA).

A second round of PCR was carried out on each cleaned first round PCR product. Reactions were prepared identically to that describe for the first round PCR, but with modified primers: forward primers contained Ion Xpress barcode sequences (341F 5′ CCATCTCATCCCTGCGTGTCTCCGACTCAG[barcode]TACGGGAGGCAGCAG) and reverse primers contained the Ion P1 adapter sequence (806R 5′CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGGACTACVSGGGTAT CTAAT). Positive and negative controls were prepared for PCR in the same manner as in first round PCR. The thermocycling conditions of the second round PCR were identical to first round, except that the primer annealing step was carried out at 65°C as opposed to 53.4°C and the program consisted of 20 cycles instead of 25 cycles. PCR amplification was again assessed on a 1% agarose gel in TAE buffer and amplicons were purified following the same methodology previously described. A final quantification of the purified amplicons was carried out using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts, USA).

2.6.3 Sequence library preparation

Each of the second-round PCR samples were pooled at equimolar concentrations. and prepared for sequencing using an Ion 520™ and Ion 530™ Kit-Chef on an Ion Chef system. Samples were sequenced on an Ion S5 XL using 400 bp chemistry (Thermo Fisher Scientific). Data was processed using AMPtk for quality filtering (Palmer et al., 2018). OTU clustering was carried out at 97% sequence identity, and taxonomy was assigned to each OTU (Palmer et al., 2018). The reads grouped to a total of 431 OTUs. The sequencing data was normalized, and out of 80 samples, 75 met the read target number of >1,500 reads.

2.7 Statistical analysis

To analyze changes in endothelial function between pre and post supplementation, we used paired t-tests to compare the absolute and relative differences between FMD_{min} at rest and FMD_{max} upon cuff deflation. To assess changes in blood pressure, paired t-tests were also used to compare systolic, diastolic, and mean arterial blood pressure between pre and post nitrate conditions. One participant was removed from the blood pressure analysis, while another was removed from the FMD analysis, as either their blood pressure or FMD data was not properly recorded.

To assess changes in the oral microbiome from pre to post nitrate conditions, analyses were carried out in R 4.0.0 (R Core Team, 2020). We assessed Bray-Curtis dissimilarity between pre

and post nitrate conditions and among sample types in individuals. From these data we produced a multidimensional scaling (MDS) plot to evaluate similarities in microbiome composition between sample types and pre and post nitrate conditions for each participant. We also carried out paired t-tests to analyze changes in the relative abundance of *Porphyromonas* from pre to post nitrate conditions in each sample type, and a one-way repeated measures ANOVA to analyze if there were any differences in the relative abundance of *Porphyromonas* between the four different sample types of saliva, floss, tongue, and centrifuged saliva.

In all analyses of the oral microbiome, one participant's pre saliva sample, two participants' post saliva samples, one participant's pre floss sample, and one participant's pre centrifuged saliva sample were removed because they had insufficient sequencing data (i.e., under 1,500 sequencing reads). The corresponding pre/post samples of the insufficiently sequenced samples were also removed in all oral microbiome analyses comparing pre and post nitrate conditions but were not removed in the repeated measures ANOVA that compared *Porphyromonas* relative abundance across sample types.

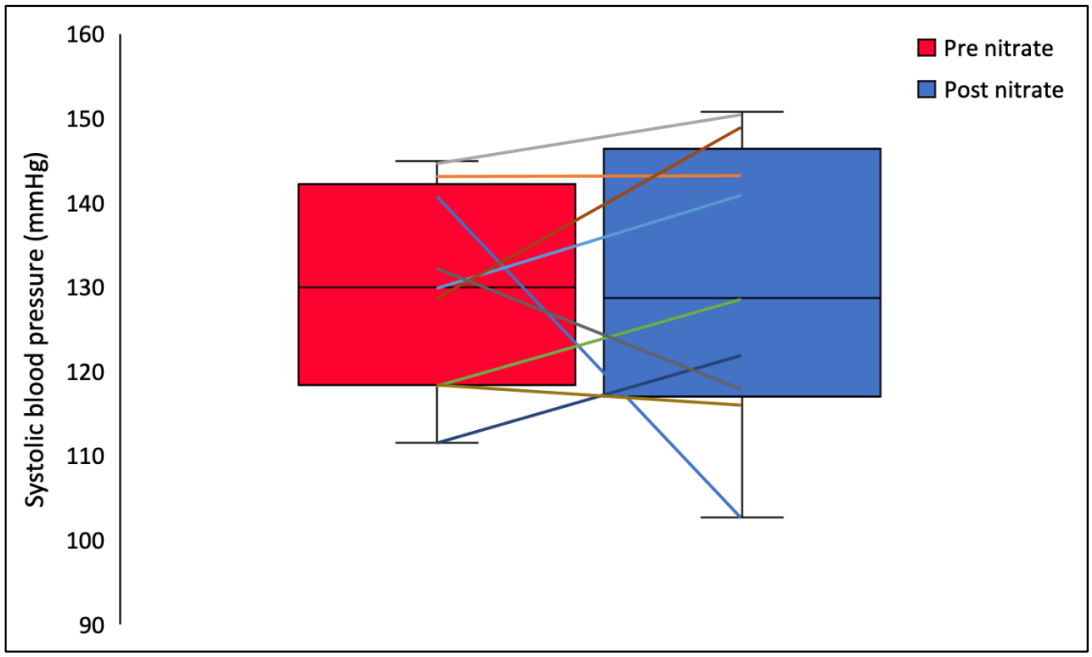
3 RESULTS

3.1 Vascular responses

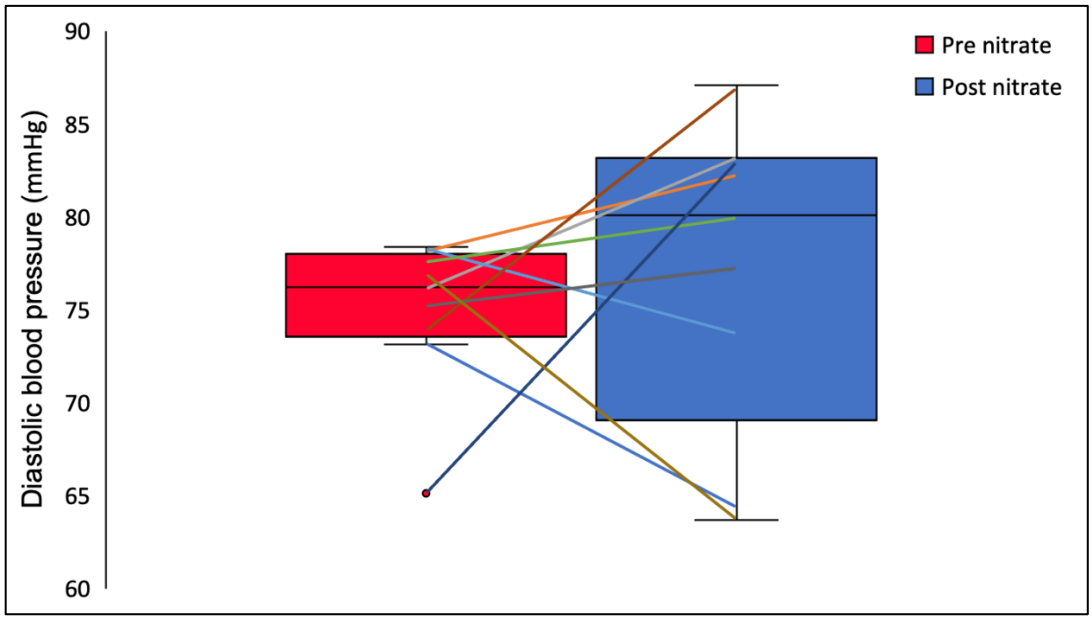
3.1.1 Blood pressure

Resting systolic, diastolic, and mean arterial blood pressure was determined for both pre and post nitrate conditions (Figure 2). Resting systolic, diastolic, and mean arterial blood pressure did not differ significantly between pre and post nitrate conditions ($p>0.05$).

a)


160
150
140
130
120
110
100
90
Systolic blood pressure (mmHg)
Pre nitrate
Post nitrate

b)


90
85
80
75
70
65
60
Diastolic blood pressure (mmHg)
Pre nitrate
Post nitrate

c)

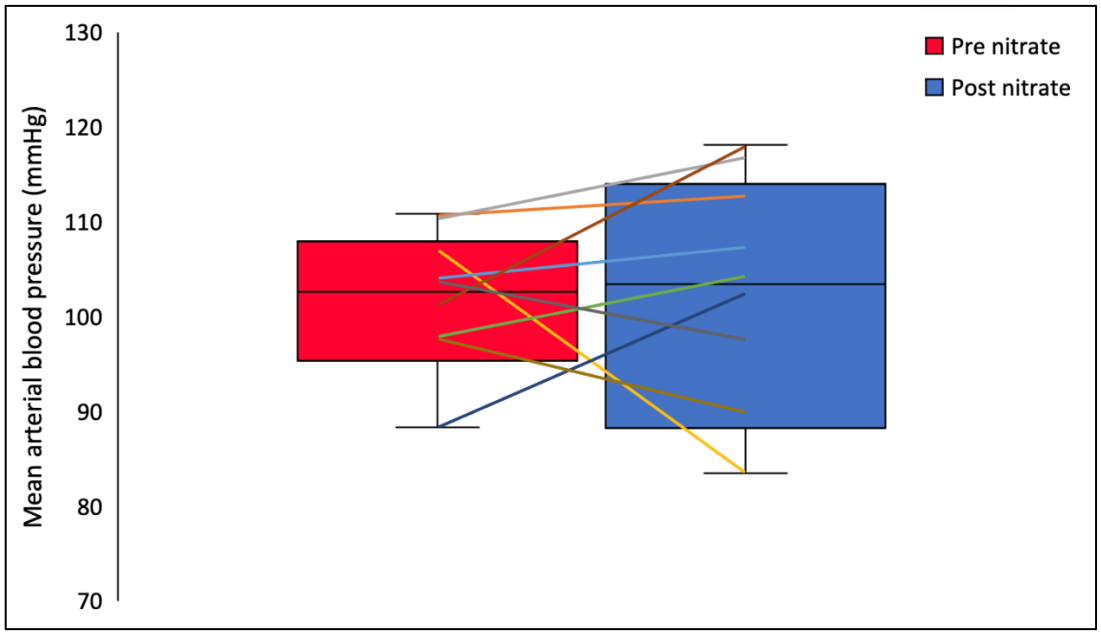


Figure 2. Mean resting blood pressure pre and post nitrate supplementation for a) systolic b) diastolic and c) mean arterial blood pressures. Lines indicate individual responses (n=9). There were no differences between conditions ($p>0.05$).

3.1.2 Endothelial function

Absolute and relative FMD responses were determined for both pre and post nitrate conditions among participants (Figures 3 and 4). Absolute and relative FMD response did not differ significantly between pre and post nitrate conditions ($p>0.05$).

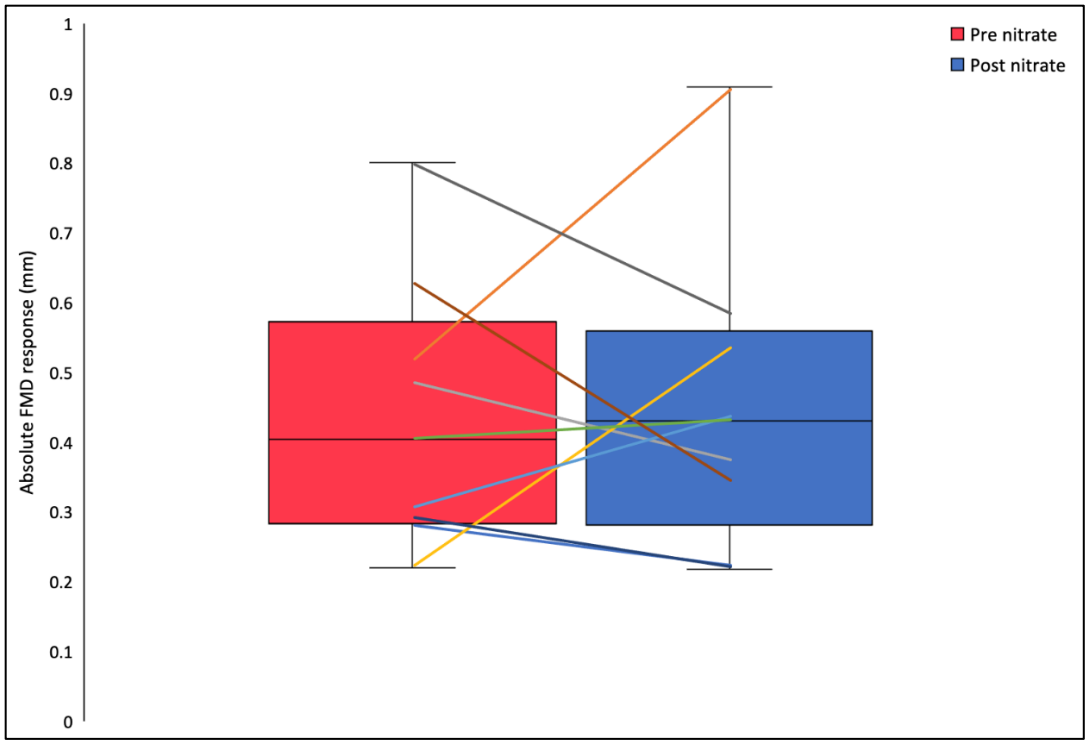


Figure 3. Mean absolute change in brachial artery FMD in pre and post nitrate conditions. Lines indicate individual responses (n=9). There were no differences between conditions (p>0.05).

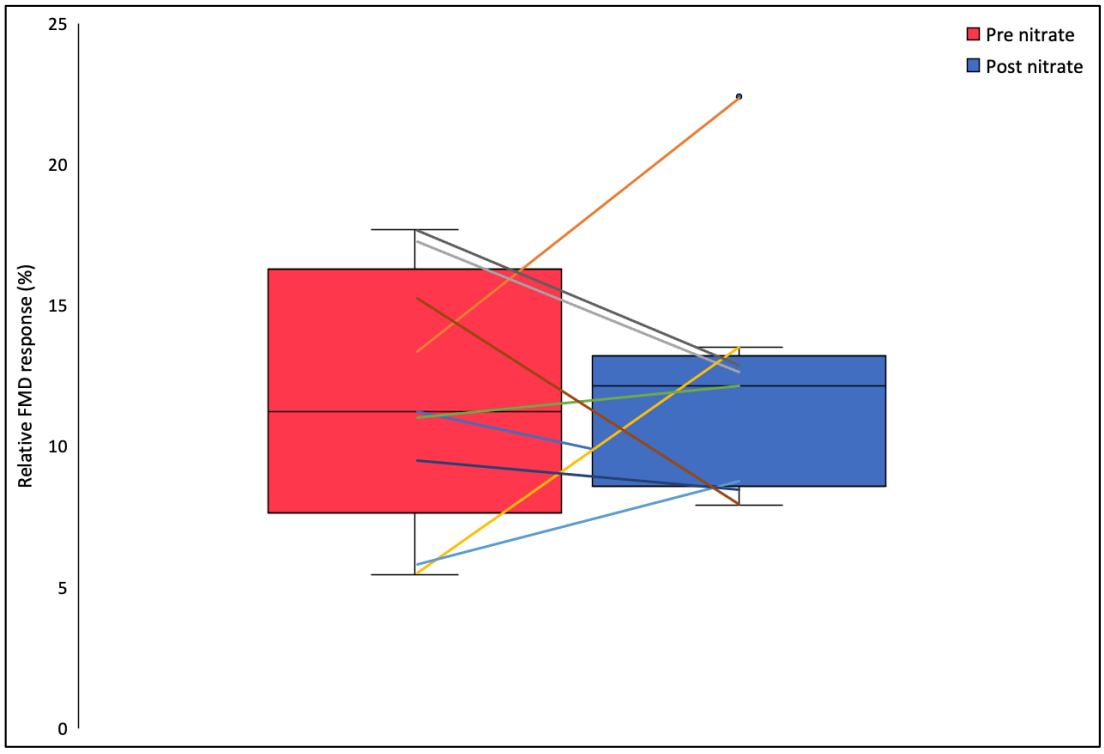


Figure 4. Mean relative change in brachial artery FMD in pre and post nitrate conditions. Lines indicate individual responses (n=9). There were no differences between conditions (p>0.05).

3.2 Oral responses

3.2.1 Oral microbiome composition

Relative abundances of each oral bacterial phylum were determined for saliva, floss, tongue, and centrifuged saliva sample types in both pre and post nitrate conditions (Figure 5). The relative abundances of each phylum within sample types did not show clear changes with nitrate supplementation. The most abundant phyla in each oral sample type were Bacteroidetes, Firmicutes, and Proteobacteria. Together these three phyla made up over 70% of the oral microbiome composition in each of the four sample types.

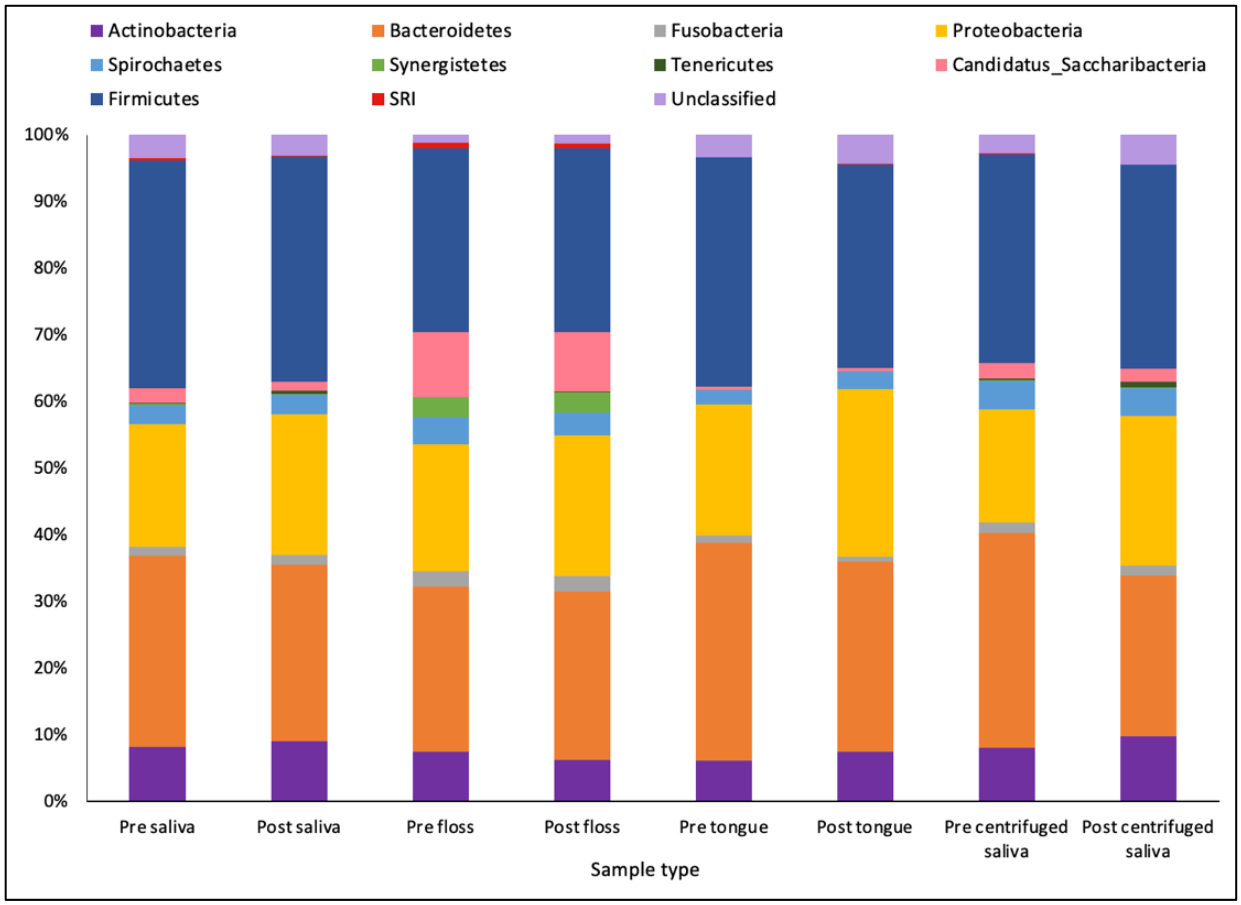


Figure 5. Relative abundance of bacterial phyla in saliva, floss, tongue, and centrifuged saliva sample types both pre and post nitrate conditions (n=8).

3.2.2 Sample structure

The Bray-Curtis dissimilarity metric was used to quantify variation in microbiome composition between samples, and a nonmetric multidimensional scaling (NMDS) plot was used to visualize trends with respect to changes in the oral microbiome composition of each individual between pre and post nitrate conditions and in each sample type (Figure 6). The microbiome associated with each sample type was closely related among individuals and did not show clear changes from pre to post nitrate conditions.

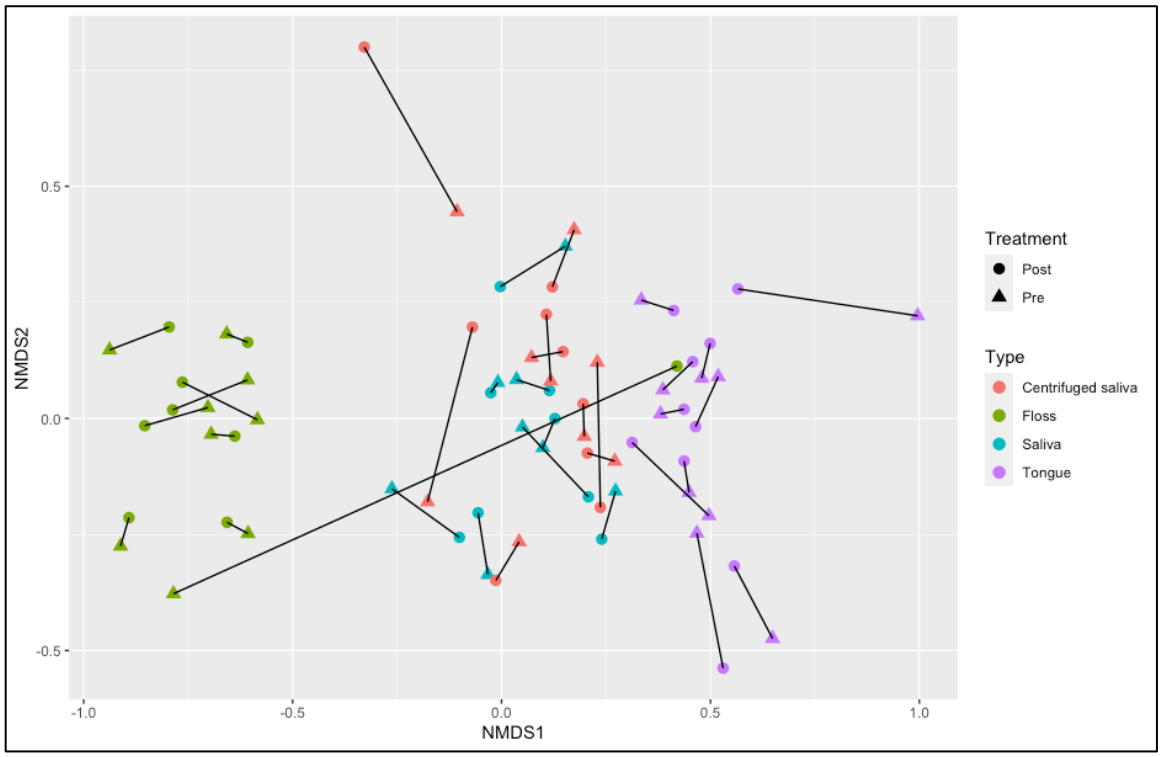
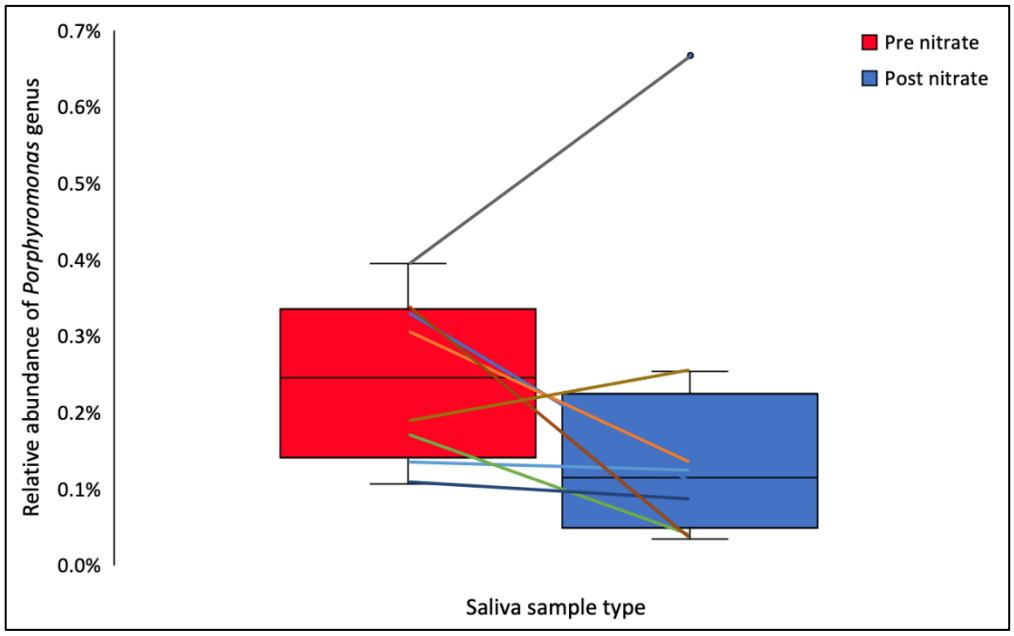


Figure 6. Multidimensional scaling (MDS) plot reflecting Bray Curtis dissimilarities in oral microbiome composition between samples. Plot shows comparisons between pre and post nitrate conditions and sample types. Lines between points show individual responses (n=8).

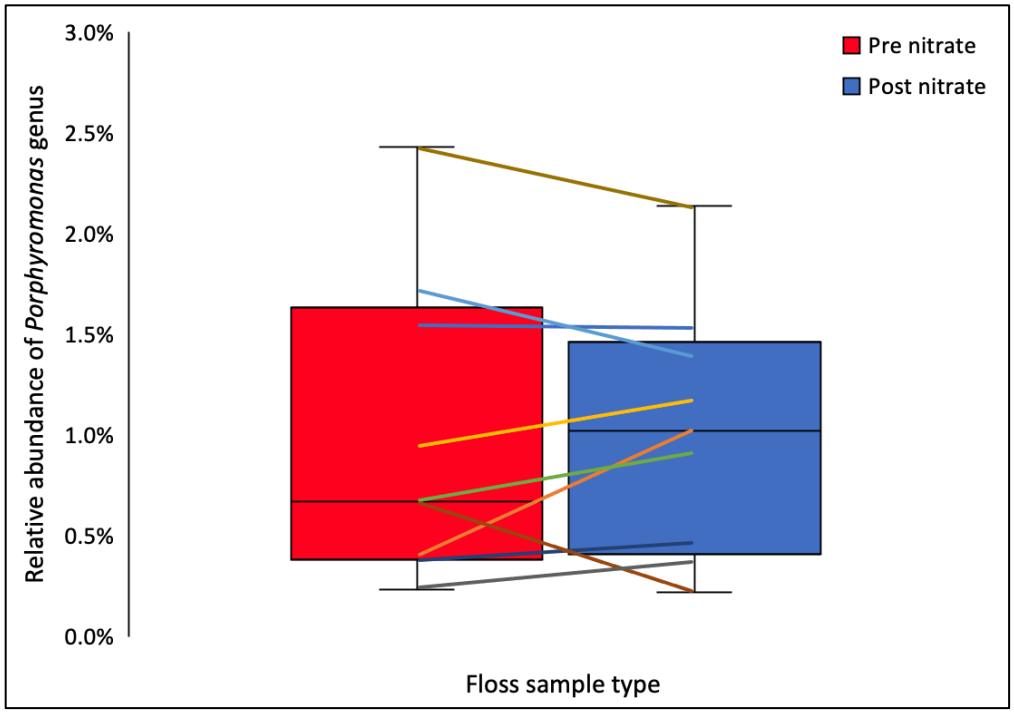
3.2.3 Abundance of *Porphyromonas*

The mean relative abundance of sequencing reads grouping to the genus *Porphyromonas* was assessed in both pre and post nitrate conditions and in the saliva, floss, tongue, and centrifuged saliva sample types (Figure 7). There were no significant differences in the relative abundance of *Porphyromonas* between pre and post nitrate condition in any sample type ($p>0.05$).

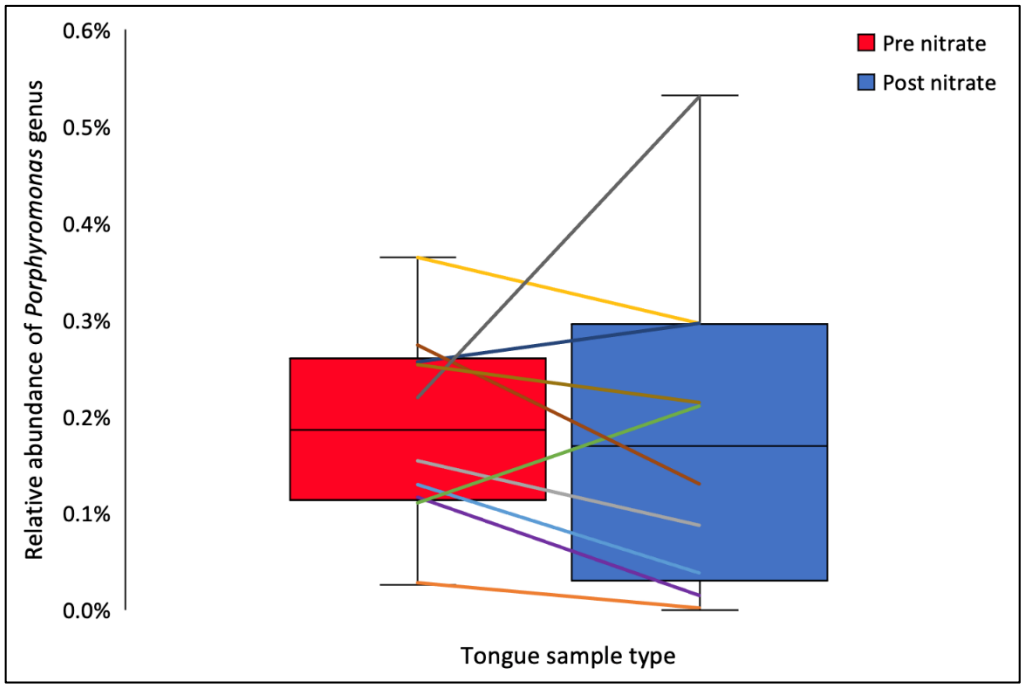
a)


0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
Relative abundance of Porphyromonas genus
Pre nitrate
Post nitrate
Saliva sample type

b)


3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Relative abundance of Porphyromonas genus
Pre nitrate
Post nitrate
Floss sample type

c)



d)

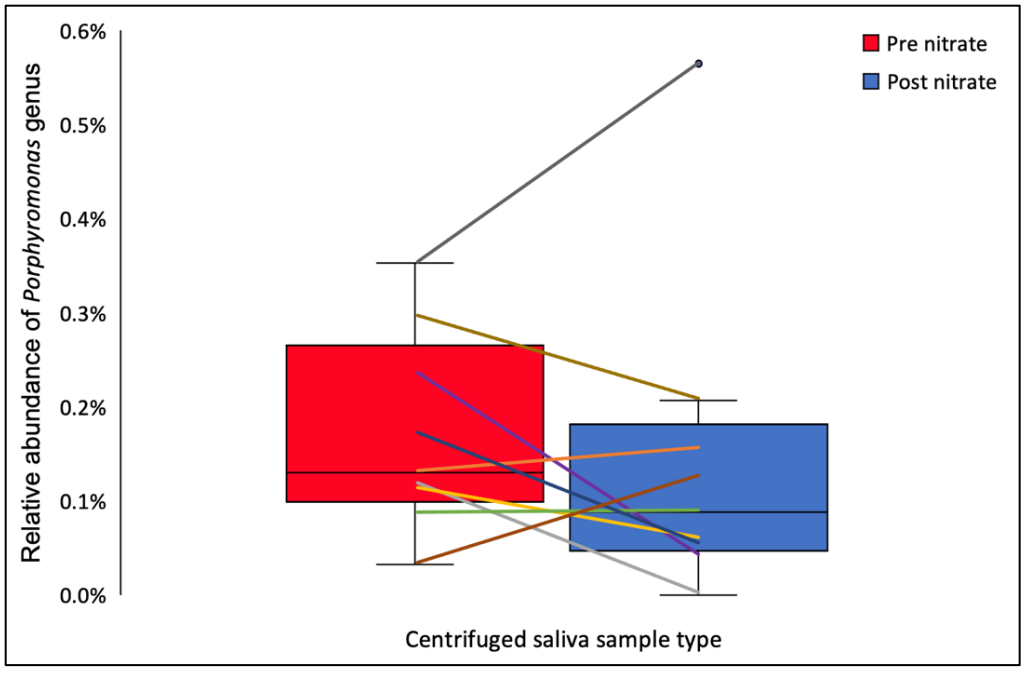


Figure 7. Mean relative abundance of *Porphyromonas* in both pre and post nitrate conditions as detected in a) saliva b) floss c) tongue and d) centrifuged saliva sample types. Lines indicate individual responses (n=8). There were no differences between conditions in any sample type ($p>0.05$).

The mean relative abundance of *Porphryomonas* was compared using a one-way repeated measures ANOVA to analyze if there were any differences between saliva, floss, tongue, and centrifuged saliva sample types (Figure 8). The mean relative abundance of *Porphryomonas* in the floss sample was statistically different from the saliva ($p<0.001$, $t=4.74$, $df=8$), tongue ($p<0.001$, $t=4.90$, $df=9$), and centrifuged saliva ($p<0.001$, $t=5.00$, $df=9$) sample types.

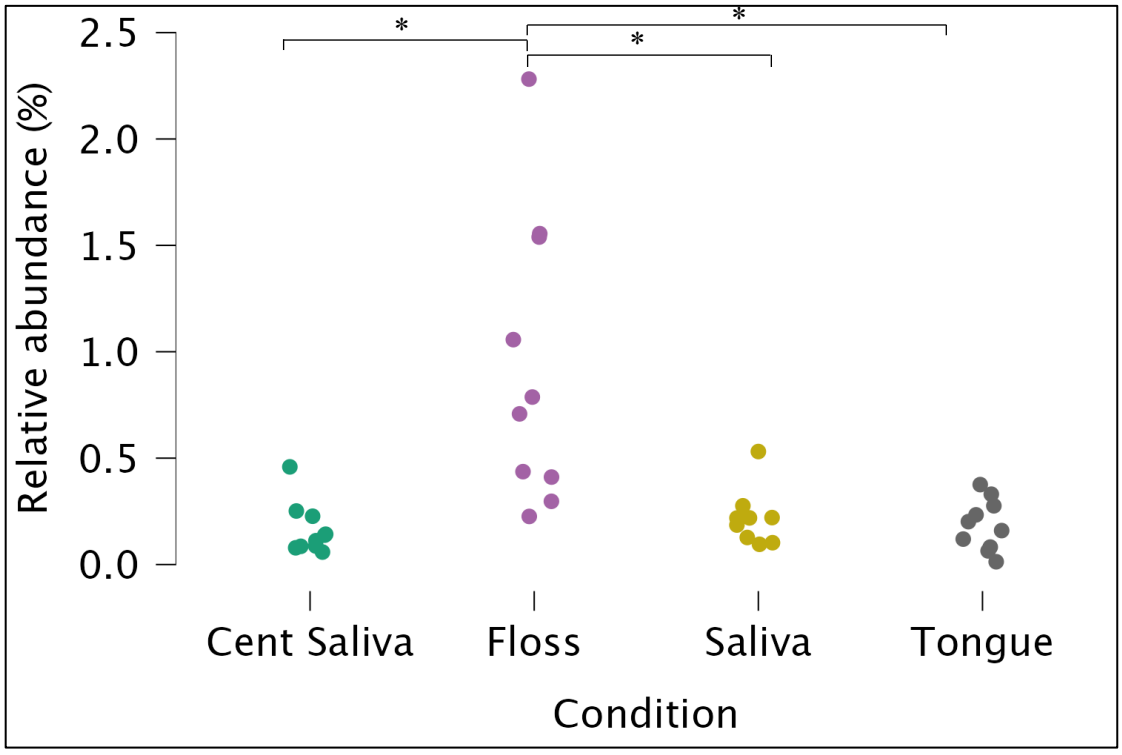


Figure 8. Relative abundance of *Porphyromonas* as detected in saliva, floss, tongue, and centrifuged saliva (cent saliva) sample types (n=9). The floss sample type was statistically different from saliva, tongue, and centrifuged saliva sample types ($p<0.001$ for all; denoted by a *).

4 DISCUSSION

This study assessed both changes in vascular health and oral microbiome composition of healthy participants in response to a 10-day inorganic dietary potassium-nitrate supplementation containing 2.42 mmol nitrate, which was supplemented twice a day for a total of 4.84 mmol nitrate each day. We found that there were no clear changes in bacterial composition and no significant shifts in the abundance of *Porphyromonas* from pre to post nitrate supplementation. Furthermore,

we found that there were no significant differences in blood pressure or flow-mediated dilation response post nitrate supplementation.

4.1 Effects of dietary nitrate on vascular response

To analyze the effects of dietary nitrate on vascular health, we took measurements both pre and post nitrate supplementation of resting blood pressure as assessed by photoplethysmography and endothelial function which we analyzed using a flow mediated dilation (FMD) test. We found no significant changes post 10-day nitrate supplementation in either relative or absolute FMD responses. Similarly, we found no significant changes from pre to post nitrate conditions of resting systolic, diastolic, and mean arterial blood pressure.

A dietary nitrate supplement is thought to provide nitrate in the oral cavity that is readily reduced to nitrite through the enterosalivary pathway, and then subsequently reduced to NO in the body (Dejam et al. 2004; Vanhalato et al. 2018; Rosier et al. 2020). In accordance with the findings of previous work, we hypothesized that a 10-day nitrate supplement would increase NO bioavailability, the degree of dilation in the brachial artery, and thus would improve the FMD response, which acts as a marker of endothelial health. In turn, we predicted blood pressure would decrease to maintain constant blood flow. However, relative and absolute FMD responses, as well as resting systolic, diastolic, and mean arterial blood pressure did not significantly change between pre and post nitrate conditions.

There are several possible factors that can impact NO bioavailability and the degree of vascular response post nitrate supplementation. First, the timing of vascular assessments post nitrate ingestion may influence the magnitude of vascular response. Assessments during peak or heightened nitrate and nitrite blood levels ensures NO availability in the circulation and may increase the chance of evoking significant responses. This factor is important to consider as the time elapsed between nitrate supplementation and vascular assessment in our study was longer than in other studies and may explain the non-significant vascular health changes seen in our study but not in previous work. Second, the concentration and duration of nitrate supplementation needs to be sufficient to induce significant vascular changes. Third, the form of nitrate (i.e., beetroot juice or nitrate salts) may impact the effect of vascular health changes post supplementation. Lastly, insufficient bioactivation of nitrate, the vascular health state of the study population, or small sample size may also play a role in the degree of vascular response induced from nitrate supplementation.

4.1.1 Sample timing

It is first important to note that the timing of vascular assessments post nitrate ingestion may influence vascular health responses. Many studies assessing the effects of nitrate supplementation on vascular health have carried out measures within a short time window post nitrate supplementation, while nitrate and nitrite levels in the blood were still elevated from the most recent dose of nitrate (Wylie et al. 2013; Kapil et al. 2010; Burleigh et al. 2019). To determine the timing of peak concentration of nitrate and nitrite post ingestion in the plasma, Wylie et al. (2013) administered 70 ml of concentrated beetroot juice containing 4.2 mmol nitrate. They found that the peak plasma nitrate concentration was reached 1-hour post administration, and progressively decreased over the next 24 hours (Wylie et al. 2013). Similarly, they found peak plasma nitrite concentration occurred 2-hours post administration, with a complete return to baseline after 12 hours (Wylie et al. 2013). Studies conducted by van Velzen et al. (2008) and Alzahrani et al. (2021) have also suggested that plasma nitrate and nitrite levels peak 1.5-1.8 and 2 hours after nitrate ingestion, respectively, and each have a half-life in the circulation of 5- or 6-hours post supplementation.

Wylie et al. (2013) found that both systolic and mean arterial blood pressure decreased significantly post administration of an acute dose of 4.2 mmol nitrate, with the peak response occurring after 4 hours for systolic pressure and 1 hour for mean arterial pressure, but neither remaining significantly reduced after 24 hours. Kapil et al. (2010) also found that systolic and diastolic blood pressure were significantly reduced 3 hours after the administration of acute doses of varying concentrations of nitrate (i.e., 2.5, 7.4, and 14.7 mmol nitrate); however, did not remain significantly reduced after 24 hours (Kapil et al. 2010). The results of the studies by Wylie et al. (2013) and Kapil et al. (2010) indicate that timing of vascular assessment post-acute nitrate supplementation may affect the degree of vascular responses. A study by Burleigh et al. (2019) confirms this notion, as they found that an acute dose of 12.4 mmol nitrate significantly improved FMD response and reduced systolic blood pressure in the hours following administration. However, these researchers found that blood pressure was not reduced after a 7-day nitrate supplementation period using the same concentration of nitrate, likely due to a 10-hour gap from the ingestion of the last nitrate dose and measurement the following day (Burleigh et al. 2019). The results of Burleigh et al. (2019) suggest that 7 days of nitrate consumption may not have been supplemented long enough to increase the abundance of nitrate-reducing bacteria in the oral cavity

and thus increase the bioavailability of NO. As a result, the timing of vascular assessments post nitrate supplementation may explain the discrepancies seen between vascular responses after an acute and chronic dose of nitrate.

In our study, participants took their last nitrate supplement on the night of the tenth day and came into the laboratory the next day for testing. Sampling start times on the eleventh day ranged from 8:00 am to 3:30 pm, and therefore varied in the amount of time since the last nitrate supplement had been consumed. Similar to the study conducted by Burleigh et al. (2019), a 10-day supplementation may not have been long enough to establish increased numbers of nitrate reducing taxa in the oral cavity and increase NO production. If this was the case, the time elapsed between the last supplement and post supplementation testing would have decreased the concentrations of nitrate and nitrite levels in the blood to levels insufficient to induce significant changes in vascular health. Therefore, it is possible that the timing of post supplementation testing in our study did not capture a sufficient concentration of nitrate or nitrite readily being converted to NO to induce significant changes in blood pressure or FMD response. Although we were not testing the effects of an acute dose of nitrate on short term vascular health responses, this explanation may provide insight into the discrepancies seen between our study and similarly designed studies analyzing vascular health responses post nitrate supplementation.

4.1.2 Nitrate concentration and duration

The concentration and duration of nitrate supplementation may also impact vascular responses post supplementation. First, supplementing greater concentrations of nitrate may induce larger vascular health responses in the long term, such as reduced blood pressure and improved endothelial function. Kapil et al. (2010) demonstrated that there is a dose-dependent reduction in blood pressure following the administration of varying concentrations of nitrate. The researchers showed that the administration of 2.5, 7.4, and 14.7 mmol nitrate significantly reduced systolic blood pressure by 2, 6, and 9 mmHg, respectively, and reduced diastolic blood pressure by 4, 4, and 6 mmHg, respectively (Kapil et al. 2010). In this study, Kapil et al. (2010) also showed that there was a dose-dependent increase in plasma nitrite by 1.3, 2, and 4-fold after administration of 2.5, 7.4, and 14.7 mmol of nitrate, respectively. Hobbs et al (2012) also tested the ingestion of beetroot juice at varying concentrations of 2.3, 5.7, and 11.4 mmol and determined that the concentration of nitrate supplements had near dose-dependent effects on lowering systolic blood pressure in the 24-hour period post ingestion. Furthermore, Webb et al. (2008) showed previously

that a highly concentrated single dose of 500 mL beet root juice containing 23 mmol nitrate reduced systolic blood pressure greatly by 10 mmHg and diastolic blood pressure by 8 mmHg. These studies suggest that higher concentrations of nitrate may induce greater vascular responses after an acute dose of nitrate. Thus, supplementing larger concentrations of nitrate in the long-term may increase the likelihood of improving vascular health outcomes. However, it should be noted that highly concentrated doses of nitrate could potentially have harmful effects to human health, such as contributing to the development of methemoglobinemia or gastrointestinal carcinogenesis, and thus should be administered with caution (Keller et al. 2020).

Several studies have also seen significant responses in vascular function after administering nitrate supplements for long durations of time, varying from 6 days to 6 weeks (Vanhatalo et al. 2018; Sobko et al. 2010; Bailey et al. 2009; Velmurugan et al. 2016). Multi-day ingestion of nitrate may favourably alter the composition of the microbiome in the oral cavity by increasing the number of nitrate-reducing taxa and thus increasing nitrite production rate (Velmurugan et al. 2016; Vanhatalo et al. 2018). Multi-day nitrate ingestion may therefore also increase NO bioavailability, by increasing the storage of nitrate in skeletal muscle and potentially improving the utilization of NO from these reserves in the longer term (Tan et al. 2022). Further work is warranted to investigate the possible storage of nitrate in muscle and utilization of NO, and how chronic supplementation of nitrate may affect these factors. It is possible that the administration of 4.84 mmol nitrate daily for 10 days was not of sufficient concentration or duration to induce significant vascular responses in our study.

4.1.3 Nitrate supplement form

Another factor that may affect the degree of vascular response to a nitrate supplementation is how the nitrate is administered (e.g., juices or salts). Sundqvist et al. (2020) and Tan et al. (2022) have suggested that beetroot juice, a commonly used nitrate supplement, may contain other compounds that influence the efficacy of the vascular response compared to other nitrate supplement forms. Beetroot contains components that may alter blood pressure, such as betalains, flavonoids, and ascorbic acid that could further increase the reduction of nitrite to NO and thus may account in part for changes in vascular responses post supplementation (Sundqvist et al. 2020; Tan et al. 2022). However, research carried out by Hobbs et al. (2012) controlled for this factor by comparing the effects of betacyanin-deficient beetroot and regular beetroot supplements on blood pressure and saw no significant differences between treatments. Many studies have seen significant

vascular health improvements, such as decreased blood pressure, upon the administration of inorganic nitrate salts, and therefore it does not seem a likely explanation in our study (Hobbs et al. 2013; Kapil et al. 2010).

4.1.4 Sample population factors

Additional factors that could explain the degree of vascular response to a nitrate supplement are the ability of oral bacteria to activate nitrate, the vascular health state of the sampled population, and the sample size. First, if oral bacteria are not able to reduce nitrate to nitrite, an individual will be unable to sufficiently activate nitrate and convert it to NO. A decreased ability to activate nitrate can result from a variety of factors and may explain non-significant vascular responses to nitrate supplementation (Sundqvist et al. 2020). However, in the context of our study, this explanation is unlikely as we controlled for factors particularly known to affect nitrate bioactivation in the oral cavity, such as antimicrobial mouthwash, antimicrobial toothpaste, and other antibiotics and drugs (Sundqvist et al. 2020). Second, if participants were already in good vascular health (e.g., had good endothelial function and blood pressure), there could have been a ceiling effect and limitations to how participants responded to the nitrate supplement. Regarding endothelial function, the baseline mean absolute FMD response was 0.44 ± 0.19 mm in our study, which can be compared to a mean value of 0.21 ± 0.14 mm in healthy participants (mean ± SD; age 29.2 ± 6.1 years) as assessed by Rakobowchuk et al. (2017). Furthermore, the baseline mean relative FMD response was 11.9 ± 4.5% in our study, which can be compared to a study analyzing 1542 healthy participants (age 44.5 ± 9.8 years) that found mean relative FMD response to be 7.5 ± 3.3% (Maruhashi et al. 2020). In comparing the baseline FMD values in our study to that of Rakobowchuk et al. (2017) and Maruhashi et al. (2020), it seems that our study population did have good baseline endothelial function. As a result, participants may have been limited as to how their endothelial function could improve once nitrate supplementation had commenced. A final factor that may contribute to the non-significant vascular responses post supplementation could be that our study was underpowered. Using a G power calculation, we determined that more than 500 participants would be needed to identify the changes from pre to post supplementation as significant.

It is important to note that previous studies have addressed many of the issues stated above and have still not seen any differences in vascular health post supplementation. Sundqvist et al. (2020) treated 231 subjects, who had systolic blood pressures between 130-159 mmHg, with a

nitrate rich diet in the form of leafy green vegetables or with 4.84 mmol nitrate supplementation twice a day for 5 weeks. After the 5-week period of nitrate supplementation, 24-hour blood pressure was monitored, and no significant differences were found, despite controlling for many of the aforementioned factors. As described by Wylie et al. (2013), there are responders and non-responders to dietary nitrate supplements, and thus the participants in our study may have been overrepresented by non-nitrate responders. However, Wylie et al. (2013) suggest that people are more likely to respond to higher doses (e.g., 16.8 mmol) rather than lower doses (e.g., 4.2 mmol), therefore highlighting an advantage of using higher concentrated nitrate supplements.

4.2 Effects of dietary nitrate on oral microbiome responses

4.2.1 Oral microbiome composition

We assessed the overall oral microbiome composition both pre and post nitrate supplementation in saliva, floss, tongue, and centrifuged saliva sample types. There was no clear change in the relative abundances of bacterial phyla in any of the oral sample types from pre to post nitrate conditions. Previous studies have shown that short-term nitrate supplementation alters the oral microbiome composition by increasing the abundance of certain health-associated bacterial phyla and decreasing the abundance of periodontal-associated bacterial phyla (Rosier et al. 2020). However, the mechanisms leading to decreases in the abundance of periodontal-associated bacteria in the oral cavity remain largely unknown (Rosier et al. 2020). Rosier et al. (2020) suggest that NO, as produced from nitrate reduction, has antimicrobial properties that may limit the growth of certain periopathogenic species (i.e., species that contribute to periodontitis) in the oral cavity. In fact, Backlund et al. (2014) found that certain periopathogenic species, such as *P. gingivalis*, are highly sensitive to NO in vitro.

Furthermore, Rosier et al. (2020) have suggested that NO-releasing materials and metal oxides may also act as antimicrobial molecules that limit the growth of periopathogenic species in the oral cavity, such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*. Metal oxides are thought to exert their antimicrobial effects by accumulating on the cell membrane and causing cell death, penetrating membranes and disrupting the cell's function, inactivating necessary proteins and enzymes, or compromising the production of ATP (Vargas-Reus et al. 2012). However, the mechanism by which NO-releasing materials and metal oxides are produced in the body after the consumption of a nitrate supplement, as well as their role in decreasing the abundance of periopathogenic species, requires further work.

A dietary nitrate supplement is also thought to promote health-associated and nitrate-reducing bacterial species growth in the oral cavity (Rosier et al. 2020; Vanhatalo et al. 2018). The bulk of nitrate-reducing bacteria are considered as facultative anaerobes that exist in the posterior dorsum of the tongue and play integral roles in the enterosalivary pathway (Koch et al. 2017). Certain species of Firmicutes (*Staphylococcus*, *Streptococcus*, and *Veillonella*), Actinobacteria (*Actinomyces),* and other taxa such as *Pasteurella, Rothia, Neisseria,* and *Haemophilus* have been identified as major nitrate reducers in the oral cavity (Koch et al. 2017; Zhurakivska et al. 2019).

Similar to the explanations of the vascular response, it is possible that the concentration and duration of the 4.84 mmol 10-day nitrate supplement in our study was insufficient to alter the oral microbiome composition, specifically the abundance of nitrate-reducing bacteria and periopathogenic species. Velmurugan et al. (2016) administered 6 mmol nitrate in the form of beetroot juice for 6 weeks in hypercholesterolemic participants and found that there was a 2.5-fold increase in plasma nitrite after 6 weeks of supplementation, but only a 2-fold increase 3 hours post-acute supplementation. The results of this study suggest that chronic nitrate supplementation over longer durations may be required to provoke significant changes in oral microbiome composition, specifically to increase nitrate-reducing taxa. Vanhatalo et al. (2018) utilized a concentrated dietary nitrate supplement of 12 mmol nitrate per day for 10 days and noted increases in the relative abundances of nitrate reducing bacteria, such as *Rothia* and *Neisseria*, as well as decreases in the relative abundance of *Prevotella.* An increased abundance of nitrate reducing bacteria as seen in Vanhatalo et al. (2018) suggests that the chronic administration of higher dietary nitrate concentrations may enable an increased capacity of nitrate reduction in the oral cavity. The results of these studies suggest that administering nitrate in larger concentrations and for longer durations may increase the nitrate reducing abilities of the oral cavity.

It is important to note that the relative abundance of bacterial phyla detected in the various oral samples seemed to accurately represent results found in previous studies. In all four pre and post nitrate oral samples, 70% of the overall community composition was comprised of the phyla Bacteroidetes, Firmicutes, and Proteobacteria. The remaining oral microbiome composition (i.e., less than 30%) showed more variability between oral sample types. The abundances of each phylum and the variation between oral samples were very similar to that found in Koch et al. (2017), which considered the average abundances compiled from various studies (Segata et al. 2012; Eren et al. 2014; Bik et al. 2010; Ahn et al. 2011; Keijser et al. 2008). Specifically, research

by Koch et al. (2017) detected the oral microbiome composition associated with the saliva, tongue dorsum, and subgingiva, which were similar to our samples of saliva, tongue, and floss, respectively. The similarity between our results and those of previous studies demonstrates robustness in our method.

4.2.2 Sample structure

Although we did not find differences in the relative abundances of bacterial phyla between pre and post nitrate conditions, we did find that the oral microbiome composition in each of the four sample types is very closely related among individuals. Furthermore, we identified that the microbiome composition associated with each sample type remains relatively stable over time in individuals, even in response to a nitrate supplement. The relative stability of the oral microbiome has been well-established in previous work (Rasiah et al. 2005; Duran-Pinedo et al. 2021). However, nitrate supplementation, as well as other interventions such as antimicrobial therapeutic approaches, probiotics, and increased consumption of glucose and other high glycemic index foods have been found to alter oral microbiome composition over time (Li and Tanner 2015; Baker and Edlund 2019; Millen et al. 2022). This suggests that the oral microbiome can adapt when subject to selective pressures or supplements, although it should be noted that the effects of these various treatments are often reversed once the intervention has ceased (Romani Vestman et al. 2015; Li and Tanner 2015). In this study, the stability of the oral microbiome in various areas of the oral cavity is an interesting and important finding for future work assessing how the oral cavity responds to nitrate supplementation.

4.2.3 Abundance of *Porphyromonas*

The relative abundance of the genus *Porphyromonas* was assessed both pre and post nitrate supplementation. We hypothesized that administering 4.84 mmol dietary nitrate per day for 10 days would reduce the abundance of *Porphyromonas* in the oral cavity. Rosier et al. (2020) previously demonstrated that treating saliva directly in vitro with 0.0016 mmol nitrate 5- and 9-hours post-extraction significantly decreased the abundance of *Porphyromonas*. However, in each of the four sample types taken from the oral cavity, we did not find significant differences between the relative abundance of *Porphyromonas* from pre to post nitrate supplementation. As mentioned, using a more concentrated nitrate supplements for longer durations may aid in reducing the abundance of *Porphyromonas* in the oral cavity.

Furthermore, our study is unique as we measured the response of three different oral cavity areas to chronic dietary nitrate supplementation. Previous work has either analyzed the salivary microbiome (Vanhatalo et al. 2018; Vanhatalo et al. 2021; Rosier et al. 2020) or tongue dorsum microbiome (Burleigh et al. 2019) in response to nitrate supplementation; however, to our knowledge, subgingival samples (i.e., floss) have not yet been assessed in this context. Given *Porphyromonas gingivalis* is a bacterium residing mainly in the gums, it is thought that sampling in the subgingival sulcus area (e.g., with floss) would demonstrate the highest prevalence of *P. gingivalis* (Griffen et al. 1998). A study conducted by Griffen et al. (1998) did detect the highest relative abundance of *Porphyromonas* in floss samples when compared to other areas in the oral cavity, which aligns with the findings of our study. Using floss samples, in addition to saliva and tongue samples, in our study provides a methodological foundation for future work to further assess the effect of nitrate on *Porphyromonas* abundance in the oral cavity.

4.3 Conclusions and future work

With increased detection of AD worldwide, identifying potential treatments and preventative strategies are crucial. *Porphyromonas gingivalis* is a bacterium that exists in the oral cavity and has been recently linked to AD pathology. A dietary nitrate supplement has been suggested to decrease the abundance of *P. gingivalis* in the oral cavity, as well as improve vascular health outcomes, and thus potentially provide protection against the onset and progression of AD (Dominy et al. 2019). In this study, we did not find significant differences in vascular health, oral microbiome composition, or the abundance of *Porphyromonas* after a 10-day nitrate supplementation, although we did develop a robust method that can be utilized in future studies. Notably, this methodology detected differences in the prevalence of *Porphyromonas* in various areas of the oral cavity. Furthermore, we did find that the oral microbiome associated with each sample type was closely related among individuals, that the oral microbiome composition remains relatively stable over time, and that the largest relative abundance of *Porphyromonas* is found in the subgingival sulcus (i.e., in floss sample types) as compared to other areas of the mouth. The methodology presented here lays a strong foundation for future work and has the potential to be further developed into a larger scale study.

The administration of a more concentrated nitrate supplement over longer durations may allow for an increased abundance of nitrate reducing bacteria to establish in the oral cavity and may impact outcomes of vascular health post supplementation. Exploring the use of different sources

of nitrate and utilizing a larger study population could provide further insight into how both vascular health and the oral microbiome respond to chronic nitrate supplementation, as well as minimize the possibility of ceiling effects. In addition, future work should also explore how salivary nitrate concentrations respond to a nitrate supplement to ensure that the desired response is attained. If future studies see improvements post supplementation in both oral microbiome composition and vascular health, the novel use of a dietary nitrate supplement could be administered to people suffering from cognitive impairment, with the hope of potentially providing protection against Alzheimer's disease.

5 REFERENCES

Ahn J, Yang L, Paster BJ, Ganly I, Morris L, Pei Z, Hayes RB. 2011. Oral microbiome profiles: 16S rRNA pyrosequencing and microarray assay comparison. PLoS One. 6(7): e22788. doi: 10.1371/journal.pone.0022788.

Alzahrani HS, Jackson KG, Hobbs DA, Lovegrove JA. 2021. The role of dietary nitrate and the oral microbiome on blood pressure and vascular tone. Nutr Res Rev. 34(2): 222-239. doi: 10.1017/S0954422420000281.

Anand KS, Dhikav V. 2012. Hippocampus in health and disease: An overview. Ann Indian Acad Neurol. 15(4): 239-246. doi: 10.4103/0972-2327.104323.

Armstrong RA. 2013. What causes Alzheimer's disease? Folia Neuropathol. 51(3): 169-188. doi: 10.5114/fn.2013.37702.

Backlund CJ, Sergesketter AR, Offenbacher S, Schoenfisch MH. 2014. Antibacterial efficacy of exogenous nitric oxide on periodontal pathogens. J Dent Res. 93(11): 1089-1094. doi: 10.1177/0022034514529974.

Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM. 2009. Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. J Appl Physiol. 107(4): 1144-1155. doi: 10.1152/japplphysiol.00722.2009.

Baker JL, Edlund A. 2019. Exploiting the Oral Microbiome to Prevent Tooth Decay: Has Evolution Already Provided the Best Tools? Front Microbiol. 9: 3323. doi: 10.3389/fmicb.2018.03323.

Battersby AJ, Khara J, Wright VJ, Levy O, Kampmann B. 2016. Antimicrobial Proteins and Peptides in Early Life: Ontogeny and Translational Opportunities. Front Immunol. 7: 309. doi: 10.3389/fimmu.2016.00309.

Beydoun MA, Beydoun HA, Hossain S, El-Hajj ZW, Weiss J, Zonderman AB. 2020. Clinical and Bacterial Markers of Periodontitis and Their Association with Incident All-Cause and Alzheimer's Disease Dementia in a Large National Survey. J Alzheimer’s Dis. 75(1): 157-172. doi: 10.3233/JAD-200064.

Bik EM, Long CD, Armitage GC, Loomer P, Emerson J, Mongodin EF, Nelson KE, Gill SR, Fraser-Liggett CM, Relman DA. 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. ISME J. 4(8): 962-974. doi: 10.1038/ismej.2010.30.

Brothers HM, Gosztyla ML, Robinson SR. 2018. The Physiological Roles of Amyloid-β Peptide Hint at New Ways to Treat Alzheimer's Disease. Front Aging Neurosci. 10: 118. doi: 10.3389/fnagi.2018.00118.

Burleigh M, Liddle L, Muggeridge DJ, Monaghan C, Sculthorpe N, Butcher J, Henriquez F, Easton C. 2019. Dietary nitrate supplementation alters the oral microbiome but does not improve the vascular responses to an acute nitrate dose. Nitric Oxide. 89: 54-63. doi: 10.1016/j.niox.2019.04.010.

Chen, G, Xu T, Yan Y, Zhou Y, Jiang Y, Melcher K, Xu HE. 2017. Amyloid beta: structure, biology and structure-based therapeutic development. Acta Pharmacol Sin. 38: 1205-1235. doi: 10.1038/aps.2017.28.

d'Errico P, Meyer-Luehmann M. 2020. Mechanisms of Pathogenic Tau and Aβ Protein Spreading in Alzheimer's Disease. Front Aging Neurosci. 12: 265. doi: 10.3389/fnagi.2020.00265.

de Bruijn RF, Ikram MA. 2014. Cardiovascular risk factors and future risk of Alzheimer's disease. BMC Med. 12: 130. doi: 10.1186/s12916-014-0130-5.

Deanfield JE, Halcox JP, Rabelink TJ. 2007. Endothelial function and dysfunction: testing and clinical relevance. Circulation. 115(10): 1285-1295. doi: 10.1161/CIRCULATIONAHA.106.652859.

Dede DS, Yavuz B, Yavuz BB, Cankurtaran M, Halil M, Ulger Z, Cankurtaran ES, Aytemir K, Kabakci G, Ariogul S. 2007. Assessment of endothelial function in Alzheimer's disease: is Alzheimer's disease a vascular disease? J Am Geriatr Soc. 55(10): 1613-1617. doi: 10.1111/j.1532-5415.2007.01378.x.

Dejam A, Hunter CJ, Schechter AN, Gladwin MT. 2004. Emerging role of nitrite in human biology. Blood Cells Mol Dis. 32(3): 423-429. doi: 10.1016/j.bcmd.2004.02.002.

Díaz-Zúñiga J, More J, Melgar-Rodríguez S, Jiménez-Unión M, Villalobos-Orchard F, Muñoz-Manríquez C, Monasterio G, Valdés JL, Vernal R, Paula-Lima A. 2020. Alzheimer's Disease-Like Pathology Triggered by *Porphyromonas gingivalis* in Wild Type Rats Is Serotype Dependent. Front Immunol. 11: 588036. doi: 10.3389/fimmu.2020.588036.

Dominy SS, Lynch C, Ermini F, Benedyk M, Marczyk A, Konradi A, Nguyen M, Haditsch U, Raha D, Griffin C, et al. 2019. *Porphyromonas gingivalis* in Alzheimer's disease brains: Evidence for disease causation and treatment with small-molecule inhibitors. Sci Adv. 5(1): 1-22. doi: 10.1126/sciadv.aau3333.

Duran-Pinedo A, Solbiati J, Teles F, Teles R, Zang Y, Frias-Lopez J. 2021. Long-term dynamics of the human oral microbiome during clinical disease progression. BMC Biol. 19(1): 240. doi: 10.1186/s12915-021-01169-z.

Eren AM, Borisy GG, Huse SM, Mark Welch JL. 2014. Oligotyping analysis of the human oral microbiome. Proc Natl Acad Sci U S A. 111(28): e2875-84. doi: 10.1073/pnas.1409644111.

Findeis MA. 2007. The role of amyloid beta peptide 42 in Alzheimer's disease. Pharmacol Ther. 116(2): 266-286. doi: 10.1016/j.pharmthera.2007.06.006.

Ghasemi A, Hedayati M, Biabani H. 2007. Protein Precipitation Methods Evaluated for Determination of Serum Nitric Oxide End Products by the Griess Assay. JMSR. 15(2): 29-32.

Glenner GG, Wong CW. 1984. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun. 120(3): 885-890. doi: 10.1016/s0006-291x(84)80190-4.

Gosztyla ML, Brothers HM, Robinson SR. 2018. Alzheimer's Amyloid-β is an Antimicrobial Peptide: A Review of the Evidence. J Alzheimers Dis. 62(4): 1495-1506. doi: 10.3233/JAD-171133.

Griffen AL, Becker MR, Lyons SR, Moeschberger ML, Leys EJ. 1998. Prevalence of *Porphyromonas gingivalis* and periodontal health status. J Clin Microbiol. 36(11): 3239-4322. doi: 10.1128/JCM.36.11.3239-3242.

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. 1986. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. J Biol Chem. 261(13): 6084-6089.

Guo Y, Nguyen K, Potempa J. 2010. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. Periodontal. 54(1): 15-44. doi: 10.1111/j.1600-0757.2010.00377.x.

Hobbs DA, Kaffa N, George TW, Methven L, Lovegrove JA. 2012. Blood pressure-lowering effects of beetroot juice and novel beetroot-enriched bread products in normotensive male subjects. Br J Nutr. 108(11): 2066-2074. doi: 10.1017/S0007114512000190.

Hobbs DA, George TW, Lovegrove JA. 2013. The effects of dietary nitrate on blood pressure and endothelial function: a review of human intervention studies. Nutr Res Rev. 26(2): 210-222. doi: 10.1017/S0954422413000188.

Hughes CG, Morandi A, Girard TD, Riedel B, Thompson JL, Shintani AK, Pun BT, Ely EW, Pandharipande PP. 2013. Association between endothelial dysfunction and acute brain dysfunction during critical illness. Anesthesiology. 118(3): 631-639. doi: 10.1097/ALN.0b013e31827bd193.

Ide M, Harris M, Stevens A, Sussams R, Hopkins V, Culliford D, Fuller J, Ibbett P, Raybould R, Thomas R, et al. 2016. Periodontitis and Cognitive Decline in Alzheimer’s Disease. PLoS One. 11(3): e0151081. doi: 10.1371/journal.pone.0151081.

Imamura T. 2003. The role of gingipains in the pathogenesis of periodontal disease. J Periodontol. 74(1): 111-118. doi: 10.1902/jop.2003.74.1.111.

Ishida N, Ishihara Y, Ishida K, Tada H, Funaki-Kato Y, Hagiwara M, Ferdous T, Abdullah M, Mitani A, Michikawa M, et al. 2017. Periodontitis induced by bacterial infection exacerbates features of Alzheimer's disease in transgenic mice. NPJ Aging Mech Dis. 3: 15. doi: 10.1038/s41514-017-0015-x.

Kamer AR, Craig RG, Dasanayake AP, Brys M, Glodzik-Sobanska L, de Leon MJ. 2008. Inflammation and Alzheimer's disease: possible role of periodontal diseases. Alzheimer's Dement. 4(4): 242-250. doi: 10.1016/j.jalz.2007.08.004.

Kametani F, Hasegawa M. 2018. Reconsideration of Amyloid Hypothesis and Tau Hypothesis in Alzheimer's Disease. Front Neurosci. 12: 25. doi: 10.3389/fnins.2018.00025.

Kanagasingam S, Chukkapalli SS, Welbury R, Singhrao SK. 2020. *Porphyromonas gingivalis* is a Strong Risk Factor for Alzheimer's Disease. J Alzheimer's Dis Rep. 4(1): 501-511. doi: 10.3233/ADR-200250.

Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, et al. 2010. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. Hypertension. 56(2): 274-281. doi: 10.1161/HYPERTENSIONAHA.110.153536.

Keijser BJ, Zaura E, Huse SM, van der Vossen JM, Schuren FH, Montijn RC, ten Cate JM, Crielaard W. 2008. Pyrosequencing analysis of the oral microflora of healthy adults. J Dent Res. 87(11): 1016-1020. doi: 10.1177/154405910808701104.

Kelleher RJ, Soiza RL. 2013. Evidence of endothelial dysfunction in the development of Alzheimer's disease: Is Alzheimer's a vascular disorder? Am J Cardiovasc Dis. 3(4): 197-226.

Keller RM, Beaver L, Prater MC, Hord NG. 2020. Dietary nitrate and nitrite concentrations in food patterns and dietary supplements. Nutr Today. 55(5): 218-226. doi: 10.1097/NT.0000000000000253

Kinane DF, Stathopoulou PG, Papapanou PN. 2017. Periodontal diseases. Nat Rev Dis Primers. 3: 17038. doi: 10.1038/nrdp.2017.38.

Koch CD, Gladwin MT, Freeman BA, Lundberg JO, Weitzberg E, Morris A. 2017. Enterosalivary nitrate metabolism and the microbiome: Intersection of microbial metabolism, nitric oxide and diet in cardiac and pulmonary vascular health. Free Radic Biol Med. 105: 48-67. doi: 10.1016/j.freeradbiomed.2016.12.015.

Krüger-Genge A, Blocki A, Franke RP, Jung F. 2019. Vascular Endothelial Cell Biology: An Update. Int J Mol Sci. 20(18): 4411. doi: 10.3390/ijms20184411.

Kumar DK, Choi SH, Washicosky KJ, Eimer WA, Tucker S, Ghofrani J, Lefkowitz A, McColl G, Goldstein LE, Tanzi RE, et al. 2017. Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. Sci Transl Med. 8(340): 340ra72. doi: 10.1126/scitranslmed.aaf1059.

Lamont RJ, Jenkinson HF. 1998. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol Mol Biol Rev. 62(4): 1244-1263. doi: 10.1128/MMBR.62.4.1244-1263.1998.

Leira Y, Domínguez C, Seoane J, Seoane-Romero J, Pías-Peleteiro JM, Takkouche B, Blanco J, Aldrey JM. 2017. Is Periodontal Disease Associated with Alzheimer's Disease? A Systematic Review with Meta-Analysis. Neuroepidemiology. 48(1-2): 21-31. doi: 10.1159/000458411.

Li Y, Tanner A. 2015. Effect of Antimicrobial Interventions on the Oral Microbiota Associated with Early Childhood Caries. Pediatr Dent. 37(3): 226-244.

Lundberg J, Weitzberg E, Gladwin M. 2008. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 7: 156–167. doi: 10.1038/nrd2466.

Lyros E, Bakogiannis C, Liu Y, Fassbender K. 2014. Molecular links between endothelial dysfunction and neurodegeneration in Alzheimer's disease. Curr Alzheimer Res. 11(1): 18-26. doi: 10.2174/1567205010666131119235254.

Maruhashi T, Kajikawa M, Kishimoto S, Hashimoto H, Takaeko Y, Yamaji T, Harada T, Han Y, Aibara Y, Mohamad Yusoff F, Hidaka T, et al. 2020. Diagnostic Criteria of Flow-Mediated Vasodilation for Normal Endothelial Function and Nitroglycerin-Induced Vasodilation for Normal Vascular Smooth Muscle Function of the Brachial Artery. J Am Heart Assoc. 9(2): e013915. doi: 10.1161/JAHA.119.013915.

Medeiros R, Baglietto-Vargas D, LaFerla FM. 2011. The role of tau in Alzheimer's disease and related disorders. CNS Neurosci Ther. 17(5): 514-524. doi: 10.1111/j.1755-5949.2010.00177.x.

Millen AE, Dahhan R, Freudenheim JL, Hovey KM, Li L, McSkimming DI, Andrews CA, Buck MJ, LaMonte MJ, Kirkwood KL, et al. 2022. Dietary carbohydrate intake is associated with the subgingival plaque oral microbiome abundance and diversity in a cohort of postmenopausal women. Sci Rep. 12(1): 2643. doi: 10.1038/s41598-022-06421-2.

Moen AE, Tannæs TM, Vatn S, Ricanek P, Vatn MH, Jahnsen J, IBD-Character Consortium. 2016. Simultaneous purification of DNA and RNA from microbiota in a single colonic mucosal biopsy. BMC Res Notes. 9: 328. doi: 10.1186/s13104-016-2110-7.

Moore EE, Liu D, Li J, Schimmel SJ, Cambronero FE, Terry JG, Nair S, Pechman KR, Moore ME, Bell SP, et al. 2021. Association of Aortic Stiffness with Biomarkers of Neuroinflammation, Synaptic Dysfunction, and Neurodegeneration. Neurology. 97(4): e329-340. doi: 10.1212/WNL.0000000000012257.

Murphy MP, LeVine H III. 2010. Alzheimer's disease and the amyloid-beta peptide. J Alzheimer's Dis. 19(1): 311-23. doi: 10.3233/JAD-2010-1221.

NIH Human Microbiome Project – Core Microbiome Sampling Protocol. 2010. Human Microbiome Project Consortium; [updated 2018 Feb 8; accessed 2022 Feb 20]. https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/document.cgi?study_id=phs000228.v3.p1&phd=3190#sec1

R Core Team. 2020. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing Available at: http://www.R-project.org/.

Raji CA, Lopez OL, Kuller LH, Carmichael OT, Becker JT. 2009. Age, Alzheimer disease, and brain structure. Neurology. 73(22): 1899-905. doi: 10.1212/WNL.0b013e3181c3f293.

Rakobowchuk M, Ritter O, Wilhelm EN, Isacco L, Bouhaddi M, Degano B, Tordi N, Mourot L. 2017. Divergent endothelial function but similar platelet microvesicle responses following eccentric and concentric cycling at a similar aerobic power output. J Appl Physiol. 122(4): 1031-1039. doi: 10.1152/japplphysiol.00602.2016

Rasiah IA, Wong L, Anderson SA, Sissons CH. 2005. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch Oral Biol. 50(9): 779-787. doi: 10.1016/j.archoralbio.2005.02.001.

Romani Vestman N, Chen T, Lif Holgerson P, Öhman C, Johansson I. 2015. Oral Microbiota Shift after 12-Week Supplementation with Lactobacillus reuteri DSM 17938 and PTA 5289; A Randomized Control Trial. PLoS One. 10(5): e0125812. doi: 10.1371/journal.pone.0125812.

Romitelli F, Santini SA, Chierici E, Pitocco D, Tavazzi B, Amorini AM, Lazzarino G, Di Stasio E. 2007. Comparison of nitrite/nitrate concentration in human plasma and serum samples measured by the enzymatic batch Griess assay, ion-pairing HPLC and ion-trap GC-MS: the importance of a correct removal of proteins in the Griess assay. J Chromatogr B Analyt Technol Biomed Life Sci. 851(1-2): 257-267. doi: 10.1016/j.jchromb.2007.02.003.

Rosier BT, Buetas E, Moya-Gonzalvez EM, Artacho A, Mira A. 2020. Nitrate as a potential prebiotic for the oral microbiome. Sci Rep. 10(1): 1-15. doi: 10.1038/s41598-020-69931-x.

Santos CY, Snyder PJ, Wu WC, Zhang M, Echeverria A, Alber J. 2017. Pathophysiologic relationship between Alzheimer's disease, cerebrovascular disease, and cardiovascular risk: A review and synthesis. Alzheimer's Dement. 7: 69-87. doi: 10.1016/j.dadm.2017.01.005.

Segata N, Haake SK, Mannon P, Lemon KP, Waldron L, Gevers D, Huttenhower C, Izard J. 2012. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. Genome Biol. 13(6): R42. doi: 10.1186/gb-2012-13-6-r42.

Sheets SM, Potempa J, Travis J, Casiano CA, Fletcher HM. 2005. Gingipains from *Porphyromonas gingivalis* W83 induce cell adhesion molecule cleavage and apoptosis in endothelial cells. Infect Immun. 73(3): 1543-1552. doi: 10.1128/IAI.73.3.1543-1552.2005.

Singhrao SK, Harding A, Poole S, Kesavalu L, Crean S. 2015. *Porphyromonas gingivalis* periodontal infection and its putative links with Alzheimer's disease. Mediators Inflamm. 137357. doi: 10.1155/2015/137357.

Singhrao SK, Harding A. 2020. Is Alzheimer's disease a polymicrobial host microbiome dysbiosis? Expert Rev Anti Infect Ther. 18(4): 275-277. doi: 10.1080/14787210.2020.1729741.

Sobko T, Marcus C, Govoni M, Kamiya S. 2010. Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers. Nitric Oxide. 22(2): 136-140. doi: 10.1016/j.niox.2009.10.007.

Soscia SJ, Kirby JE, Washicosky KJ, Tucker SM, Ingelsson M, Hyman B, Burton MA, Goldstein LE, Duong S, Tanzi RE, et al. 2010. The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. PLoS One. 5(3): e9505. doi: 10.1371/journal.pone.0009505.

Stathopoulou PG, Galicia JC, Benakanakere MR, Garcia CA, Potempa J, Kinane DF. 2009. *Porphyromonas gingivalis* induce apoptosis in human gingival epithelial cells through a gingipain-dependent mechanism. BMC Microbiol. 9: 107. doi: 10.1186/1471-2180-9-107.

Statistics Canada. 2022. Table 13-10-0394-01. Leading causes of death, total population, by age group. https://doi.org/10.25318/1310039401-eng

Stephan, BCM, Harrison SL, Keage HAD, Babateen A, Robinson L, Siervo M. 2017. Cardiovascular Disease, the Nitric Oxide Pathway and Risk of Cognitive Impairment and Dementia. Curr Cardiol Rep. 19(87). doi: 10.1007/s11886-017-0898-y.

Sundqvist ML, Larsen FJ, Carlström M, Bottai M, Pernow J, Hellénius ML, Weitzberg E, Lundberg JO. 2020. A randomized clinical trial of the effects of leafy green vegetables and inorganic nitrate on blood pressure. Am J Clin Nutr. 111(4): 749-756. doi: 10.1093/ajcn/nqaa024.

Tan R, Cano L, Lago-Rodríguez Á, Domínguez R. 2022. The Effects of Dietary Nitrate Supplementation on Explosive Exercise Performance: A Systematic Review. Int J Environ Res Public Health. 19(2): 762. doi: 10.3390/ijerph19020762.

Tettamanti L, Gaudio RM, Iapichino A, Mucchi D, Tagliabue A. 2017. Genetic susceptibility and periodontal disease: a retrospective study on a large italian sample. Oral Implantol. 10(1): 20-27. doi: 10.11138/orl/2017.10.1.020.

Tribble GD, Angelov N, Weltman R, Wang B-Y, Eswaran SV, Gay IC, Parthasarathy K, Dao D-HV, Richardson KN, Ismail NM, et al. 2019. Frequency of Tongue Cleaning Impacts the Human Tongue Microbiome Composition and Enterosalivary Circulation of Nitrate. Front Cell Infect Microbiol. 9: 39. doi: 10.3389/fcimb.2019.00039

van Velzen AG, Sips AJ, Schothorst RC, Lambers AC, Meulenbelt J. 2008. The oral bioavailability of nitrate from nitrate-rich vegetables in humans. Toxicol Lett. 181(3): 177-181. doi: 10.1016/j.toxlet.2008.07.019.

Vanhatalo A, Blackwell JR, L'Heureux JE, Williams DW, Smith A, van der Giezen M, Winyard PG, Kelly J, Jones AM. 2018. Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. Free Radic Biol Med. 124(3): 21-30. doi: 10.1016/j.freeradbiomed.2018.05.078.

Vanhatalo A, L'Heureux JE, Kelly J, Blackwell JR, Wylie LJ, Fulford J, Winyard PG, Williams DW, van der Giezen M, Jones AM. 2021. Network analysis of nitrate-sensitive oral microbiome reveals interactions with cognitive function and cardiovascular health across dietary interventions. Redox Biol. 41: 101933. doi: 10.1016/j.redox.2021.101933.

Vargas-Reus MA, Memarzadeh K, Huang J, Ren GG, Allaker RP. 2012. Antimicrobial activity of nanoparticulate metal oxides against peri-implantitis pathogens. Int J Antimicrob Agents. 40(2): 135-139. doi: 10.1016/j.ijantimicag.2012.04.012.

Velmurugan S, Gan JM, Rathod KS, Khambata RS, Ghosh SM, Hartley A, Van Eijl S, Sagi-Kiss V, Chowdhury TA, Curtis M, et al. 2016. Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. Am J Clin Nutr. 103(1): 25-38. doi: 10.3945/ajcn.115.116244.

Vogel JW, Young AL, Oxtoby NP, Smith R, Ossenkoppele R, Strandberg OT, La Joie R, Aksman LM, Grothe MJ, Iturria-Medina Y, et al. 2021. Four distinct trajectories of tau deposition identified in Alzheimer's disease. Nat Med. 27: 871-881. doi: 10.1038/s41591-021-01309-6.

Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, et al. 2008. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension. 51(3): 784-790. doi: 10.1161/HYPERTENSIONAHA.107.103523.

Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. 2013. Beetroot juice and exercise: pharmacodynamic and dose-response relationships. J Appl Physiol. 115(3): 325-336. doi: 10.1152/japplphysiol.00372.2013.

Zhurakivska K, Troiano G, Caponio VCA, Dioguardi M, Laino L, Maffione AB and Lo Muzio L. 2019. Do Changes in Oral Microbiota Correlate With Plasma Nitrite Response? A Systematic Review. Front Physiol. 10: 1029. doi: 10.3389/fphys.2019.01029

6 APPENDICES

6.1 Appendix A

Table 1. Resting mean systolic, diastolic, and mean arterial blood pressure pre and post nitrate supplementation (n=9). Data is presented as mean ± SD.

Condition	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Mean arterial blood pressure (mmHg)
Pre nitrate	130 ± 12	75 ± 4	102 ± 7
Post nitrate	130 ± 16	77 ± 8	104 ± 12

Table 2. Results of paired t-tests assessing mean systolic, diastolic, and mean arterial blood pressure between pre and post nitrate conditions (n=9). Systolic, diastolic, and mean arterial pressure did not differ significantly between pre and post nitrate conditions ($p>0.05$).

t-test statistics	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Arterial blood pressure (mmHg)
df	8	8	8
t value	2.3	2.3	2.3
p value	0.95	0.52	0.76

Table 3. Mean absolute and relative FMD responses pre and post nitrate conditions (n=9).

Condition	Mean absolute FMD (mm)	Mean relative FMD (%)
Pre nitrate	0.44 ± 0.19	11.84 ± 4.51
Post nitrate	0.45 ± 0.21	11.94 ± 4.50

Table 4. Results of paired t-tests comparing mean absolute and relative FMD responses from pre to post nitrate conditions (n=9). The mean absolute and relative FMD did not differ significantly between pre and post nitrate conditions ($p>0.05$).

t-test statistics	Absolute FMD	Relative FMD
Degrees of freedom	8	8
t value	2.6	2.6
p value	0.50	0.60

Table 5. Results of paired t-tests assessing the change in mean relative abundance of *Porphyromonas* genus between pre and post nitrate conditions (n=8). There were no significant differences between pre and post conditions in any sample types ($p>0.05$).

	Saliva	Floss	Tongue	Centrifuged saliva
df	7	8	9	8
t value	1.89	1.83	1.83	1.86
p value	0.35	0.83	0.84	0.54

Table 6. Within subject effects of the relative abundance of *Porphyromonas* in saliva, floss, tongue, and centrifuged saliva sample types. There was a significant difference between sample types ($p<0.001$).

Cases	Sum of Squares		df		Mean Square		F		p	
Sample site	4.459e-4	[a]	3	[a]	1.486e-4	[a]	11.929	[a]	< .001	[a]
Residuals	2.991e-4		24		1.246e-5					

Note. Type III Sum of Squares

[a] Mauchly's test of sphericity indicates that the assumption of sphericity is violated ($p < 0.05$).

Table 7. Between subject effects of the relative abundance of *Porphyromonas* in saliva, floss, tongue, and centrifuged saliva sample types.

Cases	Sum of Squares	df	Mean Square
Residuals	9.251e-5	8	1.156e-5

Note. Type III Sum of Squares

Table 8. Post Hoc comparisons of the mean relative abundance of *Porphyromonas* between sample types, with a Bonferroni correction. There were significant differences between the mean relative abundance of *Porphyromonas* in the floss sample type as compared to saliva, tongue, and centrifuged saliva sample types ($p<0.001$ for all).

		Mean Difference	SE	t	p_{bonf}
Saliva	Floss	-0.008	0.002	-4.739	< .001
	Tongue	2.662e-4	0.002	0.160	1.000
	Centrifuged Saliva	4.379e-4	0.002	0.263	1.000
Floss	Tongue	0.008	0.002	4.899	< .001
	Centrifuged Saliva	0.008	0.002	5.002	< .001
Tongue	Centrifuged Saliva	1.716e-4	0.002	0.103	1.000

Note. P-value adjusted for comparing a family of 6