DISSERTATION

THE EFFICACY OF RED BEETROOT JUICE SUPPLEMENTATION TO IMPROVE CARDIOMETABOLIC HEALTH IN MIDDLE-AGED/OLDER ADULTS WITH OVERWEIGHT OR OBESITY

Submitted by

Nicole S. Litwin

Department of Food Science and Human Nutrition

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2019

Doctoral Committee:

Advisor: Sarah A. Johnson

Christopher L. Gentile
Michael J. Pagliassotti
Sangeeta Rao
Douglas R. Seals

ABSTRACT

THE EFFICACY OF RED BEETROOT JUICE SUPPLEMENTATION TO IMPROVE CARDIOMETABOLIC HEALTH IN MIDDLE-AGED/OLDER ADULTS WITH OVERWEIGHT OR OBESITY

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in developed societies worldwide. Advancing age is the primary risk factor for CVD, with lifestyle factors such as diet and nutrition also playing a role. Aging results in adverse changes to the arteries including vascular endothelial dysfunction which is characterized by a decline in nitric oxide (NO)-mediated endothelium-dependent dilation, and increased stiffening of large elastic arteries. This age-associated vascular dysfunction is predominantly driven by increased oxidative stress and chronic inflammation and contributes to the development of CVD through the development of atherosclerotic plaque and hypertension.

Previous research suggests that a single high-fat meal may result in transient impairments in postprandial vascular endothelial function, which is thought to be driven by a postprandial pro-inflammatory and oxidative stress response to hypertriglyceridemia and/or hyperglycemia, resulting in a decline in NO bioavailability. This phenomenon may be exaggerated in aging individuals with overweight or obesity, though previous research findings have been inconclusive. Nonetheless, repeated high-fat meal consumption may increase CVD risk through impairments in postprandial vascular endothelial function, thus warranting further investigation. While the mechanisms of postprandial vascular endothelial dysfunction continue to be fully elucidated, an emerging area of research suggests that the oral microbiota may determine steady-state NO levels. Recent scientific discoveries indicate that the oral microbiota reduces dietary inorganic nitrate to nitrite and NO (known as the enterosalivary nitrate-nitrite-NO

pathway), thus providing a new therapeutic target for CVD risk management. Red beetroot juice is a rich source of inorganic nitrate as well as other bioactive compounds such as betalains, flavonoids, carotenoids, and ascorbic acid, and previous research suggests that it may improve several parameters of cardiometabolic health including vascular endothelial function.

The goals of this dissertation research were to 1) examine the clinical efficacy of acute and chronic red beetroot juice supplementation on postprandial vascular endothelial function after a high-fat meal challenge in middle-aged/older men and postmenopausal women with overweight or obesity, and 2) investigate the underlying mechanisms that contribute to vascular and metabolic responses to the meal challenge and supplementation, including the nitrate-dependent and -independent effects of red beetroot juice. To investigate the aforementioned, we conducted a randomized, double-blind, placebo-controlled, 4-period, crossover, clinical trial. To investigate the nitrate-dependent and -independent effects of red beetroot juice, we used 1) a placebo concentrate devoid of inorganic nitrate or polyphenols, 2) red beetroot juice concentrate, 2) nitrate-depleted red beetroot juice concentrate, and 4) a placebo concentrate with an equivalent dose of inorganic nitrate to that of red beetroot juice.

We first examined the impact of acute and chronic red beetroot juice supplementation on postprandial vascular endothelial function and other cardiometabolic responses to a high-fat meal challenge. We found that the high-fat meal led to postprandial alterations in several cardiometabolic parameters but did not impair vascular endothelial function. Significant acute and chronic increases in saliva and plasma NO metabolites were observed following consumption of red beetroot juice and the placebo plus inorganic nitrate, but these increases were not paralleled by significant changes in vascular endothelial function. Although the meal and treatments altered several other parameters of cardiometabolic health, there were no consistent effects of the treatments on those parameters.

Next, we examined the relationship between oral nitrate-reducing bacteria and NO metabolites following acute and chronic red beetroot juice supplementation to gain insight on

the impact of the oral microbiota on dietary nitrate metabolism and vascular responses to the high-fat meal. We found that red beetroot juice and inorganic nitrate salt supplementation may alter the oral microbiome to favorably affect NO metabolism and vascular endothelial function in this population.

Taken together, these results suggest that although red beetroot juice did not modulate postprandial vascular endothelial function, it may be a promising dietary intervention for targeting the enterosalivary nitrate-nitrite-NO pathway to increase NO bioavailability in middle-aged/older adults with overweight or obesity. Further research is needed to evaluate the potential of red beetroot juice as an oral microbiota targeted therapy for improving NO bioavailability and overall cardiovascular health. Additionally, further research is needed to better understand the impact of high-fat meal consumption on cardiometabolic health.

ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my advisor and mentor, Dr. Sarah A. Johnson. My academic and professional success, as well as the completion of my dissertation, would not have been possible without the unwavering support and guidance of Dr. Johnson. I thank you, Dr. Johnson, for your invaluable advice and experiences over the years. I am forever grateful for your profound belief in my academic work and professional abilities.

Second, I would like to express my appreciation to my doctoral committee – Dr. Gentile, Dr. Pagliassotti, Dr. Rao, and Dr. Seals. I appreciate the time, guidance and unparalleled knowledge and support that you all have provided me with during my doctoral career. I would like to personally thank Dr. Rao for the statistical analysis training she provided me with, which was pivotal in my studies and completion of my dissertation research. I am also grateful to Dr. Chris Melby for his trainings in conducting successful clinical research. I would also like to thank Dr. Tiffany Weir for her teachings on the human microbiome and helping me to understand methodologies and interpretation of microbiota data. I would also like to thank Yuren (Rosie) Wei for her time and hard work completing several of the biochemical assays included in my dissertation. I cannot express my thankfulness enough to Yuren. I would also like to acknowledge Dr. Kimberly Cox-York for her support in my pursuit of a non-academic scientist career and always offering valuable advice.

Lastly, I would like to gratefully acknowledge and thank my lab mates for their hard work and continued support. This dissertation would not be possible without the blood, sweat and tears that all of you (Hannah Van Ark, Shannon Hartley, Kiri Michell, Allegra Vazquez, and Scott Wrigley) have put in to conduct and complete this clinical study (and all the others). Much appreciation also goes to the study participants for their commitment to this study – this dissertation certainly would not be possible without all of you!

DEDICATION

This dissertation is dedicated to my exceptional and always encouraging aunts, Erika and Shelly, who have never stopped believing in me and told me to follow my dreams, despite what odds were against me. My perseverance, bravery, resilience, initiative and tenacity come from you both. Words cannot express my love and gratitude for the two of you.

I also dedicate this dissertation to my smart, dedicated and outrageously loving and supportive fiancé, Aaron, and my exuberant, sweet, kind-hearted and precocious step-daughter, Madeleine. I love you both and I could not have done this without your patience and endless support, love and affection.

Lastly, this dissertation is dedicated in loving memory to my grandmother, Sandra Louise Boles, a smart and joyous woman who taught me strength and the value of hard work. Thank you and I miss you.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in developed countries worldwide with advancing age as the primary risk factor for CVD.[1] Aging is associated with a decline in various physiological processes in the human body and is thought to increase CVD risk due to adverse changes to the arteries.[2–4] These changes include the development of vascular endothelial dysfunction and large elastic arterial stiffening. Central mediators to these age-related manifestations include vascular oxidative stress and chronic inflammation.[2]

Aside from aging, numerous risk factors can increase the risk for developing CVD including diet.[1] For instance, a single high-fat meal has been shown to induce transient metabolic disturbances (e.g., hyperlipidemia and hyperglycemia) and a concurrent loss of nitric oxide (NO)-mediated endothelium-dependent dilation which is referred to as postprandial vascular endothelial dysfunction.[5] This phenomenon has been proposed to result from decreased NO bioavailability, which may be driven by increased oxidative stress and inflammation resulting from hypertriglyceridemia and/or hyperglycemia that may occur in the postprandial state following a high-fat meal.[6–11] It has been shown that overweight or obese individuals have exaggerated postprandial metabolic responses to a high-fat meal including significantly elevated levels of triglycerides, glucose, insulin, inflammatory and oxidative stress markers.[12,13] Given that much of the day is spent in the postprandial state and that the Western diet largely consists of high-fat meals,[14] repeated high-fat meal consumption may further increase CVD risk in aging overweight/obese individuals. Indeed, even mildly elevated postprandial triglyceride and glucose levels have been linked to increased CVD risk.[15] Therefore, therapeutic interventions that attenuate these postprandial responses are needed.

One promising dietary intervention is red beetroot juice supplementation. Red beetroot (*Beta vulgaris*) is a rich source of inorganic nitrate, providing a natural means of increasing NO

bioavailability *in vivo* through inorganic nitrate-dependent mechanisms, and thus has been of recent scientific attention as a potential therapeutic intervention to prevent and manage conditions associated with reduced NO bioavailability such as vascular endothelial dysfunction.[16,17] Red beetroot juice contains other bioactive compounds aside from inorganic nitrate including ascorbic acids, flavonoids (e.g., anthocyanins), phenolic acids, carotenoids and betalains.[16] These compounds and their downstream metabolites possess strong anti-oxidant and anti-inflammatory capabilities and therefore may also improve NO bioavailability through inorganic nitrate-independent mechanisms.[18]

Moreover, the conversion of dietary inorganic nitrate to NO occurs via the enterosalivary nitrate-nitrite-NO pathway.[19] This pathway provides an alternative means of producing NO, complementing the traditional, endogenous synthesis of NO via the L-arginine-endothelial NO synthase pathway. The enterosalivary nitrate-nitrite-NO pathway is dependent upon the oral microbiota due to the unique ability of specific oral commensal bacteria to enzymatically reduce inorganic nitrate to nitrite.[19–21] The newly formed nitrite can enter circulation and be further reduced to NO in the vasculature, thereby increasing NO bioavailability as well as eliciting endothelium-dependent vasodilation.[17,19,22] This pathway is of utmost therapeutic potential when NO bioavailability is diminished, such as that that occurs after high dietary fat intake and with aging. In summary, red beetroot juice offers therapeutic potential for alleviating inflammation and oxidative stress and reduced NO bioavailability associated with postprandial vascular endothelial dysfunction.

Research Objectives

The goals of this dissertation research were to examine the clinical efficacy of acute and chronic red beetroot juice supplementation on postprandial endothelial function in middle-aged/older men and postmenopausal women with overweight or obesity, and to investigate the underlying mechanisms contributing to clinical responses including nitrate-dependent and -

independent effects of red beetroot juice. First (Chapter 3), we examined the impact of both acute and chronic red beetroot juice supplementation on vascular endothelial function and other cardiometabolic responses to a high-fat meal challenge. Second (Chapter 4), we examined the relationship between oral nitrate-reducing bacteria and nitric oxide metabolites following acute and chronic red beetroot juice supplementation to gain insight on the impact of the oral microbiota on dietary nitrate metabolism and vascular responses including postprandial vascular endothelial function.

REFERENCES

1. Benjamin, E. J. *et al.* Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. *Circulation* **137**, e67–e492 (2018).
2. Donato Anthony J., Machin Daniel R. & Lesniewski Lisa A. Mechanisms of Dysfunction in the Aging Vasculature and Role in Age-Related Disease. *Circ. Res.* **123**, 825–848 (2018).
3. North Brian J. & Sinclair David A. The Intersection Between Aging and Cardiovascular Disease. *Circ. Res.* **110**, 1097–1108 (2012).
4. Johnson, S. A., Litwin, N. S. & Seals, D. R. Age-Related Vascular Dysfunction: What Registered Dietitian Nutritionists Need to Know. *J. Acad. Nutr. Diet.* (2019). doi:10.1016/j.jand.2019.03.016
5. Vogel, R. A., Corretti, M. C. & Plotnick, G. D. Effect of a single high-fat meal on endothelial function in healthy subjects. *Am. J. Cardiol.* **79**, 350–354 (1997).
6. Huang, K. *et al.* The short-term consumption of a moderately high-fat diet alters nitric oxide bioavailability in lean female Zucker rats. *Can. J. Physiol. Pharmacol.* **89**, 245–257 (2011).
7. Martins, M. A. *et al.* High fat diets modulate nitric oxide biosynthesis and antioxidant defence in red blood cells from C57BL/6 mice. *Arch. Biochem. Biophys.* **499**, 56–61 (2010).
8. Razny, U. *et al.* Increased nitric oxide availability attenuates high fat diet metabolic alterations and gene expression associated with insulin resistance. *Cardiovasc. Diabetol.* **10**, 68 (2011).
9. Stancu, C. S., Toma, L. & Sima, A. V. Dual role of lipoproteins in endothelial cell dysfunction in atherosclerosis. *Cell Tissue Res.* **349**, 433–446 (2012).
10. Hall, W. L. Dietary saturated and unsaturated fats as determinants of blood pressure and vascular function. *Nutr. Res. Rev.* **22**, 18–38 (2009).

11. Esposito, K. & Giugliano, D. Diet and inflammation: a link to metabolic and cardiovascular diseases. 6
12. Blackburn, P. *et al.* Postprandial hyperlipidemia: another correlate of the "hypertriglyceridemic waist" phenotype in men. *Atherosclerosis* **171**, 327–336 (2003).
13. Couillard, C. *et al.* Postprandial triglyceride response in visceral obesity in men. *Diabetes* **47**, 953–960 (1998).
14. Mente, A., Koning, L. de, Shannon, H. S. & Anand, S. S. A Systematic Review of the Evidence Supporting a Causal Link Between Dietary Factors and Coronary Heart Disease. *Arch. Intern. Med.* **169**, 659–669 (2009).
15. Lefèbvre, P. J. & Scheen, A. J. The postprandial state and risk of cardiovascular disease. *Diabet. Med. J. Br. Diabet. Assoc.* **15 Suppl 4**, S63-68 (1998).
16. Clifford, T., Howatson, G., West, D. J. & Stevenson, E. J. The Potential Benefits of Red Beetroot Supplementation in Health and Disease. *Nutrients* **7**, 2801–2822 (2015).
17. Lidder, S. & Webb, A. J. Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway: Vascular effects of dietary nitrate. *Br. J. Clin. Pharmacol.* **75**, 677–696 (2013).
18. Zhang, H. & Tsao, R. Dietary polyphenols, oxidative stress and antioxidant and anti-inflammatory effects. *Curr. Opin. Food Sci.* **8**, 33–42 (2016).
19. Lundberg, J. O., Weitzberg, E. & Gladwin, M. T. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* **7**, 156–167 (2008).
20. A. J. Smith, N. Benjamin, D. A. Wee. The Microbial Generation of Nitric Oxide in the Human Oral Cavity. *Microb. Ecol. Health Dis.* **11**, 23–27 (1999).
21. Gao, L. *et al.* Oral microbiomes: more and more importance in oral cavity and whole body. *Protein Cell* **9**, 488–500 (2018).
22. Bondonno, C. P., Croft, K. D. & Hodgson, J. M. Dietary Nitrate, Nitric Oxide, and Cardiovascular Health. *Crit. Rev. Food Sci. Nutr.* **56**, 2036–2052 (2016).

23. Burleigh, M. C. *et al.* Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria. *Free Radic. Biol. Med.* **120**, 80–88 (2018).
24. Zweier, J. L., Li, H., Samouilov, A. & Liu, X. Mechanisms of Nitrite Reduction to Nitric Oxide in the Heart and Vessel Wall. *Nitric Oxide Biol. Chem. Off. J. Nitric Oxide Soc.* **22**, 83–90 (2010).
25. Pereira, C., Ferreira, N. R., Rocha, B. S., Barbosa, R. M. & Laranjinha, J. The redox interplay between nitrite and nitric oxide: From the gut to the brain. *Redox Biol.* **1**, 276–284 (2013).
26. Bryan, N. S. & Grisham, M. B. Methods to Detect Nitric Oxide and its Metabolites in Biological Samples. *Free Radic. Biol. Med.* **43**, 645–657 (2007).
27. Webb Andrew J. *et al.* Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate via Bioconversion to Nitrite. *Hypertension* **51**, 784–790 (2008).

CHAPTER 2: REVIEW OF THE LITERATURE

Cardiovascular disease (CVD), including coronary heart disease (CHD), hypertension (HTN) and stroke, is the leading cause of mortality worldwide, accounting for 17.9 million deaths per year in 2015 and is expected to reach 23.6 million by the year 2030.[1] Age is the strongest independent predictor of CVD risk.[1] As a result of population aging, the number of older adults with CVD risk factors is increasing as life span extends. For instance, in the United States, 43.9% of the adult population is projected to have some form of CVD by the year 2030 with associated total direct health care costs reaching $918 billion.[1] Thus, cost-effective, evidence-based interventions are needed to prevent, delay and/or reverse the development of aging-associated CVD.

The link between aging and CVD risk is largely attributable to vascular dysfunction which is primarily characterized by two physiological changes: vascular endothelial dysfunction and large elastic arterial stiffening.[2] The onset and progression of processes associated with vascular dysfunction, namely oxidative stress and inflammation, can be favorably modified by dietary habits and lifestyle approaches[3], making this an important therapeutic target.

Age-Related Vascular Dysfunction and Cardiovascular Disease

Various measures can be used to assess vascular endothelial function and arterial stiffness non-invasively in humans with adequate repeatability and reproducibility. These measures have been shown to be significant, independent predictors of long-term clinical CVD outcomes and thus can be used as a diagnostic and prognostic measurement tool.[4] Additionally, these measures have been shown to be positively modulated by dietary constituents.[3,5,6]

Vascular Endothelial Function

The vascular endothelium is a monolayer of cells that line the innermost layer of all blood vessels (veins, arteries, and microvessels). The endothelium represents an interface between circulating blood in the vessel interior (referred to as the lumen) and the outer layers of the vessel wall. The vascular endothelium is a metabolically active and responsive "surface" that regulates various physiological activities that contribute to the overall health and function of the vascular system.[7] The endothelium serves as a barrier that selectively controls the movement of fluid, oxygen, nutrients, metabolites, and other molecules between the circulating blood and underlying tissues through constant cell-cell signaling and communication via endothelial cell surface receptors and inter-endothelial junction[7,8]. The endothelium is responsible for synthesizing and secreting vasoactive molecules that act together within the endothelium and underlying vascular smooth muscle cells to regulate vascular tone (i.e., degree of vasoconstriction relative to vasodilation), blood flow, and blood pressure.[9] In a healthy endothelium, a balance among these molecules exists, promoting vasodilation and inhibiting oxidation, coagulation, cellular adhesion, smooth muscle cell proliferation, and vessel wall inflammation.[10] However, with advancing age, the endothelium shifts to a pro-vasoconstrictive, pro-thrombotic, pro-fibrinolytic, proliferative, pro-oxidative and pro-inflammatory state.[11] This phenotypic state is referred to as vascular endothelial dysfunction and is recognized as the initial step in the development of atherosclerosis and is antecedent to most cardiovascular diseases.[11,12]

The Role of Nitric Oxide in Vascular Endothelial Function

Among the endothelium-derived vasoactive molecules, nitric oxide (NO) is the most potent endogenous vasodilator, plays an essential role in maintaining vascular health, and homeostasis and is a key determinant of a properly functioning endothelium.[13] NO is synthesized in endothelial cells from the oxidation of L-arginine to L-citrulline via the enzymatic

actions of endothelial NO synthase (eNOS; also known as NOS-3) under the influence of chemical agonists acting on specific endothelial chemoreceptors or by mechanical forces on mechanoreceptors, such as shear stress.[13] This conversion of L-arginine to NO requires the presence of oxygen and the cofactors nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH_4).[13] eNOS is constitutively expressed and therefore continuously produces NO in healthy endothelial cells. eNOS is bound to the protein caveolin which is located in small invaginations in the cell membrane called caveolae, and its activation is calcium-dependent.[14] When intracellular calcium levels increase in response to NO agonists (e.g., acetylcholine, bradykinin, serotonin, thrombin), eNOS is released from caveolin and is activated. These agonists displace calcium from the endoplasmic reticulum (ER), in which calcium freely attaches to the protein calmodulin in the cytoplasm of the cell after it undergoes structural changes to allow it to bind to and activate eNOS.[13,15,16] It is important to highlight that this mechanism of NO synthesis is dependent on the levels of intracellular calcium in the ER, as well as calcium that diffuses into the cell from extracellular stores. A reduction in calcium causes the calcium-calmodulin complex to dissociate from eNOS, allowing eNOS to bind to its inhibitor, caveolin, and thus, becoming inactivated.[14,16] When intracellular calcium levels become depleted, additional mechanisms are triggered to maintain continuous basal release of NO. One such mechanism is the phosphorylation of eNOS via protein kinases (i.e., protein kinase A, cGMP protein kinase dependent II).[13,15] Increased shear stress on the vessel wall can also induce eNOS phosphorylation via protein kinase B (Akt) and activation of calcium-potassium ion channels on the endothelial cell surface.[16]

Once produced, NO diffuses to the vascular smooth muscle cells, binding to and activating soluble guanylate cyclase (sGC).[15] The now activated sGC enzyme increases the conversion rate of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), resulting in vascular smooth muscle cell relaxation.[9,13,15] Again, the mechanisms described

above are continuously active and produce NO to maintain basal vasodilation in a healthy and functional endothelium.

Apart from vasodilation, NO at physiologic levels provides other protective cellular functions in the vasculature such as anti-inflammatory and anti-atherogenic properties.[15,17] NO prevents platelet and leukocyte adhesion to the vessel wall by inhibiting endothelial cell expression of cytokine-induced monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and E- and P-selectin via inactivation of the pro-inflammatory transcription factor NFkB.[15,17,18] NO also directly inhibits vascular smooth muscle cell proliferation and migration by decreasing the expression and activity of cell cycle regulatory proteins (cyclin A, p21, cdk2).[19]

Non-Invasive Clinical Assessment of Vascular Endothelial Function

Flow-Mediated Dilation of the Brachial Artery

In humans, vascular endothelial function is commonly assessed by measuring endothelium-dependent dilation (EDD), or the degree to which a blood vessel dilates in response to a chemical or mechanical stimulus that evokes NO production via the activation of eNOS.[9,20] When produced and released by the endothelium, NO diffuses to the underlying vascular smooth muscle and induces vasorelaxation and vasodilation of the artery, leading to a subsequent increase in blood flow, which can be assessed by several non-invasive methods.[9] In clinical research studies, the current, non-invasive gold-standard method to assess EDD in the macrovasculature (i.e., large peripheral conduit arteries such as brachial, radial, femoral) is flow-mediated dilation (FMD) of the brachial artery using ultrasonography[21]. During FMD, vasodilation of the brachial artery occurs after an acute increase in blood flow induced by temporary forearm ischemia. This reactive hyperemic response is recognized to be largely NO dependent.[22] Using ultrasonography, the diameter of the brachial artery is captured at baseline, and during both occlusion and reactive hyperemia. FMD is calculated as the peak diameter of

the artery during reactive hyperemia relative to baseline artery diameter.[20,21] A low FMD response is suggested to be indicative of low NO bioavailability and is independently and inversely associated with an increased risk of CVD and CVD-related events, with greater associations in diseased populations.[23] Additionally, FMD of the brachial artery has been shown to correlate well with coronary endothelial function and the extent and severity of coronary atherosclerosis.[24]

Although FMD is widely used in clinical research settings, it is not yet recommended for routine diagnostic use due to its expense, difficulty and methodological limitations.[20,25] Despite well-established guidelines, inter-individual FMD responses can be highly variable with little reproducibility, due to differences in methodology.[26] For instance, subject preparation, different occlusion durations, cuff and ultrasound probe positions, as well as operator-dependent technique and analysis skill can contribute to within-person measurement error of FMD and reduced repeatability and reproducibility.[20,27] Environmental factors such as room temperature, lighting, noise, etc. also play a role in FMD measurement error and variability and must be accounted for during clinical assessment.[20]

Digital Peripheral Arterial Tonometry (PAT)

In contrast to FMD of the brachial artery, non-invasive peripheral artery tonometry (PAT) is easy to perform and operator-independent, and thus has better reproducibility and minimal variability and measurement error.[28–30] PAT is a fast, semi-automated method, introduced by Itamar Medical (Israel), that assesses peripheral microvascular endothelial function with a device called the EndoPAT-2000.[28–30] The EndoPAT uses two fingertip pneumatic probes that measure arterial pulse wave amplitudes. After baseline recording, the brachial artery of the non-dominant arm is occluded with a blood pressure cuff inflated to suprasystolic pressure for 5 minutes and then the cuff is released. The resulting hyperemic response is recorded for an additional 5 minutes, and the device software automatically calculates a reactive hyperemia

index (RHI; also called EndoScore).[28–30] An RHI value of <1.67 is indicative of endothelial dysfunction.[31]

RHI/PAT has been shown to correlate with brachial artery FMD,[28,32–35] coronary microvascular function,[31] multiple cardiovascular risk factors,[31,36] and is an independent predictor of future adverse cardiovascular events.[37,38] However, it is important to note that although correlations between FMD and PAT have been observed, results are equivocal due to vast methodological and physiological differences in the two measurements. For instance, FMD assesses vasodilatory responses in the macrovasculature, whereas PAT assesses vasodilatory responses in the microvasculature, representing different vascular beds and thereby, different aspects of vascular endothelial function. As such, unlike FMD of the brachial artery, digital/fingertip reactive hyperemia, is not entirely NO-dependent.[32] It is purported that NO is released during reactive hyperemia in the finger.[29] Significant reductions (approximately 46%) in fingertip reactive hyperemia have been demonstrated after pharmacological infusion with a NOS-inhibitor (N^G-nitro-l-arginine methyl ester, L-NAME) in the brachial artery prior to EndoPAT assessment in healthy adults.[39] In the same study, infusion with phenylephrine, a vasoconstrictor, had no effect on pulse wave amplitude during reactive hyperemia, but significantly reduced resting/baseline pulse wave amplitude. This suggests that the observed reduction in pulse wave amplitude during reactive hyperemia by L-NAME is specifically mediated by NO and not by non-specific vasoconstriction.[39] This also suggests that in the peripheral microvasculature, there is a central dependence of NO in the PAT pulse wave amplitude signal during reactive hyperemia and that this measurement may be used to assess NO bioavailability and EDD.[39] Nonetheless, there seems to be NO-independent vasodilatory factors that contribute to peripheral microvascular endothelial function.

For instance, peripheral microvasculature tone is highly responsive to the autonomic and sympathetic nervous systems.[36,39,40] Thus, fluctuations in the PAT signal/finger pulse wave amplitude may occur from induced stress from uncomfortable room temperature, light, noise,

etc. These environmental stressors must be controlled for when performing EndoPAT.[28] Additionally, the fingertip probes are sensitive to movement which can cause artefacts in the pulse wave recordings and therefore fixation of the probe to the finger is dire to ensure accurate measurements.[28,29] The finger probes need to be replaced with each use of the EndoPAT and result in continuous expense.

Arterial Stiffness

Stiffening of the central vasculature (i.e., large, elastic arteries such as the aorta) is a consequence of biological aging and is also a significant contributor to the development of CVDs in older individuals.[41,42] In general, arterial stiffness refers to the loss of vascular compliance (i.e., volume-pressure relationship in a vessel) and alterations to the vessel wall.[42,43] A healthy, young aorta is highly compliant due to greater elastin content relative to collagen (scaffolding proteins that provide elasticity and structural integrity), which buffers the pulsatility of ventricular ejection, thereby reducing pulse pressure. Additionally, the reflected pulse wave that is generated during each heartbeat is slow and returns to the heart during diastole, increasing diastolic pressure and improving coronary perfusion.[43] On the other hand, aortic stiffening that occurs with age leads to a faster pulse wave velocity (PWV) resulting in an early return of the reflected wave which reaches the heart during systole (rather than diastole in a young aorta). This results in an increase in systolic pressure, while causing an increased cardiac workload (particularly left ventricular) and a reduction in diastolic blood pressure (thus increasing pulse pressure) and coronary perfusion.[43–45] Over time, this can lead to lead to left ventricular hypertrophy and an increased risk of myocardial infarction and heart failure.[2]

Mechanisms of Central Arterial Stiffness

Age-related stiffening of large, elastic central arteries is mediated by both structural and functional changes to the vessel wall. Structural changes include extracellular matrix remodeling

that result from increased collagen deposition and elastin fragmentation and degradation, and the formation of advanced glycation end products (AGE) which non-enzymatically cross-link collagen and elastin leading to glycation damage and additional changes in elastin and collagen properties.[41,42,45,46] In a healthy elastic artery, there is a tightly regulated balance between elastin and collagen production and degradation and enzymatic cross-linking of the two which is initiated by the enzyme lysyl oxidase (LOX). Elastin and collagen are also regulated by metalloproteases (MMPs), which degrade the extracellular matrix (ECM) by producing weak collagen and broken/frayed elastin.[42] Thus, a balance must exist between LOX and MMP activity to provide a stable ECM and maintain vascular compliance. This balance can be disrupted, as seen with aging, when there is increased MMP expression and activity occurs from infiltration of pro-inflammatory cells (e.g., macrophages) into the vessel wall.[42,45–49] Additionally, AGEs can stimulate inflammatory responses and cell signaling resulting in increased expression of NFkB, the formation of ROS and oxidative stress, and production of pro-inflammatory cytokines, and vascular cell adhesion molecules, which can activate MMPs resulting in increased arterial stiffness as well as vascular endothelial dysfunction and atherosclerotic plaque formation.[45–49] Moreover, functional changes associated with aging that contribute to arterial stiffening result from alterations in vascular smooth muscle tone and imbalances between endothelium-derived vasoconstrictors and vasodilators that occur during vascular endothelial dysfunction. Arterial stiffness and endothelial dysfunction have a bi-directional relationship in that arterial stiffening leads to endothelial disturbances and endothelial disturbances may lead to or worsen arterial stiffening.[43,45,46]

Non-Invasive Clinical Assessment of Central Arterial Stiffness

Carotid-Femoral Pulse Wave Velocity

The most commonly used non-invasive technique to determine stiffness along an arterial segment is the assessment of PWV.[43] PWV is obtained by recording the time taken by an

arterial pulse wave form between two anatomical sites (proximal and distal to one another) that are a measured distance apart. Measuring PWV between the common carotid and common femoral arteries (known as carotid-femoral PWV) is most often used in clinical research as the two vessels are relatively superficial and easy to identify.[43] Additionally, the distance between the carotid and femoral arteries is comparable to the length of the aorta, and thereby is used as a surrogate marker for aortic PWV (aPWV).[43,45] Elasticity of a vessel wall is known to determine the speed or velocity of which a pressure pulse generated from ventricular ejection takes to propagate along the arterial tree. As such, PWV has been shown to increase in parallel with age and arterial stiffness.[41,42] PWV of the carotid-femoral region consists of the time taken for the arterial pulse to propagate from the carotid to the femoral artery and calculated as PWV (m/s) = distance (m) / time (s).[43,45] PWV can be measured in any arterial segment; however, carotid-femoral PWV, also referred to as aPWV, is considered the current gold-standard, and has been shown to be predictive for CVD risk, events, and mortality.[50]

Several devices have been developed that measure aPWV such as the SphygmoCor® (AtCor Medical), which uses tonomoter probes and cuffs with pressure sensors placed on the carotid and femoral arteries, respectively, that record the pulse pressure waveforms (referred to as applanation tonometry). These devices are clinically relevant as they are relatively fast and easy to use, require little technical training, are repeatable and reproducible, and have been validated in healthy and disease populations.[51]

Augmentation Index (AIx)

Augmentation index (AIx) is an additional, albeit indirect, measure of aortic or central arterial stiffness. It is influenced by vessel compliance and is defined as the percentage of the central (aortic) pulse pressure attributed to the reflected pulse wave. Therefore, AIx is dependent on the timing and magnitude of the reflected waveform, which appears early (i.e., in systole rather than diastole) in stiff, older vessels. AIx has been shown to increase with age and

is associated with CVD risk and events.[10,52,53] AIx can be obtained from pressure waveforms via applanation tonometry of the brachial artery such as with the SphygmoCor® device, as well as from digital pulse wave volume amplitudes by PAT. However, it is important to note that the AIx obtained by PAT cannot be used interchangeably with AIx obtained by applanation tonometry since they are derived from two different vascular beds. AIx can be influenced by heart rate. A slower heart rate will cause the reflected wave peak to occur early in systole, which will increase the AIx. On the other hand, a faster heart rate will cause the reflected wave to arrive late in systole or during diastole, causing a decrease in the AIx. Therefore, it has been suggested that Aix be normalized to a standard heart rate of 75 beats per minute (bpm) to account for variability in heart rate between individuals. Conveniently, the SphygmoCor® device provides AIx normalized to the patient's heart rate as well as corrected to a heart rate of 75 bpm.[51] However, it has been shown that this normalization approach is not generalizable to all populations and both sexes, thus both values (AIx and AIx@75) should be considered and reported.[54,55]

The Role of Oxidative Stress and Inflammation in Age-Related Vascular Dysfunction

Oxidative stress and chronic, low-grade inflammation are recognized as central mechanisms contributing to age-related changes in the vasculature. In healthy young arteries there is tight regulation of and a balance between inflammatory and oxidative pathways which promotes vascular homeostasis. Advancing age results in dysregulation of these pathways due to upregulation of oxidative stress and inflammation, disrupting vascular homeostasis and ultimately result in the development of age-related vascular dysfunction.

Oxidative Stress in Age-Related Vascular Dysfunction

It is generally well-accepted that oxidative stress gradually develops with age, and that aging itself is associated with systemic oxidative stress as evidenced by elevated circulating oxidative stress markers in aging/older adults.[56,57] Oxidative stress results from an imbalance

between the rate of production of reactive oxygen species (ROS) relative to antioxidant defenses, and causes cellular dysfunction and damage. Oxidative stress in the vasculature is a central, underlying mechanism of age-related reductions in NO-bioavailability and EDD, as well as increased stiffness in large elastic arteries.[58] A characteristic feature of vascular oxidative stress is increased arterial ROS. [58-60]

A major ROS involved in vascular oxidative stress is superoxide anion, which is produced from mitochondrial respiration and multiple enzymatic systems including NADPH oxidase, xanthine oxidase, dysfunctional/uncoupled eNOS, cytochrome *P*-450 and myeloperoxidases (MPO) in vascular cells.[58–60] It has been proposed that the predominant source of superoxide in large peripheral vessels is NADPH oxidase, with its activity being regulated by pro-inflammatory cytokines, growth factors (e.g., platelet-derived growth factor), vasoactive agents (e.g., angiotensin-II), and mechanical forces (e.g., shear stress, pulsatile stretch and strain), all of which are implicated in the pathogenesis of vascular diseases.[59,60] Under normal physiological conditions, superoxide and other ROS are produced in a controlled manner and function as cell signaling molecules that help maintain vascular tone and homeostasis. However, when produced in excess, superoxide reacts with NO to produce peroxynitrite (ONOO-), a pro-inflammatory and cytotoxic reactive oxygen and nitrogen species, resulting in NO degradation.[59–61] ONOO- can oxidize BH_4 (essential cofactor for eNOS) to biologically inactive BH_3 causing uncoupling of eNOS in which superoxide radicals are produced instead of NO and contributes further to oxidative stress. This decrease in NO production and/or NO degradation decreases the bioavailability of NO and impairs EDD[58–61].

Experimental studies have demonstrated that NADPH oxidase-derived superoxide directly contributes to age-related vascular endothelial dysfunction as evidenced by restoration of EDD and NO bioavailability after pharmacologically inhibiting NADPH oxidase with the drug apocynin in cannulated carotid arteries of old mice.[62] Studies also support that NADPH oxidase and downstream eNOS uncoupling are the primary sources of superoxide in aged vessels and

vascular endothelial cells, indicating that contribution of other oxidant enzymes to vascular ROS/oxidative stress is less compared with that of NADPH oxidase. For example, no age-related increases in xanthine oxidase or cytochrome *P*-450 expression/activity have been found in older adult humans or animals, nor has pharmacological inhibition of xanthine oxidase or cytochrome *P*-450 been effective at improving age-related, oxidative-stress mediated suppression of EDD.[63,64] Further, in older humans and animals, uncoupling of eNOS and subsequent production of superoxide can occur when there are deficient/limited amounts of its cofactor BH_4. It has been demonstrated that after acute supplementation with a high oral dose of commercially available BH_4, EDD (as measured by brachial artery FMD) was improved in older healthy men, but did not affect EDD/FMD in younger healthy men.[65] This restoration of EDD was likely mediated through "recoupling of eNOS" resulting in decreased superoxide formation and increased NO production due to a more functional eNOS enzyme, and also establishes the role of BH_4 deficiency in age-related, oxidative stress-mediated suppression or loss of EDD. These findings have also been corroborated in various studies demonstrating improvements in forearm blood flow after intra-arterial infusion of BH_4 in older adults and in individuals with disease states associated with increased vascular oxidative stress (e.g., those with CAD risk factors, hypertension, chronic heart failure, chronic smokers).[66–69]

Superoxide radicals are normally removed by the antioxidant enzyme, superoxide dismutase (SOD), which has three isoforms located in the mitochondria (manganese SOD, MnSOD), cytoplasm (copper-zinc SOD, Zn/Cu SOD) and extracellular compartments (extracellular SOD, eSOD).[70] SOD rapidly converts superoxide to hydrogen peroxide when the concentration of SOD is high relative to superoxide.[59,60] However, in aging when superoxide production is increased, SOD activity (all 3 isoforms) has not been shown to increase in response to elevated superoxide.[58] In fact, SOD activity has been shown to be about 50% lower in the aorta of old mice compared to young control mice and was associated with greater superoxide production.[71] Expression of SOD (particularly MnSOD) and has also been shown to

be lower or unchanged in aged arteries of older mice compared to that of younger counterparts.[62,71–73] This insufficient SOD response with aging may not only contribute to excess superoxide which scavenges NO and reduces its bioavailability, but may also contribute to reduced hydrogen peroxide production, further contributing to impaired EDD.[58] At physiologically low concentrations, hydrogen peroxide is a stable cell-permanent ROS that is involved in NO-dependent EDD in large vessels via eNOS activation, and also as an endothelium-dependent hyperpolarizing factor (EDHF) in small/resistance vessels.[74–76] When produced in excess (by NADPH oxidase/superoxide and mitochondrial dysfunction), hydrogen peroxide becomes detrimental to cells and can impair vascular endothelial function.[75,76] Additionally, like SOD, the expression of the enzyme catalase (reduces hydrogen peroxide to oxygen and water) has also been shown to decrease with age in mice.[77] This decreased expression and activity of catalase and SOD with advancing age, combined with increased superoxide production, can lead to excessive hydrogen peroxide levels further impairing EDD. Acute infusion with a supraphysiological dose of ascorbic acid (a direct scavenger of SOD) has been shown to restore EDD in older men and postmenopausal women to that of younger healthy controls.[65,78]

Preclinical studies have shown that increased superoxide is associated with age-related increases in collagen deposition and AGE accumulation, as well as reduced elastin in arteries of aging mice, all of which are associated with increased aPWV.[72,79,80] Reductions in superoxide levels have been shown using short-term treatment with an SOD-mimetic (TEMPOL) in older mice, which also ameliorated age-related increases in aPWV and impaired EDD by restoring NO bioavailability (i.e., increased eNOS expression) and reducing levels of arterial collagen, nitrotyrosine, and expression of NADPH oxidase and aortic pro-inflammatory cytokines comparable to that of young control mice.[79] These preclinical studies in combination with human trials[81] confirm that age-related vascular stiffness is indeed related to superoxide-driven oxidative stress in the vessel wall. In summary, superoxide-driven oxidative stress in the

vasculature increases with age and is a primary contributor to the development of age-related vascular dysfunction.

Inflammation in Age-Related Vascular Dysfunction

Aging itself is associated with a state of chronic low-grade inflammation and thus has been coined "inflammaging".[82] Circulating levels of pro-inflammatory markers such as TNF-α, C-reactive protein (CRP), and IL-6 have been shown to increase with age and to be positively related to aPWV, early wave reflection and arterial elasticity,[34,83,84] as well as impaired EDD.[85]

Activation of the pro-inflammatory transcription factor nuclear factor kappa-light chain-enhancer of activated B cells (NFκB) by superoxide and other ROS (e.g., hydrogen peroxide) in the vasculature plays a crucial role in age-related vascular dysfunction.[86,87] In non-stimulated cells, the NFκB complex resides in the cytoplasm in its' inactive from due to the actions of its inhibitory protein IκB. Upon cell stimulation via ROS, pro-inflammatory cytokines (e.g., TNF-α, IL-1) or microbial products (e.g., lipopolysaccride, LPS), IκB is phosphorylated (via IκB kinases, IKK), ubiquitinated and degraded by proteases. The degradation of IκB frees/enables NFκB to translocate to the nucleus of the cell where it induces the transcription of its target genes.[87] Additional activators of NFκB beyond inflammatory/pathogenic stimuli that pertain to vascular inflammation include angiotensin-II[88], oxidized LDL[89], high glucose concentrations[90], and disturbed flow/shear stress.[91,92] Furthermore, NFκB activation induces pro-inflammatory gene expression resulting in increased production of cytokines [e.g., IL-1β, IL-6, IL-12, interferon γ (INFγ), TNF-α], chemokines (e.g., monocyte chemotactic protein-1, MCP-1), cell adhesion molecules (e.g., ICAM-1, VCAM-1, E-selectin, P-selectin) and oxidant enzymes (e.g., NADPH oxidase).[93] Increased expression of endothelial cell adhesion molecules leads to recruitment of immune cells that further exacerbate inflammation and oxidative stress in the vasculature through the production of cytokines (INF-γ and TNF-α) and superoxide anions. This creates a vicious cycle between NFκB-dependent inflammation and oxidative stress in that ROS can

activate NFκB leading to pro-inflammatory signaling, but also pro-inflammatory signaling induced by NFκB can lead to ROS production from increased expression of NADPH oxidase, as well as from newly recruited immune cells and inflammatory cytokines. As such, leads to a pro-atherogenic environment in the vessel. In fact, it has been shown that NFκB activation in the vasculature is critically involved in the initiation and development of atherosclerosis.[94]

NFκB activation can also induce expression of matrix metalloproteinases (MMPs) and transforming growth factor-β (TGF-β) which increase collagen content and degrade or decrease elastin production, changing the structural properties of the vessel wall, thereby resulting in arterial stiffening.[58] Older adults with age-related impaired EDD have been shown to have increased endothelial cell protein levels of several NFκB-dependent pro-inflammatory mediators such as IL-6, TNF-α, MCP-1. Increased expression of NFκB has also been observed in vascular endothelial cells biopsied from older[95,96] and obese[97] humans, in which the increase in NFκB expression was positively associated with endothelial cell nitrotyrosine (marker of cellular oxidative stress) abundance.[96] Inhibition of NFkB activity via salsalate was associated with improvements in EDD and lower endothelial cell expression of NADPH oxidase ($p47^{phox}$ subunit) and nitrotyrosine in overweight and obese middle-aged and older adults.[98] Short term treatment with salsalate has also been shown to improve aPWV in middle-aged and older sedentary adults, while aPWV was not affected in young sedentary controls.[99] Inhibition of TNF-α (which is NFκB-dependent) also improves age-related loss of EDD[100]. These studies further establish that NFκB-dependent inflammation contributes to age-related vascular dysfunction by inducing oxidative stress and vice versa. Taken together, aging leads to the development of chronic inflammation and oxidative stress, which induce and exacerbate one another in a vicious cycle that negatively impacts vascular function with NFκB acting a central mediator.

Postprandial Vascular Endothelial Dysfunction as a Cardiovascular Risk Factor

Consumption of high-fat and/or high-carbohydrate meals often results in transient postprandial hyperglycemia and hyperlipidemia, even in healthy individuals, which may lead to transient impairments in vascular endothelial function. [101–103] It has been suggested that oxidative stress and inflammation are central contributors to impairments in postprandial endothelial function likely due to reductions in NO bioavailability.[101–103] Vascular oxidative stress and inflammation resulting from postprandial hyperglycemia and hyperlipidemia (also frequently termed "postprandial dysmetabolism) are suggested to stem predominately from increased mitochondrial ROS production due to heightened fatty acid influx into vascular endothelial cells resulting in increased fatty acid oxidation in the mitochondria. As a result, the mitochondria overproduces electron donors (NADH, FADH) and overloads the mitochondrial electron transport chain which leads to downstream superoxide production.[102] This increase in mitochondrial superoxide production can lead to activation of NFκB-dependent pro-inflammatory cytokines and enzymes (e.g., NADPH oxidase) as well as uncoupling of eNOS and impaired eNOS activity due to oxidation of BH_4.[101–103] Postprandial endotoxemia has been suggested to contribute to increased inflammation after consumption of a high-fat meal likely due to dietary fats (particularly saturated fat) promoting the intestinal absorption of bacterial endotoxins (e.g., lipopolysaccharide, LPS) from the gut into circulation partly through transport by chylomicrons.[144-148] Once LPS is in the circulation, it binds to LPS-binding protein (LBP) via the receptor CD14 present in a membrane-bound state (mCD14) or in a circulating soluble form (soluble cluster of differentiation 14, sCD14). The LPS–LBP–CD14 complex can then bind to toll-like receptor-4 (TLR-4) on endothelial cells and monocytes/macrophages, which results in the release of proinflammatory cytokines and ROS (e.g., superoxide), and increases the expression of vascular cell surface adhesion molecules, which can lead to postprandial endothelial dysfunction, and over time, atherosclerotic plaque formation, oxidation of LDLs, and thrombogenesis.[149]

Moreover, repeated consumption of high-fat and/or high-carbohydrate meals, which are often present in the Western dietary pattern, can not only lead to a continuous state of postprandial hyperglycemia, hyperlipidemia and endotoxemia, but also continuous insults to the vascular endothelium.[102, 149] In healthy males, consumption of two consecutive high-fat meals (i.e., breakfast and lunch, 4 hours apart) resulted in oxidative stress-induced impairments in EDD that were paralleled by elevations in plasma glucose and triglycerides, particularly after the second meal.[104] Impairments in vascular endothelial function either measured by RHI or brachial artery FMD are typically seen 2-4 hours after a meal and are inversely associated with peak postprandial glycemic and lipemic responses in healthy and diabetic individuals.[102,105–110] Additionally, high-fat, high-carbohydrate meals have also been shown to induce a prolonged and exaggerated lipemic and glycemic response as well as a greater oxidative and inflammatory response in obese individuals.[111] Postprandial levels of glucose, lipids, and endotoxins have been suggested to better predict CVD risk and future cardiac events than fasting levels, and as such, are emerging as primary CVD risk factors.[112,149] Experimental studies have shown that blunting postprandial increases in glucose and lipids improves inflammation, oxidative stress and vascular endothelial function, demonstrating the role of postprandial dysmetabolism in CVD risk.[25,112] It should be noted that normal weight individuals and individuals with overweight or obesity have been shown to have similar degrees of hyperlipidemia and hyperglycemia following a high-fat meal and also have similar vascular reactivity responses in which meal-related changes in endothelial function are undetectable between the two groups.[142] Currently, research remains unclear on whether single high-fat meals can induce postprandial vascular endothelial dysfunction, particularly in overweight or obese individuals. Therefore, further research is needed to better understand the impact of a high-fat meal on postprandial endothelial function in general, and in those with overweight or obesity. Nonetheless, considering that research has implicated high-fat meal consumption in postprandial vascular endothelial function, repeated high-fat meal consumption may increase CVD risk. Thus,

therapeutic interventions that aim to reduce postprandial vascular endothelial dysfunction should be evaluated as they may have important implications in CVD risk reduction and prevention of future CVD.

Therapeutic Potential of Red Beetroot Juice

In recent years, the therapeutic potential of red beetroot (*Beta vulgaris)* has gained much attention due to its potential vascular-protective health effects including blood pressure reduction and improvements on vascular endothelial function, which have been shown to occur in both healthy populations and in disease states associated with high blood pressure and vascular dysfunction.[113–116] These vascular effects have been largely attributed to beetroot's high inorganic nitrate content, which provides an alternative means of generating NO *in vivo.* Once ingested, dietary nitrate is rapidly absorbed in the stomach and upper small intestine (shown to be almost 100% absorption following digestion) and subsequently enters systemic circulation.[117,118] It is suggested that about 25% of circulating nitrate is sequestered by the salivary glands and concentrated in the saliva which is called enterosalivary circulation of nitrate.[117,118] The remaining 75% of nitrate in circulation is excreted in the urine by the kidneys. When nitrate is released by the salivary glands into the mouth/oral cavity, oral commensal facultative anaerobic bacteria that reside on the tongue's surface reduce nitrate to nitrite. These bacteria have nitrate reductase enzymes that mammalian cells lack, and as such, nitrate reduction to nitrite depend on the presence of these bacteria which include *Veillonella, Prevotella, Neisseria, Haemophilus, Fusobacterium, Pophryomonas, Actinomyces, Granulicatella, Leptotichia, Rothia,* and *Strepotococcus*.[119,120] These bacteria can initially convert nitrate to nitrite before it is swallowed; however, this is very minimal. Once nitrite in the saliva is swallowed and reaches the stomach, nitrite is non-enzymatically reduced to NO due to chemical acidification and can stimulate mucosal blood flow.[117,118] Additional reactions between nitrite and polyphenolic compounds and ascorbic acid in the stomach can generate NO.[118] Any remaining

nitrite in the stomach can be absorbed and enter circulation in which peak nitrite levels in plasma are seen within 2-3 hours after dietary nitrate intake.[116,121] Once in circulation, heme proteins such as hemoglobin and myoglobin in red blood cells can reduce nitrite to NO in the blood and blood vessels, which ultimately leads to vasodilation.[117] This reduction of nitrite to NO is known to be enhanced during hypoxic/ischemic conditions.[117] This alternative pathway for NO can be disrupted by the use of antibacterial mouthwash which destroys the oral bacteria capable of reducing nitrate.[123–126] Sex differences in this pathway have also been implicated.[127]

This enterosalivary nitrate-nitrite-NO pathway can be targeted to increase endogenous NO production independently of NOS and represents a plausible mechanism for the beneficial vascular effects of dietary nitrate.[117,118,121] The first study to demonstrate blood pressure reduction after and a single oral dose of inorganic nitrate was Larsen *et al.* in 2006.[143] Soon after this, Webb *et al.* investigated whether a single dose of red beetroot juice (500 mL, 23 mmol nitrate) with similar nitrate content as the Larsen study was also capable of blood pressure reduction, and indeed brachial systolic and diastolic blood pressure reduced by 10 and 8 mmHg, respectively, with peak effects occurring 2-3 hours after ingestion and corresponded with increased plasma nitrite levels, which were still evident 24 hours after red beetroot juice intake.[122] Since then, there has been an overwhelming amount of clinical studies investigating the hypotensive and vasodilatory effects of dietary nitrate, either in the form of red beetroot juice or as inorganic nitrate salt supplements (e.g., potassium or sodium nitrate). A meta-analysis of these trials (total of 16) by Siervo *et al.*[113] showed significant and similar reductions in systolic blood pressure between red beetroot juice or inorganic nitrate (-4.5 and -4.2 mmHg, respectively) after short-term (2-15 days) supplementation. No significant changes in diastolic blood pressure were observed after red beetroot juice or inorganic nitrate supplementation.[113] The meta-analysis also showed that changes in systolic blood pressure in the two intervention groups were directly associated with the daily dose of inorganic nitrate but was not associated with plasma nitrite concentrations.[113] The authors suggested that similar effects seen on systolic

blood pressure between red beetroot juice and inorganic nitrate supplementation is due to comparable amounts of inorganic nitrate (mean nitrate: beetroot, 12.0 ± 13.2 mmol/dose; inorganic nitrate salts, 15.5 ± 9.2 mmol/dose) and minimal additive hypotensive effects of beetroots' other bioactive constituents.[113] A later meta-analysis by Ashor *et al.*[128] examined whether medium- and longer-term clinical studies (duration: 1-6 weeks) with red beetroot juice (total of 11 studies with dose/day: 70-500 mL; nitrate/dose: 5.2-10 mmol) or nitrate salts (one study with 0.15 mmol/kg body weight sodium nitrate) had similar effects on blood pressure, and contrary to Siervo *et al.* it was discovered that > 1 week supplementation with higher daily doses of inorganic nitrate did not result in greater vascular benefits, and in fact, was associated with a significantly lower effect size for both systolic and diastolic blood pressure.[128] This indicates that there may be a threshold dose of inorganic nitrate for blood pressure reduction and potentially vasodilation. It should be noted that these two meta-analyses should be interpreted with caution as the studies included were of relatively small sample size (< 60 subjects), short duration, and primarily consisted of young, healthy men. Both of these reviews[113,128] stated that inorganic nitrate appears to be less effective in reducing systolic blood pressure but remains inconclusive at this time due to the limited studies in older populations, thus demonstrating a need for further research in aging individuals.

As mentioned above, inorganic nitrate is not the only bioactive compound in red beetroot that can elicit beneficial vascular-protective health effects. Red beetroot is also a rich source of several phytochemical compounds including ascorbic acid, betalains, carotenoids, and phenolics including flavonoids (e.g., anthocyanins), and phenolic acids (e.g.,chlorogenic, caffeic, ferulic, cinnamic and *p*-coumaric acids).[129,130] These compounds and their downstream metabolites have been shown to have anti-inflammatory and antioxidant effects *in vitro,* and have also been shown to have decent bioavailability, thereby producing biological effects *in vivo.*[130] The anti-inflammatory and antioxidant effects of these phytochemicals have been shown to play a role in increasing NO bioavailability and improvements in vasodilation and blood

pressure through a variety of mechanisms at the endothelial cell level including, but not limited to, activation of eNOS to increase NO production, inhibition of NADPH oxidase to decrease superoxide production, direct scavenging of superoxide and other free radicals, increased antioxidant capacity by increasing synthesis of SOD and other oxidant enzymes, and inhibition of NFkB-signaling thereby repressing the NFkB-dependent synthesis/expression of pro-inflammatory cytokines, chemokines, and vascular cell adhesion molecules.[115,130,131]

A recent systematic review and meta-analysis by Bahadoran *et al.*[132] investigated the nitrate-dependent and -independent effects of red beetroot juice on blood pressure by only including clinical trials with beetroot juice as their intervention (total of 43; excluding nitrate salts). They observed that systolic and diastolic blood pressure were significantly lower (-3.55 mmHg and -1.32 mmHg; $P < 0.001$, respectively) in the red beetroot juice supplemented groups than in the controls. Additionally, a greater reduction in systolic blood pressure was observed in nitrate-depleted beetroot juice controls than in controls such as water, juice, or low-nitrate diets (-4.51 mmHg vs. -3.09 mmHg, $P = 0.037$).[132] It was also shown that red beetroot juice supplementation with the lowest nitrate content (<150 mg/100 mL) resulted in a larger reduction in both systolic and diastolic blood pressure than with the highest nitrate-containing beetroot supplement (≥250 mg/dL/100 mL). These findings suggest that red beetroot juice also exerts its blood pressure-lowering effects through its other bioactive constituents, and not just through inorganic nitrate.

There are a limited number of human intervention studies solely investigating the effects of red beetroot (i.e., excluding inorganic nitrate salt studies) on vascular endothelial function (**Table 2.1**). Out of the 9 studies conducted, 6 demonstrated improvements in vascular endothelial function after red beetroot juice supplementation. Specifically, two of these studies, by Webb *et al.*[122] and Kapil *et al.*[133] demonstrated that red beetroot juice acutely attenuated suppression of vascular endothelial function induced by an ischemic insult (20 min brachial artery occlusion), as evidenced by FMD responses remaining at pre-ischemic levels after

beetroot juice supplementation and compared to controls (water). This attenuated ischemia-induced endothelial dysfunction was attributed to increased plasma nitrite levels, as nitrite reduction to NO is enhanced under hypoxic conditions. Other studies showed improvements in FMD of the brachial artery after 4 weeks of daily beetroot juice consumption in hypertensive individuals[134] and in healthy older adults with slight cardiovascular risk,[135] and after in individuals with hypercholesterolemia but were otherwise healthy after 6 weeks daily beetroot juice consumption (250 mL/d; ~6 mmol/d).[136] On the other hand, two of the studies did not demonstrate improvements in FMD after an acute dose of beetroot in individuals with peripheral artery disease[137] and type 2 diabetes[138] after two weeks of daily beetroot consumption. These studies should be interpreted with caution as the doses of beetroot (70-500 mL) and inorganic nitrate content vary (5-42 mmol) among studies, as well as the study sizes, durations and patient populations (healthy vs. diseased, young vs. old), and lack "true placebos".

Only one study to date (shown in **Table 2.1**) has investigated whether red beetroot juice supplementation would attenuate postprandial impairments in vascular endothelial function induced by a Western mixed macronutrient meal in overweight, slightly obese men [139] In that study, it was shown that after consuming a mixed-macronutrient meal, FMD decreased by about 1.6% in the control group, while pre-treatment with red beetroot juice (two 70 mL concentrated beetroot juice shots) before the meal prevented any decline in FMD. The authors attributed this protective effect of beetroot on postprandial vascular endothelial dysfunction to improvements in NO bioavailability via the nitrate-nitrite-NO pathway.[139]

Very few studies have examined the effects of red beetroot juice on arterial stiffness. Studies have not shown acute improvements in aPWV, despite reductions in aortic systolic blood pressure following a single dose of red beetroot juice[140]. Reductions in aPWV have been shown following 4 weeks and 6 weeks of daily supplementation with red beetroot juice (shown in **Table 2.1**).[134,136] However, these improvements were observed with concomitant decreases in

systolic blood pressure, which may account for the lower aPWV. Further research on the effects of red beetroot juice on central arterial stiffness is needed.

In summary, it is evident that targeting the dietary nitrate-nitrite-NO pathway offers much therapeutic potential for improving vascular health due to increases in the bioavailability of NO, which occur independently of the traditional L-arginine/eNOS pathway. Red beetroot juice is a practical, affordable, easily-acceptable and promising functional food that can be used as a dietary strategy to increase NO bioavailability, and therefore prevent and manage conditions associated with decreased NO bioavailability such as vascular dysfunction. The current body of data indicate that there are both nitrate-dependent and -independent vascular health effects of beetroot. The bioactive compounds in red beetroot, especially inorganic nitrate, appear to be well-absorbed, bioavailable and have high biological activity; however, there is limited data regarding the bioavailability of betalains. Independent, additive and synergistic effects of these compounds and their downstream metabolites regulate beetroots' cardioprotective effects; however, research is limited on the relative contribution or synergistic relationship of beetroots' bioactive constituents. There is modest, albeit convincing, clinical evidence from acute and chronic (medium to long-term) supplementation studies demonstrating the blood pressure lowering effects of red beetroot juice, whereas clinical evidence for improvements in vascular endothelial function remain limited. These vascular benefits rely, at least in part, on enterosalivary circulation of nitrate and the efficiency of oral bacteria to endogenously convert nitrate to nitrite. Currently, there is not an identified optimal dose of red beetroot juice that offers the desired therapeutic effects, though, research to date has not shown any adverse side effects from varying red beetroot juice doses. Current clinical evidence does not typically include appropriate study designs that examine the nitrate-dependent and -independent effects of red beetroot juice. It also remains difficult at this time to generalize findings from current clinical beetroot intervention studies to general and patient populations, as majority of studies conducted have been in healthy men and in relatively small samples of short duration. There

are many limited and unexplored areas of clinical research in which red beetroot juice supplementation might confer benefits, such as postprandial vascular endothelial dysfunction. Additional longer-term (≥4 weeks), well-designed clinical studies are clearly warranted, and especially in individuals at risk for CVD.

Tables

Table 2.1 Summary of human intervention studies examining both acute and chronic effects of red beetroot juice on vascular endothelial function.

Reference	Study Population	Study Design & Duration	Beetroot Dose & Nitrate Content	Placebo-Control Used	Vascular Outcome & Effects	Other Findings
Webb *et al*. (2008)[122]	Healthy; n=10 (4 M/6 F); Age: 26.6 ± 7.4 Mean BMI: 21.3	Open-label, crossover Acute: took RBJ 2 hours prior to FMD testing; returned 7 days after; asked to avoid high-nitrate foods & PA 12-hr prior	500 mL; 45.0±2.6 mmol single dose	None	FMD_{BA} before & after I/R-insult. RBJ prevented I/R induced ED. RBJ did not alter pre-ischemia FMD_{BA}	Plasma nitrate peaked at 1.5 hr & remained ↑ for 6 hrs, while nitrite peaked at 3 hrs and remained ↑ for 5 hrs in RBJ Peak SBP ↓ was 2.5 hrs, & peak DBP ↓ was 3 hrs after RBJ Δ SBP was inversely related to Δ plasma nitrite
Kapil *et al.*[133] (2010)	Healthy; n=12 (M/F: not reported; age: 24.7; BMI: not reported)	Open-label, crossover, placebo-controlled RCT Acute: 3 hrs; took RBJ 90 min prior to FMD	250 mL; 5.5 mmol single dose	None	FMD_{BA} before & after I/R-insult. RBJ prevented I/R induced ED.	Separate 3-hr study with 250mL RBJ, SBP ↓, plasma nitrate peaked at 1 hr & remained ↑, plasma nitrite

		testing; asked not to alter diet & PA				peaked at 2.5 hr & no Δ in DBP
Kapil *et al.* (2014)[134]	Hypertensive; N=64 (drug-naïve: n=34; drug-treated: n=34) NF-RBJ: n=32 (10 M/22 F; age: 56.3±16.4; BMI: 26.5±4.0) RBJ: n=32 (16 M/16 F; age: 57.6±13.9, BMI: 26.8±5.0)	Parallel, double-blind, placebo-controlled RCT Chronic: 4 weeks suppl. Control of diet and PA not reported	250 mL/d; 6.4 mmol/d	250 mL/d NF-RBJ; ~0.007 mmol/d	% Δ FMD_{BA} from baseline to 4 wk within & b/w groups ↑ FMD_{BA} of 1% in RBJ after 4 wks	aPWV ↓ 0.59 m/s in RBJ compared to baseline & ↓ 0.58 m/s compared to NF-RBJ AIx ↓ 5.2% in RBJ compared with baseline & ↓ 6.1% compared to NF-RBJ SBP and DBP ↓ & plasma & saliva nitrite ↑
Gilchrest *et al.* (2013)[138]	Type 2 Diabetes; n=27 (18 M/ F 9; age: 67.2±4.9; BMI: 30.8±3.2; duration of diabetes: 13.6±8.1 years)	Crossover, double-blind, placebo-controlled RCT. Chronic: 2 wks suppl. with 4-wk washout period Asked not to alter diet & PA	250 mL/d; 7.5 mmol/d	250 mL/d NF-RBJ; ~0.002 mmol/d	% Δ FMD_{BA} from baseline to 2 wk within & b/w groups FMD_{BA} was unchanged after RBJ and compared to NF-RBJ	Plasma NOx levels ↑ in RBJ, but did not lower SBP, DBP or improve microvascular EF
Kenjale *et al.* (2011)[137]	Peripheral arterial disease; n=8 (4 M/4 F;	Crossover, open-label, RCT Acute: 2 hrs	500 mL; 18 mmol single dose	500 mL orange juice single dose (low nitrate, calorie-matched,	% Δ FMD_{BA} b/w RBJ and PBO	Plasma nitrate peaked 2 hrs and plasma nitrite peaked at

	age: 67 ± 13; BMI: 28.6 ± 5.8)	FMD testing performed ~150 min after RBJ or PBO; Testing separated by 7-14 days Instructed not to use anti-bacterial mouthwash, PPI's, & PA 12-hr prior		similar antioxidant content)	No difference in FMD_{BA} responses were seen b/w RBJ and PBO	3 hrs in RBJ, and both remained elevated. No change in NOx levels in PBO
Ashor *et al.* (2014)[114]	Overweight or obese older adults; N = 21 RBJ: n=10 (7 M/3 F; age: 62.7±4.9; BMI: 30.5±4.4) PBO: n=11 (age: 61.5±4.4; BMI: 29.4±4.2)	Parallel, open label, placebo-controlled RCT Chronic: 3 wks suppl. Immediately followed by 1 wk washout	70 mL; ~300-400 mg/d	200 mL black currant juice; <5 mg/d	Δ in microvascular EF via forearm skin post-occlusive reactive hyperemia (PORH) with laser Doppler after 3-wk RBJ and PBO suppl.	PORH index were not different b/w RBJ and PBO after 3-wks of suppl. Nor after 1-wk washout Saliva & plasma nitrate ↑ after 3-wks, while SBP & DBP were unchanged
Velmurugan *et al.* (2016)[136]	Hypercholesteremic, but healthy; N=65 RBJ: n=33 (12 M/21 F; age: 53±10; BMI: 26.8±4.9)	Parallel, double-blind, placebo-controlled RCT Acute: 3 hr (subgroup of first 34 enrolled)	250 mL/d; 6 mmol/d (taken once for acute study; taken once/d for 6 wks for chronic study)	250 mL/d NF-RBJ; ~0.001 mmol/d	% Δ FMD_{BA} from baseline to 3 hr & 6 wks within & b/w groups Acute: ↑ FMD_{BA} in RBJ at 3 hr (P=0.01) & no Δ	aPWV improved in RBJ (↓ 0.22 m/s) Oral bacteria (*Neisseria flavescens* & *Rothia mucilaginosa*) ↑

	NF-RBJ: n=34 (12 M/22 F; age:53±12; BMI: 26.7±5.1)	Chronic: 6 wks suppl. Asked to consume low-nitrate diet			in NF-RBJ *(*b/w group *P* =0.05) Chronic: FMD_{BA} ↑ 1.1% in RBJ vs. ↓ 0.3% in NF-RBJ after 6 wks (*P*<0.0001)	after RBJ but not NF-RBJ Acute & chronic ↑ plasma & saliva NOx in RBJ both
Jones *et al.* (2019)[135]	Healthy older adults; N=18 (M/F not reported; postmenopausal status reported) RBJ: n=11 (age: 65±8; BMI: 26.2±6.3) PBO: n=7 (age: 61±5; BMI:26.9±2.1)	Parallel, placebo-controlled, pilot RCT Chronic: 28 (± 7 days) suppl. FMD testing performed at baseline, 2 & 4 wks Asked not to alter diet	70 mL; 400 mg/d	Prune juice (amount not reported)	%FMD_{BA} Δ from baseline to wk 2 and 4 within & b/w groups FMD_{BA} ↑ 1.5% from baseline to 2 wks and remained ↑ till 4 wks in RBJ. No Δ in FMD_{BA} in PBO, and no significant b/w group Δ at wk 2 or wk 4.	Plasma nitrate levels were highest at wk 2, but declined at wk 4 SBP and DBP ↓ over 4-weeks with greatest reduction seen at wk 2 No changes in microvascular EF were seen.
Joris & Mensick (2013)[139]	Overweight, slightly obese ; n=20 (all men; age: 61±7; BMI: 30.1±1.9)	Crossover RCT. Acute: 3 hrs FMD testing performed at baseline and 2 hrs after meal (56.6 g fat) Asked to avoid high-fat food and PA prior	140 mL; 500 mg	140 mL isocaloric drink	FMD_{BA} pre- and post-RBJ/PBO and meal FMD_{BA} ↓ after meals w/ PBO; RBJ prevented ↓ FMD_{BA} after meal compared w/ PBO	No differences were seen w/ AIx and aPWV Plasma NOx ↑ in RBJ FMD did not correlate w/ postprandial triglycerides or glucose

Abbreviations: AIx: augmentation index; BMI: body mass index (kg/m^2); DBP: diastolic blood pressure; d:day; ED: endothelial dysfunction; EF: endothelial function; F: female; FMD_{BA}: flow-mediated dilation of the brachial artery (ultrasound based); hr: hours I/R: ischemia/reperfusion; M: male; NF-RBJ: nitrate-free red beetroot juice; NOx: nitrate and nitrite; PBO: placebo; PPIs: proton pump inhibitors; PA: physical activity; RBJ: red beetroot juice; SBP: systolic blood pressure; wks: weeks

REFERENCES

1. J., B. E. *et al.* Heart Disease and Stroke Statistics—2017 Update: A Report From the American Heart Association. *Circulation* **135**, e146–e603 (2017).
2. G., L. E. & Daniel, L. Arterial and Cardiac Aging: Major Shareholders in Cardiovascular Disease Enterprises. *Circulation* **107**, 139–146 (2003).
3. Rossman, M. J., LaRocca, T. J., Martens, C. R. & Seals, D. R. Healthy lifestyle-based approaches for successful vascular aging. *J. Appl. Physiol.* **125**, 1888–1900 (2018).
4. N., C. J., A., Q. A., K., H. N. & A., J. K. Surrogate Markers for Cardiovascular Disease. *Circulation* **109**, IV-31-IV–46 (2004).
5. Brown, A. A. & Hu, F. B. Dietary modulation of endothelial function: implications for cardiovascular disease. *Am. J. Clin. Nutr.* **73**, 673–686 (2001).
6. Pase, M. P., Grima, N. A. & Sarris, J. The effects of dietary and nutrient interventions on arterial stiffness: a systematic review. *Am. J. Clin. Nutr.* **93**, 446–454 (2010).
7. Cahill, P. A. & Redmond, E. M. Vascular endothelium – Gatekeeper of vessel health. *Atherosclerosis* **248**, 97–109 (2016).
8. Wallez, Y. & Huber, P. Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. *Biochim. Biophys. Acta BBA - Biomembr.* **1778**, 794–809 (2008).
9. Sandoo, A., van Zanten, J. J. C. S. V., Metsios, G. S., Carroll, D. & Kitas, G. D. The endothelium and its role in regulating vascular tone. *Open Cardiovasc. Med. J.* **4**, 302–312 (2010).
10. Janner, J. H., Godtfredsen, N. S., Ladelund, S., Vestbo, J. & Prescott, E. The association between aortic augmentation index and cardiovascular risk factors in a large unselected population. *J. Hum. Hypertens.* **26**, 476 (2011).

11. Seals, D. R., Jablonski, K. L. & Donato, A. J. Aging and vascular endothelial function in humans. *Clin. Sci. Lond. Engl. 1979* **120**, 357–375 (2011).

12. Widmer, R. J. & Lerman, A. Endothelial dysfunction and cardiovascular disease. *Glob. Cardiol. Sci. Pract.* **2014**, 291–308 (2014).

13. Feletou, M. *The Endothelium, Part I: Multiple Functions of the Endothelial Cells -- Focus on Endothelium-Derived Vasoactive Mediators*. (2011). doi:https://doi.org/10.4199/C00031ED1V01Y201105ISP019

14. G., F. P., E., W. S., S., P. D. & P., L. M. Caveolin, Caveolae, and Endothelial Cell Function. *Arterioscler. Thromb. Vasc. Biol.* **23**, 1161–1168 (2003).

15. Luiking, Y. C., Engelen, M. P. K. J. & Deutz, N. E. P. Regulation of nitric oxide production in health and disease. *Curr. Opin. Clin. Nutr. Metab. Care* **13**, 97–104 (2010).

16. Ulrich, F. & Thomas, M. Endothelial Nitric Oxide Synthase in Vascular Disease. *Circulation* **113**, 1708–1714 (2006).

17. Naseem, K. M. The role of nitric oxide in cardiovascular diseases. *Mol. Aspects Med.* **26**, 33–65 (2005).

18. Carreau, A., Kieda, C. & Grillon, C. Nitric oxide modulates the expression of endothelial cell adhesion molecules involved in angiogenesis and leukocyte recruitment. *Exp. Cell Res.* **317**, 29–41 (2011).

19. C., T. F. *et al.* Nitric Oxide Modulates Expression of Cell Cycle Regulatory Proteins. *Circulation* **101**, 1982–1989 (2000).

20. Thijssen, D. H. J. *et al.* Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am. J. Physiol. Heart Circ. Physiol.* **300**, H2–H12 (2011).

21. A., H. R., K., N. S., Walter, W. D. & S., R. R. Ultrasound Assessment of Flow-Mediated Dilation. *Hypertension* **55**, 1075–1085 (2010).

22. J., G. D., A., D. E., M.M., G. H., Helen, J. & H.J., T. D. Is Flow-Mediated Dilation Nitric Oxide Mediated? *Hypertension* **63**, 376–382 (2014).

23. Ras, R. T., Streppel, M. T., Draijer, R. & Zock, P. L. Flow-mediated dilation and cardiovascular risk prediction: A systematic review with meta-analysis. *Int. J. Cardiol.* **168**, 344–351 (2013).

24. Neunteufl, T. *et al.* Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. *Atherosclerosis* **129**, 111–118 (1997).

25. Donald, A. E. *et al.* Methodological Approaches to Optimize Reproducibility and Power in Clinical Studies of Flow-Mediated Dilation. *J. Am. Coll. Cardiol.* **51**, 1959–1964 (2008).

26. Hijmering, M. L. *et al.* Variability of flow mediated dilation: consequences for clinical application. *Atherosclerosis* **157**, 369–373 (2001).

27. Johnson, S. A., Litwin, N. S. & Seals, D. R. Age-Related Vascular Dysfunction: What Registered Dietitian Nutritionists Need to Know. *J. Acad. Nutr. Diet.* (2019). doi:https://doi.org/10.1016/j.jand.2019.03.016

28. Kuvin, J. T. *et al.* Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. *Am. Heart J.* **146**, 168–174 (2003).

29. Hedetoft, M. & Olsen, N. V. Evaluation of endothelial function by peripheral arterial tonometry and relation with the nitric oxide pathway. *Nitric Oxide* **42**, 1–8 (2014).

30. Bruno, R. M., Gori, T. & Ghiadoni, L. Endothelial function testing and cardiovascular disease: focus on peripheral arterial tonometry. *Vasc. Health Risk Manag.* **10**, 577–584 (2014).

31. Bonetti, P. O. *et al.* Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. *J. Am. Coll. Cardiol.* **44**, 2137–2141 (2004).

32. Dhindsa, M. *et al.* Interrelationships among noninvasive measures of postischemic macro- and microvascular reactivity. *J. Appl. Physiol.* **105**, 427–432 (2008).

33. Onkelinx, S. *et al.* Reproducibility of different methods to measure the endothelial function. *Vasc. Med.* **17**, 79–84 (2012).

34. Schnabel, R. B. *et al.* Noninvasive vascular function measurement in the community: cross-sectional relations and comparison of methods. *Circ. Cardiovasc. Imaging* **4**, 371–380 (2011).

35. Wilk, G. *et al.* Endothelial function assessment in atherosclerosis: comparison of brachial artery flow-mediated vasodilation and peripheral arterial tonometry. *Pol. Arch. Intern. Med.* **123**, 443–452 (2013).

36. Hamburg, N. M. *et al.* Cross-sectional relations of digital vascular function to cardiovascular risk factors in the Framingham Heart Study. Circulation 2008, 117: 2467–2474. *IX Bibliogr.*

37. Rubinshtein, R. *et al.* Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. *Eur. Heart J.* **31**, 1142–1148 (2010).

38. Matsuzawa, Y., Kwon, T.-G., Lennon, R. J., Lerman, L. O. & Lerman, A. Prognostic Value of Flow-Mediated Vasodilation in Brachial Artery and Fingertip Artery for Cardiovascular Events: A Systematic Review and Meta-Analysis. *J. Am. Heart Assoc.* **4**, e002270 (2015).

39. Nohria, A. *et al.* Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *J. Appl. Physiol.* **101**, 545–548 (2006).

40. Coffman, J. D. Effects of endothelium-derived nitric oxide on skin and digital blood flow in humans. *Am. J. Physiol.-Heart Circ. Physiol.* **267**, H2087–H2090 (1994).

41. Mikael, L. de R. *et al.* Vascular Aging and Arterial Stiffness. *Arq. Bras. Cardiol.* **109**, 253–258 (2017).

42. Lyle, A. N. & Raaz, U. Killing Me Unsoftly: Causes and Mechanisms of Arterial Stiffness. *Arterioscler. Thromb. Vasc. Biol.* **37**, e1–e11 (2017).

43. Quinn, U., Tomlinson, L. A. & Cockcroft, J. R. Arterial stiffness. *JRSM Cardiovasc. Dis.* **1**, cvd.2012.012024 (2012).

44. O'Rourke, M. F. & Hashimoto, J. Mechanical Factors in Arterial Aging: A Clinical Perspective. *J. Am. Coll. Cardiol.* **50**, 1–13 (2007).

45. Shirwany, N. A. & Zou, M. Arterial stiffness: a brief review. *Acta Pharmacol. Sin.* **31**, 1267–1276 (2010).

46. J., Z. S., Vojtech, M. & A., K. D. Mechanisms, Pathophysiology, and Therapy of Arterial Stiffness. *Arterioscler. Thromb. Vasc. Biol.* **25**, 932–943 (2005).

47. Manicone, A. M. & McGuire, J. K. Matrix metalloproteinases as modulators of inflammation. *Semin. Cell Dev. Biol.* **19**, 34–41 (2008).

48. Shirai, T., Hilhorst, M., Harrison, D. G., Goronzy, J. J. & Weyand, C. M. Macrophages in vascular inflammation--From atherosclerosis to vasculitis. *Autoimmunity* **48**, 139–151 (2015).

49. S., G. Z. & J., K. J. Matrix Metalloproteinases in Vascular Remodeling and Atherogenesis. *Circ. Res.* **90**, 251–262 (2002).

50. Zhong, Q. *et al.* Carotid–Femoral Pulse Wave Velocity in the Prediction of Cardiovascular Events and Mortality: An Updated Systematic Review and Meta-Analysis. *Angiology* **69**, 617–629 (2017).

51. Butlin, M. & Qasem, A. Large Artery Stiffness Assessment Using SphygmoCor Technology. *Pulse Basel Switz.* **4**, 180–192 (2017).

52. Nürnberger, J. *et al.* Augmentation index is associated with cardiovascular risk. *J. Hypertens.* **20**, 2407–2414 (2002).

53. Vlachopoulos, C. *et al.* Prediction of cardiovascular events and all-cause mortality with central haemodynamics: a systematic review and meta-analysis. *Eur. Heart J.* **31**, 1865–1871 (2010).

54. Wilkinson, I. B. *et al.* The influence of heart rate on augmentation index and central arterial pressure in humans. *J. Physiol.* **525 Pt 1**, 263–270 (2000).

55. Stoner, L. *et al.* Should the Augmentation Index be Normalized to Heart Rate? *J. Atheroscler. Thromb.* **21**, 11–16 (2014).

56. Junqueira, V. B. C. *et al.* Aging and oxidative stress. *Mol. Aspects Med.* **25**, 5–16 (2004).

57. Liguori, I. *et al.* Oxidative stress, aging, and diseases. *Clin. Interv. Aging* **13**, 757–772 (2018).

58. J., D. A., R., M. D. & A., L. L. Mechanisms of Dysfunction in the Aging Vasculature and Role in Age-Related Disease. *Circ. Res.* **123**, 825–848 (2018).

59. Hua, C. & G., H. D. Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. *Circ. Res.* **87**, 840–844 (2000).

60. Higashi, Y., Noma, K., Yoshizumi, M. & Kihara, Y. Endothelial Function and Oxidative Stress in Cardiovascular Diseases. *Circ. J.* **73**, 411–418 (2009).

61. Pacher, P., Beckman, J. S. & Liaudet, L. Nitric oxide and peroxynitrite in health and disease. *Physiol. Rev.* **87**, 315–424 (2007).

62. Durrant, J. R. *et al.* Voluntary wheel running restores endothelial function in conduit arteries of old mice: direct evidence for reduced oxidative stress, increased superoxide dismutase activity and down-regulation of NADPH oxidase. *J. Physiol.* **587**, 3271–3285 (2009).

63. Donato, A. J. *et al.* Cytochrome P-450 2C9 signaling does not contribute to age-associated vascular endothelial dysfunction in humans. *J. Appl. Physiol. Bethesda Md 1985* **105**, 1359–1363 (2008).

64. Eskurza, I., Kahn, Z. D. & Seals, D. R. Xanthine oxidase does not contribute to impaired peripheral conduit artery endothelium-dependent dilatation with ageing. *J. Physiol.* **571**, 661–668 (2006).

65. Eskurza, I., Myerburgh, L. A., Kahn, Z. D. & Seals, D. R. Tetrahydrobiopterin augments endothelium-dependent dilatation in sedentary but not in habitually exercising older adults. *J. Physiol.* **568**, 1057–1065 (2005).

66. Higashi, Y. *et al.* Tetrahydrobiopterin enhances forearm vascular response to acetylcholine in both normotensive and hypertensive individuals*. *Am. J. Hypertens.* **15**, 326–332 (2002).

67. Heitzer, T. *et al.* Tetrahydrobiopterin improves endothelium-dependent vasodilation in chronic smokers: evidence for a dysfunctional nitric oxide synthase. *Circ. Res.* **86**, e36–e41 (2000).

68. Setoguchi, S., Mohri, M., Shimokawa, H. & Takeshita, A. Tetrahydrobiopterin improves endothelial dysfunction in coronary microcirculation in patients without epicardial coronary artery disease. *J. Am. Coll. Cardiol.* **38**, 493–498 (2001).

69. Setoguchi, S., Hirooka, Y., Eshima, K., Shimokawa, H. & Takeshita, A. Tetrahydrobiopterin improves impaired endothelium-dependent forearm vasodilation in patients with heart failure. *J. Cardiovasc. Pharmacol.* **39**, 363–368 (2002).

70. Fukai, T. & Ushio-Fukai, M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid. Redox Signal.* **15**, 1583–1606 (2011).

71. Sindler, A. L. *et al.* Nitrite supplementation reverses vascular endothelial dysfunction and large elastic artery stiffness with aging. *Aging Cell* **10**, 429–437 (2011).

72. Fleenor, B. S. *et al.* Curcumin ameliorates arterial dysfunction and oxidative stress with aging. *Exp. Gerontol.* **48**, 269–276 (2013).

73. Rippe, C. *et al.* Short-term calorie restriction reverses vascular endothelial dysfunction in old mice by increasing nitric oxide and reducing oxidative stress. *Aging Cell* **9**, 304–312 (2010).

74. Drummond, G. R., Cai, H., Davis, M. E., Ramasamy, S. & Harrison, D. G. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ. Res.* **86**, 347–354 (2000).

75. Cai, H. Hydrogen peroxide regulation of endothelial function: Origins, mechanisms, and consequences. *Cardiovasc. Res.* **68**, 26–36 (2005).

76. Satoh, K., Godo, S., Saito, H., Enkhjargal, B. & Shimokawa, H. Dual roles of vascular-derived reactive oxygen species—With a special reference to hydrogen peroxide and cyclophilin A—. *J. Mol. Cell. Cardiol.* **73**, 50–56 (2014).

77. Donato, A. J. *et al.* Life-long caloric restriction reduces oxidative stress and preserves nitric oxide bioavailability and function in arteries of old mice. *Aging Cell* **12**, 772–783 (2013).

78. L., M. K., M., G. K., E., P. A. & R., S. D. Ascorbic Acid Selectively Improves Large Elastic Artery Compliance in Postmenopausal Women. *Hypertension* **45**, 1107–1112 (2005).

79. Fleenor, B. S., Seals, D. R., Zigler, M. L. & Sindler, A. L. Superoxide-lowering therapy with TEMPOL reverses arterial dysfunction with aging in mice. *Aging Cell* **11**, 269–276 (2012).

80. Fleenor, B. S. *et al.* Sodium nitrite de-stiffening of large elastic arteries with aging: role of normalization of advanced glycation end-products. *Exp. Gerontol.* **47**, 588–594 (2012).

81. Delles, C. *et al.* Vascular stiffness is related to superoxide generation in the vessel wall. *J. Hypertens.* **26**, (2008).

82. Sanada, F. *et al.* Source of Chronic Inflammation in Aging. *Front. Cardiovasc. Med.* **5**, 12 (2018).

83. Lieb, W. *et al.* Multimarker approach to evaluate correlates of vascular stiffness: the Framingham Heart Study. *Circulation* **119**, 37–43 (2009).

84. Duprez, D. A. *et al.* Relationship between C-reactive protein and arterial stiffness in an asymptomatic population. *J. Hum. Hypertens.* **19**, 515 (2005).

85. A., V. J. *et al.* Brachial Artery Vasodilator Function and Systemic Inflammation in the Framingham Offspring Study. *Circulation* **110**, 3604–3609 (2004).

86. Morgan, M. J. & Liu, Z. Crosstalk of reactive oxygen species and NF-κB signaling. *Cell Res.* **21**, 103–115 (2011).

87. Karin, M. & Delhase, M. The IκB kinase (IKK) and NF-κB: key elements of proinflammatory signalling. in *Seminars in immunology* **12**, 85–98 (Elsevier, 2000).

88. Pueyo, M. E. *et al.* Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-κB activation induced by intracellular oxidative stress. *Arterioscler. Thromb. Vasc. Biol.* **20**, 645–651 (2000).

89. Brand, K. *et al.* Dysregulation of monocytic nuclear factor-κB by oxidized low-density lipoprotein. *Arterioscler. Thromb. Vasc. Biol.* **17**, 1901–1909 (1997).

90. Morigi, M. *et al.* Leukocyte-endothelial interaction is augmented by high glucose concentrations and hyperglycemia in a NF-κB-dependent fashion. *J. Clin. Invest.* **101**, 1905–1915 (1998).

91. Mohan, S., Mohan, N. & Sprague, E. A. Differential activation of NF-kappa B in human aortic endothelial cells conditioned to specific flow environments. *Am. J. Physiol.-Cell Physiol.* **273**, C572–C578 (1997).

92. Partridge, J. *et al.* Laminar shear stress acts as a switch to regulate divergent functions of NF-κB in endothelial cells. *FASEB J.* **21**, 3553–3561 (2007).

93. Liu, T., Zhang, L., Joo, D. & Sun, S.-C. NF-κB signaling in inflammation. *Signal Transduct. Target. Ther.* **2**, 17023 (2017).

94. P.J., de W. M., Edwin, K., Georg, K. & H., H. M. Nuclear Factor κB Signaling in Atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **25**, 904–914 (2005).

95. Donato, A. J., Black, A. D., Jablonski, K. L., Gano, L. B. & Seals, D. R. Aging is associated with greater nuclear NFκB, reduced IκBα, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. *Aging Cell* **7**, 805–812 (2008).

96. Donato, A. J. *et al.* Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-κB. *Circ. Res.* **100**, 1659–1666 (2007).

97. E., S. A. *et al.* Overweight and Obese Humans Demonstrate Increased Vascular Endothelial NAD(P)H Oxidase-p47phox Expression and Evidence of Endothelial Oxidative Stress. *Circulation* **115**, 627–637 (2007).

98. Pierce, G. L., Lesniewski, L. A., Lawson, B. R., Beske, S. D. & Seals, D. R. Nuclear factor-{kappa}B activation contributes to vascular endothelial dysfunction via oxidative stress in overweight/obese middle-aged and older humans. *Circulation* **119**, 1284–1292 (2009).
99. Jablonski, K. L. *et al.* Reduced large elastic artery stiffness with regular aerobic exercise in middle-aged and older adults: potential role of suppressed nuclear factor κ B signalling. *J. Hypertens.* **33**, 2477–2482 (2015).
100. Moreau, K. L., Deane, K. D., Meditz, A. L. & Kohrt, W. M. Tumor necrosis factor-α inhibition improves endothelial function and decreases arterial stiffness in estrogen-deficient postmenopausal women. *Atherosclerosis* **230**, 390–396 (2013).
101. Mah, E. & Bruno, R. S. Postprandial hyperglycemia on vascular endothelial function: mechanisms and consequences. *Nutr. Res.* **32**, 727–740 (2012).
102. Wallace, J. P., Johnson, B., Padilla, J. & Mather, K. Postprandial lipaemia, oxidative stress and endothelial function: a review. *Int. J. Clin. Pract.* **64**, 389–403 (2010).
103. Lacroix, S., Des Rosiers, C., Tardif, J.-C. & Nigam, A. The role of oxidative stress in postprandial endothelial dysfunction. *Nutr. Res. Rev.* **25**, 288–301 (2012).
104. TUSHUIZEN, M. E. *et al.* Two consecutive high-fat meals affect endothelial-dependent vasodilation, oxidative stress and cellular microparticles in healthy men. *J. Thromb. Haemost.* **4**, 1003–1010 (2006).
105. Vogel, R. A., Corretti, M. C. & Plotnick, G. D. Effect of a Single High-Fat Meal on Endothelial Function in Healthy Subjects. *Am. J. Cardiol.* **79**, 350–354 (1997).
106. Antonio, C. *et al.* Evidence for an Independent and Cumulative Effect of Postprandial Hypertriglyceridemia and Hyperglycemia on Endothelial Dysfunction and Oxidative Stress Generation. *Circulation* **106**, 1211–1218 (2002).
107. Torimoto, K., Okada, Y., Mori, H. & Tanaka, Y. Relationship between fluctuations in glucose levels measured by continuous glucose monitoring and vascular endothelial dysfunction in type 2 diabetes mellitus. *Cardiovasc. Diabetol.* **12**, 1 (2013).

108. Kawano, H. *et al.* Hyperglycemia rapidly suppresses flow-mediated endothelium-dependent vasodilation of brachial artery. *J. Am. Coll. Cardiol.* **34**, 146–154 (1999).

109. Bae, J.-H. *et al.* Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. *Atherosclerosis* **155**, 517–523 (2001).

110. Anderson, R. A. *et al.* The relationships between post-prandial lipaemia, endothelial function and oxidative stress in healthy individuals and patients with type 2 diabetes. *Atherosclerosis* **154**, 475–483 (2001).

111. Patel, C. *et al.* Prolonged Reactive Oxygen Species Generation and Nuclear Factor-κB Activation after a High-Fat, High-Carbohydrate Meal in the Obese. *J. Clin. Endocrinol. Metab.* **92**, 4476–4479 (2007).

112. O'Keefe, J. H. & Bell, D. S. H. Postprandial Hyperglycemia/Hyperlipidemia (Postprandial Dysmetabolism) Is a Cardiovascular Risk Factor. *Am. J. Cardiol.* **100**, 899–904 (2007).

113. Siervo, M., Lara, J., Ogbonmwan, I. & Mathers, J. C. Inorganic Nitrate and Beetroot Juice Supplementation Reduces Blood Pressure in Adults: A Systematic Review and Meta-Analysis. *J. Nutr.* **143**, 818–826 (2013).

114. Lara, J. *et al.* Effects of inorganic nitrate and beetroot supplementation on endothelial function: a systematic review and meta-analysis. *Eur. J. Nutr.* **55**, 451–459 (2016).

115. Hobbs, D. A., George, T. W. & Lovegrove, J. A. The effects of dietary nitrate on blood pressure and endothelial function: a review of human intervention studies. *Nutr. Res. Rev.* **26**, 210–222 (2013).

116. Kapil, V., Weitzberg, E., Lundberg, J. O. & Ahluwalia, A. Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health. *Nitric Oxide* **38**, 45–57 (2014).

117. Lundberg, J. O. & Govoni, M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic. Biol. Med.* **37**, 395–400 (2004).

118. Lundberg, J. O., Weitzberg, E. & Gladwin, M. T. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* **7**, 156 (2008).

119. Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M. & Allaker, R. P. Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur. J. Oral Sci.* **113**, 14–19 (2005).

120. Hyde, E. R. *et al.* Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis. *PloS One* **9**, e88645–e88645 (2014).

121. Lidder, S. & Webb, A. J. Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway. *Br. J. Clin. Pharmacol.* **75**, 677–696 (2013).

122. J., W. A. *et al.* Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate via Bioconversion to Nitrite. *Hypertension* **51**, 784–790 (2008).

123. Govoni, M., Jansson, E. Å., Weitzberg, E. & Lundberg, J. O. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* **19**, 333–337 (2008).

124. Woessner, M. *et al.* A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load. *Nitric Oxide* **54**, 1–7 (2016).

125. Kapil, V. *et al.* Physiological role for nitrate-reducing oral bacteria in blood pressure control. *Free Radic. Biol. Med.* **55**, 93–100 (2013).

126. Bondonno, C. P. *et al.* Antibacterial Mouthwash Blunts Oral Nitrate Reduction and Increases Blood Pressure in Treated Hypertensive Men and Women. *Am. J. Hypertens.* **28**, 572–575 (2014).

127. Kapil, V. *et al.* Sex differences in the nitrate-nitrite-NO• pathway: Role of oral nitrate-reducing bacteria. *Free Radic. Biol. Med.* **126**, 113–121 (2018).

128. Ashor, A. W., Lara, J. & Siervo, M. Medium-term effects of dietary nitrate supplementation on systolic and diastolic blood pressure in adults: a systematic review and meta-analysis. *J. Hypertens.* **35**, 1353–1359 (2017).

129. Chhikara, N., Kushwaha, K., Sharma, P., Gat, Y. & Panghal, A. Bioactive compounds of beetroot and utilization in food processing industry: A critical review. *Food Chem.* **272**, 192–200 (2019).

130. Clifford, T., Howatson, G., West, D. J. & Stevenson, E. J. The potential benefits of red beetroot supplementation in health and disease. *Nutrients* **7**, 2801–2822 (2015).

131. Maaliki, D., Shaito, A. A., Pintus, G., El-Yazbi, A. & Eid, A. H. Flavonoids in hypertension: a brief review of the underlying mechanisms. *Curr. Opin. Pharmacol.* **45**, 57–65 (2019).

132. Bahadoran, Z., Mirmiran, P., Kabir, A., Azizi, F. & Ghasemi, A. The Nitrate-Independent Blood Pressure–Lowering Effect of Beetroot Juice: A Systematic Review and Meta-Analysis. *Adv. Nutr.* **8**, 830–838 (2017).

133. Kapil, V. *et al.* Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension* **56**, 274–281 (2010).

134. Vikas, K., S., K. R., Amy, R., J., C. M. & Amrita, A. Dietary Nitrate Provides Sustained Blood Pressure Lowering in Hypertensive Patients. *Hypertension* **65**, 320–327 (2015).

135. Jones, T. *et al.* The Effects of Beetroot Juice on Blood Pressure, Microvascular Function and Large-Vessel Endothelial Function: A Randomized, Double-Blind, Placebo-Controlled Pilot Study in Healthy Older Adults. *Nutrients* **11**, 1792 (2019).

136. Velmurugan, S. *et al.* Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study. *Am. J. Clin. Nutr.* **103**, 25–38 (2015).

137. Kenjale, A. A. *et al.* Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *J. Appl. Physiol. Bethesda Md 1985* **110**, 1582–1591 (2011).

138. Gilchrist, M. *et al.* Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. *Free Radic. Biol. Med.* **60**, 89–97 (2013).

139. Joris, P. J. & Mensink, R. P. Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. *Atherosclerosis* **231**, 78–83 (2013).

140. Kukadia, S. *et al.* A Double-Blind Placebo-Controlled Crossover Study of the Effect of Beetroot Juice Containing Dietary Nitrate on Aortic and Brachial Blood Pressure Over 24 h . *Frontiers in Physiology* **10**, 47 (2019).

141. Strijdom, H., Chamane, N. & Lochner, A. Nitric oxide in the cardiovascular system: a simple molecule with complex actions. *Cardiovasc. J. Afr.* **20**, 303–310 (2009).

142. Ayer JG, Harmer JA, Steinbeck K, Celermajer DS. Postprandial vascular reactivity in obese and normal weight young adults. Obesity (Silver Spring) 2010;18(5):945-51. doi: 10.1038/oby.2009.331.

143. Larsen et al., Effects of dietary nitrate on blood pressure in healthy volunteers. N Engl J Med 2006; 355:2792-2793

144. Cani PD, Amar J, Iglesias MA, *et a*.. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes . 2007;56:1761–1772.

145. Erridge C, Attina T, Spickett CM, Webb DJ. A high-fat meal induces low-grade endotoxemia: Evidence of a novel mechanism of postprandial inflammation. Am J Clin Nutr . 2007;86:1286–1292.

146. Laugerette F, Vors C, Geloen A, *et al*. Emulsified lipids increase endotoxemia: Possible role in early postprandial low-grade inflammation. J Nutr Biochem . 2011;22:53–59.

147. Ghanim H, Abuaysheh S, Sia CL, *et al*. Increase in plasma endotoxin concentrations and the expression of Toll-like receptors and suppressor of cytokine signaling-3 in mononuclear cells after a high-fat, high-carbohydrate meal: Implications for insulin resistance. Diabetes Care . 2009;32:2281–2287.

148. Cécile Vors, Gaëlle Pineau, *et al*. Postprandial Endotoxemia Linked With Chylomicrons and Lipopolysaccharides Handling in Obese Versus Lean Men: A Lipid Dose-Effect Trial, The Journal of Clinical Endocrinology & Metabolism. 2015; (100)9:3427–3435.

149. Melania Manco, Lorenza Putignani, Gian Franco Bottazzo. Gut Microbiota, Lipopolysaccharides, and Innate Immunity in the Pathogenesis of Obesity and Cardiovascular Risk, *Endocrine Reviews*, 2010;(31)6:817–844,

CHAPTER 3: IMPACT OF RED BEETROOT JUICE ON VASCULAR ENDOTHELIAL FUNCTION AND CARDIOMETABOLIC RESPONSES TO A HIGH-FAT MEAL IN MIDDLE-AGED/OLDER ADULTS WITH OVERWEIGHT AND OBESITY

Summary

Background: Previous research suggests that high-fat meal (HFM) consumption may induce transient postprandial atherogenic responses, including significant impairments in vascular endothelial function, in individuals with overweight and obesity. Red beetroot juice (RBJ) may modulate vascular endothelial function and other measures of cardiometabolic health, though clinical effects have been equivocal. *Objective:* This study investigated the impact of acute and chronic RBJ consumption, including nitrate–dependent and –independent effects, on postprandial vascular endothelial function and other cardiometabolic responses to a HFM. *Design:* Fifteen men and postmenopausal women (mean ± SEM, range age: 53 ± 2, 42-65 years, 7 men and 8 women) with overweight and obesity (mean ± SEM, range BMI in kg/m^2: 29.8 ± 0.9, 26.2–36.4) were enrolled in this randomized, double-blind, placebo-controlled, 4-period, crossover clinical trial. Following an overnight fast, participants underwent baseline assessment of vascular endothelial function (reactive hyperemia index, RHI), pulse wave analysis, blood pressure, and biological sample collection. In random order, participants consumed one of the following 70 mL treatments (acute visit), 1) RBJ, 2) nitrate-free RBJ (NF-RBJ), 3) placebo + nitrate (PBO+NIT), or 4) placebo (PBO), followed immediately by a HFM. RHI was measured again 4 hours post-HFM, and hemodynamic assessment and biological sample collection were performed 1, 2, and 4 hours post-HFM consumption. Participants then consumed treatments daily for 4 wks (chronic visit), and all assessments were repeated before and after the HFM but without consuming treatments. *Results:* HFM consumption did not lead to significant impairments in postprandial endothelial function, assessed as RHI, in the PBO group.

No significant differences in the primary outcome, RHI, were detected across treatment groups following acute and chronic exposure, despite significant increases in circulating nitrate/nitrite (NOx) levels in the RBJ and PBO+NIT group compared to PBO and NF-RBJ ($P < 0.0001$ for all time points at the acute visit; $P < 0.05$ for all time points at the chronic visit). Although the HFM led to significant alterations in several of the secondary outcomes, there were no consistent effects of the treatments on postprandial cardiometabolic responses to a HFM. *Conclusions:* The results of this study suggest that consumption of a HFM did not significantly impair postprandial vascular endothelial function in this population. In addition, acute and chronic RBJ exposure did not significantly alter postprandial vascular endothelial function or other outcomes assessed despite significantly increasing plasma and saliva NOx concentrations.

Introduction

Advancing age is the primary risk factor for atherosclerotic cardiovascular disease (CVD), largely due to adverse effects on the arteries [1, 2] including vascular endothelial dysfunction, which is characterized by impaired endothelium-dependent vasodilation. Nitric oxide (NO) is critical to cardiovascular health as it promotes vasodilation, blood flow, anti-thrombotic and anti-inflammatory effects. A central driver of vascular endothelial dysfunction is reduced NO bioavailability secondary to oxidative stress.[3] Inflammation also promotes vascular endothelial dysfunction through a bidirectional relationship with oxidative stress.[2,3] The progression of atherosclerosis is characterized by chronic oxidative stress, activation of pro-inflammatory pathways, and recruitment and adhesion of immune cells to the endothelium.[4] Previous research suggests that consumption of a single high-fat meal (HFM) may induce transient postprandial atherogenic responses including impairments in vascular endothelial function, hypertriglyceridemia, hyperglycemia, inflammation and oxidative stress[5-10] that are exacerbated in individuals with overweight and obesity.[8] Indeed, even mildly elevated postprandial glucose and triglyceride levels have been linked to the development of

atherosclerosis and other CVDs in the general population.[5,11] As such, repeated HFM consumption may accelerate atherogenesis in aging individuals with overweight and obesity, and dietary interventions that prevent or attenuate these responses may contribute to the preservation of cardiovascular health.

Consumption of red beetroot juice (RBJ) has emerged as a potential therapeutic approach for reducing CVD risk. Research has demonstrated antioxidant[12-14], anti-inflammatory[15,16], antihypertensive[17-19], and cardiometabolic-protective[17, 19-22] effects of RBJ and its bioactive components in animals and humans, though results have been equivocal. Cardiometabolic-protective effects of RBJ have been primarily attributed to RBJs' high inorganic nitrate content, as inorganic nitrate is reduced via the enterosalivary nitrate-nitrite-NO pathway to NO in an endothelium-independent manner.[17,23] Hence, dietary inorganic nitrate may be an effective approach for improving vascular health in individuals or situations in which vascular endothelial dysfunction is present. Underappreciated is the fact that RBJ is also rich in other bioactive compounds including flavonoids, betalains, carotenoids, and ascorbic acid, which also have antioxidant, anti-inflammatory, and cardiometabolic-protective effects.[24] In fact, a previous meta-analysis observed similar blood pressure outcomes when comparing nitrate-rich RBJ with nitrate-depleted RBJ, suggesting that RBJ may have nitrate-independent effects as well.[25] In addition, polyphenols and ascorbic acid can enhance the reduction of nitrate to nitrite and to NO.[26,27] The purpose of this randomized, double-blind, placebo-controlled, 4-period crossover clinical trial was to investigate the impact of both acute and chronic RBJ consumption on vascular endothelial function and other cardiometabolic responses to HFM consumption. This study also aimed to investigate underlying mechanisms contributing to clinical responses, including nitrate–dependent and –independent effects of RBJ. To achieve the latter, we used 1) a placebo (PBO) concentrate devoid of nitrate or polyphenols, 2) RBJ concentrate, 2) nitrate-depleted RBJ concentrate, and 4) a PBO concentrate with an equivalent dose of nitrate to that

of the RBJ. To our knowledge, this is the first clinical trial designed to isolate the effects of inorganic nitrate in RBJ on cardiometabolic health.

Methods

Study Population

Men and postmenopausal women (≥ 1 y absence of menses) 40 to 65 y of age and with a BMI (kg/m^2) between 25 and 39.9 were recruited to participate in this trial. Exclusion criteria included taking nitrate, anti-hypertensive, lipid-lowering, acid reflux, hypoglycemic, phosphodiesterase 5 inhibitor, or hormone replacement medications, triglyceride levels ≥ 250 mg/dL, hemoglobin A1c ≥ 6.5%, diagnosed hypertension or a blood pressure greater than 139/89 mmHg, CVD, diabetes, cancer, kidney, liver, or pancreatic disease, participating in a weight loss program or actively trying to lose weight, smokers, heavy drinkers (> 3 drinks on any given occasion and/or > 7 drinks/wk for women, or > 4 drinks on any given occasion and/or > 14 drinks/wk for men), allergy to test meals or treatments, or consuming > 2 servings RBJ/wk.

Participant Recruitment

Participants were recruited from the greater Fort Collins, Colorado area through advertisements in local newspapers, Colorado State University webpages and email, flyer distribution, direct mailers, and clinicaltrials.gov between November 2016 and December 2017. Individuals sent an email or called to indicate interest and were then asked a series of questions regarding their health history to determine eligibility through a phone prescreening. Qualified individuals were invited for an onsite screening visit where they provided written informed consent, and inclusion and exclusion criteria were confirmed. Specifically, a detailed health history was obtained from the participant, followed by seated rest in a quiet room for 10 min prior to blood pressure assessment. Seated brachial blood pressure was measured in triplicate, with each measurement separated by one min, using an automatic device (Omron Healthcare,

Inc.). A finger stick blood draw was performed to assess lipid profiles (Alere Cholestech LDX® Analyzer, Abbott) and hemoglobin A1c (Alere Afinion Analyzer System, Abbott). Anthropometric measurements (i.e. height, weight, and waist and hip circumferences) were performed.

A detailed schematic of participant recruitment and enrollment for the study is provided in **Figure 3.1**. A total of 240 individuals responded to advertisements, 37 of which met inclusion criteria through the phone prescreening and completed the onsite screening visit. Of those, 23 met inclusion criteria, agreed to partake in the study, and were randomly assigned. Eight participants withdrew or were excluded from the study, and therefore data are reported for 15 participants who completed all protocol-specified procedures. This trial was conducted in accordance with the Declaration of Helsinki, was approved by the Colorado State University Institutional Review Board (16-6495HH) and is registered at clinicaltrials.gov as NCT02949115.

Study Design and Interventions

This was a randomized, double-blind, placebo-controlled, 4-period crossover trial in which participants completed two postprandial challenges during each treatment period, where each testing period lasted ~5-6 hours (times varied for intravenous (IV) catheter placement and blood sample collection). Overall study design, schedule of study visits, and a schematic of the test day timeline for data collection and measurements are presented in **Figure 3.2**. Study treatments included 70 mL of: 1) RBJ; 2) nitrate-free RBJ concentrate (NF-RBJ); 3) PBO concentrate; and 4) PBO concentrate + potassium nitrate (PBO+NIT). Nutrient compositions of the treatments are presented in **Supplemental Table 3S.1** in **Appendix 1**. The PBO was devoid of polyphenols and nitrate, had similar degrees of sweetness, flavor and color to the RBJ. Prepackaged RBJ and NF-RBJ concentrates were purchased from James White Drinks, Ltd., pharmaceutical grade potassium nitrate was purchased from Spectrum Pharmacy Products, and PBO powder was purchased from Flavor Dynamics, Inc. PBO and PBO+NIT concentrates were prepared, packaged, and labelled by staff in the Kendall Reagan Nutrition

Center in the Department of Food Science and Human Nutrition at Colorado State University. All treatments were packaged in individual bottles with the same packaging (from James White Drinks, Ltd.) to ensure blinding. Randomization permutations of treatments were created using the second generator at Randomization.com (www.randomization.com). Participants were assigned to randomization sequences in order of qualification and enrollment into the study. Bottles were labeled with participant ID and sequentially numbered based on randomization sequences.

The two postprandial challenges occurred on the first and last day of each treatment period, with 4 weeks of daily treatment consumption in between. Each treatment period was separated by a 4-week washout. During the first test day of each treatment period (acute test visit), preprandial assessments were performed followed by consumption of respective treatments 10 min prior to consuming a HFM to assess the acute impact of treatments on postprandial responses. The HFM was a breakfast meal consisting of one bagel, one tablespoon of butter, two tablespoons of cream cheese, one tablespoon of apple or peach jelly, two boiled eggs, and one cup of whole milk. Nutrient composition of the test meal is presented in **Supplemental Table 3S.2** in **Appendix 1.** Following the first test day of each treatment period, participants consumed respective treatments daily until returning to the clinical research facility 4 weeks later for a follow-up test visit (chronic test visit). During the follow-up test visit, preprandial assessments were performed and subjects did not consume their respective treatment prior to consuming a HFM in order to assess the chronic effects of the treatment on postprandial responses.

Enrolled participants were provided a 2-wk supply of treatments at a time and asked to consume one 70 mL bottle in the morning daily for 4 weeks. Treatment compliance was assessed by asking study participants to 1) return empty and/or unused treatment bottles at their next visit, and 2) record the date and time their treatment was consumed each day, and to document missing doses and reason for missing the dose (e.g. sick, fell asleep, forgot) in a daily

dosing diary. Non-compliance was defined as missing ≥ 1 dose per wk. Participants agreed to adhere to their usual dietary habits, to avoid use of antibacterial mouthwash, and to maintain their physical activity level during the course of the study. They also agreed to abstain from caffeine, alcohol, prescription and over-the-counter medications, dietary supplements, brushing their teeth during the 12 hours before the start of all test visits, and to avoid intense physical activity for 24 hours prior to their test visit. All vascular and hemodynamic measurements were performed in a quiet, dimly-lit, temperature-controlled room (20-25° C).

The primary outcome measure for this trial was reactive hyperemia index (RHI), a validated measure of microvascular endothelial function that is predictive of atherosclerosis and future cardiovascular events.[28-30] Secondary outcome measures included arterial stiffness (augmentation index [AIx] and AIx@75), hemodynamics (brachial and aortic systolic blood pressure, diastolic blood pressure, pulse pressure, heart rate, mean arterial pressure, and augmented pressure), biochemical markers of cardiovascular health, metabolism, inflammation, oxidative stress, and endoplasmic reticulum (ER) stress (blood triglycerides, glucose, insulin, and plasma and saliva nitrate/nitrite [NOx]), peripheral blood mononuclear cell (PBMC) gene expression (*NADPH oxidase, NFκB, TLR-4, TNF-α, GADD34,* and *XBP1s*), and endothelial cell protein expression (NADPH oxidase).

Anthropometrics

Height without shoes was measured using a scale-mounted stadiometer to the nearest 0.5 cm and weight was assessed using a digital scale (Health o Meter Professional, Sunbeam Products, Ibc). BMI was calculated as weight in kilograms divided by height in meters^2. Midabdominal waist circumference and hip circumference were measured using a Gulick fiberglass measuring tape with a tension handle (Creative Health Products, Inc.).

Vascular Endothelial Function

Digital artery endothelium-dependent vasodilation was assessed using a non-invasive, reproducible plethysmographic method (EndoPAT2000, Itamar Medical, Ltd) as previously described[30,31] and in accordance with conditions specified by the manufacturer. After 10 min of supine rest, pneumatic finger-tip probes were placed on each index finger and a blood pressure cuff was placed on the experimental (non-dominant) upper arm, while the other arm served as the contralateral control, with both arms at rest on arm supports. After an equilibration period (i.e. baseline recording of pulse amplitude for 5 min on each arm), the cuff on the experimental arm was inflated to 200 mmHg or 60 mmHg higher than the participants' systolic blood pressure (whichever was higher) for 5 min to occlude the brachial artery. The cuff was then deflated to induce reactive hyperemia and post-occlusion peripheral arterial tonometry (PAT)-signals were recorded for an additional five min in both arms. RHI, an index of flow-mediated dilation, was derived as the ratio of the average pulse wave amplitude during hyperemia (60 to 120 sec of post-occlusion period) to the average pulse wave amplitude during baseline in the occluded hand, divided by the same value in the control hand and then multiplied by a baseline correction factor. Framingham RHI (F-RHI), which uses a different post-occlusion period (90-120 seconds) without baseline correction and has a natural logarithmic transformation applied to the resulting ratio, is also reported. RHI and F-RHI have been shown to correlate with CVD risk.[28,32]

Hemodynamics and Arterial Stiffness

Brachial pulse pressure was calculated as the difference between mean systolic blood pressure and diastolic blood pressure. Central aortic blood pressure and related hemodynamic parameters (e.g. aortic mean arterial pressure, aortic pulse pressure) were derived from brachial pressure waveforms using a validated transfer function and automatically recorded. Aortic AIx, a measure of pulse wave reflection and arterial stiffness, was automatically calculated as the ratio between augmented aortic pressure (i.e. difference between the first and second derived aortic systolic peaks) and aortic pulse pressure. Both AIx and AIx normalized to

a heart rate of 75 beats per min (AIx@75) are reported, as AIx can be influenced by heart rate, though this approach may not be generalizable to all populations.[33,34]

At the screening visit, aortic stiffness was assessed by measuring carotid-femoral pulse wave velocity (aPWV) in the supine position (SphygmoCor XCEL, AtCor Medical, Inc.).[35] Carotid and femoral waveforms were simultaneously captured using applanation tonometry above the carotid artery, and a femoral blood pressure cuff. Distance between the sternal notch and the carotid artery, the sternal notch to the top of the femoral cuff, and the femoral artery to the top of the femoral cuff was measured with a nonelastic measuring tape. The distance traveled and transit time were automatically determined by the SphygmoCor system and used to calculate aPWV, which is expressed as distance over transit time (i.e. meters per second). Three measurements were obtained and averaged for analysis. At the beginning of each testing visit and following 10 min of supine rest, brachial and aortic blood pressure, and AIx were measured in the non-dominant arm (SphygmoCor XCEL, AtCor Medical, Inc.) and the mean value of three measurements was used in analyses.

Blood and Saliva Collection and Biochemical Analyses

Following baseline vascular and hemodynamic assessments, an IV catheter was placed into an antecubital vein. Blood was collected in vacutainers with EDTA (BD) for plasma separation, centrifuged according to the manufacturers' instructions, aliquoted, and stored at -80°C until analysis. A slow saline drip was then initiated to keep the line patent for serial blood draws. Saliva was collected directly into cryovials using a saliva collection aid (SalivaBio, Inc.) and stored at -80°C until analysis.

Plasma triglyceride, glucose, and insulin concentrations were analyzed using an AU480 Automated Chemistry Analyzer (Beckman Coulter) at the University of Colorado-Denver Colorado Clinical and Translational Sciences Institute. The Homeostatic Model Assessment of

insulin resistance (HOMA-IR) was calculated using the HOMA2 Calculator v2.2.3 based on initial fasting baseline values insulin and glucose measurements.[28] Plasma samples were filtered using 30 kDa molecular weight cut-off filters (Millipore Sigma) to reduce the presence of hemoglobin prior to NOx analysis. Plasma and saliva NOx concentrations were measured using commercially available colorimetric assay kits according to the manufacturers' instructions (Cayman Chemical).

PBMC Isolation and Gene Expression Analyses

A portion of venous blood collected in EDTA plasma vacutainers was used for PBMC isolation. Whole blood was transferred into 50 mL conical tubes and diluted with an equal amount of phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Diluted blood was added to a SepMate 50 mL tube containing a density gradient medium (Lymphoprep, STEMCELL Technologies), and PBMCs were isolated per the manufacturer's protocol. Briefly, after centrifugation at 1200 x g for ten min with the brake on, the top layer containing the enriched PBMCs was poured into a fresh conical tube and washed twice with PBS containing 2% FBS at room temperature. The cell pellet was resuspended with PBS+2% FBS at room temperature. After cell counting, PBMCs were cryopreserved in a cryopreservation medium (CryoStor® CS10, STEMCELL Technologies) at 5×10^6 cells per 1 mL and placed inside a Nalgene® Mr. Frosty® Cryo 1°C Freezing Container (Thermo Fisher Scientific) container at −80°C for 24 hours. Cells were then transferred into liquid nitrogen where they were stored until analysis.

Total RNA was extracted with Trizol reagent according to the manufacturer's protocol (Invitrogen). For real-time PCR, reverse transcription was performed using 0.5 µg of DNase-treated RNA, Superscript II RnaseH- and random hexamers. PCR reactions were performed in

96-well plates using transcribed cDNA and IQ-SYBR green master mix (Bio Rad Laboratories). Primer sets are provided in **Supplemental Table 3S.3** in **Appendix 1**. PCR efficiency was between 90% and 105% for all primer and probe sets and linear over 5 orders of magnitude. The specificity of products generated for each set of primers was examined for each amplicon using a melting curve and gel electrophoresis. Reactions were run in triplicate and data were calculated as the change in cycle threshold (ΔCT) for the target gene relative to the ΔCT for β2-microglobulin (control/reference gene) according to the procedures of Muller *et al.* (36).

Endothelial Cell Biopsy and Protein Expression Analyses

Endothelial cell collection and protein expression analyses were performed as previously described (37, 38). Endothelial cells were biopsied from the antecubital vein as venous endothelial cell protein expression correlates with arterial endothelial cell protein expression (39). Briefly, endothelial cells were biopsied using sterile 0.025 inch J-wires (GuideRight™, St. Jude Medical) advanced through an IV catheter ~4 cm beyond the tip of the catheter and withdrawn. The distal portion of the wire was transferred to a 50 mL conical tube containing a buffer solution, and cells were recovered by centrifugation, fixed with formaldehyde, plated to microscope slides, and stored at -80°C until analysis.

Slides were stained for NADPH oxidase/p47 subunit (Sigma-Aldrich), and a complementary fluorescent secondary Alexafluor 555 antibody (Invitrogen). Slides were also stained for vascular endothelial-cadherin (Abcam) for positive identification of endothelial phenotype and DAPI (4', 6'-diamidino-2-phenylindole hydrochloride; Vector Laboratories) for nuclear integrity. Images were digitally captured and analyzed using cellSens Software (Olympus Corporation). Values are reported as ratios of subject endothelial cell protein expression to human umbilical vein endothelial cell (HUVEC; control cells) protein expression.

This ratio is reported to minimize the possible confound of differences in staining intensity among different staining sessions.

Sample Size Estimation and Statistical Analyses

Sample size was estimated with a minimum anticipated difference between the RBJ and PBO groups of 0.3 with a standard deviation of 0.392, and 0.25 change in the treatment group and 0.1 in the control group with a standard deviation of 0.1 and 0.05, respectively, from baseline to 4 weeks. A crossover design was considered and hence a moderate intra-class correlation of 0.3 was used for calculation. A final sample size of 15 subjects in the study was estimated to provide a statistical power > 90% and a confidence of 99% with 2-tailed hypothesis. Collected data were stored electronically using Research Electronic Data Capture (REDCap) for secure data management.[40,41] As a measure of quality control, data were double-entered by two individuals and evaluated for consistency by a third person. Subject characteristics were analyzed using descriptive statistics from data collected at the screening visit. For each treatment and exposure (acute vs. chronic) arm, data were tested for normality using Shapiro-Wilks tests (PROC UNIVARIATE, SAS, version 9.4, SAS Institute) and confirmed using QQ-plot observations. Data not conforming to normal distribution were natural log-transformed before statistical analysis to accommodate assumptions of normality. Outlier removal may have resulted in fewer evaluable subjects for primary and secondary outcomes. Thus, we visually inspected the residual plots and excluded observations of extreme outliers (> 5 times the SD from the mean). AUC was calculated for postprandial glucose, insulin, triglyceride, and NOx concentrations using the linear and log-linear trapezoidal rule, and incremental AUC (iAUC) was calculated for glucose, insulin, and triglycerides in the same way after controlling for baseline. Differences in AUC between treatment groups for each postprandial value were assessed using a one-way ANOVA (PROC GLM, SAS) with Tukey's test for multiple comparisons for repeated measures. A linear mixed model (PROC MIXED,

SAS) was used to assess main and interaction effects of treatment (PBO, RBJ, PBO+NIT, NF-RBJ) and time (0, 1, 2, 4 hours postprandial) on primary and secondary outcomes, time, treatment and time*treatment interaction were set at fixed effects, and subject and treatment order were set as random effects. Age, sex, and BMI were included in the models as covariates. For gene expression analysis, differences in relative expression (expressed as fold-change) within each treatment group were analyzed with a mixed model (PROC MIXED, SAS) to assess the magnitude of change over time. The 0 hour time point at the acute visit was set at 1.0 for all treatment groups, and fold-change comparisons are relative to 1.0 within each treatment group. For endothelial cell protein expression, baseline/preprandial (0 hr) differences between the acute and chronic test visits were assessed by a linear mixed model (PROC MIXED, SAS). For both gene and endothelial cell protein expression, the same fixed effects, random effects and +covariates were used as previously stated. Results are presented as least squares mean ± SEMs. Statistical significance was set at a two-sided α level of 0.05.

Results

Baseline Characteristics

Screening and baseline characteristics of participants who completed the study are presented in **Table 3.1.**

Acute Treatment Effects on Postprandial Blood and Saliva Biomarkers

There were no significant effects of time, treatment, or the interaction effect of time*treatment for glucose for the acute test visit. At the 2 hour time point, plasma glucose levels were significantly lower in the PBO+NIT group than the NF-RBJ group (91.78 ± 1.03 vs. 97.35 ± 1.03 mg/dL, respectively; time*treatment $P = 0.0269$, **Figure 3.6A**). There were significant main effects of time ($P < 0.0001$) and treatment ($P = 0.0386$) for plasma insulin

levels. At the 1 hour time point, plasma insulin levels were significantly lower in the NF-RBJ group than the PBO and PBO+NIT groups (23.05 and 24.35 uIU/mL vs. 16.35 uIU/mL; time*treatment $P = 0.0193$ and 0.0068, respectively; **Figure 3.6B**). There were no significant main effects of time or time*treatment interactions. There was a significant main effect of time *(P* < 0.001) and treatment ($P = 0.0392$) for plasma triglycerides but no significant differences between treatment groups were observed at any time point for postprandial triglyceride concentrations (**Figure 3.6C**). There were no significant differences in glucose, insulin, or triglyceride iAUC between treatment groups (**Figure 3.6D-E**).

There were significant main effects of time, treatment, and time*treatment (all $P <$ 0.0001) for plasma NOx and saliva NOx. At the 1, 2, and 4 hour time points, plasma and saliva NOx levels in the RBJ and PBO+NIT groups were significantly higher (time*treatment $P <$ 0.0001 for all time points) than the PBO and NF-RBJ groups whose values remained unchanged throughout the 4 hour testing period (**Figure 3.7A and B**). Similarly, plasma and saliva NOx AUC for the RBJ and PBO+NIT groups were significantly higher than PBO and NF-RBJ groups *(P* < 0.05, **Figure 3.7C and D**).

Acute Treatment Effects on PBMC Gene Expression

Gene expression results at the acute test visit are presented in **Supplemental Table 3S.6** in **Appendix 1**. There were no significant main effects of time on the gene expression markers of oxidative stress (i.e. *NADPH oxidase-p47phox*), inflammation (*i.e. NFκB-p65*), and ER stress (i.e. *XBP1s*) in PBMCs for any treatment group. There was a significant main effect of time for the ER stress marker, *GADD34*, in the PBO, RBJ and NF-RBJ groups ($P < 0.05$). There were significant main effects of time for the pro-inflammatory markers TNF-α in the NF-RBJ group ($P = 0.0223$), and *TLR-4* in the RBJ group ($P = 0.004$), but not in the other treatment groups for either marker. In the PBO group, there was a 1.9-fold increase in TLR-4 at the 4-hour time point from baseline ($P = 0.0096$). In the RBJ group, there was a 1.5-fold increase of

p47phox (*P* = 0.0469), a 1.7-fold increase of *TLR-4* (*P* = 0.0214), and 0.6-fold decrease of *GADD34* at the 4-hour time point from baseline. In the NF-RBJ group, there was a 0.6-fold decrease in TNF-α (*P* = 0.0128) and *GADD34* (*P* = 0.0282) at the 4-hour time point from baseline. No other within treatment group differences were observed at the 4-hour time point relative to baseline at the acute visit.

Chronic treatment effects on preprandial and postprandial vascular endothelial function

There were no significant main effects of time, treatment, or their interaction for RHI or F-RHI (**Figure 3.8**) following 4 weeks of daily treatment consumption. Postprandial change scores, evaluated by subtracting baseline values from 4-hour values, were not significantly different between groups (data not shown). Individual RHI responses are shown in **Figure 3.9**. There were no significant preprandial or postprandial differences within treatment groups at 4 weeks compared to 0 weeks (data not shown).

Chronic Treatment Effects on Preprandial and Postprandial Hemodynamics

Preprandial and postprandial hemodynamic parameters at the chronic test visit are presented in **Supplemental Table 2S.5** in **Appendix 1**. There were significant main effects of time for brachial systolic blood pressure, diastolic blood pressure, brachial pulse pressure, aortic systolic blood pressure, aortic diastolic blood pressure, heart rate, aortic mean arterial pressure, augmented pressure, AIx, and AIx@75 (brachial pulse pressure *P* = 0.009, all others *P* < 0.0001). There were significant main effects of treatment for brachial systolic blood pressure (*P* = 0.0002), brachial diastolic blood pressure (*P* = 0.0027), aortic systolic blood pressure *(P = 0.0037),* aortic diastolic blood pressure (*P* = 0.0128), and aortic mean arterial pressure (*P* = 0.0041). There was a significant preprandial difference within the RBJ group at 4 weeks compared to 0 weeks for augmented pressure (11 ± 1% vs. 14 ± 1%, respectively, time*treatment *P* = 0.0125). There was a significant decrease in preprandial AIx@75 within the

PBO+NIT group at 4 weeks compared to 0 weeks (25 ± 2% vs. 20 ± 2%, respectively, time*treatment *P* = 0.0363). There was a significant postprandial increase in aortic pulse pressure at the 4-hour time point within the PBO+NIT group at 4 weeks compared to 0 weeks (32 ± 1 vs. 39 ± 1, respectively, time*treatment *P* = 0.02), and a significant postprandial decease in AIx within the PBO group at 4 weeks compared to 0 weeks (30 ± 2% vs. 26 ± 2%, time*treatment *P* = 0.0426). There were no significant preprandial or postprandial differences within treatment groups at 4 weeks compared to 0 weeks for the remaining parameters (data not shown).

Chronic Treatment Effects on Preprandial and Postprandial Blood and Saliva Biomarkers

As expected, there were significant main effects of time (*P* = 0.0355) for plasma glucose, time (*P* < 0.0001) and treatment (*P* = 0.0386) for plasma insulin, and time (*P* < 0.0001) and treatment (*P* = 0.002) for plasma triglycerides at the chronic test visit. There were no significant preprandial or postprandial differences within treatment groups at 4 weeks compared to 0 weeks for plasma glucose, insulin, or triglycerides (data not shown) except for PBO group which had a significant preprandial increase at 4 weeks compared to 0 weeks for triglycerides (91 ± 1 vs. 79 ± 1, time*treatment *P = 0.0361).* Postprandial glucose, insulin and triglyceride concentrations and iAUC did not differ between treatment groups following 4 weeks of chronic treatment consumption (**Figure 3.11A-F**).

There was a significant main effect of time (*P* = 0.0129) and treatment (*P* < 0.001) for plasma NOx, and a significant main effect of time (*P* = 0.0006) and treatment (*P* < 0.0001) for saliva NOx. There were significant preprandial differences within the RBJ group at 4 weeks compared to 0 weeks for plasma NOx (70.8 ± 1.2 µmol/L vs. 26.2 ± 1.2 µmol/L, respectively, time*treatment *P* < 0.001) and saliva NOx (1214.2 ± 1.3 µmol/L vs. 423.7 ± 1.2 µmol/L, respectively, time*treatment *P* < 0.05), and within the PBO+NIT group at 4 weeks compared to 0 weeks for plasma NOx (61.4 ± 1.2 µmol/L vs. 27.9 ± 1.2 µmol/L, respectively, time*treatment

P < 0.001) and saliva NOx (1002.9 ± 1.3 µmol/L vs. 471.2 ± 1.2 µmol/L, respectively, time*treatment *P* < 0.05). At the 1, 2, and 4 hour time points, plasma NOx levels in the RBJ and PBO+NIT groups were significantly higher (time*treatment *P* < 0.01 for all time points) than the PBO and NF-RBJ groups whose levels remained unchanged throughout the 4 hour testing period (**Figure 3.12A**). Saliva NOx levels were significantly higher in the RBJ group than the PBO and NF-RBJ group at the 1, 2, and 4 hour time points (all time points, time*treatment *P* < 0.001), while saliva NOx levels in the RBJ and PBO+NIT group were significantly higher than both PBO and NF-RBJ groups only at the 1 hour time point (time*treatment *P* < 0.001) (**Figure 3.12B**). Plasma NOx AUC was significantly higher in the RBJ group compared to the PBO group, and saliva NOx AUC was significantly higher in the RBJ group compared to the PBO and NF-RBJ groups (time*treatment *P* < 0.05, **Figure 3.12C and D**).

Chronic Treatment Effects on Preprandial and Postprandial PBMC Gene Expression

The main effect of time for the gene expression markers at the chronic visit are identical to those for the acute treatment effects on PBMC gene expression (**Supplemental Table 3S.6** in **Appendix 1**). In the PBO group, there was a significant difference in relative expression of *TNF-α* and *GADD34* between 0 and 4 hour time points at the chronic visit (1.2 ± 0.2 vs. 0.6 ± 0.2, *P* = 0.0357; and 1.7 ± 0.3 vs. 0.4 ± 0.3, *P* = 0.058, respectively). In the RBJ group, there was a significant difference in relative expression of *TLR-4* and *GADD34* between 0 and 4 hour time points at the chronic visit (1.0 ± 0.2 vs. 1.9 ± 0.2, *P* = 0.0037; and 0.8 ± 0.1 vs. 0.4 ± 0.1, *P* = 0.0102, respectively). In the PBO+NIT group, there was a significant difference in relative expression of *GADD34* between 0 and 4 hour time points (1.7 ± 0.3 vs. 0.7 ± 0.3, *P* = 0.0218). In the NF-RBJ group, there was a significant difference in relative expression of *TLR-4* and *GADD34* between the 0 and 4 hour time points (1.1 ± 0.4 vs. 2.4 ± 0.4, *P* = 0.0311; and 1.1 ± 0.1 vs. 0.7 ± 0.1, *P* = 0.0375). No other significant differences between 0 and 4 hour time points within treatment groups were observed at the chronic visit. There were few significant

differences in relative expression at the 4 hour time point when compared to baseline, such as a 0.5-fold decrease of *XBP1s* in the PBO group (P = 0.0105), a 1.9-fold increase of *TLR-4* (P = 0.004) and 0.4-fold decrease of *GADD34* (P < 0.001) in the RBJ group, a 0.6-fold decrease of *TNF-α* in the PBO+NIT group (P = 0.0391), and a 2.4-fold increase in *TLR-4* (P = 0.0285) in the NF-RBJ group.

Chronic Treatment Effects on Preprandial Endothelial Cell Protein Expression

There were no significant main effects of time, treatment and time*treatment interaction effect for the endothelial cell protein marker of oxidative stress, NADPH oxidase/phox47 subunit**.** There were no significant preprandial differences within treatment groups at 4 weeks compared to 0 weeks for NADPH oxidase/phox47 subunit protein expression **(Figure 3.13).**

Discussion

To our knowledge, this is the first randomized controlled trial to investigate the acute and chronic effects of RBJ supplementation, including nitrate-dependent and -independent effects, on vascular endothelial function and other cardiometabolic responses to HFM consumption in middle-aged/older adults with overweight and obesity. We found that acute and chronic RBJ and PBO+NIT supplementation increased saliva and plasma NOx concentrations compared to PBO and NF-RBJ, but these increases were not paralleled by significant differences in postprandial vascular endothelial function across treatment groups. Postprandial vascular endothelial function was not altered by the PBO+NIT and NF-RBJ treatments, ruling out any sole contribution of inorganic nitrate or other bioactive compounds in RBJ on this outcome in the present study. There were no significant within-group declines in RHI following HFM and PBO consumption, suggesting that the HFM did not significantly impair postprandial vascular endothelial function even though it led to significant changes in several hemodynamic parameters, and in plasma insulin and triglyceride concentrations.

The premise that consumption of a HFM leads to exacerbated postprandial impairments in vascular endothelial function in individuals with overweight and obesity is not supported by our current findings, which are in agreement with others. Ayer *et al.*[42] did not observe significant within- or between-group differences in measures of vascular endothelial function including RHI, brachial artery flow-mediated dilation (FMD), or hyperemic forearm blood flow (FBF) at 1- and 3-hours following consumption of a HFM (1000 kcal, 60 g fat) in young adults with obesity vs. a normal body weight. In other studies with healthy adults, transient impairments in FMD at 2, 3, and 4 hours following HFM consumption were strongly associated with the magnitude of postprandial triglyceride concentrations, as well as leukocyte superoxide production.[9,10] In a dose-response study, Schwander *et al.*[43] observed significant increases in plasma triglyceride iAUC over a 6-hour period in men with obesity following consumption of HFMs containing 1000 kcal (68 g fat) or 1500 kcal (102 g fat), and in serum IL-6 concentrations but only following consumption of the 1500 kcal HFM. They did not observe significant increases in these parameters following consumption of a 500 kcal HFM (34 g fat) in men with obesity or following consumption of any of the meals in normal weight men. Vascular endothelial function was not assessed in that study; however, their data suggest that a higher caloric and fat challenge may be needed to induce postprandial inflammation, and thus likely oxidative stress and impairments in vascular endothelial function. Indeed, while we did observe a moderate increase in postprandial triglycerides, consumption of the HFM did not provoke postprandial hyperglycemia, inflammation, oxidative stress, or ER stress. Because those processes are believed to be major contributors to postprandial impairments in vascular endothelial function, the lack of an effect on postprandial RHI may be explained by the neutral glycemic, pro-inflammatory, oxidative stress, and ER stress responses to the HFM in our study. In general, the effect of a HFM on vascular endothelial function (and other cardiometabolic responses) is uncertain due to significant inter–individual and –group variability. Studies investigating the type of fat given in a HFM (e.g. saturated vs. unsaturated) have not consistently demonstrated postprandial impairments in

measures of vascular endothelial function with different fat types.[44-48] Major discrepancies among studies in this area, including the present study, are the different techniques used to assess vascular endothelial function (e.g. EndoPat assessed RHI vs. ultrasound assessed FMD, FBF, etc.), as well as different time points chosen to assess cardiometabolic parameters and the duration of the postprandial testing period. We therefore cannot rule out the fact that the techniques chosen to measure vascular endothelial function, the times for performing measurements, or the postprandial testing period duration may be factors contributing to our findings. Nonetheless, considering the discrepant findings among published studies in this area, robust human studies designed specifically to evaluate causes of inter–individual and –group variability in men and women are needed to better understand the impact of HFM consumption on CVD risk.

Several studies investigating the efficacy of RBJ, primarily as a rich source of inorganic nitrate, have shown promising but mixed results on measures of cardiovascular health.[24,49,50] Inorganic nitrate supplementation may compensate for disrupted endothelium-dependent pathways for NO production and bioavailability that contribute to vascular endothelial dysfunction, hypertension, and other CVD risk factors.[51] RBJ increases NO bioavailability via the enterosalivary nitrate-nitrite-NO endothelium-independent pathway which is complementary to the L-arginine-NO synthase endothelium-dependent pathway.[52]) Since transient impairments in vascular endothelial function have been observed following HFM consumption in several previous studies, we hypothesized that acute and chronic supplementation of RBJ would increase NO bioavailability, in part, through the enterosalivary nitrate-nitrite-NO pathway, and therefore increase postprandial RHI compared to PBO. After acute and chronic ingestion of RBJ or PBO+NIT in the present study, plasma and saliva NOx concentrations increased significantly, but were not paralleled by significant effects on postprandial RHI values. To our knowledge, only one previous study has evaluated the impact of acute RBJ consumption on postprandial vascular endothelial function following consumption of a HFM (1122 kcal, 57 g fat).[22] Contrary to

our study, Joris and Mensink[22] showed attenuated impairments in postprandial FMD following consumption of 140 mL of concentrated RBJ in men with overweight and obesity. That study differed from ours in that they measured brachial artery FMD vs. RHI, vascular endothelial function was assessed 2 hours postprandially vs. 4 hours, the study included only men, and the RBJ dose provided was double that provided in our study. The neutral effect of RBJ on postprandial RHI in the present study may be due, in part, to the lack of a major effect of the HFM on RHI. It is important to note that the cardiovascular effects of inorganic nitrate can vary due to individual differences in oral hygiene and the oral microbiota, including the abundance of nitrate-reducing oral bacteria.[53]

Several studies have investigated the acute vasodilatory effects of a single dose of RBJ (in the absence of a HFM), and have found increased FMD to be associated with increased circulating NOx levels.[17,18,54,55] There are many differences among those studies and the present study, including experimental design, volume of RBJ (i.e. 140 – 500 mL), RBJ type (i.e. concentrate vs. juice), dose of inorganic nitrate provided (i.e. 1.1 to 45 mmol/L), placebo used (e.g. equivalent volumes of water, potassium nitrate in water, NF-RBJ, and juice), and study population (i.e. healthy young lean men, hypertensive men, overweight and obese men, healthy men and women). It is possible that any potential for RBJ to increase RHI following acute consumption was negated by consumption of the HFM in the present study, or any of the aforementioned variables.

Hemodynamic parameters and AIx are reduced following meal consumption, potentially due to the vasodilatory effect of insulin or other direct effects on the vascular tissue.[56,57] As expected, insulin peaked 1 hour postprandially which coincided with reductions in several hemodynamic parameters and AIx. Overall, there were no clear or consistent effects of any one treatment on hemodynamic parameters following acute or chronic consumption. Considering blood pressure values were relatively normal at baseline, we did not expect to see major changes in these values. In fact, these data suggest that consumption of RBJ or other nitrate-

containing supplements (at the dose provided in this study) is safe for normotensive individuals to ingest without exerting major hypotensive effects. On the other hand, AIx is a measure of pulse wave reflection and arterial stiffness. A postprandial decrease in AIx is suggested to be a protective hemodynamic response reflective of lower systolic loading.[58] We observed significant decreases in AIx 4 hours post-HFM consumption in the RBJ and PBO+NIT groups compared to PBO following acute treatment exposure. It is possible that this represents reversible changes in arterial stiffness, presumably from smooth muscle relaxation.[56] There were small but significant differences between RBJ and PBO 4 hours after acute treatment ingestion suggesting that RBJ may exert mild effects on insulin sensitivity. After adjusting for heart rate, these effects were no longer significant. Heart rate has been shown to have a linear relationship with AIx which may be related to alterations in timing of the reflected pressure wave.[59] Thus, AIx is often standardized at 75 beats per minute; however, this has been argued to be a physiologically inappropriate approach as it may not apply to all populations[34] and thus both values should be considered.

Based on previous work[7,60,61] we expected a response to HFM consumption reflective of pro-inflammation, oxidative stress, and ER stress that would be detected in PBMCs. In line with the overall results of the present study, we did not observe substantial or consistent responses in PBMC gene expression to the HFM or the treatments. Postprandial pro-inflammatory responses are believed to be caused in part by absorption of lipopolysaccharide by enterocytes during triglyceride-rich chylomicron secretion and circulating triglyceride and glucose concentrations following HFM consumption.[62,63] Given that the HFM did not provoke statistically significant impairments in RHI or robust metabolic derangements, these findings are not surprising. Baseline health status (e.g. composition of the gut microbiota, degree of insulin resistance) may be a contributing factor to variability in postprandial responses to a meal.[64] RBJ also had no influence on endothelial cell NADPH oxidase protein expression, suggesting that RBJ did not reduce vascular superoxide production following chronic treatment consumption.

There are several strengths of the present study include the randomized, double-blind, placebo-controlled crossover design, the exploration of nitrate-dependent and –independent effects and potential synergy of the bioactive compounds in RBJ, as well as the acute and chronic effects of treatment consumption on postprandial responses to a HFM. In addition, our study included an aging population of both men and postmenopausal women at risk for developing CVD. We used a dose of RBJ that would be practical for daily consumption, whereas most other research has used at least 140 mL (the equivalent of two doses of RBJ concentrate). Lastly, we utilized a HFM challenge representative of the typical meal composition consumed in the Western diet.

There are several limitations of the present study. Although inclusion of both men and women should be considered a strength, it is possible that there were sex differences in meal and treatment responses and our study was not powered to evaluate that. The study population included was a metabolically healthy overweight/obese study population (i.e. without the presence of hypertension, diabetes, etc.). The multiple exclusion criteria, which were warranted to minimize confounding variables, may have resulted in a sample of relatively metabolically healthy, middle-aged/older adults with overweight or obesity, whose metabolic flexibility minimized or preserved the effects of the HFM. The challenge meal used may have lacked adequate calories and fat to induce postprandial vascular endothelial dysfunction in this population. Previous research has adjusted caloric and fat content of a HFM challenge to individual body weight, body surface area, or resting metabolic rate, whereas we chose to standardize the caloric and fat content of the meal to represent typical Western intake. In addition, the postprandial duration and timing of the measurements may not have been sufficient to capture responses to the meal and treatments. It is also possible that the volume of RBJ may not have provided a large enough dose of inorganic nitrate or other bioactive compounds to modulate the outcomes studied. Lastly, measurement of NOx does not distinguish between the amount of nitrate and nitrite, and thus any influence on nitrate reduction

to nitrite cannot be determined. Future research studies are planned to examine differences in the oral and gut microbiota among the study participants in the present study and their relationship with treatment responses and non-responses.

Conclusion

In summary, this study confirms that the nitrate-nitrite NO pathway is intact in healthy, middle-aged/older adults with overweight and obesity following acute and chronic consumption of concentrated RBJ. Although consumption of the HFM led to postprandial alterations in several cardiometabolic parameters, we were unable to detect HFM-induced impairments in vascular endothelial function. Acute nor chronic RBJ or other treatment supplementation did not consistently modulate postprandial cardiometabolic responses to a HFM. Additional research in this area is needed, particularly with respect to inter-individual and -group variability in response to HFM and RBJ consumption.

Figures and Tables

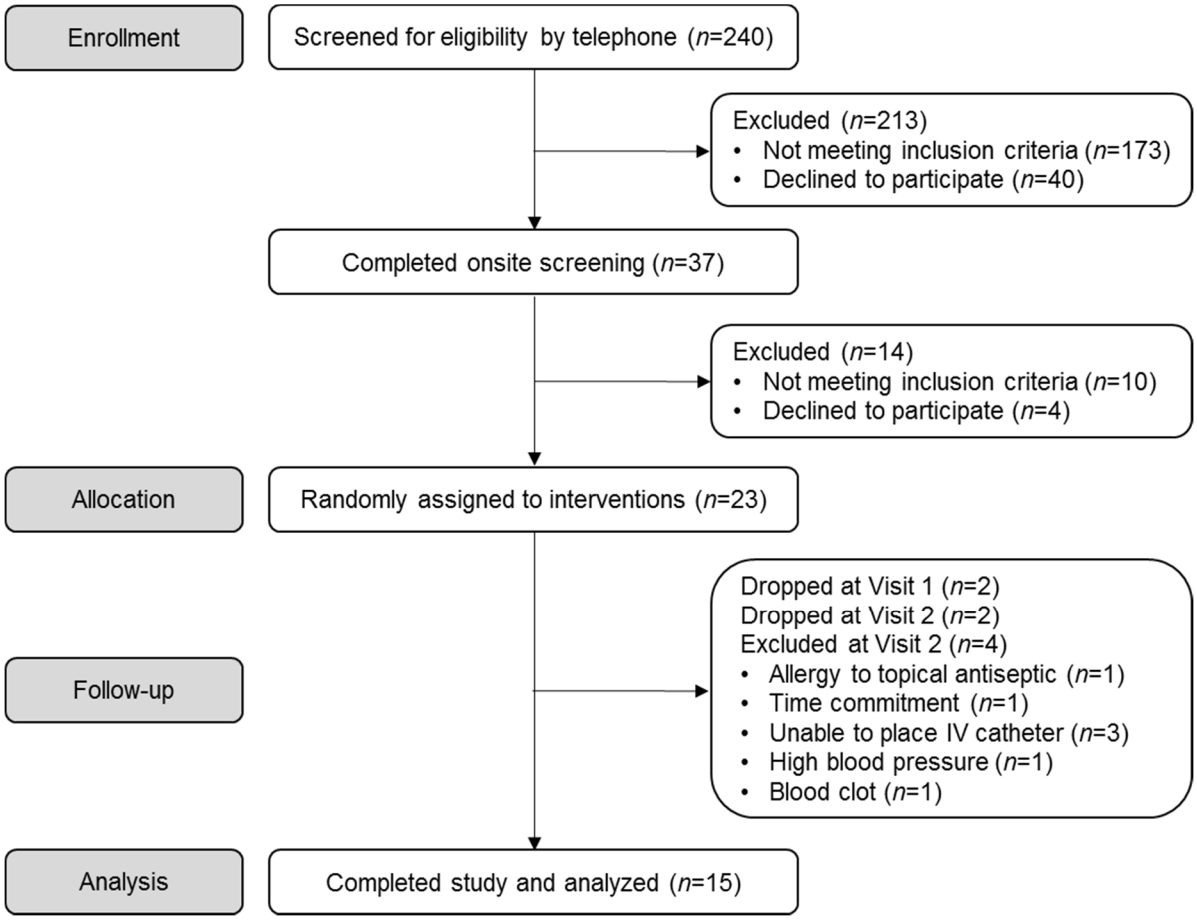


Figure 3.1 CONSORT flow diagram of participants through the trial.

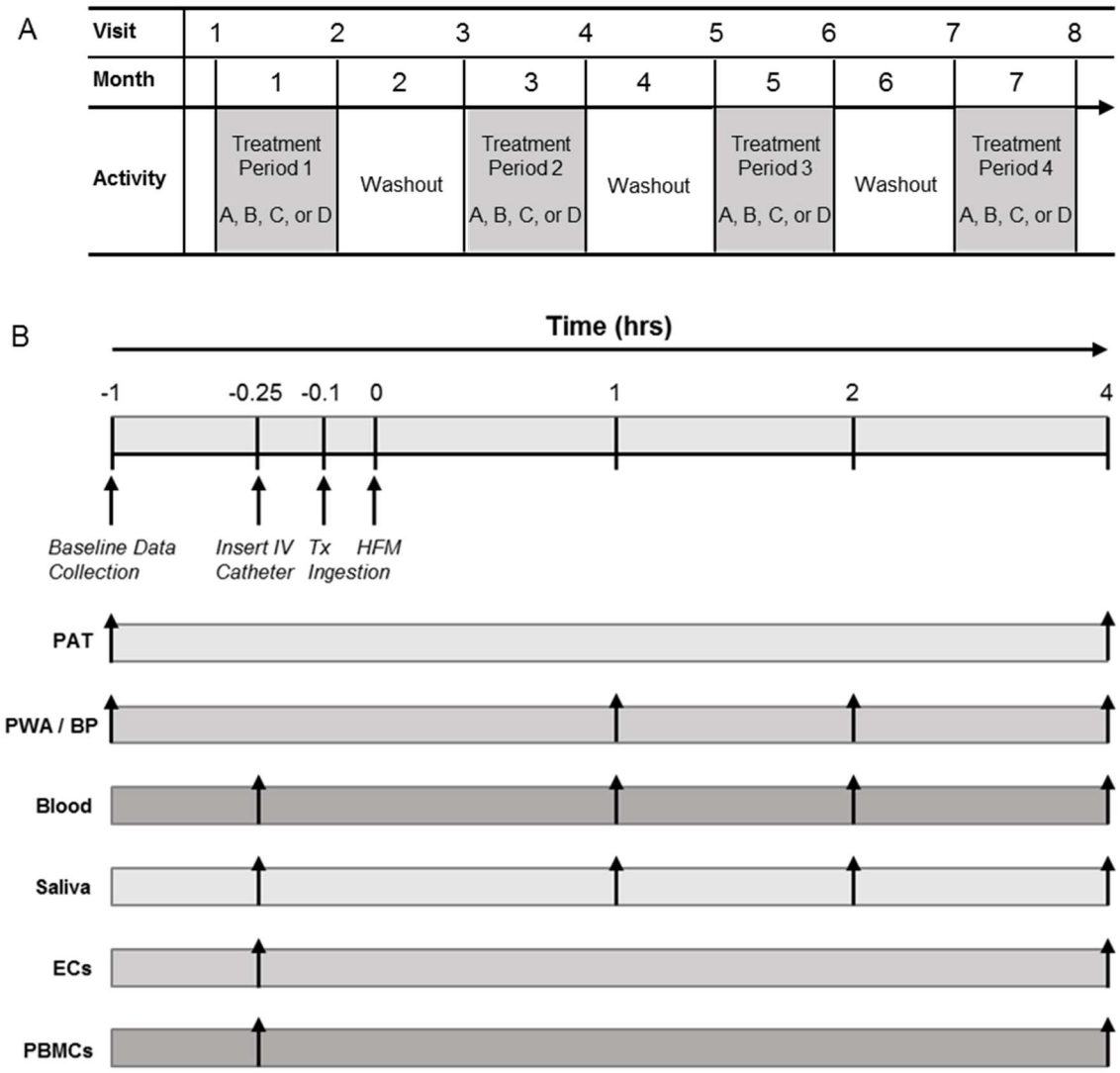


Figure 3.2 (A) Overall study design and schedule of participant study visits. After enrollment, participants were randomized to receive four 70 mL treatments in random order: 1) placebo (PBO), 2) red beetroot juice (RBJ), 3) placebo + nitrate (PBO+NIT), and 4) nitrate-free RBJ (NF-RBJ). Each treatment period consisted of two postprandial challenges (i.e. first and last day of each 4-week treatment period), followed by 4 weeks of daily treatment consumption. Each treatment period was separated by a 4-week washout period. Participants were enrolled in the trial for an 8-month period. **(B)** Schematic of the test day timeline for data collection and measurements. Abbreviations: BP, blood pressure; ECs, endothelial cells; HFM, high-fat meal; IV, intravenous; PAT, peripheral arterial tonometry; PBMCs, peripheral blood mononuclear cells; Tx, treatment. Participants were randomized to treatments A, B, C, or D for each treatment period in random order.

Table 3.1 Screening characteristics of participants who completed the study (n = 15).

	Mean ± SEM (Range)
Age, years	53 ± 2 (42 – 65)
Years postmenopausal (women only)	5 ± 1 (1 – 8)
Sex, M:F (n)	7:8
BMI, kg/m^2	29.8 ± 0.9 (26.2 – 36.4)
Waist-to-hip ratio	0.86 ± 0.02 (0.76 – 1.00)
Total cholesterol, mg/dL	202 ± 11 (100 – 278)
HDL, mg/dL	59 ± 4 (17 – 85)
LDL, mg/dL	131 ± 0 (86 – 181)
Triglycerides, mg/dL	112 ± 16 (45 – 249)
HDL:LDL ratio	0.48 ± 0.05 (0.27 – 0.86)
Hemoglobin A1c, %	5.3 ± 0.1 (5.0 – 6.0)
HOMA-IR	0.79 ± 0.11 (0.21 – 1.52)
SBP, mmHg	121 ± 3 (97 – 136)
DBP, mmHg	78 ± 2 (62 – 89)
RHI*	1.74 ± 0.09 (1.26 – 2.38)
PWV, m/s	7.2 ± 0.4 (4.6 – 10.0)

Values are mean ± SEM (ranges). *RHI was measured at the baseline visit. Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PWV, pulse wave velocity; RHI, reactive hyperemia index; SBP, systolic blood pressure; WC, waist circumference; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance.

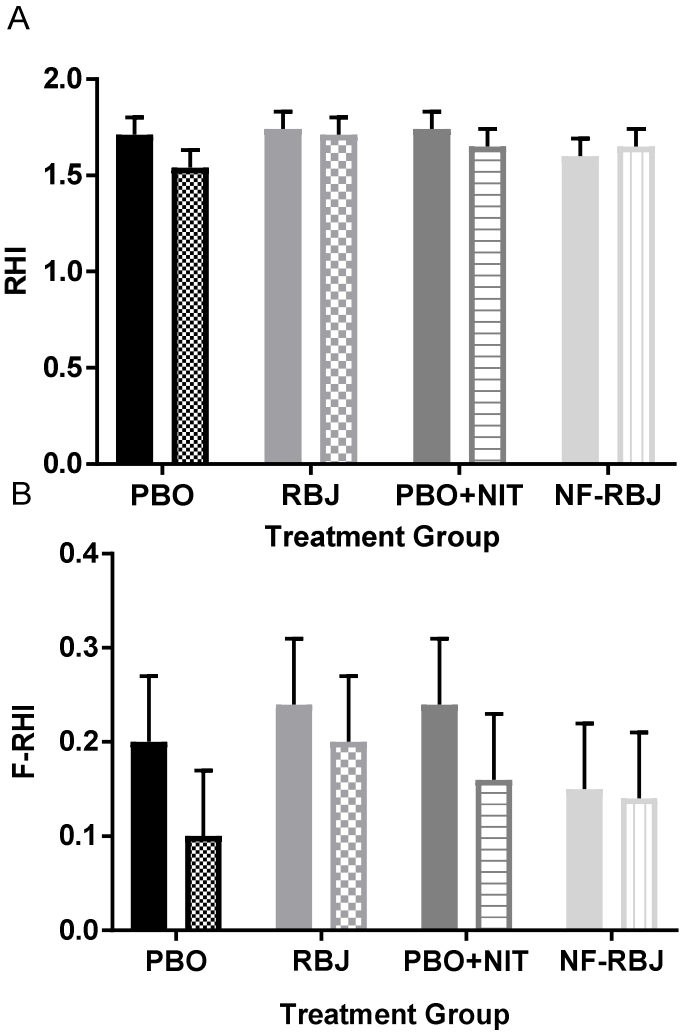


Figure 3.3 Effects of PBO, RBJ, PBO+NIT, and NF-RBJ on RHI (A) and F-RHI (B) before (0 hours) and 4 hours after high-fat meal consumption at the acute visit. Data are least square means ± SEM, n = 15. Means were compared with the use of the PROC MIXED procedure in SAS version 9.4. There were no significant main effects of time, treatment, or main interaction effect of time*treatment for RHI or F-RHI in the model. There were no significant differences in pre- and post-meal RHI and F-RHI values within treatment groups. Solid color = before meal (T0), and patterned color = 4 hours after meal and treatment consumption (T4). Abbreviations: RHI, reactive hyperemia index, F-RHI, Framingham-RHI; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

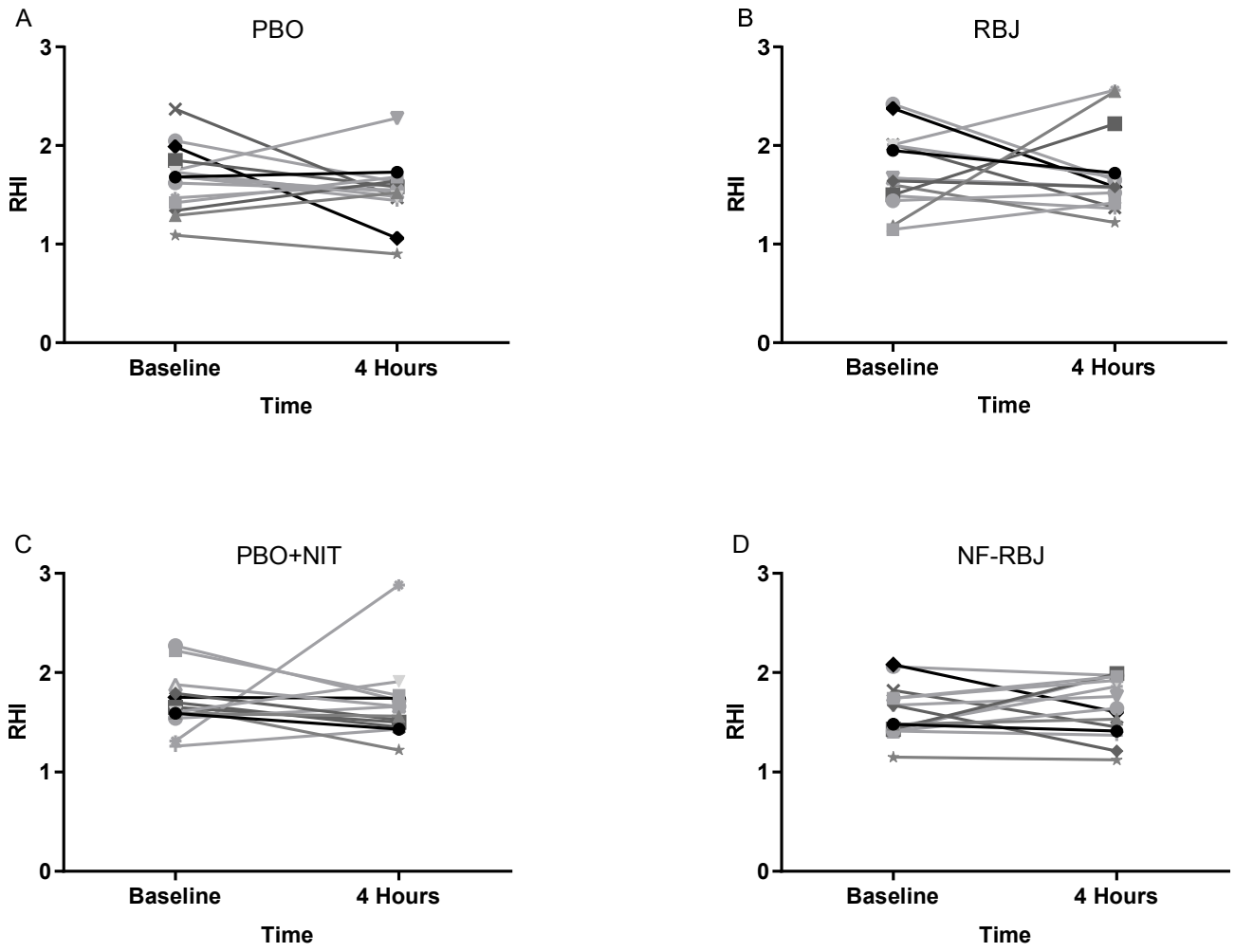


Figure 3.4 Individual effects of PBO (A), RBJ (B), PBO+NIT (C), and NF-RBJ (D) on RHI before (0 hours) and 4 hours after high-fat meal consumption at the acute visit. Data are least square means ± SEM, n = 15. Values were obtained with the use of the PROC MIXED procedure in SAS version 9.4. Abbreviations: RHI, reactive hyperemia index; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

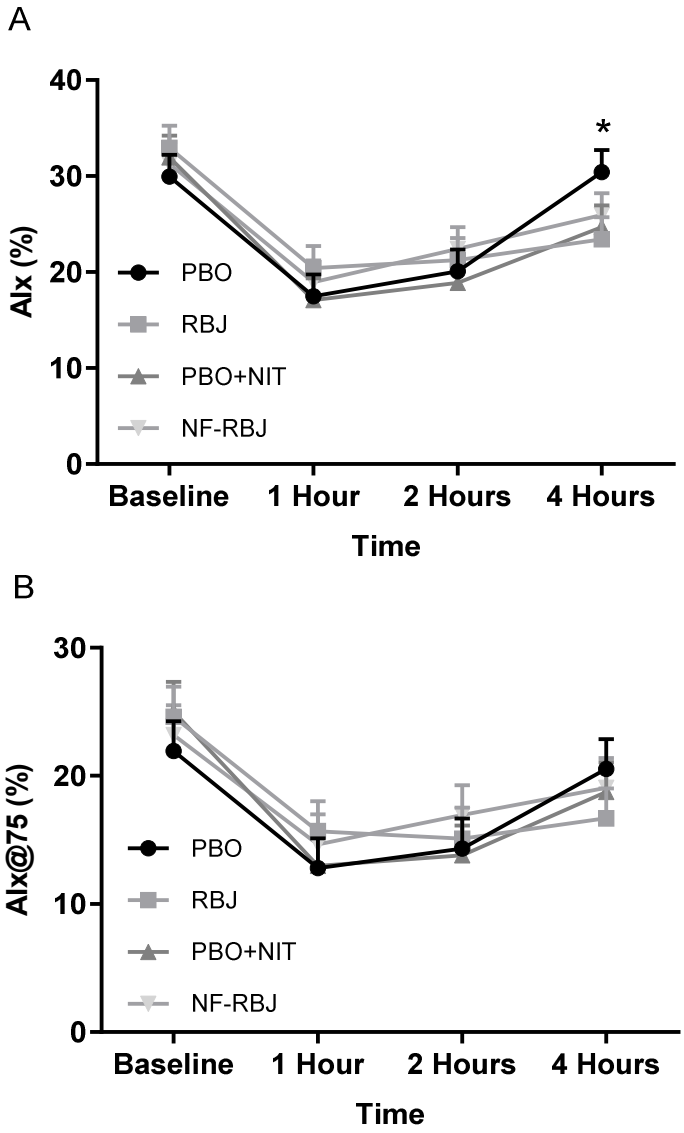


Figure 3.5 Effects of PBO, RBJ, PBO+NIT, and NF-RBJ on AIx (A) and Aix@75 (B) before (0 hours) and 1, 2, and 4 hours after high-fat meal consumption at the acute visit. Data are least square means ± SEM, n = 15. Values were compared with the use of the PROC MIXED procedure in SAS version 9.4. There was a significant main effect of time for AIx and AIx@75 ($P < 0.0001$) in the models, but no significant main effect of treatment on AIx or AIx@75. Time points annotated with symbols represent a significant time*treatment interaction between treatment groups. *PBO significantly different than RBJ and PBO+NIT (both $P < 0.05$). No other time*treatment interactions for AIx or AIx@75 were observed. Abbreviations: AIx, augmentation index; AIx@75, augmentation index at 75 beats per min; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

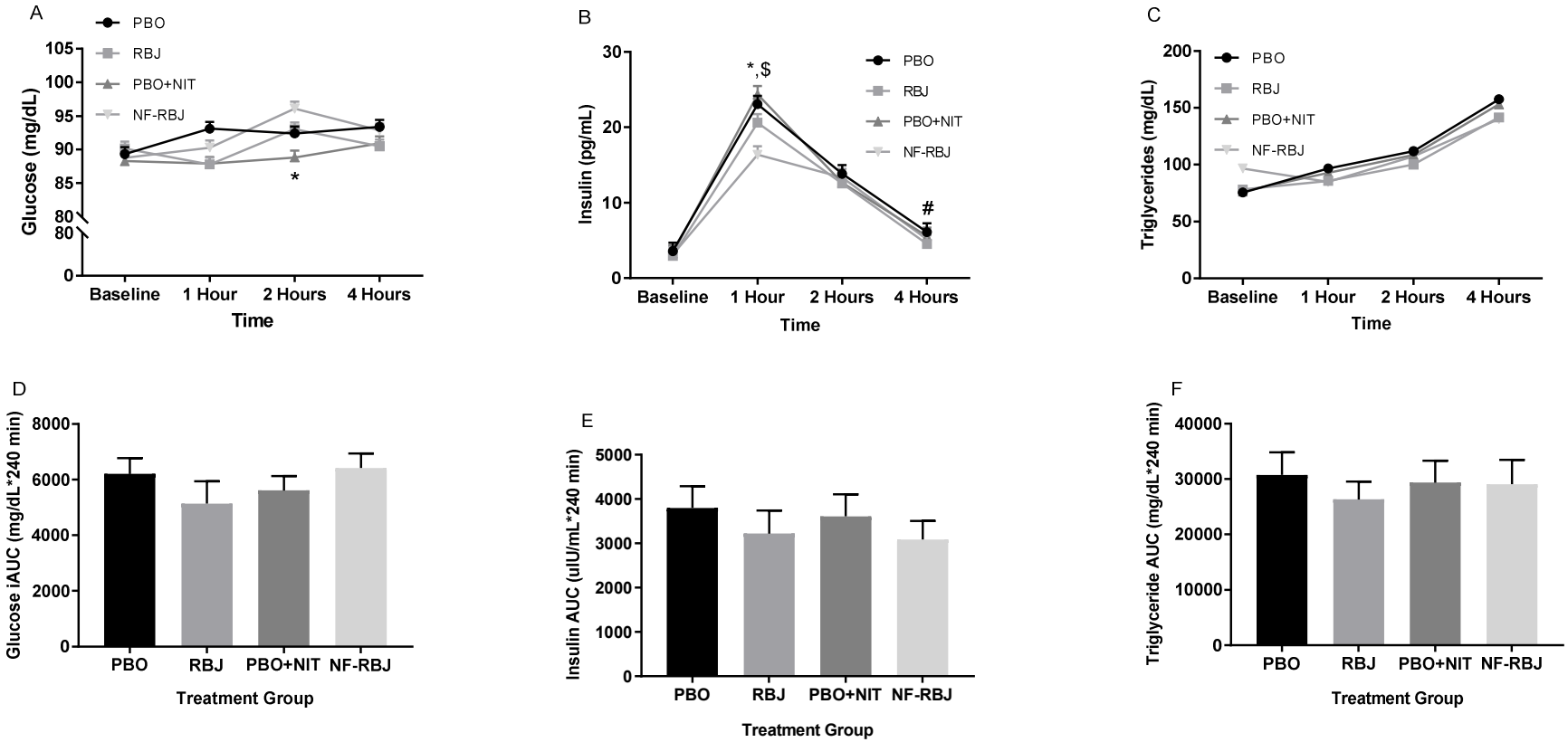


Figure 3.6 Plasma concentrations of glucose (A), insulin (B), and triglycerides (C) at baseline (0 hours), and 1, 2, and 4 hours after HFM and PBO, RBJ, PBO+NIT, and NF-RBJ treatment ingestion, and postprandial (0-240 min) glucose (D), insulin (E) and triglyceride (F) incremental area under the curve (iAUC) at the acute visit. Data in A-C are presented as log-transformed least square means ± SEM, n = 15. Data in D-F are presented as untransformed mean ± SEM. Values in A-C were compared with the use of the PROC MIXED procedure, while iAUC values in D-F were compared by use of PROC GLM procedure with Tukey's multiple comparison test in SAS version 9.4. All time points were significantly different from baseline for plasma insulin and triglycerides among all treatment groups. There were no significant main effects of time, treatment, or time*treatment for plasma glucose, whereas there was a significant main effect of time ($P < 0.0001$) and treatment ($P = 0.0386$) for plasma insulin and a significant main effect of treatment ($P = 0.0392$) for plasma triglycerides. Time points annotated with symbols represent significant time*treatment interaction between treatment groups. *PBO+NIT significantly different than NF-RBJ; $PBO+NIT significantly different than PBO; #RBJ significantly different than PBO, all $P < 0.05$. No significant differences between treatment groups at any time point for postprandial triglyceride concentrations were observed. There were no significant differences in postprandial glucose, insulin, or triglyceride AUC between treatment groups. Abbreviations: PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

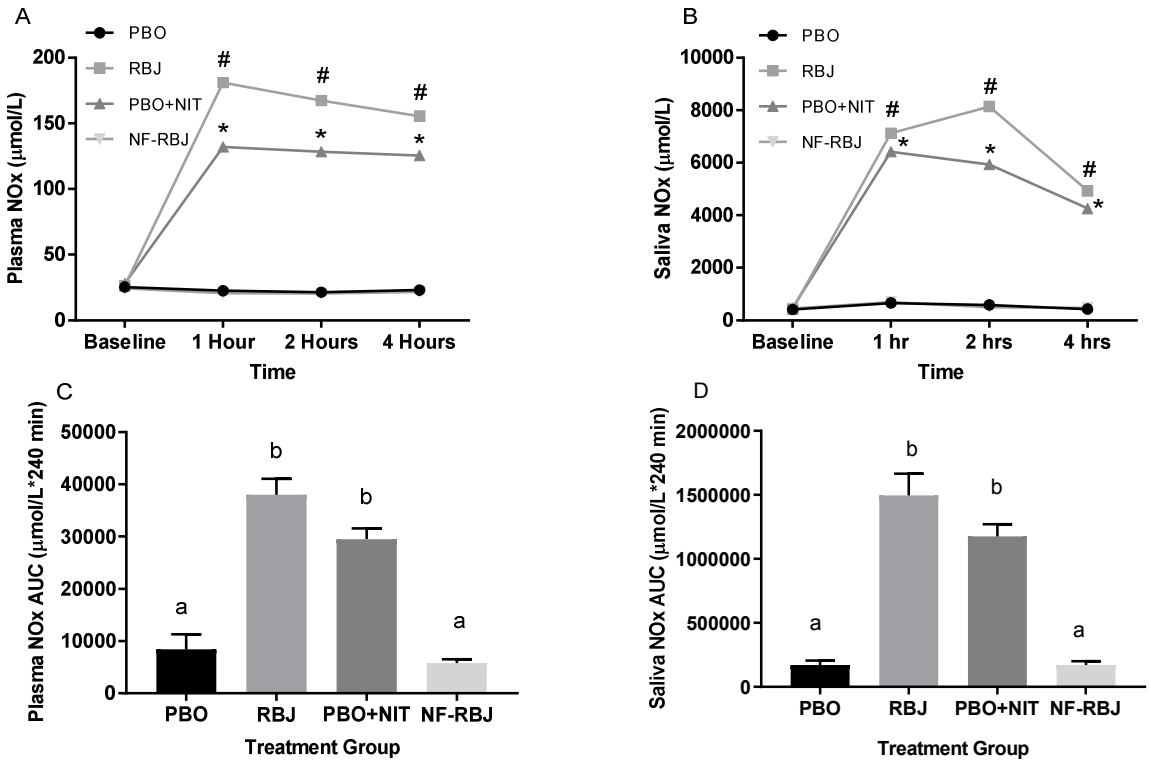


Figure 3.7 Plasma NOx concentrations (A) and saliva NOx concentrations (B) at baseline (0 hours) and 1, 2, and 4 hours after high-fat meal and PBO, RBJ, PBO+NIT, and NF-RBJ treatment ingestion, and postprandial (0-240 min) plasma NOx (C) and saliva NOx (F) area under the curve (AUC) at the acute visit. Data in A-C are presented as log-transformed least square means ± SEM, n = 15. Data in D-F are presented as untransformed mean ± SEM. Values in A-C were compared with the use of the PROC MIXED procedure, while AUC values in D-F were compared by use of the PROC GLM procedure with Tukey's multiple comparison test in SAS version 9.4. All time points were significantly different than baseline for plasma and saliva NOx concentrations in the RBJ and PBO+NIT groups. There was a significant main effect of time, treatment, and time*treatment interaction (all $P < 0.0001$) for plasma and saliva NOx in the models. Time points annotated with symbols represent significant time*treatment interactions between treatment groups. #RBJ significantly different from PBO and NF-RBJ, $P < 0.0001$. *PBO+NIT significantly different from PBO and NF-RBJ, $P < 0.0001$. Treatment groups annotated with different letters were significantly different from one another, $P < 0.05$. Abbreviations: NOx, nitrate/nitrite; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RB.

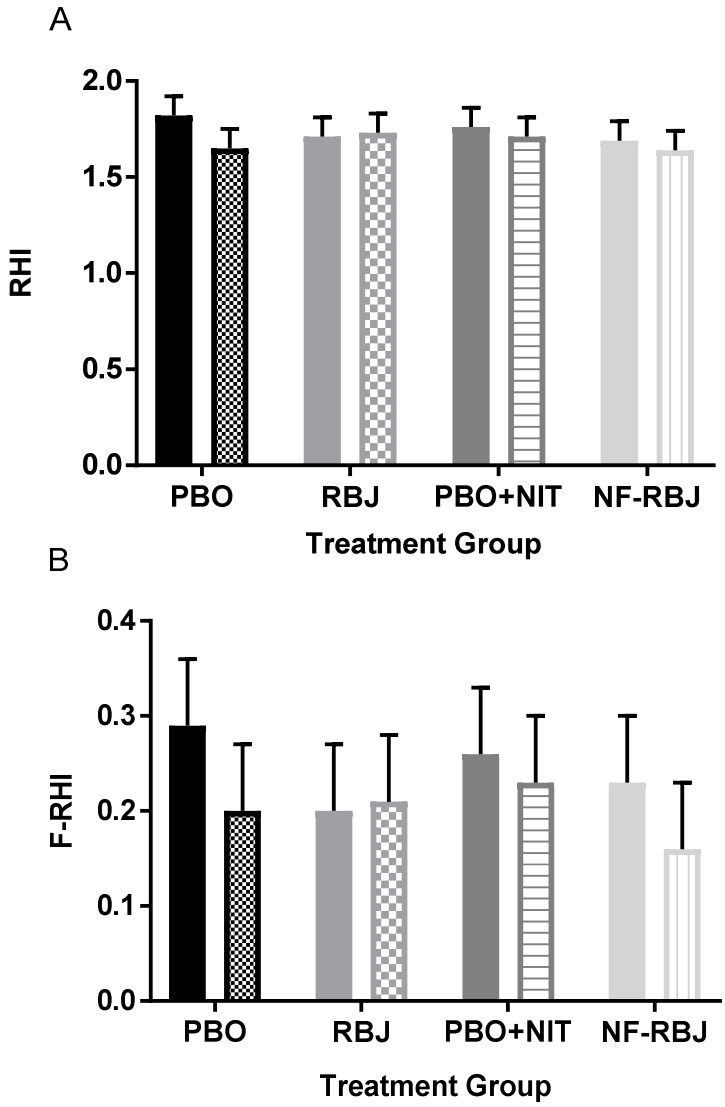


Figure 3.8 Effects of PBO, RBJ, PBO+NIT, and NF-RBJ on RHI (A) and F-RHI (B) after 4 weeks treatment ingestion at baseline (0 hours) and 4 hours after high-fat meal consumption at the chronic visit. Data are least square means ± SEM, n = 15. Means were compared with the use of the PROC MIXED procedure in SAS version 9.4. There were no significant main effects of time, treatment, or main interaction effect of time*treatment for RHI or F-RHI in the model. There were no significant differences in pre- and post-meal RHI and F-RHI values within treatment groups. Solid color = before meal (T0), and patterned color = 4 hours after meal and treatment consumption (T4). Abbreviations: RHI, reactive hyperemia index, F-RHI, Framingham-RHI; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

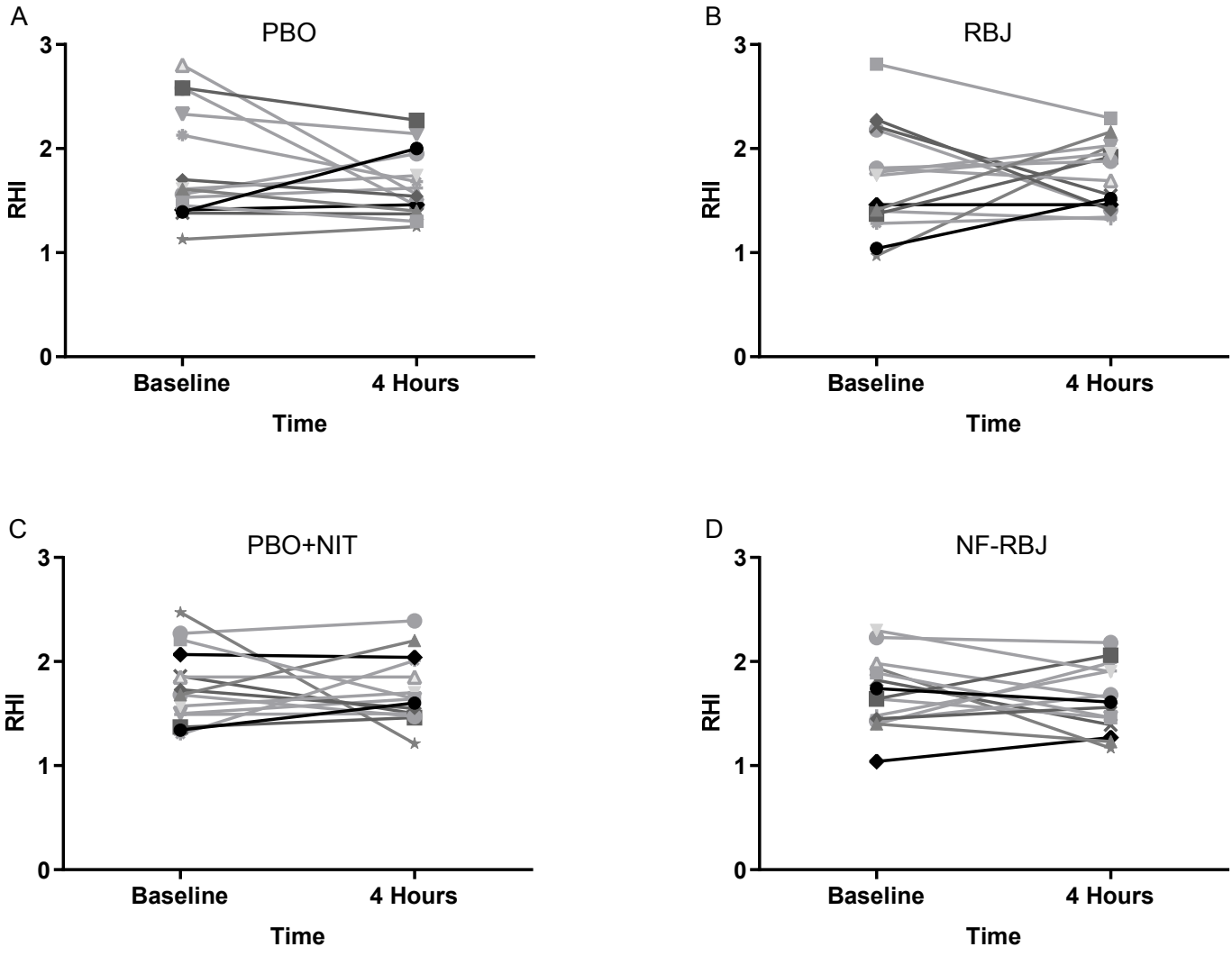


Figure 3.9 Individual effects of PBO (A), RBJ (B), PBO+NIT (C), and NF-RBJ (D) on RHI after 4 weeks treatment ingestion at baseline (0 hours) and 4 hours after high-fat meal consumption before at the chronic visit. Data are least square means ± SEM, n = 15. Values were obtained with the use of the PROC MIXED procedure in SAS version 9.4. Abbreviations: RHI, reactive hyperemia index; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

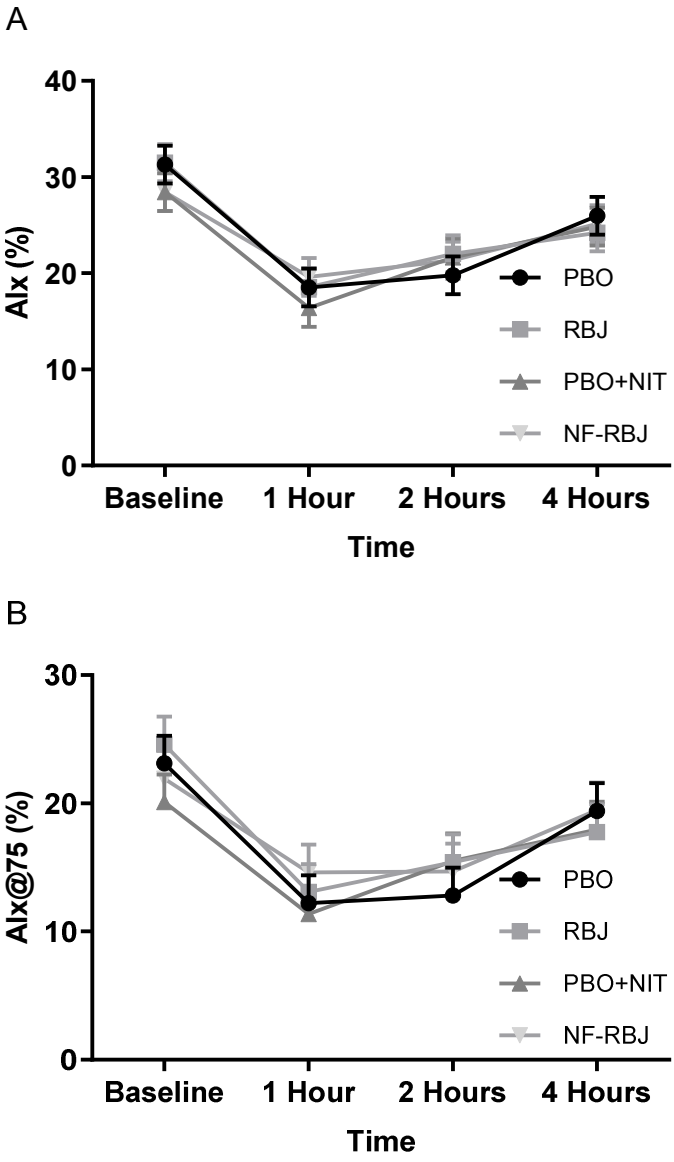


Figure 3.10 AIx (A) and Aix@75 (B) after 4 weeks PBO, RBJ, PBO+NIT, and NF-RBJ treatment ingestion at baseline (0 hours) and 1, 2, and 4 hours after high-fat meal consumption at the chronic visit. Data are least square means ± SEM, n = 15. Values were compared with the use of the PROC MIXED procedure in SAS version 9.4. For both outcomes, there were no significant main effects of time, treatment, or main interaction effect of time*treatment in the models. No significant differences between treatment groups at any time point were observed. Abbreviations: AIx, augmentation index; AIx@75, augmentation index at 75 beats per min; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

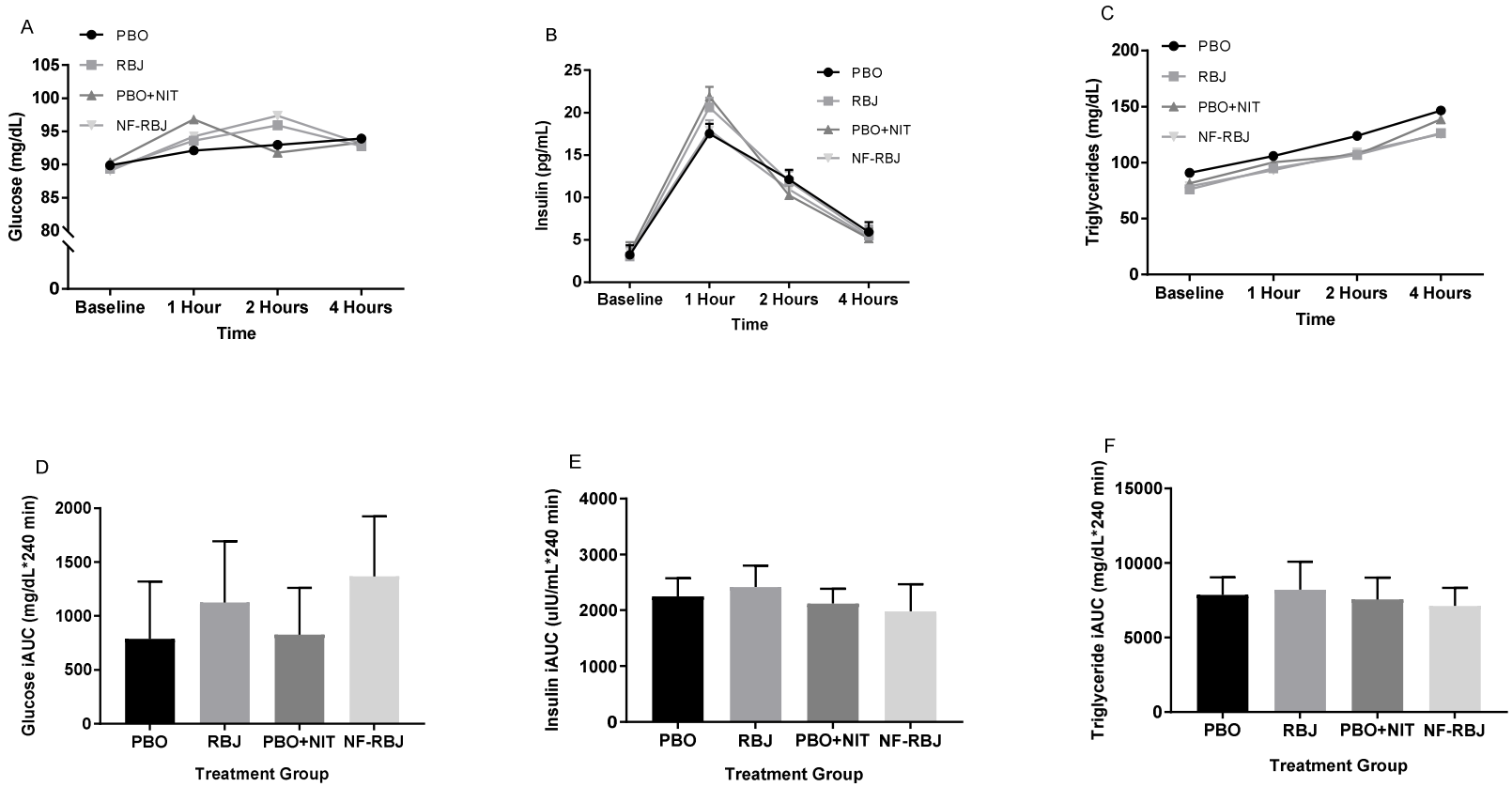


Figure 3.11 Plasma concentrations of glucose (A), insulin (B), and triglycerides (C) after 4 weeks PBO, RBJ, PBO+NIT, and NF-RBJ treatment ingestion at baseline (0 hours) and 1, 2, and 4 hours after high-fat meal consumption, and postprandial (0-240 min) glucose (D), insulin (E) and triglyceride (F) incremental area under the curve (iAUC) at the chronic visit. Data in A-C are presented as log-transformed least square means ± SEM, n = 15. Data in D-F are presented as untransformed mean ± SEM. Values in A-C were compared with the use of the PROC MIXED procedure, while iAUC values in D-F were compared by use of the PROC GLM procedure with Tukey's multiple comparison test in SAS version 9.4. All time points were significantly different from baseline for plasma insulin and triglyceride concentrations among all treatment. Groups. For plasma glucose, insulin and triglyceride responses, there were no significant main effects of time, treatment, or main interaction effect of time*treatment, as well as no significant differences among treatment groups at any time point. There were no significant differences in postprandial glucose, insulin, or triglyceride AUC between treatment groups. Abbreviations: PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

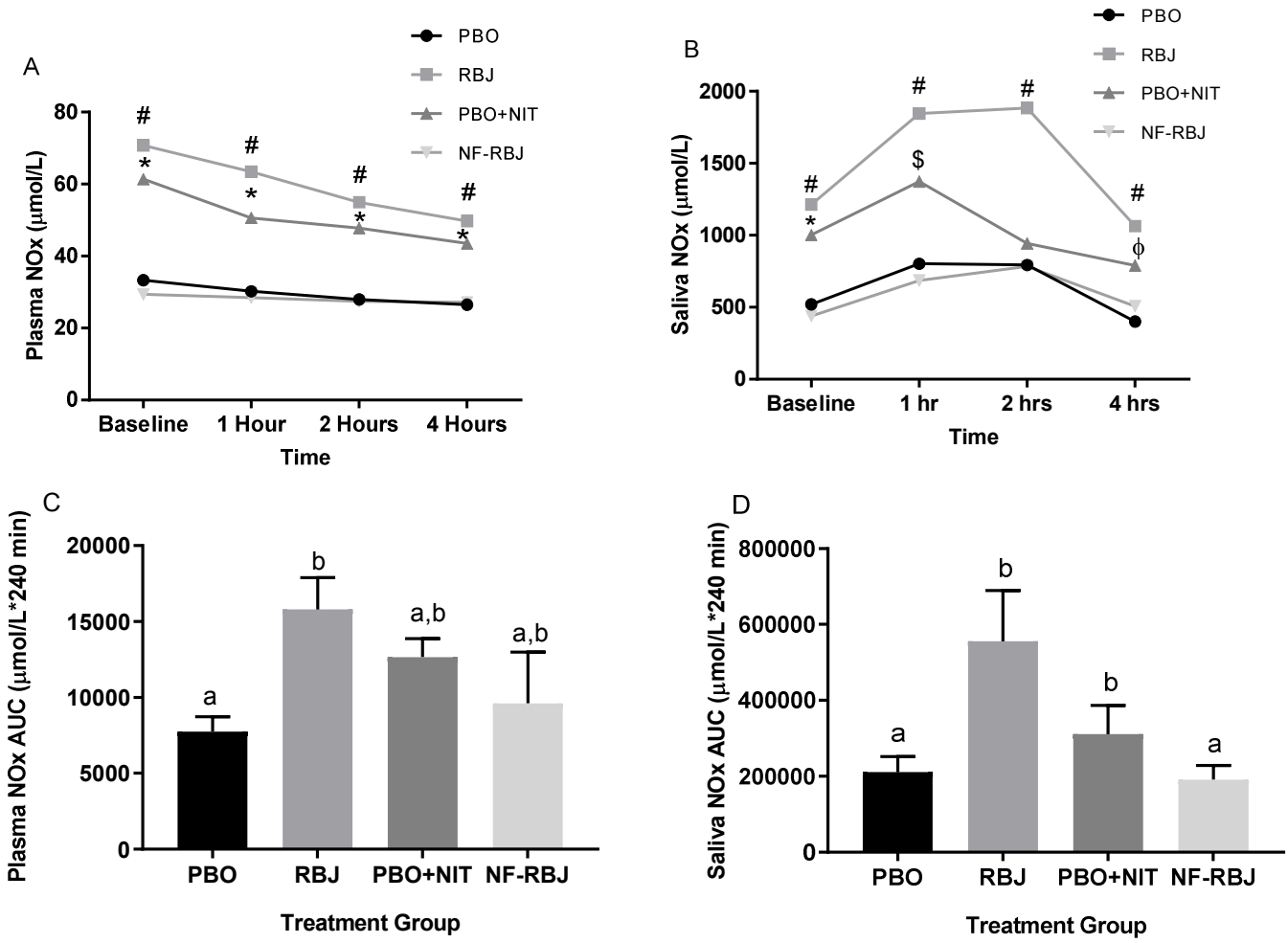


Figure 3.12 Plasma NOx concentrations (A) and saliva NOx concentrations (B) after 4 weeks PBO, RBJ, PBO+NIT, and NF-RBJ treatment ingestion at baseline (0 hours) and 1, 2, and 4 hours after high-fat meal consumption, and postprandial (0-240 min) plasma NOx (C) and saliva NOx (F) area under the curve (AUC) at the chronic visit. Data in A-C are presented as log-transformed least square means ± SEM, n = 15. Data in D-F are presented as untransformed mean ± SEM. Values in A-C were compared with the use of the PROC MIXED procedure, while AUC values in D-F were compared by use of the PROC GLM procedure with Tukey's multiple comparison test in SAS version 9.4. All time points were significantly different than baseline for plasma NOx concentrations in the RBJ and PBO+NIT groups. There was a significant main effect of time, treatment, and time*treatment interaction (all $P < 0.0001$) for plasma NOx, while there was only a significant main effect of time ($P = 0.0006$) and treatment ($P < 0.0001$) for saliva NOx in the models. Time points annotated with symbols represent significant time*treatment interactions between treatment groups. #RBJ significantly different from PBO and NF-RBJ; *PBO+NIT significantly different from PBO and NF-RBJ; $PBO+NIT significantly different than NF-RBJ; all $P < 0.01$. Treatment groups annotated with different letters were significantly different from one another, $P < 0.05$. Abbreviations: NOx, nitrate/nitrite; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

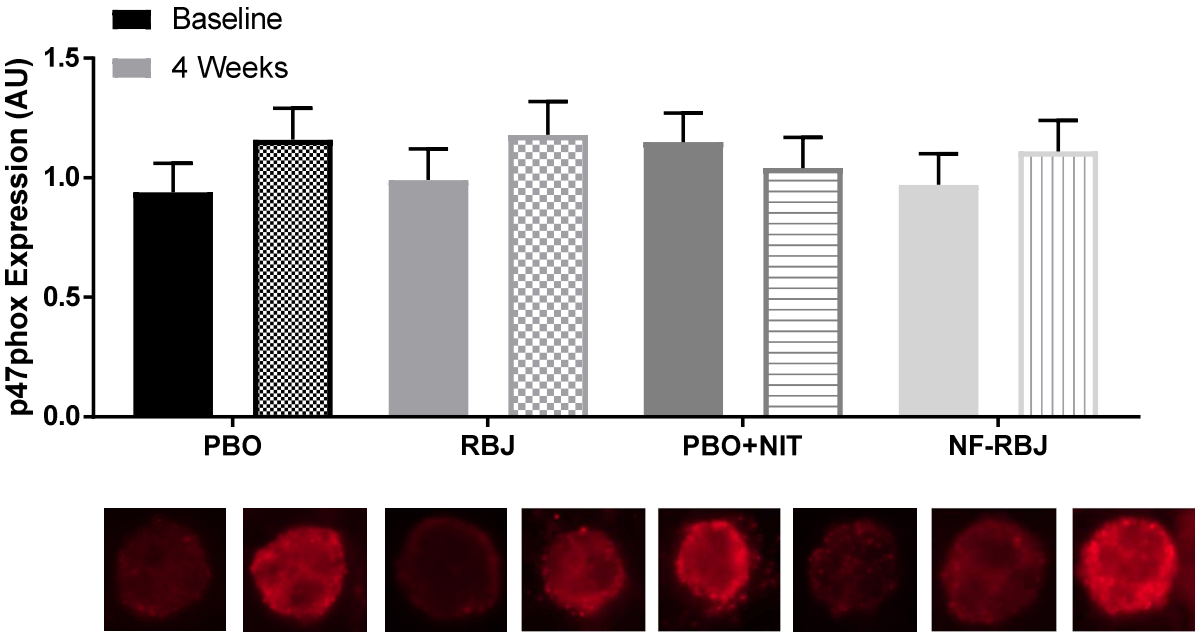


Figure 3.13 Mean endothelial cell protein expression at baseline and after 4 weeks of PBO, RBJ, PBO+NIT, and NF-RBJ consumption. Data are normalized to human umbilical vein endothelial cell protein expression via immunofluorescence. Values represent least square mean AU ± SEM, n = 15. Values were compared with the use of the PROC MIXED procedure in SAS version 9.4. There were no significant main effects of time, treatment, or main interaction effect of time*treatment in the model. There were no significant differences in endothelial protein expression of p47phox within or between treatment groups at any time point. Solid color = before treatment consumption (baseline) and patterned color = 4 weeks after treatment consumption (4 weeks). Abbreviations: AU, arbitrary units; PBO, placebo; RBJ, red beetroot juice; PBO+NIT, PBO+nitrate; NF-RBJ, nitrate-free-RBJ.

REFERENCES

1. Lakatta EG. Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part III: cellular and molecular clues to heart and arterial aging. Circulation 2003;107(3):490-7.
2. Rossman MJ, LaRocca TJ, Martens CR, Seals DR. Healthy lifestyle-based approaches for successful vascular aging. J Appl Physiol (1985) 2018;125(6):1888-900. doi: 10.1152/japplphysiol.00521.2018.
3. Seals DR, Kaplon RE, Gioscia-Ryan RA, LaRocca TJ. You're only as old as your arteries: translational strategies for preserving vascular endothelial function with aging. Physiology (Bethesda) 2014;29(4):250-64. doi: 10.1152/physiol.00059.2013.
4. Botham KM, Wheeler-Jones CP. Postprandial lipoproteins and the molecular regulation of vascular homeostasis. Prog Lipid Res 2013;52(4):446-64. doi: 10.1016/j.plipres.2013.06.001.
5. O'Keefe JH, Bell DS. Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor. Am J Cardiol 2007;100(5):899-904. doi: 10.1016/j.amjcard.2007.03.107.
6. de Koning EJ, Rabelink TJ. Endothelial function in the post-prandial state. Atheroscler Suppl 2002;3(1):11-6.
7. Herieka M, Erridge C. High-fat meal induced postprandial inflammation. Mol Nutr Food Res 2014;58(1):136-46. doi: 10.1002/mnfr.201300104.
8. Jonk AM, Houben AJ, Schaper NC, de Leeuw PW, Serne EH, Smulders YM, Stehouwer CD. Obesity is associated with impaired endothelial function in the postprandial state. Microvasc Res 2011;82(3):423-9. doi: 10.1016/j.mvr.2011.08.006.

9. Bae JH, Bassenge E, Kim KB, Kim YN, Kim KS, Lee HJ, Moon KC, Lee MS, Park KY, Schwemmer M. Postprandial hypertriglyceridemia impairs endothelial function by enhanced oxidant stress. Atherosclerosis 2001;155(2):517-23.
10. Marchesi S, Lupattelli G, Schillaci G, Pirro M, Siepi D, Roscini AR, Pasqualini L, Mannarino E. Impaired flow-mediated vasoactivity during post-prandial phase in young healthy men. Atherosclerosis 2000;153(2):397-402.
11. Ceriello A, Genovese S. Atherogenicity of postprandial hyperglycemia and lipotoxicity. Reviews in Endocrine and Metabolic Disorders 2016;17(1):111-6.
12. Kanner J, Harel S, Granit R. Betalains--a new class of dietary cationized antioxidants. J Agric Food Chem 2001;49(11):5178-85.
13. Kujawska M, Ignatowicz E, Murias M, Ewertowska M, Mikolajczyk K, Jodynis-Liebert J. Protective effect of red beetroot against carbon tetrachloride- and N-nitrosodiethylamine-induced oxidative stress in rats. J Agric Food Chem 2009;57(6):2570-5. doi: 10.1021/jf803315d.
14. Sakihama Y, Maeda M, Hashimoto M, Tahara S, Hashidoko Y. Beetroot betalain inhibits peroxynitrite-mediated tyrosine nitration and DNA strand cleavage. Free Radic Res 2012;46(1):93-9. doi: 10.3109/10715762.2011.641157.
15. Zielinska-Przyjemska M, Olejnik A, Dobrowolska-Zachwieja A, Grajek W. In vitro effects of beetroot juice and chips on oxidative metabolism and apoptosis in neutrophils from obese individuals. Phytother Res 2009;23(1):49-55. doi: 10.1002/ptr.2535.
16. Martinez RM, Longhi-Balbinot DT, Zarpelon AC, Staurengo-Ferrari L, Baracat MM, Georgetti SR, Sassonia RC, Verri WA, Jr., Casagrande R. Anti-inflammatory activity of betalain-rich dye of Beta vulgaris: effect on edema, leukocyte recruitment, superoxide anion and cytokine production. Arch Pharm Res 2015;38(4):494-504. doi: 10.1007/s12272-014-0473-7.

17. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, *et al.* Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension 2008;51(3):784-90. doi: 10.1161/HYPERTENSIONAHA.107.103523.

18. Kapil V, Khambata RS, Robertson A, Caulfield MJ, Ahluwalia A. Dietary nitrate provides sustained blood pressure lowering in hypertensive patients: a randomized, phase 2, double-blind, placebo-controlled study. Hypertension 2015;65(2):320-7. doi: 10.1161/HYPERTENSIONAHA.114.04675.

19. Hobbs DA, Goulding MG, Nguyen A, Malaver T, Walker CF, George TW, Methven L, Lovegrove JA. Acute ingestion of beetroot bread increases endothelium-independent vasodilation and lowers diastolic blood pressure in healthy men: a randomized controlled trial. J Nutr 2013;143(9):1399-405. doi: 10.3945/jn.113.175778.

20. Lara J, Ashor AW, Oggioni C, Ahluwalia A, Mathers JC, Siervo M. Effects of inorganic nitrate and beetroot supplementation on endothelial function: a systematic review and meta-analysis. Eur J Nutr 2015. doi: 10.1007/s00394-015-0872-7.

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

22. Joris PJ, Mensink RP. Beetroot juice improves in overweight and slightly obese men postprandial endothelial function after consumption of a mixed meal. Atherosclerosis 2013;231(1):78-83. doi: 10.1016/j.atherosclerosis.2013.09.001.

23. Rathod KS, Velmurugan S, Ahluwalia A. A 'green' diet-based approach to cardiovascular health? Is inorganic nitrate the answer? Mol Nutr Food Res 2016;60(1):185-202. doi: 10.1002/mnfr.201500313.

24. Clifford T, Howatson G, West DJ, Stevenson EJ. The potential benefits of red beetroot supplementation in health and disease. Nutrients 2015;7(4):2801-22. doi: 10.3390/nu7042801.

25. Bahadoran Z, Mirmiran P, Kabir A, Azizi F, Ghasemi A. The Nitrate-Independent Blood Pressure-Lowering Effect of Beetroot Juice: A Systematic Review and Meta-Analysis. Adv Nutr 2017;8(6):830-8. doi: 10.3945/an.117.016717.

26. Lundberg JO, Gladwin MT, Weitzberg E. Strategies to increase nitric oxide signalling in cardiovascular disease. Nat Rev Drug Discov 2015;14(9):623-41. doi: 10.1038/nrd4623.

27. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, *et al.* Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol 2009;5(12):865-9. doi: 10.1038/nchembio.260.

28. Rubinshtein R, Kuvin JT, Soffler M, Lennon RJ, Lavi S, Nelson RE, Pumper GM, Lerman LO, Lerman A. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. Eur Heart J 2010;31(9):1142-8. doi: 10.1093/eurheartj/ehq010.

29. Bonetti PO, Pumper GM, Higano ST, Holmes DR, Jr., Kuvin JT, Lerman A. Noninvasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. J Am Coll Cardiol 2004;44(11):2137-41. doi: 10.1016/j.jacc.2004.08.062.

30. McCrea CE, Skulas-Ray AC, Chow M, West SG. Test-retest reliability of pulse amplitude tonometry measures of vascular endothelial function: implications for clinical trial design. Vasc Med 2012;17(1):29-36. doi: 10.1177/1358863X11433188.

31. Brant LC, Barreto SM, Passos VM, Ribeiro AL. Reproducibility of peripheral arterial tonometry for the assessment of endothelial function in adults. J Hypertens 2013;31(10):1984-90. doi: 10.1097/HJH.0b013e328362d913.

32. Hamburg NM, Keyes MJ, Larson MG, Vasan RS, Schnabel R, Pryde MM, Mitchell GF, Sheffy J, Vita JA, Benjamin EJ. Cross-sectional relations of digital vascular function to cardiovascular risk factors in the Framingham Heart Study. Circulation 2008;117(19):2467-74. doi: 10.1161/CIRCULATIONAHA.107.748574.

33. Wilkinson IB, MacCallum H, Flint L, Cockcroft JR, Newby DE, Webb DJ. The influence of heart rate on augmentation index and central arterial pressure in humans. J Physiol 2000;525 Pt 1:263-70.

34. Stoner L, Faulkner J, Lowe A, Lambrick DM, Young JM, Love R, Rowlands DS. Should the augmentation index be normalized to heart rate? Journal of Atherosclerosis and Thrombosis 2014;21(1):11-6.

35. Hwang MH, Yoo JK, Kim HK, Hwang CL, Mackay K, Hemstreet O, Nichols WW, Christou DD. Validity and reliability of aortic pulse wave velocity and augmentation index determined by the new cuff-based SphygmoCor Xcel. J Hum Hypertens 2014;28(8):475-81. doi: 10.1038/jhh.2013.144.

36. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 2002;32(6):1372-4, 6, 8-9.

37. Donato AJ, Black AD, Jablonski KL, Gano LB, Seals DR. Aging is associated with greater nuclear NF kappa B, reduced I kappa B alpha, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans. Aging Cell 2008;7(6):805-12. doi: 10.1111/j.1474-9726.2008.00438.x.

38. Donato AJ, Gano LB, Eskurza I, Silver AE, Gates PE, Jablonski K, Seals DR. Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. Am J Physiol Heart Circ Physiol 2009;297(1):H425-32. doi: 10.1152/ajpheart.00689.2008.

39. Silver AE, Christou DD, Donato AJ, Beske SD, Moreau KL, Magerko KA, Seals DR. Protein expression in vascular endothelial cells obtained from human peripheral arteries and veins. J Vasc Res 2010;47(1):1-8. doi: 10.1159/000231715.

40. Obeid JS, McGraw CA, Minor BL, Conde JG, Pawluk R, Lin M, Wang J, Banks SR, Hemphill SA, Taylor R, *et al.* Procurement of shared data instruments for Research Electronic Data Capture (REDCap). J Biomed Inform 2013;46(2):259-65. doi: 10.1016/j.jbi.2012.10.006.

41. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. J Biomed Inform 2009;42(2):377-81. doi: 10.1016/j.jbi.2008.08.010.

42. Ayer JG, Harmer JA, Steinbeck K, Celermajer DS. Postprandial vascular reactivity in obese and normal weight young adults. Obesity (Silver Spring) 2010;18(5):945-51. doi: 10.1038/oby.2009.331.

43. Schwander F, Kopf-Bolanz KA, Buri C, Portmann R, Egger L, Chollet M, McTernan PG, Piya MK, Gijs MA, Vionnet N, *et al.* A dose-response strategy reveals differences between normal-weight and obese men in their metabolic and inflammatory responses to a high-fat meal. J Nutr 2014;144(10):1517-23. doi: 10.3945/jn.114.193565.

44. Berry SE, Tucker S, Banerji R, Jiang B, Chowienczyk PJ, Charles SM, Sanders TA. Impaired postprandial endothelial function depends on the type of fat consumed by healthy men. The Journal of nutrition 2008;138(10):1910-4.

45. Steer P, Sarabi DM, Karlström B, Samar B, Berne C, Vessby B, Lars L. The effect of a mixed meal on endothelium-dependent vasodilation is dependent on fat content in healthy humans. Clinical Science 2003;105(1):81-7.

46. Nicholls SJ, Lundman P, Harmer JA, Cutri B, Griffiths KA, Rye K-A, Barter PJ, Celermajer DS. Consumption of saturated fat impairs the anti-inflammatory properties of

high-density lipoproteins and endothelial function. Journal of the American College of Cardiology 2006;48(4):715-20.

47. Raitakari OT, Lai N, Griffiths K, McCredie R, Sullivan D, Celermajer DS. Enhanced peripheral vasodilation in humans after a fatty meal. Journal of the American College of Cardiology 2000;36(2):417-22.

48. Dow CA, Stauffer BL, Greiner JJ, DeSouza CA. Influence of habitual high dietary fat intake on endothelium-dependent vasodilation. Applied Physiology, Nutrition, and Metabolism 2015;40(7):711-5.

49. Lara J, Ashor AW, Oggioni C, Ahluwalia A, Mathers JC, Siervo M. Effects of inorganic nitrate and beetroot supplementation on endothelial function: a systematic review and meta-analysis. Eur J Nutr 2016;55(2):451-9. doi: 10.1007/s00394-015-0872-7.

50. Bonilla Ocampo DA, Paipilla AF, Marin E, Vargas-Molina S, Petro JL, Perez-Idarraga A. Dietary Nitrate from Beetroot Juice for Hypertension: A Systematic Review. Biomolecules 2018;8(4). doi: 10.3390/biom8040134.

51. Munzel T, Daiber A. Inorganic nitrite and nitrate in cardiovascular therapy: A better alternative to organic nitrates as nitric oxide donors? Vascul Pharmacol 2018;102:1-10. doi: 10.1016/j.vph.2017.11.003.

52. Lidder S, Webb AJ. Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway. British journal of clinical pharmacology 2013;75(3):677-96.

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

54. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, *et al.* Inorganic nitrate supplementation

lowers blood pressure in humans: role for nitrite-derived NO. Hypertension 2010;56(2):274-81. doi: 10.1161/HYPERTENSIONAHA.110.153536.

55. Kenjale AA, Ham KL, Stabler T, Robbins JL, Johnson JL, Vanbruggen M, Privette G, Yim E, Kraus WE, Allen JD. Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. J Appl Physiol (1985) 2011;110(6):1582-91. doi: 10.1152/japplphysiol.00071.2011.

56. Ahuja KD, Robertson IK, Ball MJ. Acute effects of food on postprandial blood pressure and measures of arterial stiffness in healthy humans. The American journal of clinical nutrition 2009;90(2):298-303.

57. Greenfield JR, Samaras K, Chisholm DJ, Campbell LV. Effect of postprandial insulinemia and insulin resistance on measurement of arterial stiffness (augmentation index). International journal of cardiology 2007;114(1):50-6.

58. Phillips L, Peake J, Zhang X, Hickman I, Kolade O, Sacre J, Huang B, Simpson P, Li S, Whitehead J. The effect of a high-fat meal on postprandial arterial stiffness in men with obesity and type 2 diabetes. The Journal of Clinical Endocrinology & Metabolism 2010;95(9):4455-9.

59. Wilkinson IB, MacCallum H, Flint L, Cockcroft JR, Newby DE, Webb DJ. The influence of heart rate on augmentation index and central arterial pressure in humans. The Journal of physiology 2000;525(1):263-70.

60. Sies H, Stahl W, Sevanian A. Nutritional, dietary and postprandial oxidative stress. J Nutr 2005;135(5):969-72. doi: 10.1093/jn/135.5.969.

61. Rangel-Zuniga OA, Haro C, Perez-Martinez P, Delgado-Lista J, Marin C, Quintana-Navarro GM, Tinahones FJ, Malagon MM, Lopez-Segura F, Lopez-Miranda J, *et al.* Effect of frying oils on the postprandial endoplasmic reticulum stress in obese people. Mol Nutr Food Res 2014;58(11):2239-42. doi: 10.1002/mnfr.201400401.

62. Vors C, Pineau G, Drai J, Meugnier E, Pesenti S, Laville M, Laugerette F, Malpuech-Brugere C, Vidal H, Michalski MC. Postprandial Endotoxemia Linked With Chylomicrons and Lipopolysaccharides Handling in Obese Versus Lean Men: A Lipid Dose-Effect Trial. J Clin Endocrinol Metab 2015;100(9):3427-35. doi: 10.1210/JC.2015-2518.

63. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: role of oxidative stress. Circulation 2002;106(16):2067-72.

64. Zeevi D, Korem T, Zmora N, Israeli D, Rothschild D, Weinberger A, Ben-Yacov O, Lador D, Avnit-Sagi T, Lotan-Pompan M. Personalized nutrition by prediction of glycemic responses. Cell 2015;163(5):1079-94.

CHAPTER 4: ACUTE AND CHRONIC EFFECTS OF RED BEETROOT JUICE AND INORGANIC NITRATE SUPPLEMENTATION ON ORAL NITRATE-REDUCING BACTERIA AND THEIR RELATIONSHIP WITH NITRIC OXIDE METABOLITES IN MIDDLE-AGED/OLDER ADULTS WITH OVERWEIGHT AND OBESITY

Summary

Background: Dietary inorganic nitrate from foods such as red beetroot juice (RBJ) can contribute to nitric oxide (NO) bioavailability through the enterosalivary nitrate-nitrite-NO pathway. A critical step in this pathway is the reduction of nitrate to nitrite by oral nitrate-reducing bacteria. *Objective:* We investigated whether acute and chronic inorganic nitrate supplementation, either in the form of RBJ or potassium nitrate (PBO+NIT), would influence the oral microbiota, and the relationship of the oral microbiota with postprandial circulating NO metabolites and digital vascular endothelial function. *Methods:* We measured the abundance of oral nitrate-reducing bacteria in saliva samples from 15 overweight/obese, middle-aged/older adults using 16 rRNA sequencing, and observed for 4 hours (after acute and chronic supplementation) the physiological responses to dietary nitrate via measurement of saliva and plasma nitrate/nitrite (NOx), plasma nitrite levels, and digital reactive hyperemia index (RHI). *Results:* A significant decrease in the alpha diversity metric, Pileou's Evenness, was detected after 4 weeks of PBO+NIT (0.69 ± 0.05 at baseline vs. 0.65 ± 0.05 at 4 weeks, respectively; $P < 0.05$, while the RBJ group was trending for a significant decline (0.69 ± 0.05 at the acute visit vs. 0.65 ± 0.05 at the chronic visit; $P = 0.08$). No significant differences in the abundance of nitrate-reducing bacteria were observed after chronic supplementation with any of the treatment groups, although abundance of the species *Neisseria subflava* was trending toward a significant increase after chronic supplementation in the RBJ group (10.8% at the acute visit vs. 12.2% at the chronic visit; $P = 0.07$). Plasma and saliva NOx increased from baseline and remained

elevated for the 4-hour testing period after acute and chronic RBJ and PBO+NIT supplementation (all $P < 0.05$), while plasma nitrite only peaked at two hours in the RBJ group after acute supplementation and was significantly higher than PBO+NIT group ($P < 0.01$). No significant correlations between total abundance of nitrate-reducing bacteria and peak change in plasma nitrite or plasma and saliva NOx were observed, though several significant correlations between individual species and these parameters were observed. A significant positive correlation between RHI change from baseline (T0 to T4) and total abundance of nitrate-reducing species was observed after chronic RBJ supplementation ($r = 0.5$; $P = 0.05$). *Conclusions:* Acute and chronic RBJ and PBO+NIT supplementation increases NO metabolites and may alter the oral microbiome to favorably effect vascular endothelial function in overweight/obese, middle-aged and older adults. Further research is needed to evaluate the potential of RBJ as an oral microbiota targeted therapy for improving NO bioavailability and overall vascular health.

Introduction

Nitric oxide (NO) plays a central role in the maintenance of vascular homeostasis and integrity and is thus vital for cardiovascular health.[1]-In the vasculature, NO has a variety of crucial functions including regulating vascular tone and blood pressure by promoting endothelium-dependent vasodilation.[2] The two main sources of NO in the vasculature include the traditional endogenous synthesis of NO via the L-arginine-endothelial NO synthase (eNOS) pathway, and through the diet via the enterosalivary nitrate-nitrite-NO pathway.[1] The traditional pathway for endogenous NO production can become dysfunctional with age and in the presence of CVD risk factors, ultimately leading to a reduction in NO production and bioavailability, thereby disrupting vascular homeostasis.[3,4] The enterosalivary nitrate-nitrite-NO pathway has been suggested to provide an alternative source of generating NO at times when

endogenous NO production and bioavailability are compromised, and thus represents a therapeutic target for improving cardiovascular health.[5]

Once ingested, dietary nitrate can initially be reduced to nitrite in the mouth during mastication by oral commensal facultative anaerobic bacteria to nitrite; however this is minimal and most of the nitrate is absorbed in the intestine (~100%) and subsequently released into circulation. About 25% of this circulating nitrate is then sequestered by the salivary glands via the actions of sialin, an active nitrate-transporter.[6,7] Once in the saliva, nitrate is converted to nitrite by oral facultative anaerobic bacteria that reside in the crypts on the dorsal surface of the tongue and possess nitrate-reducing enzymes that mammalian cells lack. Salivary nitrite is then swallowed and can be further reduced to NO, either enzymatically or non-enzymatically under certain physiological conditions such as low oxygen or low pH, or nitrite can be stored in the blood and tissues for when endogenous synthesis of NO via eNOS is limited/disturbed.[5]

This pathway is referred to as enterosalivary circulation of nitrate, and underpins the vasodilatory effects seen after intake of dietary inorganic nitrate.[5,8,9] Sources of inorganic nitrate include root vegetables such beetroot, and green leafy vegetables such as arugula and spinach.[9] It has been proposed that increased inorganic nitrate intake either through foods such as beetroot or inorganic nitrate salts may alter oral bacterial composition and confer greater vascular responses (e.g., enhanced nitrate reduction, greater plasma nitrite concentrations, and greater reductions in blood pressure).[10–12] However, current clinical findings remain equivocal due to some studies reporting no change in vascular responses to red beetroot juice/inorganic nitrate supplementation, despite favorable changes in the oral microbiota and circulating NO metabolites.[11,12] The lack of vascular responsiveness has been attributed to study durations, red beetroot juice doses used, oral baseline health, and healthy subject populations.[10–12]

Currently no studies have explored the influence of inorganic nitrate on the oral microbiota and physiologic responses to inorganic nitrate in overweight/obese, middle-aged and older adults, a population known to have an increased risk for CVD. Therefore, the primary

objective of this study was to investigate the relationship between the abundance of nitrate-reducing oral bacteria, NO metabolites, and postprandial endothelial function following acute and chronic inorganic nitrate supplementation (i.e. red beetroot juice and potassium nitrate) in middle-aged/older men and postmenopausal women with overweight or obesity. A secondary objective was to explore possible sex differences. We hypothesized that abundance of known oral nitrate-reducing bacteria would be related to NO metabolite concentrations in saliva and plasma. We also hypothesized that the abundance of oral nitrate-reducing bacteria would increase following chronic inorganic nitrate supplementation, and that these changes would be associated with greater levels of NO metabolites in saliva and plasma. We also investigated the influence of nitrate-free red beetroot juice on oral nitrate-reducing bacteria and circulating NO metabolites, as red beetroot juice contains other bioactive compounds (e.g., polyphenols, ascorbic acid) known to enhance nitrite reduction and independently increase NO production and bioavailability.

Methods

Study Population

A total of fifteen middle-aged/older (mean ± SEM: age 53 ± 2 yrs; age range 42-65 yrs) men and postmenopausal women with a BMI (kg/m^2) between 25 and 39.9 participated in this clinical trial. Participant characteristics, separated by sex, are shown in **Table 4.1**. Inclusion and exclusion criteria are provided in greater detail in Chapter 2.[13]

Study Design and Intervention

As this is a secondary analysis of a clinical trial, the study design and interventions remain the same and can be found in Chapter 2.-[13]

Blood and Saliva Collection

Venous blood (total of ~10 mL) was collected via an intravenous catheter, and immediately deposited into vacutainers with EDTA (BD Vacutainer, Plymouth, UK) for plasma separation, centrifuged according to the manufacturers' instructions, aliquoted, and stored at -80°C until analysis. Using the passive drool method, saliva was collected directly into 2 mL cryovials using a saliva collection aid (SalivaBio, Inc., Salvimetrics, Carlsbad, CA, USA) and stored at -80°C until analysis. The saliva samples collected at each time point (total of 4 time points; hours 0, 1, 2 and 4) were pooled for analysis of the oral microbiome.

Nitrate and Nitrite Analysis

Given the difficulty in measuring NO directly, plasma and saliva nitrate/nitrite and nitrite concentrations are used to assess the efficacy of enterosalivary conversion of dietary nitrate and are established surrogates for the bioavailability of NO.[14–16] Plasma samples were filtered using 30 kDa molecular weight cut-off filters (Millipore Sigma) to reduce the presence of hemoglobin prior to nitrate/nitrite and nitrite analysis. Plasma and saliva nitrate/nitrite and nitrite concentrations were measured using commercially available colorimetric assay kits according to the manufacturers' instructions (Cayman Chemical, Ann Arbor, Michigan, USA).

Oral Bacteria – 16s rRNA Gene Data Analysis

DNA from saliva samples were extracted using the FastDNA® Kit (MP Biomedicals, #116540400) following the written protocol provided by the manufacturer. Negative extraction controls, following the same protocol as the saliva samples, were ran to account for user-error or extraction-kit impurity error. Amplification of the V4 16S rA region through qPCR was completed utilizing the Earth Microbiome Project protocol and the 515F-806R primer set (forward: 5'GTGYCAGCMGCCGCGGTAA 3'; reverse 5' GGACTACNVGGGTWTCTAAT 3') {Caporaso, 2012 #3574}. Unique 12bp error correcting barcodes were incorporated in the build of the forward primer. Cycling conditions using the Biorad CFX96 thermal cycler were as follows: 94°C for 3min and then 35 cycles of 94°C 45s, 50°C 60s, 72°C 90s followed by 72°C for

10min. Paired-end sequencing libraries of the V4 region were then created by purifying amplicons utilizing AmPure beads and quantifying and pooling equimolar ratios of each sample library. The pooled library was quantified by qPCR and sequenced on an Illumina MiSeq at the Next-Generation Sequencing Facility at Colorado State University.

Following qPCR the forward reads, reverse reads, and barcodes were imported into QIIME2 (version 2019.4) for downstream analysis. The sequence reads were demultiplexed with QIIME2's '*qiime demux'* plugin to examine the overall sequence quality. Forward and reverse reads were paired for each sample using the '*qiime dada2'* plugin. Sequences were trimmed to 210 base pairs for the forward reads and 230 base pairs for the reverse reads based on a Phred score of 30, to preserve the highest quality sequences for downstream analysis. A feature table was generated using a taxonomic assignment based on the GreenGenes version 13.8-reference database by training a classifier with the '*qiime feature-classifier*' plugin. Sequences were filtered to remove any undesired mitochondrial or chloroplast DNA from the dataset using the *'qiime feature-table'* plugin. Phylogenetic trees were created using the '*qiime phylogeny'* plugin using the FastTree2 method.

Alpha diversity was analyzed using phylogenetic and non-phylogenetic metrics through the '*qiime diversity'* plugin. Evaluations of phylogenetic diversity was evaluated with Faith's Phylogenetic Diversity Index and species evenness was evaluated with Pileou's Index of Evenness. In addition, both richness and evenness in samples was determined using the alpha diversity Shannon's Index.

Vascular Endothelial Function Assessed by Digital Reactive Hyperemia Index (RHI)

The measurement of the increase in digital pulse wave amplitude via peripheral arterial tonometry (PAT) during temporary reactive hyperemia (relative to baseline pulse wave amplitude) is widely used as a measurement of NO-mediated endothelium-dependent dilation.[17]

Digital reactive hyperemia index (RHI) was assessed via PAT using the EndoPAT® 2000 device. Methods for PAT are previously described in Chapter 2.[13]

Statistical Analysis

SAS 9.4 Software (SAS Institute, Cary, North Carolina, USA), was used for all statistical analyses, except for the non-parametric correlations which were analyzed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, USA). The distributions of data were evaluated with Shapiro Wilk test (PROC UNIVARIATE, SAS) and non-parametric tests were used for data that were not normally distributed. A linear mixed model (PROC MIXED, SAS) was used to assess the main effects of time (baseline and 4-week) and treatment (PBO, RBJ, PBO+NIT, NF-RBJ) and time x treatment interaction effects for plasma and salivary NOx and plasma nitrite. Time, treatment and time*treatment interaction were set at fixed effects, while subject and treatment order were set as random effects. Data for the mixed model are presented as least squares mean ± SEMs. AUC plasma and saliva NOx and plasma nitrite concentrations were calculated using the trapezoidal rule. Differences in NOx and nitrite AUC between treatment groups were assessed using a one-way repeated measures ANOVA (PROC GLM, SAS).

The association between the abundance of nitrate-reducing bacteria genera and species and peak delta values of plasma and saliva levels of NOx, plasma nitrite, and corresponding AUC were analyzed using Spearman's rank correlations. Peak delta values were calculated as the highest change from baseline. The association between abundance of nitrate-reducing bacteria genera and species and reactive hyperemia index (RHI) change from baseline were calculated using the same method.

Differences in bacterial abundances (genus and species) from baseline (acute visit) to 4-weeks (chronic visit), as well as sex differences, were assessed using a linear mixed model (PROC MIXED, SAS) with the same fixed and random effects as stated above. Within treatment group (baseline vs. 4-week) in bacterial diversity and evenness indices were assessed using

Kruskal Wallis in the qiime software. A mixed, repeated measures model of two-way ANOVA (sex and time) was used to compare differences between bacterial diversity and evenness indices within treatment groups. Data for all analyses (except PROC MIXED) are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted as $P < 0.05$.

Results

Global Changes in the Oral Microbiota Pre- to Post-Supplementation

Alpha and beta diversity metrics pre- and post-supplementation are shown in **Table 4.2**. No baseline differences in the alpha and diversity metrics were observed among treatment groups. No statistically significant within-group differences in Faith's Phylogenetic Diversity index (measure of taxa richness) pre- to post-supplementation were observed among treatment groups, though the RBJ group was trending toward a significant decline from baseline in Faith's Phylogenetic Diversity Index at 4-weeks (9.59 ± 1.08 vs. 9.19 ± 1.25; $P = 0.08$). A statistically significant decrease in Pileou's Index of Evenness (measure of species evenness) was observed in the PBO+NIT group from baseline to 4-weeks (0.69 ± 0.05 vs. 0.65 ± 0.05, respectively; $P < 0.05$). The RBJ group was trending toward a significant decline from baseline in Pileou's Index of Evenness at 4-weeks relative to baseline (0.69 ± 0.05 vs. 0.65 ± 0.05, respectively; $P = 0.06$). No statistical difference in Shannon's Index (a measure of species diversity and evenness) in the RBJ group were observed from baseline to 4-weeks, though the RBJ group was approaching a statistically significant decline from baseline in Shannon's Index at 4-weeks (4.74 ± 0.44 vs. 4.41 ± 0.45; $P = 0.06$).

Sex differences in the bacterial indices were also explored and are shown in **Table 4.2**. There was a baseline sex difference in Pileou's Index of Evenness in the PBO+NIT group

(Females: 0.66 ± 0.05 vs. Males: 0.72 ± 0.03; $P = 0.02$). No other significant baseline sex differences for the remaining treatment groups were observed. In males, there was a significant reduction in Pileou's Evenness from baseline to 4-weeks in the PBO+NIT group (0.72 ± 0.03 vs. 0.67 ± 0.05; $P < 0.05$). In females, there was a significant reduction in Shannon's Index from baseline to 4-weeks in the PBO+NIT group (4.63 ± 0.55 vs. 4.36 ± 0.51; $P < 0.05$). No other statistically significant sex differences were observed; however, all three bacterial indices in the RBJ group were trending toward statistically significant reductions in males, but not females, at 4 weeks compared to baseline (**Table 4.2**).

Abundance of Oral Nitrate-reducing Bacteria after Acute and Chronic Supplementation

A total of 13 genera previously implicated in nitrate reduction was detected in saliva samples of each treatment group at both acute and chronic visits. The relative abundance of these genera presented in **Tables 4.3 and 4.4** and **Figure 4.1**. The most abundant genera (>1% relative abundance) among all treatment groups at both visits were *Prevotella, Veillonella, Haemophilus, Neisseria, Fusobacterium, Pophyromonas, Rothia, Leptotrichia,* and *Granulicatella.* Specifically, at the acute visit, *Prevotella* was the first most abundant genera in all treatment groups, while *Veillonella* was the second most abundant genus in the PBO, PBO+NIT, and NF-RBJ with *Neisseria* being the second most abundant in the RBJ group. At the chronic visit, *Prevotella* remained the top most abundant genus for all treatment groups, while *Neisseria* was the second most abundant genus in the PBO, RBJ and PBO+NIT groups with *Veillonella* being the second most abundant genus in the NF-RBJ group. There were no statistically significant between group differences for any of the genera detected at the acute or chronic visit. Similarly, there were no significant within group differences in genera abundance from the acute visit to the chronic visit. As such, no significant main effects of time, treatment or time*treatment interactions were observed. Sex differences in the total relative abundance of nitrate-reducing genera per treatment group and visit were also explored and are presented in

Figure 4.2. When separated by sex, no statistically significant differences either within or between sexes, in genera abundance from the acute to chronic visit was observed for any of the treatment groups.

At the species level, we detected 8 bacterial nitrate-reducing species that have previously been identified as having a nitrate reduction gene.[18,19] Relative abundance of these species per treatment group at the acute and chronic visits are presented in **Tables 4.5 and 4.6**, respectively. The most prevalent species (>1% relative abundance) among all treatment groups at the both visits include *Prevotella melaninogenica, Veillonella parvula, Veillonella dispar, Rothia mucilaginosa, and Rothia dentocariosa* and are shown in **Figure 4.3**. No statistically significant within group differences in species abundance were observed from the acute to chronic visits, and as such, there were no significant main effects of time, treatment or time*treatment interactions. Sex differences in the relative abundance of oral nitrate-reducing species per treatment group and visit were explored and are shown in **Figure 4.4**. No statistically significant differences, either within or between sexes, in the nitrate-reducing species from the acute to chronic visit were observed in any of the treatment groups.

Total abundance of nitrate-reducing species (i.e., sum of all nitrate-reducing species) was analyzed per treatment group and no statistical difference was observed in the total abundance of nitrate-reducing species from the acute to chronic visit within any of the treatment groups. Sex differences in the total abundance of nitrate-reducing species was also explored and no statistically significant differences in total nitrate-reducing species between sexes was observed for any of the treatment groups (data not shown).

Plasma and Saliva NOx levels and Plasma Nitrite Levels after Acute and Chronic Supplementation

Results for postprandial plasma and saliva NOx levels and AUC at the acute visit and the chronic visit can be found in Chapter 2.[13] At the acute visit, there was a significant main

effect of treatment (P = 0.0002) for plasma nitrite. No significant main effects of time or time*treatment were observed. Baseline differences in plasma nitrite levels between the PBO+NIT and NF-RBJ groups were observed (371.4 ± 95.6 vs. 539.1 ± 95.6, P < 0.05, **Figure 4.5A**). The PBO+NIT group exhibited the lowest levels of plasma nitrite levels compared to the other 3 groups across the 4-hour sampling period. At the 1, 2 and 4 hr time points, plasma nitrite levels in the PBO+NIT group were significantly lower than the NF-RBJ group (P < 0.05 for all timepoints; **Figure 4.5A**). At the 2 hr time point, plasma nitrite levels peaked in the RBJ group and were significantly higher than PBO+NIT (P < 0.01; **Figure 4.5A**). No other between group differences were observed at any timepoint. No significant between group differences were observed for plasma nitrite AUC at the acute visit (**Figure 4.5C).**

At the chronic visit, there was a significant main effect of treatment (P < 0.0001) for plasma nitrite, while no significant main effects of time or time*treatment were observed. Baseline differences in plasma nitrite were observed between the PBO and PBO+NIT group, (526.4 vs. 381.0; P < 0.05, respectively), while RBJ was approaching statistical difference compared to the PBO at baseline (526.4 vs. 396.2; P = 0.06; **Figure 4.5B).** Again, the PBO+NIT group exhibited the lowest levels of plasma nitrite levels compared to the other 3 groups across the 4-hour sampling period with the exception of similar levels in the RBJ group at the 1 hour timepoint. The PBO group had the highest plasma nitrite levels, which was significantly different than the PBO+NIT group at the 2 and 4 hr time point (P < 0.05; **Figure 4.5B**). No significant between group differences were observed for plasma nitrite AUC at the chronic visit (**Figure 4.5C).** Sex differences in plasma nitrite and plasma and saliva NOx concentrations at both the acute and chronic test visits are shown in **Supplemental Figures 4S.1 and 4S.2 in Appendix 2.**

Relationship between the Abundance of Nitrate-Reducing Bacteria Species on Plasma and Saliva NOx and Plasma Nitrite Levels

No statistically significant relationships were identified with the correlation analysis of the total (i.e., sum of) nitrate-reducing species (those identified in **Table 4.5 and 4.6**) and the peak delta change in plasma nitrite and plasma and saliva NOx at either the acute or chronic visit (shown in **Supplemental Figures 4S.3-8 in Appendix 2**). Additionally, no significant correlations between total species abundance and AUC for plasma nitrite and plasma and saliva NOx were observed (data not shown). Correlations for plasma nitrite and plasma and saliva NOx AUC with individual bacteria species are presented in **Table 4.7**.

At the individual species level, correlation analyses revealed several statistically significant correlations, of which those are reported. At the acute visit, a positive relationship between the change in saliva NOx and the abundance of *Haemophilus parainfluenzae* (r = 0.6; *P* = 0.02; **Figure 4.6A**) was observed in the PBO group, as well as a positive relationship between the change in saliva NOx and abundance of *Rothia dentocariosa* in the PBO+NIT group (r = 0.5; *P* = 0.05; **Figure 4.6B**). At the chronic visit, an inverse relationship between the change in plasma NOx and *Rothia mucilaginosa* was observed in the PBO group (r = -0.56; *P* = 0.03**; Figure 4.7A**), along with a positive correlation with change in plasma NOx and the abundance of *Veillonella dispar* (r = 0.56; *P* = 0.03; **Figure 4.7B**). A negative correlation between the abundance of *Veillonella parvula* and the change in plasma nitrite was observed in the RBJ group (r = -0.68; *P* = 0.02**; Figure 4.7C**), as well as a negative correlation between abundance of *Prevotella melaninogenica* and change in plasma NOx in the PBO+NIT group (r = 0.57; *P* < 0.03; **Figure 4.7D**).

Sex Differences in the Relationship between Abundance of Nitrate-Reducing Bacteria Species on Plasma Nitrite Levels

Sex differences in bacterial abundance and plasma nitrite levels were explored to see if there were varying responses among males and females, and only plasma nitrite responses were explored as it is the delivery source of NO.[14,20] At the acute visit, an inverse relationship

between the total abundance of nitrate-reducing species and change in plasma nitrite was observed in both females (r = -0.9, *P* = 0.005; **Figure 4.8A**) males (r = -0.9; *P* = 0.007, **Figure 4.9B**) in the PBO+NIT group. At both the acute and chronic visits, total abundance of nitrate-reducing species was positively related to change in plasma nitrite levels in males only in the PBO group (r = 0.8; *P* = 0.05 vs. r = 0.99; *P* = 0.0008; **Figure 4.9A and 4.10A**, respectively). No significant correlations between total nitrate species and change in plasma nitrite were observed for females at the chronic visit (shown in **Figure 4.11**).

At the species level, two significant correlations were observed in females, and one significant correlation was observed in males. In females, the abundance of *Prevotella melaninogenica* was inversely correlated with change in plasma nitrite in the PBO+NIT group (r = -0.9; *P* = 0.01, **Figure 4.12A**), whereas at the chronic visit, abundance of *Veillonella dispar* was inversely related to change in plasma nitrite in the RBJ group (r = -0.9; *P* = 0.009, **Figure 4.12B**). In males, the abundance of *Prevotella melaninogenica* was positively associated with change in plasma nitrite in the PBO group at the acute visit (r = 0.9; *P* = 0.02, **Figure 4.13**)

Impact of Nitrate-Reducing Bacteria Species on Digital Reactive Hyperemia Index (RHI)

The impact of total nitrate-reducing species on digital vascular response to reactive hyperemia (assessed via EndoPAT) was explored as this test is used as means assessing of endothelial function and NO bioavailability.[17] No significant correlations between total nitrate-reducing bacteria and RHI were observed at the acute visit (data not shown). At the chronic visit, total nitrate-reducing species was positively correlated with RHI change from baseline in the RBJ group (r = 0.05; *P* = 0.05; **Figure 4.14B**). No other significant correlations were observed for the remaining treatment groups. Sex differences in digital hyperemic response and relationship with total nitrate-reducing species were also evaluated; however, no significant correlations were observed (data not shown).

Discussion

As expected, this study demonstrates that acute and chronic red beetroot juice/inorganic nitrate supplementation in middle-aged/older adults with overweight or obesity increased circulating levels of NO metabolites and resulted in detectable alterations in the oral microbiota, and specifically, oral nitrate-reducing bacteria previously implicated in the enterosalivary nitrate-nitrite-NO pathway.[18,19] In this study, we provide descriptive data at both the genus and species level in a population that has yet to be explored. To the best of our knowledge, this is the second longest intervention with RBJ to date.

Impact of Acute and Chronic Red Beetroot Juice and Inorganic Nitrate Supplementation on the Oral Microbiota

Alpha diversity metrics for all bacterial genera and species detected in our samples per treatment group are shown in **Table 4.1** No baseline differences for all three of the Alpha diversity metrics were detected for any of the treatment groups, indicating that no carry-over effects were observed, and our washout period of 4-weeks was effective. We observed a significant decline in Pileou's Index of Evenness in the PBO+NIT group from baseline to 4-weeks. This change in evenness may have been driven by slight, but non-significant, changes in nitrate-reducing genera that occurred within each treatment group from the acute to chronic visit. There were no significant changes in Shannon's Index observed among the treatment groups indicating that the treatment interventions did not alter community evenness and richness of the bacterial species. This is in concordance with other studies,[10,11,21] also observing no change in Shannon's Index after acute (7 and 10 days) of RBJ supplementation in healthy adults. It should be noted that in the present study, the RBJ group was trending toward a significant decline in Shannon's Index from baseline to 4-weeks (i.e., acute to chronic visit; P = 0.06). The lack of statistical significance may likely due to uneven oral microbiota samples in the RBJ group (n = 12 at week 0 and n = 15 at week 4), and we speculate that with an even sample

size, the change in Shannon's Index from baseline to 4-weeks would have been statistically significant. In line with this, Pileou's Index of Evenness and Faith's Phylogenetic Diversity Index from baseline to 4-weeks in the RBJ group was also approaching statistical significance (P = 0.08 and 0.09, respectively) which, again, may have been met with an even biological sample size. These alpha diversity changes in the RBJ may be driven by slight attenuations in nitrate-reducing genera and species (some increases and some decreases, but not of statistical significance) following chronic supplementation. Additionally, changes in other bacterial genera and species not implicated in nitrate-reduction may also be a contributing factor.

When exploring sex differences within each treatment group in the three Alpha diversity metrics, a significant decrease in Pileou's Eveness Index from baseline to 4-weeks was observed only in males in the PBO+NIT group, which may due to shifts, albeit non-significant, in nitrate-reducing genera from the acute to chronic visit (shown in **Figure 4.2C**). Additionally, in the PBO+NIT group, a significant decrease in Shannon's index from baseline to 4-weeks was observed only in females, which may also be driven by (non-significant) shifts in nitrate-reducing genera from the acute to chronic visit (**Figure 4.2C**). This suggests that females responded differently to the PBO+NIT treatment and experienced changes in both evenness and richness of oral bacterial communities, while males only experienced changes in species evenness.

Impact of Acute and Chronic Red Beetroot Juice and Inorganic Nitrate Supplementation on Oral Nitrate-Reducing Bacteria

At the genus level, all genera detected in our samples have been previously implicated in nitrate reduction,[18,19] and surprisingly, had similar relative abundance levels across all four treatment groups at both time points. As such, no statistically significant differences in any of the genera were detected after acute and chronic supplementation, which is contrary to previous studies.[10–12,21,22] It should be noted that the relative of abundance of *Neisseria* was approaching a statistically significant increase from the acute to chronic visit in the RBJ group (P = 0.07).

This, again, may have reached statistical significance if there were even samples between the two timepoints as previously mentioned. There have been previous reports of significant increases in *Neisseria* and *Rothia* in saliva samples after short-term (10 days) and long-term (6 weeks) of RBJ supplementation in young and old healthy adults and hypercholesterolemic patients, respectively.[10,22]

Likewise, in the RBJ group, the relative abundance of all nitrate-reducing genera detected (total of 8) after acute and chronic RBJ supplementation are consistent with that seen of others using a RBJ intervention.[10,11,11,21] Previous research suggests that *Veillonella* is the most abundant and main contributing genus in nitrate reduction.[18,19] However, in the present study, *Prevotella* was found to the most prevalent genus with abundance levels almost twice that of *Veillonella* in the RBJ, PBO+NIT and NF-RBJ groups at both visits (not statistically significant). This dissimilarity has also been shown by others after acute RBJ supplementation (single dose of 140 mL; ~12.4 mmol nitrate) in young, healthy adults.[12,21] Though direct comparison with these studies cannot be made as oral bacteria was collected directly from the tongue dorsum and populations differ (young healthy adults vs. middle-aged/older adults with overweight or obesity).

After chronic (4-week daily) consumption of RBJ and PBO+NIT, mean relative abundance of *Prevotella and Veillonella* decreased, albeit non-significantly, by 5 and 3% (respectively) in the RBJ group and 1.1 and 1.3% (respectively) in the PBO+NIT group from the acute to the chronic visit. These decreases are supported by Vanhatalo *et al.*[10] who reported significant decreases in *Prevotella* (~60%) and *Veillonella* (~65%) in saliva samples from both young (18-22 years) and old (70-79 years) healthy adults following 10 days of RBJ supplementation (140 mL/d; ~12.4 mmol/d nitrate), and compared to 10 days of an equal volume NF-RBJ supplement. Our findings also agree with a study by Burleigh *et al.*[11] who detected significant reductions in the abundance of *Prevotella* in tongue scrape samples from young healthy males after 7 days of RBJ supplementation (140 mL/d; ~12.2 mmol/d nitrate) with

no change in *Prevotella* seen after 7 days of NF-RBJ supplementation, also of equal volume. These reductions in *Prevotella* following RBJ supplementation can be attributed to changes in the pH of the oral cavity.[18] Salivary nitrite, especially in excess, can be acidified to NO under low pH conditions which often occurs in the mouth when bacterial species ferment dietary carbohydrates resulting in the production of strong acid byproducts.[18,23] *In vitro* studies have shown that NO formed from acidification of nitrite exerts bactericidal effects and inhibits oral bacterial acid formation, which would decrease the amount of acid in the mouth, and thus increase oral pH which is known to halt/limit the growth of acidogenic bacteria.[24] This, of course, is all dependent on proper enterosalivary circulation of inorganic nitrate, and an individuals' oral health status. Thus, it is logical to postulate that the observed reductions in *Prevotella* (which is acidogenic) after intake of RBJ/inorganic nitrate is due to increased acidification of nitrite to NO resulting in increased oral pH levels and subsequent reductions in acidogenic bacteria abundance. However, this is speculation as oral pH was not measured in the present study. In support of this, the study by Burleigh *et al.*[11] which had similar reductions in *Prevotella*, also demonstrated increased saliva pH (7.13 to 7.39) after just one week of RBJ supplementation.[11] In a similar, but separate study, saliva pH also increased (7.0 to 7.5) after 2 weeks of RBJ supplementation (100 mL/d; 400 mg/d nitrate) in young healthy adults (18-35 years).[25] These studies suggest that RBJ may have beneficial effects on oral health including anti-cariogenic properties and demonstrate the need to assess saliva pH concurrently with changes in the oral bacterial community after RBJ/inorganic nitrate supplementation.

In line with the genera abundance, the species *Prevotella melaninogenica and Veillonella parvula* were the first and second most abundant in all treatment groups at both the acute and chronic visit. *Prevotella melaninogenica* has been reported by others to be the top most abundant nitrate-reducing species followed by *Veillonella dispar,*[12,21] which surprisingly was our fourth most abundant species with abundance of ~1% in each treatment group at both visits. *Rothia muciliginosa* was detected as our third most abundant species across treatment

groups. All of the species detected in our samples are of comparable abundance with that reported by studies of similar work.[10–12,21,22] As expected, the abundance of *Prevotella and Veillonella* species decreased (non-significantly) from the acute to chronic visit in our active nitrate treatment groups. Reductions in *Prevotella melaninogenica* after short-term (7-day) RBJ supplementation have been reported by others[11] and this decline may be due to changes in saliva/oral pH following RBJ supplementation as this cariogenic species thrives in a lower pH.[26] There was a non-significant increase in the abundance of *Prevotella melaninogenica* in the NF-RBJ group, which could also be attributed to changes in saliva/oral pH as others have demonstrated reductions in saliva pH following 7 days of NF-RBJ supplementation, potentially creating an optimal environment for *Prevotella melaninogenica* to propagate.[11] However, this remains purely speculative at this time.

In the RBJ group, the relative abundance of *Rothia mucilaginosa* increased by 1% (non-significantly) from the acute to chronic visit, and nevertheless, increases in this species have been reported by others after long-term (6-weeks) consumption of RBJ compared to NF-RBJ.[22] A surprising finding in the present study is that *Neisseria subflava* was not one of the most abundant species, especially in the RBJ group, given that the abundance of *Neisseria* at the genus level was approaching a statistically significant difference after 4 weeks of supplementation. The relative abundance for this species remained at <0.1% in the RBJ group at both visits, indicating that a different *Neisseria* species (not implicated in nitrate-reduction) was contributing to the overall genus abundance levels. This is contrary to most studies reporting substantial increases in this species post-RBJ supplementation,[10,12,21,22] with one of these studies reporting an unexpected increase in *Neisseria subflava* in the NF-RBJ group after 7 days of supplementation suggesting that this species may be responding to the other bioactive compounds in RBJ.[11] In the present study after 4-weeks of NF-RBJ, *Neisseria subflava* was diminished/undetectable.

The present data suggest that acute and chronic supplementation with RBJ and inorganic nitrate may impact alpha-diversity of the oral microbiome; however, definitive conclusions cannot be made at this time and in this population as the magnitude of treatment-related change in the relative abundance of genera and species implicated in nitrate-reduction was not statistically significant. This may be due to intra-individual variability as an individual's overall oral microbiota composition can drift over both short and long periods of time,[27] and specifically the abundance of genera and species implicated in nitrate-reduction can also profoundly vary within person.[21] Despite this variability, a core microbiome has been identified and includes *Streptococcus, Veillonella, Granulicatella, Neisseria, Haemophilus, Corynebacterium, Rothia, Actinomyces, Prevotella, Capnocytophaga, Porphyromonas,* and *Fusobacterium.*[27,28] This may be why in the present study genera and species abundance were similar across treatment groups.

Impact of Acute and Chronic Red Beetroot Juice and Inorganic Nitrate Supplementation on Plasma and Saliva NOx and Plasma Nitrite Levels

As expected, plasma and saliva NOx levels were significantly elevated after acute and chronic RBJ and PBO+NIT supplementation, compared to nitrate-free controls (PBO and NF-RBJ). Unexpectedly, plasma nitrite levels did not follow the same trend. At the acute visit, the PBO+NIT group had the lowest nitrite levels. At the two hour time point, plasma nitrite levels in the RBJ group peaked and were significantly higher than all other treatment groups, which is consistent with other studies demonstrating peaks around 2-3 hours after a single dose of RBJ.[8,29–33] After 4-weeks of consumption, we expected plasma nitrite levels in the RBJ and PBO+NIT levels to be higher than what was measured at the acute visit and also to be elevated compared to the nitrate-free treatment groups. However, at the chronic visit, the PBO group exhibited the highest plasma nitrite levels, and the PBO+NIT group exhibited the lowest levels of plasma nitrite levels with RBJ having comparable levels to that PBO+NIT. It is not apparent

why plasma nitrite levels were not elevated following chronic RBJ and PBO+NIT supplementation. This is contrary to what has been demonstrated in previous long-term RBJ supplementation studies.[22] However, it has been speculated by Burleigh *et al*.[23] that excess plasma nitrite may be excreted via the kidneys, suggesting that there may be a threshold for circulating nitrite in which excess nitrite is excreted to prevent excessive drops in blood pressure. Future studies that include urine analysis of nitrite levels could test this posit. It could also be purported that nitrite is being more efficiently converted to NO, which could be confirmed with direct measurement of NO via electron paramagnetic resonance (EPR) techniques, though this is typically not feasible in human studies due to the short half-life of NO.[24–26]

Given the crucial role of oral nitrate-reducing bacteria in the nitrate-nitrite-NO pathway, it was hypothesized that abundance of these bacteria would be positively associated with circulating NO metabolites following RBJ and inorganic nitrate supplementation. Contrarily, the total abundance of nitrate-reducing species was not associated with peak change in plasma nitrite and NOx or saliva NOx after acute or chronic RBJ or PBO+NIT supplementation in the present study. This is in agreement with Burleigh *et al*.[12] who also demonstrated that the abundance of nitrate-reducing bacteria is not associated with the change in plasma nitrite, nor did having a high (>50%) overall abundance of nitrate-reducing bacteria result in an enhanced plasma nitrite response compared to those with low (<50%) overall abundance. Additionally, a recent systematic review indicated that the influence of oral bacteria on plasma nitrite is unclear, with studies either reporting positive, negative or no correlation at all between oral nitrate-reducing bacteria and changes in plasma nitrite.[35] The review highlighted that the relationship between oral bacteria and salivary nitrite is unclear. Likewise, Burleigh *et al.*[12] demonstrated that the total abundance of nitrate-reducing species is positively related to peak increases in salivary nitrite levels and having higher abundance of these bacteria results in earlier increases of nitrite in the saliva suggesting a faster rate of reduction of nitrate to nitrite compared to those

with lower abundance levels. Unfortunately, we were unable to measure salivary nitrite in the present study and thus are limited in our ability to make conclusions.

Significant correlations with individual species abundance and peak changes in NO metabolites after acute and chronic RBJ and PBO+NIT supplementation were observed. These correlations lend insight into the role that abundance of these species may have in nitrate-reduction following RBJ and PBO+NIT supplementation; however, it should be acknowledged that these correlations were of "moderate" strength (r = 0.5-0.6) and also do not necessarily imply "cause and effect". It has been suggested that metabolic activity of these species, rather than abundance, has a greater influence on nitrate reduction and thus NO metabolite bioavailability. We cannot determine in the present study whether abundance of these species is related to a greater capacity to generate salivary nitrite as nitrite levels in saliva was not measured and we do not have nitrate-only data in plasma and saliva, thus we are limited in our ability to draw conclusions. Aside from oral-nitrate reducing bacteria, other factors that play a role in nitrate reduction and can affect NO metabolite bioavailability include abundance and activity of gut microbiota, gastric emptying and absorption rates, stomach pH, availability of sialin (active nitrate transporter in saliva/salivary glands) and salivary flow rates.[6,7,36,37]

Impact of Changes in the Oral Microbiome on Vascular Function Following Acute and Chronic RBJ and Inorganic Nitrate Supplementation

It was hypothesized in this study that an increased abundance of nitrate-reducing bacteria would increase NO metabolites after acute and chronic RBJ and inorganic nitrate supplementation, which would then enhance vascular responses to dietary nitrate. A positive, linear relationship with the total abundance of nitrate-reducing species and RHI change from baseline (T0 to T4) was observed in the RBJ group at the chronic visit. This suggests that potential alterations to the oral microbiota following chronic RBJ supplementation might enhance vascular function/responsiveness to dietary nitrate. This may be due to increases in

NO bioavailability resulting from a heightened ability of the bacteria to reduce nitrate to nitrite after chronic RBJ supplementation. Only one other study has explored this, but did not demonstrate enhanced vascular function (as measured by brachial artery FMD) after noticeable changes in nitrate-reducing bacteria abundance following 7 days of RBJ supplementation.[11] It should be mentioned that the effects of RBJ and inorganic nitrate on vascular endothelial function have been suggested to be reduced in older adults and in those with greater cardiometabolic risk, so our results, which are correlative in nature, should be interpreted with caution.[38]

Limitations

Although findings in the present study seem to be in broad agreement with others of similar work,[10–12,21,22] comparisons with previous studies are difficult to make due to study population and methodological dissimilarities. For instance, sequencing platforms differ across studies, as well as oral bacteria sampling sites (tongue vs. saliva). Study populations vary including heterogenous groups of healthy young men (21-44 years), healthy men and women (27-34 years), hypercholesterolemic patients, or separate groups of healthy young (18-22 years) and older (70-79 years) adults. Additionally, doses of red beetroot juice with varying nitrate content are used (either 140 mL with ~12.4 mmol nitrate or 250 mL with ~6 mmol nitrate), and exposure time varies (either acute/single does or 7 days, 10 days or 6 weeks).

The present study presents its own methodological considerations. Bacterial analyses were performed using saliva samples, and although saliva samples represent a composite of bacteria from all oral cavity sites, it has been suggested that bacteria from saliva is less metabolically active compared to bacteria collected from the dorsal surface of the tongue. Scrapes of the tongue dorsum have been shown to have the highest nitrate-reduction capacity than all other oral cavity sites. Total nitrate and nitrite in plasma and saliva was analyzed and used as a means of assessing the circulating NO pool. Separation of nitrate and nitrite in saliva

may have yielded different results and limits our ability to draw conclusions as we do not know if saliva nitrate reduction to nitrite was evident and/or enhanced. We also inferred oral nitrate reduction from plasma nitrite. It has been suggested that the enterosalivary circulation of nitrate is disrupted when spitting out saliva over the course of a few hours, [27], which may be why we did not observe a rise in plasma nitrite (but not nitrate) concentration in our active nitrate groups. Additionally, the RBJ and nitrate dose used in this study (70 mL, ~300 mg) is considerably lower than that used in studies of similar design and objectives[10–12,21,22] and we may have seen similar results to those studies if a larger dose containing more nitrate was used. We cannot rule out inter-individual variability in oral bacteria and NO metabolites as profound differences in these have been shown following acute RBJ consumption. Additionally, oral health status of our study participants was not determined/collected, and thus they may have been in good oral health meaning that their microbiota was already capable of efficient nitrate-reduction and may not be sensitive to manipulation with dietary supplementation. We also did not ask participants to limit nitrate-rich foods in their diet as the oral microbiota is highly responsive to dietary stimuli and may be why the placebo group had comparable abundance levels to the active nitrate groups. However, we did restrict teeth brushing and use of mouthwash on testing days and restricted the use of mouthwash for the entire duration of the study (8 months), but we do not know if participants were compliant with this restriction despite verbal statements confirming such. It should be noted that the population studied may be less responsive to RBJ as aging and increased cardiometabolic risk (i.e. overweight and obesity) has been associated with a reduced capacity to convert inorganic nitrate into nitrite.[34]

Conclusion

The present study demonstrates that acute and chronic RBJ and inorganic nitrate supplementation increases circulating NO metabolites and may alter oral microbiota composition in overweight/obese, middle-aged and older adults. We demonstrated differences

in the alpha-diversity of the oral microbiota following chronic RBJ and inorganic nitrate supplementation. We showed that *Prevotella, Veillonella, Haemophilus, and Neisseria* were the top most abundant nitrate-reducing genera and that *Prevotella melaninogenica, Veillonella parvula and Rothia dentocariosa* were the most abundant nitrate-reducing species after acute and chronic RBJ and inorganic nitrate supplementation. Contrary to other studies, we did not detect any relationship between total nitrate reducing species and peak change in circulating NO metabolites. We did, however, demonstrate that total nitrate reducing species was positively related to vascular responsiveness/function following chronic RBJ supplementation. Further research in different populations including those of greater cardiovascular risk and those with oral dysbiosis should be conducted to explore the potential of RBJ as an oral-microbiota targeted therapy for improving NO bioavailability and overall vascular health.

Tables and Figures

Table 4.1 Screening participant characteristics by sex

	Male (n = 7)	**Female (n = 8)**
Age	53.4 ± 7.6	54 ± 8
Years postmenopausal	-	5 ± 3
BMI, kg/m^2	29.8 ± 3.3	29.92 ± 3.4
Waist-to-hip ratio	0.9 ± 0.1	0.9 ± 0.1
Total cholesterol, mg/dL	202 ± 44	203 ± 45
HDL, mg/dL	59 ± 16	59 ± 17
LDL, mg/dL	130 ± 30	132 ± 32
Triglycerides, mg/dL	113 ± 62	115 ± 63
HDL:LDL ratio	0.5 ± 0.2	0.5 ± 0.2
Hemoglobin A1c, %	5.3 ± 0.3	5.3 ± 0.3
HOMA-IR	0.6 ± 0.4	0.9 ± 0.4
SBP, mmHg	121 ± 11	120 ± 11
DBP, mmHg	79 ± 10	79 ± 10
RHI*	1.63 ± 0.3	1.83 ± 0.4
PWV, m/s	7.2 ± 1.5	7.2 ± 1.5

Table 4.2 Pre- and post-supplementation bacterial diversity and evenness metrics after each treatment period and sex differences among treatment periods.

	Faith's Phylogenetic Diversity Index		Pileou's Index of Evenness		Shannon's Index	
Tx	**Acute**	**Chronic**	**Acute**	**Chronic**	**Acute**	**Chronic**
PBO	9.51 ±1.79	9.74 ±1.45	0.66 ±0.06	0.68 ±0.04	5.53 ± 0.47	4.72 ± 0.35
RBJ	9.59 ±1.08	9.19 ±1.25[€]	0.69 ±0.05	0.65 ±0.05[£]	4.74 ± 0.44	4.41 ± 0.45[¥]
PBO+NIT	9.40 ±1.42	9.29 ±1.30	0.69 ±0.05	0.65 ±0.05*	4.71 ± 0.47	4.41 ± 0.52
NF-RBJ	9.91 ±1.53	9.82 ±1.52	0.67 ±0.09	0.69 ±0.05	4.63 ± 0.77	4.66 ± 0.48
Sex:Tx						
F:PBO	9.88 ±2.22	9.84 ±1.74	0.67 ± 0.07	0.68± 0.05	4.59 ± 0.48	4.71 ± 0.4
M:PBO	9.08 ±1.17	9.62 ±1.08	0.66 ± 0.04	0.70 ± 0.04	4.46 ± 0.48	4.73 ± 0.28
F:RBJ	9.60 ±1.17	9.21 ±1.64	0.66 ± 0.05	0.62 ± 0.05	4.56 ± 0.43	4.26 ± 0.18
M:RBJ	9.57 ±1.07	9.16 ±0.58[£]	0.72 ± 0.04	0.68 ± 0.04 [£]	4.98 ± 0.35	4.58 ±0.34[¥]
F:PBO+NIT	9.38 ±1.56	9.48 ±1.53	0.66 ± 0.05	0.63 ± 0.05	4.63 ± 0.55	4.36 ± 0.51*
M:PBO+NIT	9.42 ±1.36[$]	9.08 ±1.07	0.72 ± 0.03	0.67 ± 0.05*	4.83 ± 0.33	4.48 ± 0.57
F:NF-RBJ	9.96 ±1.82	10.01 ±1.83	0.64 ± 0.09	0.68 ± 0.03	4.42 ± 0.65	4.69 ± 0.37
M:NF-RBJ	9.85 ±1.20	9.60 ±1.17	0.70 ± 0.08	0.68 ± 0.07	4.87 ± 0.82	4.61 ± 0.68

Data are presented as mean ± SD, n=15, unless otherwise noted. Kruskal-Wallis H test was performed to examine within treatment group differences from baseline to 4-weeks and was performed in QIIME2 (version 2019.4) online software.

*Denotes significant difference from baseline, $P < 0.05$.

[$]Denotes significant baseline difference compared to females,

[¥]Approaching significance, $P = 0.06$.

[€]Approaching significance, $P = 0.08$.

[£]Approaching, $P = 0.09$. Data are mean ± SD.

Table 4.3 Total abundance of genera (%) that have been previously implicated as nitrate reducers in pooled saliva samples at the acute visit. Data are presented as mean ± SD; min, max (%). Top 4 most abundant genera highlighted in blue.

OUT ID	PBO	RBJ	PBO+NIT	NF-RBJ
Prevotella	18.8 ± 12.0; (4.5, 45.7)	18.7 ± 8.9; (5.2, 32.8)	17.1 ± 9.7; (2.6, 36.6)	17.1 ± 9.4; (6.3, 40.1)
Veillonella	9.7 ± 5.1; (10.3, 21.5)	9.9 ± 4.04; (3.9, 17.7)	9.7 ± 4.01; (2.3, 18.0)	8.1 ± 3.3; (1.8, 14.0)
Haemophilus	8.9 ± 7.5; (0.3, 33.0)	7.1 ± 5.0; (2.2, 16.8)	8.8 ± 4.5; (3.3, 16.9)	7.7 ± 8.6; (1.4, 31.0)
Neisseria	6.32 ± 8.44; (0, 31.8)	10.8 ± 7.7; (0, 20.3)	8.7 ± 10.1; (0.5, 32.1)	6.6 ± 5.9; (0.4, 20.1)
Fuscobacterium	3.57 ± 1.9; (0.8, 8.8)	3.7 ± 1.9; (0.6, 7.3)	3.6 ± 2.5; (0.5, 9.9)	3.4 ± 1.6; (1.4, 6.9)
Porphyromonas	3.4 ± 3.5; (0.2, 12.9)	3.02 ± 2.2; (0.1, 8.1)	3.2 ± 2.7; (0.2, 8.5)	2.8 ± 2.8; (0.5, 10.0)
Rothia	1.9 ± 1.0; (0.3, 4.0)	2.8 ± 0.3; (0.5, 7.5)	3.5 ± 3.0; (0.9, 12.3)	1.8 ± 1.2; (0.3, 4.3)
Leptotrichia	1.85 ± 1.35; (0.32, 4.89)	2.9 ± 2.4; (0.5, 10.1)	2.7 ± 2.5; (0.6, 10.3)	2.6 ± 2.3; (0.4, 8.5)
Granulicatella	1.8 ± 1.1; (0.6, 4.1)	1.4 ± 1.0; (0, 3.3)	1.9 ± 1.5; (0.6, 6.7)	1.6 ± 0.8; (0.7, 3.2)
Actinomyces	1.1 ± 1.0; (0.1, 3.8)	1.0 ± 0.9; (0.1, 2.8)	0.9 ± 0.8; (0.2, 2.7)	0.8 ± 0.4; (0.3, 1.4)
Campylobacter	0.5 ± 0.4; (0.08, 1.3)	0.5 ± 0.5; (0.05, 1.6)	0.5 ± 0.3; (0.1, 1.3)	0.6 ± 0.5; (0.09, 0.8)
Eikenella	0.2 ± 0.2; (0, 0.7)	0.4 ± 0.4; (0, 1.2)	0.5 ± 0.9; (0, 3.7)	0.3 ± 0.3; (0, 1.2)
Selenomonas	0.4 ± 0.3; (0.03, 1.3)	0.3 ± 0.3; (0.04, 0.8)	0.4 ± 0.4; (0, 1.3)	0.3 ± 0.3; (0.02; 1.0)

Table 4.4 Total relative abundance of genera (%) that have been previously implicated as nitrate reducers in pooled saliva samples at chronic visit. Data are presented as mean ± SD; min, max (%). Top 4 most abundant genera highlighted in blue.

OUT ID	PBO	RBJ	PBO+NIT	NF-RBJ
Prevotella	17.6 ± 9.3; (6.5, 36.3)	13.8 ± 10.9; (1.8, 41.3)	16.04 ± 12.01; (1.7, 42.4)	19.6 ± 9.1; (7.9, 39.2)
Veillonella	10.3 ± 5.03; (1.5, 21.0)	6.9 ± 4.2; (2.3, 18.0)	8.4 ± 4.1; (2.4, 14.7)	10.2 ± 5.3; (2.0, 23.0)
Haemophilus	9.1 ± 8.1; (1.1, 29.0)	6.8 ± 5.0; 7.1 (0.3, 15.0)	9.1 ± 8.5; (1.3, 35.7)	7.9 ± 10.3; (0.08, 42.0)
Neisseria	10.38 ± 10.1; (0.1, 30.0)	12.2 ± 9.1; (0.7, 28.7)[¥]	11.2 ± 8.1; (0.5, 27.5)	8.1 ± 9.4; (0, 26.4)
Fuscobacterium	4.0 ± 2.2; (1.5, 8.4)	2.9 ± 1.4; (0.7, 5.6)	3.0 ± 1.9; (0.6, 7.5)	4.2 ± 2.5; (0.5, 8.2)
Porphyromonas	3.5 ± 3.4; (0, 11.2)	2.3 ± 2.2; (0.2, 8.0)	2.9 ± 3.5; (0.2, 13.8)	3.3 ± 4.2; (0.2, 15.6)
Leptotrichia	3.2 ± 3.4; (0, 11.8)	1.8 ± 1.3; (0.3, 5.3)	1.6 ± 1.9; (0.2, 7.6)	2.3 ± 1.9; (0.3, 7.3)
Rothia	1.6 ± 1.2; (0.3, 4.9)	3.8 ± 3.4; (0.2, 12.0)	2.5 ± 1.7; (0.8, 6.6)	1.6 ± 1.2; (0.3, 4.9)
Granulicatella	1.7 ± 1.1; (0.6, 3.8)	2.0 ± 1.4; (0.5, 6.3)	1.7 ± 0.8; (0.5, 2.9)	1.6 ± 1.0; (0.4, 3.5)
Actinomyces	0.9 ± 0.6; (0.2, 2.1)	0.8 ± 0.7; (0.2, 3.2)	0.8 ± 0.6; (0.1, 2.1)	1.0 ± 0.8; (0.2, 3.2)
Campylobacter	0.7 ± 0.6; (0.1, 2.2)	0.6 ± 0.5; (0.1, 2.1)	0.4 ± 0.2; (0.09, 0.8)	0.6 ± 0.3; 0.1 (0.1, 1.05)
Eikenella	0.3 ± 0.3; (0, 1.1)	0.5 ± 0.4; (0, 1.1)	0.3 ± 0.4; (0, 1.3)	0.2 ± 0.1; (0, 0.4)
Selenomonas	0.5 ± 0.6; (0, 2.0)	0.3 ± 0.4; (0, 1.5)	0.4 ± 0.3; (0, 0.9)	0.5 ± 0.2; 0.6 (0, 0.8)

[¥]Compared to acute visit value, approaching significance (p=0.07)

Table 4.5 Total relative abundance (%) of nitrate-reducing species in pooled saliva samples at acute visit. Data are presented as mean ± SD; min, max (%). Top 3 most abundant species highlighted in blue.

Species	PBO	RBJ	PBO+NIT	NF-RBJ
Prevotella melaninogenica	11.6 ± 10.3; (0.8, 33.3)	12.1 ± 8.1; (0.5, 24.5)	11.6 ± 8.5; (0.8, 30.5)	10.3 ± 8.2; (0.2, 31.4)
Veillonella parvula	4.4 ± 4.7; (0.2, 16.4)	4.3 ± 4.5; (0.3, 15.8)	3.6 ± 3.0; (0.4, 10.6)	2.9 ± 2.9; (0.4, 8.8)
Rothia muciliaginosa	1.6 ± 1.1; (0.2, 4.6)	2.1 ± 1.5; (0.7, 6.4)	2.8 ± 2.2; (0.9, 9.1)	1.4 ± 0.9; (0.3, 3.6)
Veillonella dispar	0.8 ± 1.1; (0, 4.8)	0.6 ± 0.6; (0, 2.4)	1.0 ± 1.2; (0, 5.1)	0.7 ± 0.9 (0, 3.1)
Rothia dentocarisoa	0.3 ± 0.2; (0. 0.6)	1.0 ± 1.0; (0.1, 1.8)	1.0 ± 1.0; (0.05, 2.4)	0.3 ± 0.3; (0.03, 1.3)
Haemophilus parainfluenza	0.1 ± 0.3; (0, 0.8)	0.02 ± 0.1; (0, 0.3)	0.1 ± 0.2; (0, 0.5)	0.1 ± 0.2; (0, 0.9)
Neisseria subflava	-	0.1 ± 0.4; (0, 1.5)	0.03 ± 0.13; (0, 0.5)	0.1 ± 0.2; (0, 0.8)
Selenomonas noxia	0.1 ± 0.1; (0, 0.6)	0.03 ± 0.06; (0, 0.2)	0.1 ± 0.2; (0, 0.6)	0.1 ± 0.1; (0, 0.4)

Table 4.6 Total relative abundance (%) of nitrate-reducing species in pooled saliva samples at chronic visit. Data are presented as mean ± SD; min, max (%). Top 3 most abundant species highlighted in blue.

Species	PBO	RBJ	PBO+NIT	NF-RBJ
Prevotella melaninogenica	11.0 ± 8.0; (1.1, 26.0)	8.6 ± 9.4; (0.8, 31.1);	10.0 ± 10.01; (0.8, 33.3)	12.0 ± 8.4; (2.4, 31.9)
Veillonella parvula	3.8 ± 4.1; (0.4, 12.6)	2.8 ± 3.2; (0.05, 11.4)	3.1 ± 2.8; (0.7, 9.5)	3.8 ± 3.2; (0.4, 9.5)
Rothia muciliaginosa	1.3 ± 1.1; (0.2, 4.3)	3.1 ± 2.5; (0.5, 8.2)	1.9 ± 0.9; (0.7, 4.0)	1.5 ± 1.1; (0.4, 4.4)
Veillonella dispar	0.9 ± 1.6; (0, 6.2)	1.0 ± 1.0; (0, 2.5)	1.0 ± 1.0; (0, 3.8)	1.4 ± 3.7; (0, 14.8)
Haemophilus parainfluenza	0.1 ± 0.3; (0, 0.8)	0.1 ± 0.1; (0, 0.5)	0.1 ± 0.1; (0, 0.4)	0.1 ± 0.2; (0, 0.08)
Rothia dentocarisoa	0.2 ± 0.2; (0, 0.7)	1.0 ± 1.0; (0.03, 1.7)	0.3 ± 0.3; (0.05, 1.4)	0.2 ± 0.16; (0, 0.6)
Neisseria subflava	0.04 ± 0.17; (0, 0.7)	0.1 ± 0.3; (0, 0.9)	0.1 ± 0.23; (0, 0.9)	-
Selenomonas noxia	0.1 ± 0.15; (0, 0.6)	0.1 ± 0.17; (0, 0.7)	0.1 ± 0.03; (0, 0.1)	0.04 ± 0.1; (0, 0.1)

Table 4.7 Correlation coefficients for relationships between selected nitrate-reducing species (% relative abundance) at the acute and chronic visit for plasma and saliva NOx and plasma nitrite AUC per treatment group. Only significant correlations between individual species and AUC for plasma or saliva NOx and plasma nitrite at the acute and chronic visit are shown.

	PBO		**RBJ**		**PBO+NIT**		**NF-RBJ**	
	Acute	**Chronic**	**Acute**	**Chronic**	**Acute**	**Chronic**	**Acute**	**Chronic**
Plasma NOx AUC	-	*H. parainfluenzae* r = -0.54 *P* = 0.02	-	-	-	-	-	-
Saliva NOx AUC	-	-	-	-	-	*V. dispar* r = 0.57 *P* = 0.02	*S. noxia* r = -0.57 *P* = 0.02	-
Plasma nitrite AUC	-	-	-	*S. noxia r = -0.52* *P* = 0.05	-	-	*V. dispar* r = -0.53 *P* = 0.05	-

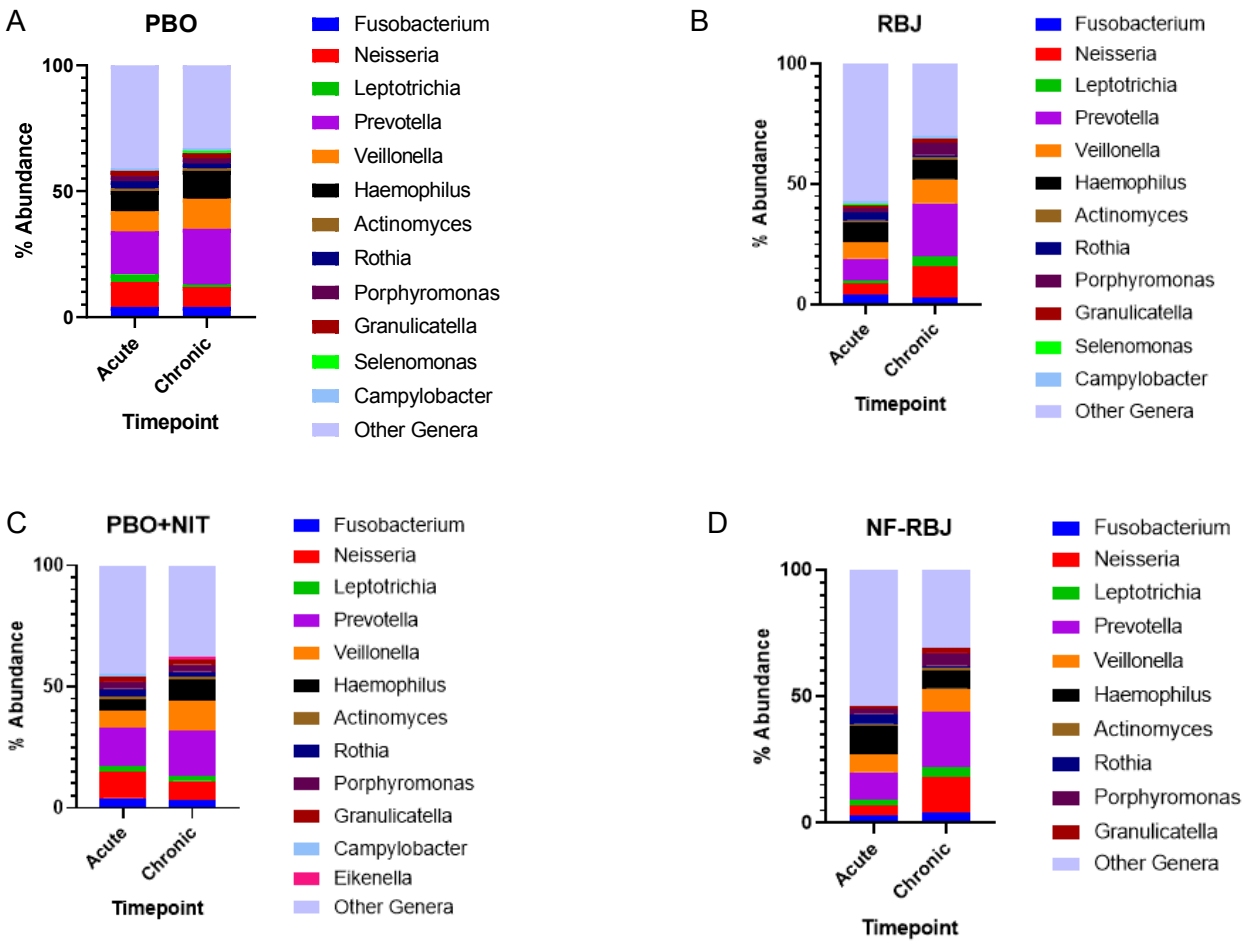


Figure 4.1 A comparison of the total relative abundance of genera implicated in nitrate reduction at the acute and chronic visit for each treatment period (A-D). Data are presented as group mean with SD excluded for clarity, n = 15.

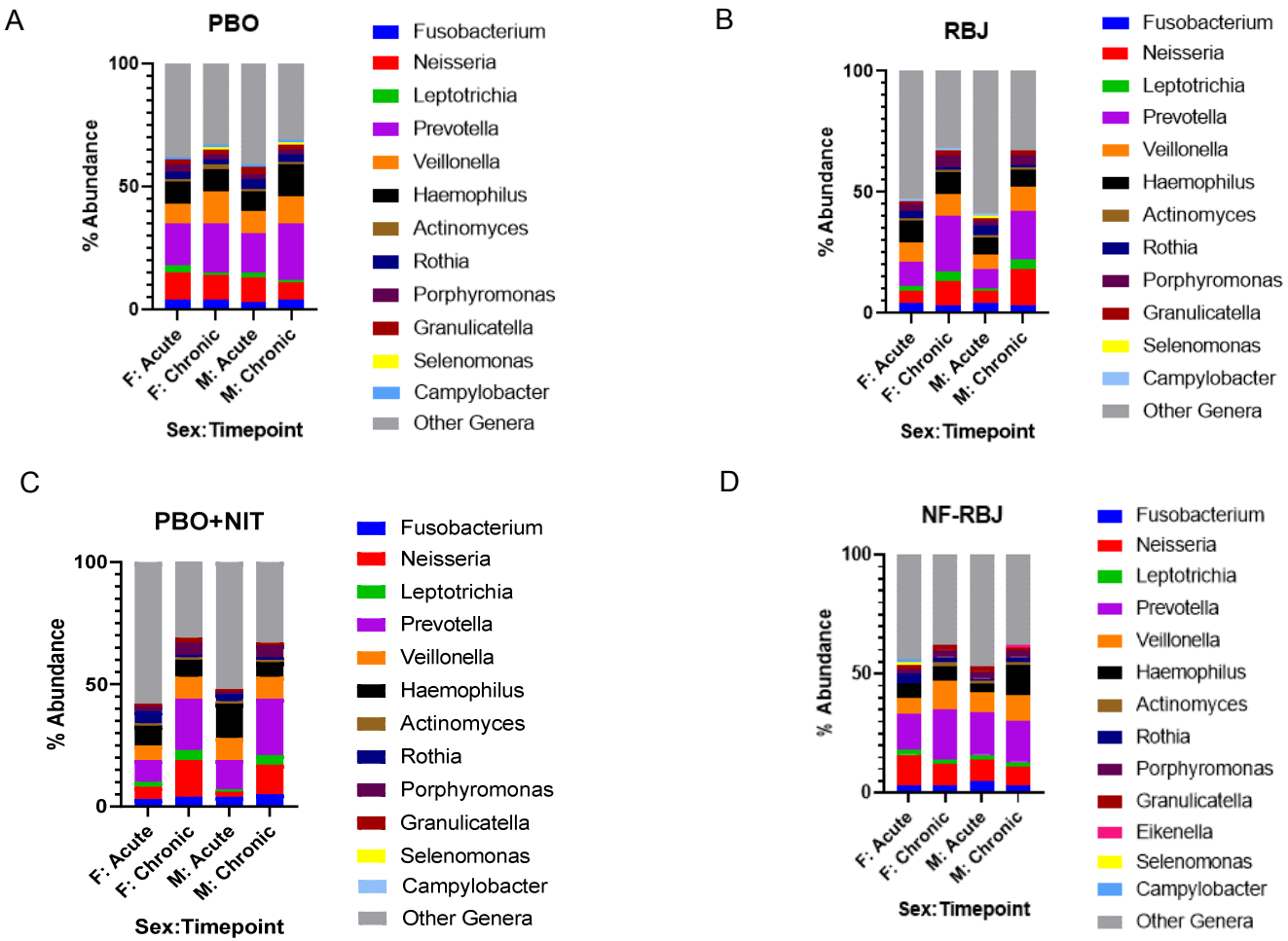


Figure 4.2 A comparison of the sex differences in the total relative abundance of genera implicated in nitrate reduction at the chronic visit for each treatment period (A-D). Data are presented as group mean with SD excluded for clarity, n = 15.

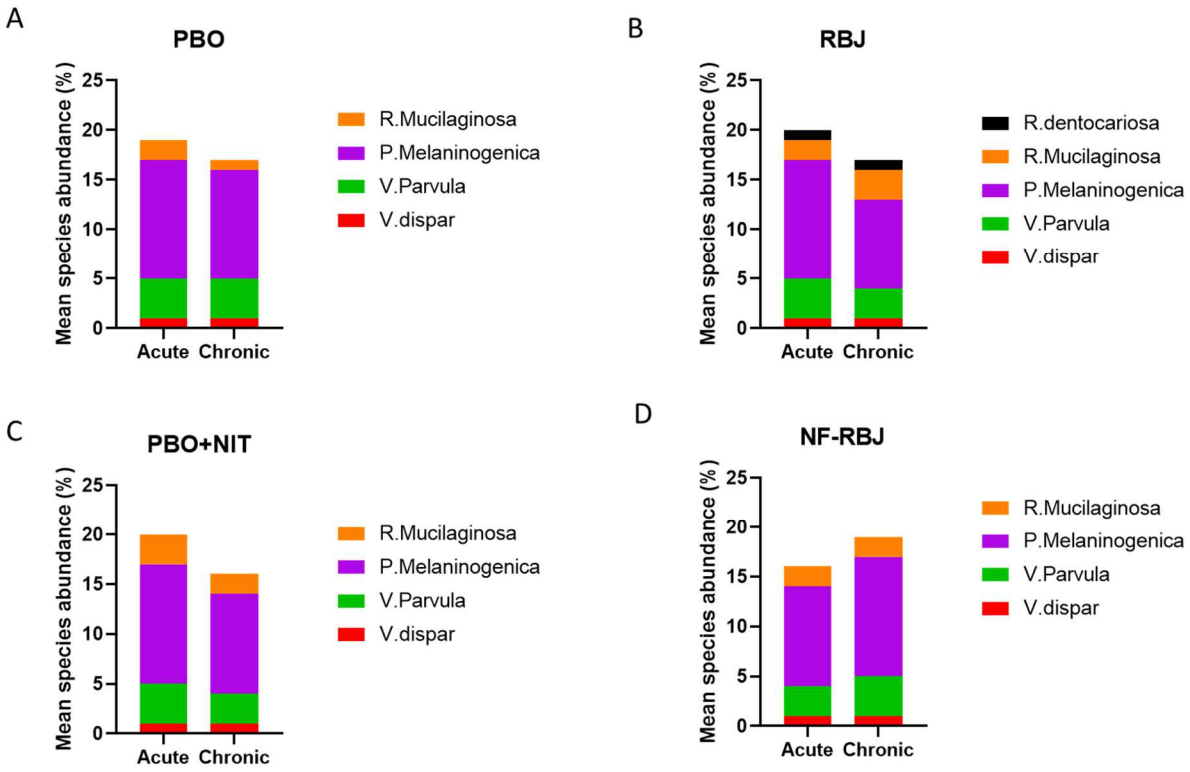


Figure 4.3 Total relative abundance of the most prevalent nitrate-reducing species at the acute and chronic visit of each treatment period (A-D). Data are presented as group mean with SD excluded for clarity, n = 15.

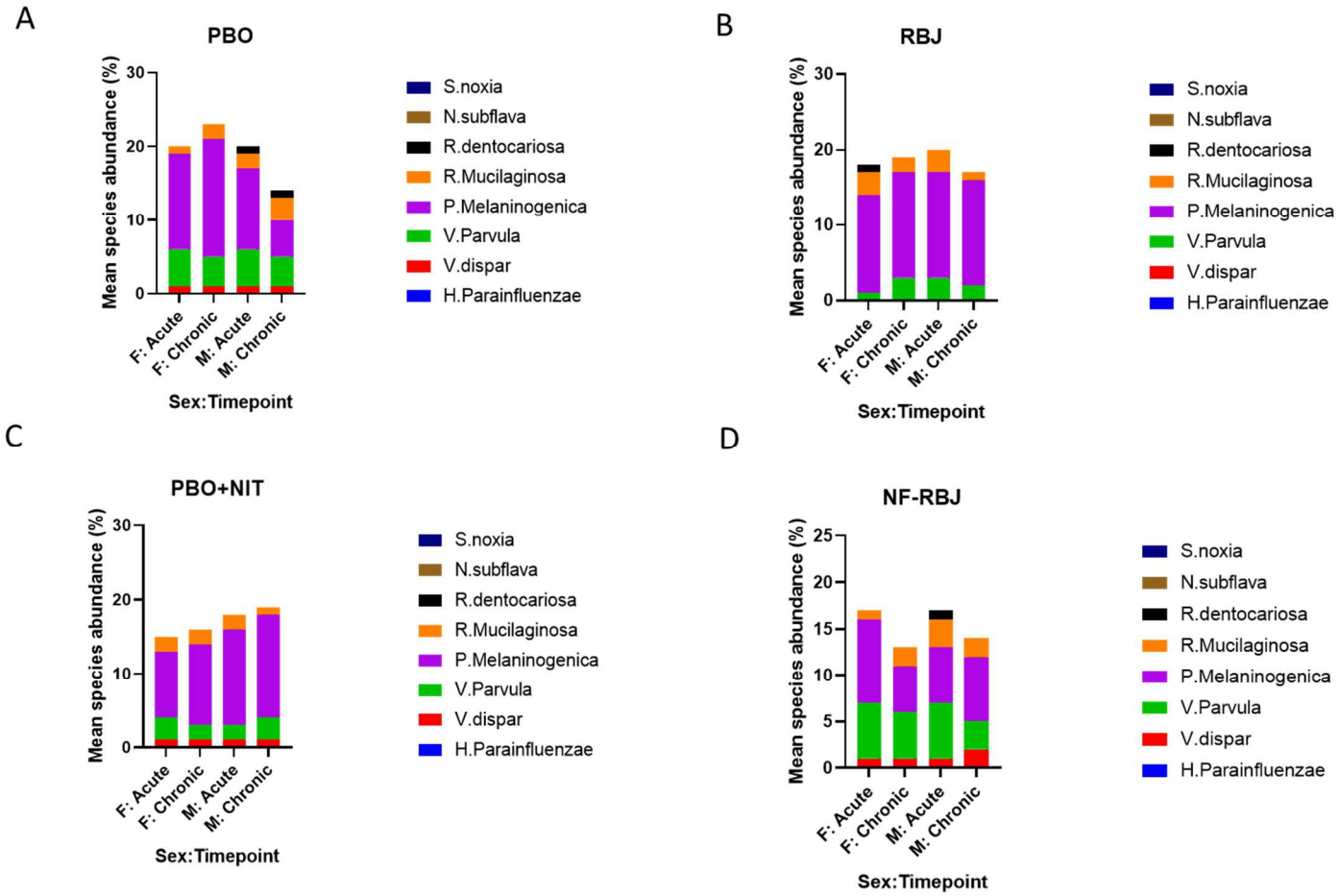


Figure 4.4 A comparison of the sex differences in total abundance of species implicated in nitrate reduction at the acute and chronic visit per treatment group (A-D). Data are presented as group mean with SD excluded for clarity, n = 15.

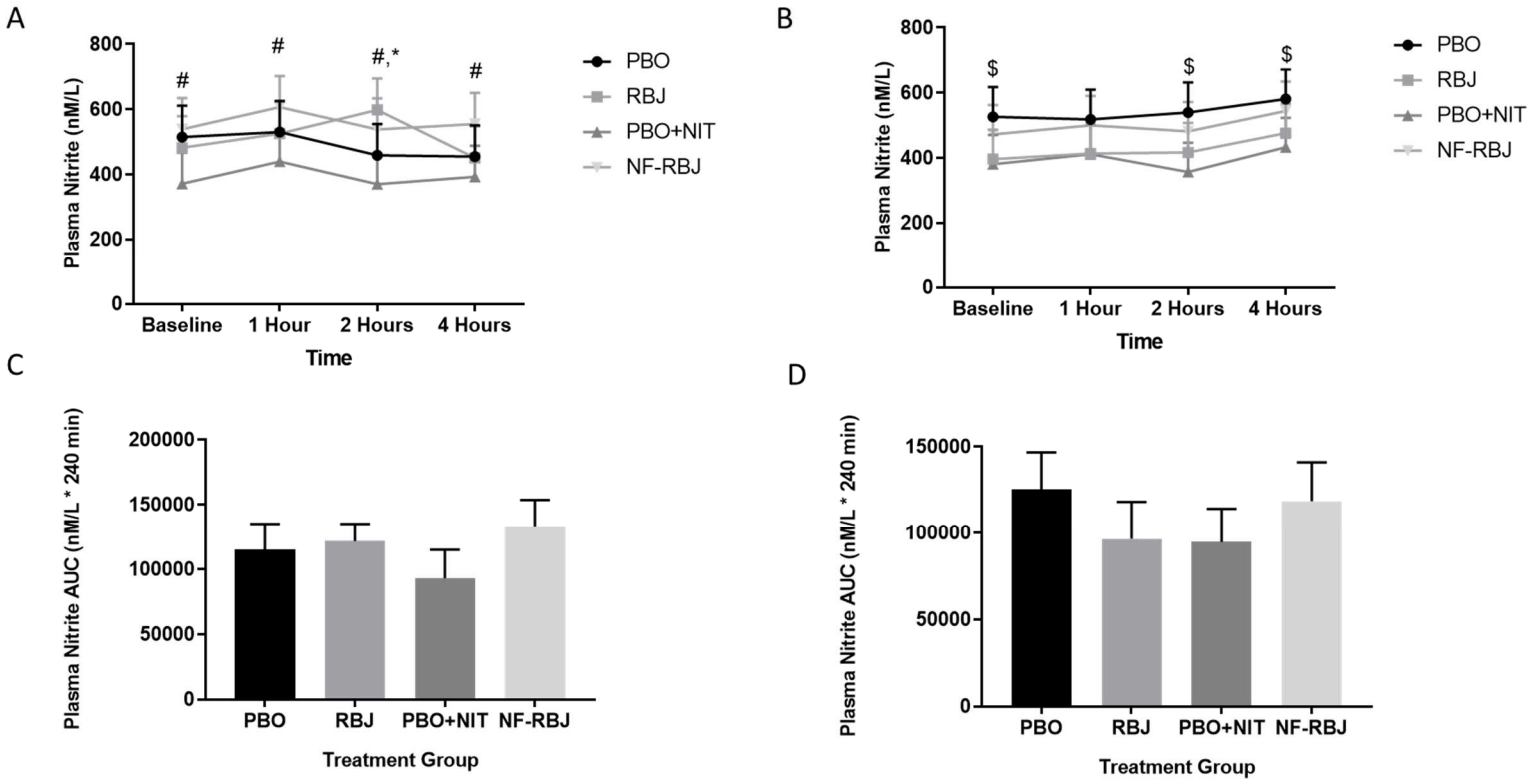
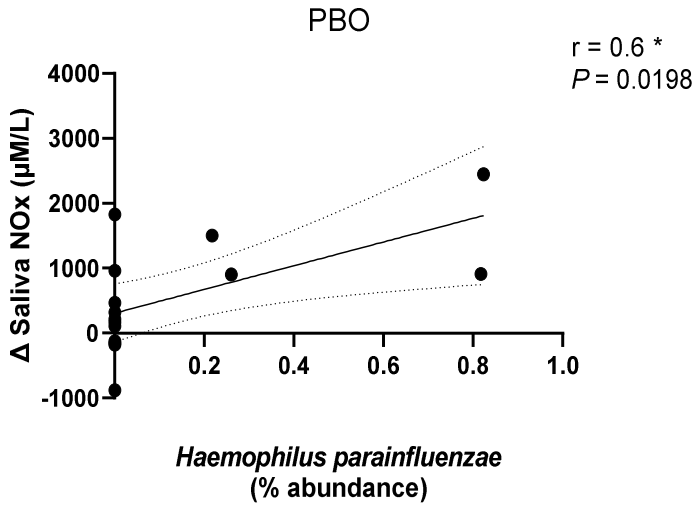


Figure 4.5 Plasma nitrite concentrations at the acute visit (A) and the chronic visit (B) at baseline, 1, 2 and 4 hours after high-fat meal and PBO, RBJ, PBO+NIT and NF-RBJ treatment consumption, and postprandial (0-240 min) plasms nitrite area under the curve (AUC) at the acute visit (C) and chronic visit (D). Time points annotated with symbols represent significant time*treatment interactions between treatment groups. #PBO+NIT significantly different than NF-RBJ, $P<0.05$. *RBJ significantly different than PBO+NIT, $P < 0.01$. $PBO significantly different than PBO+NIT, $P < 0.05$. No significant differences were observed for plasma nitrite AUC between treatment groups.

A



B

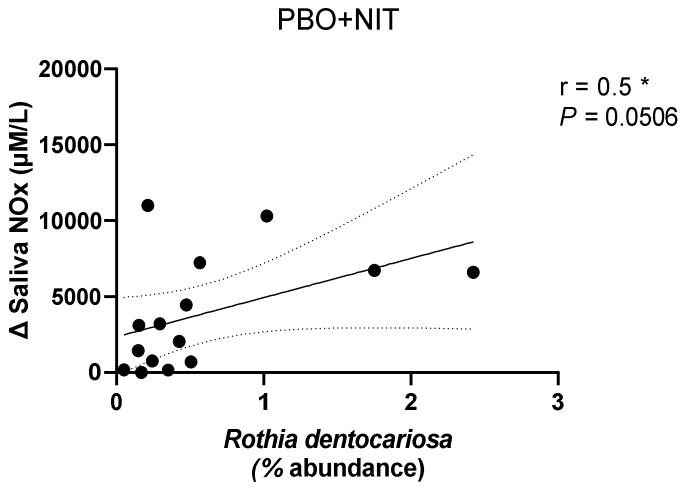


Figure 4.6 Correlations between *Haemophilus parainfluenzae* in the PBO group (A) and *Rothia dentocariosa* in the PBO+NIT group (B) with peak change in saliva NOx at the acute visit, n = 15. *denotes statistical significance, $P < 0.05$. Only significant correlations between individual species and change in saliva NO are shown.

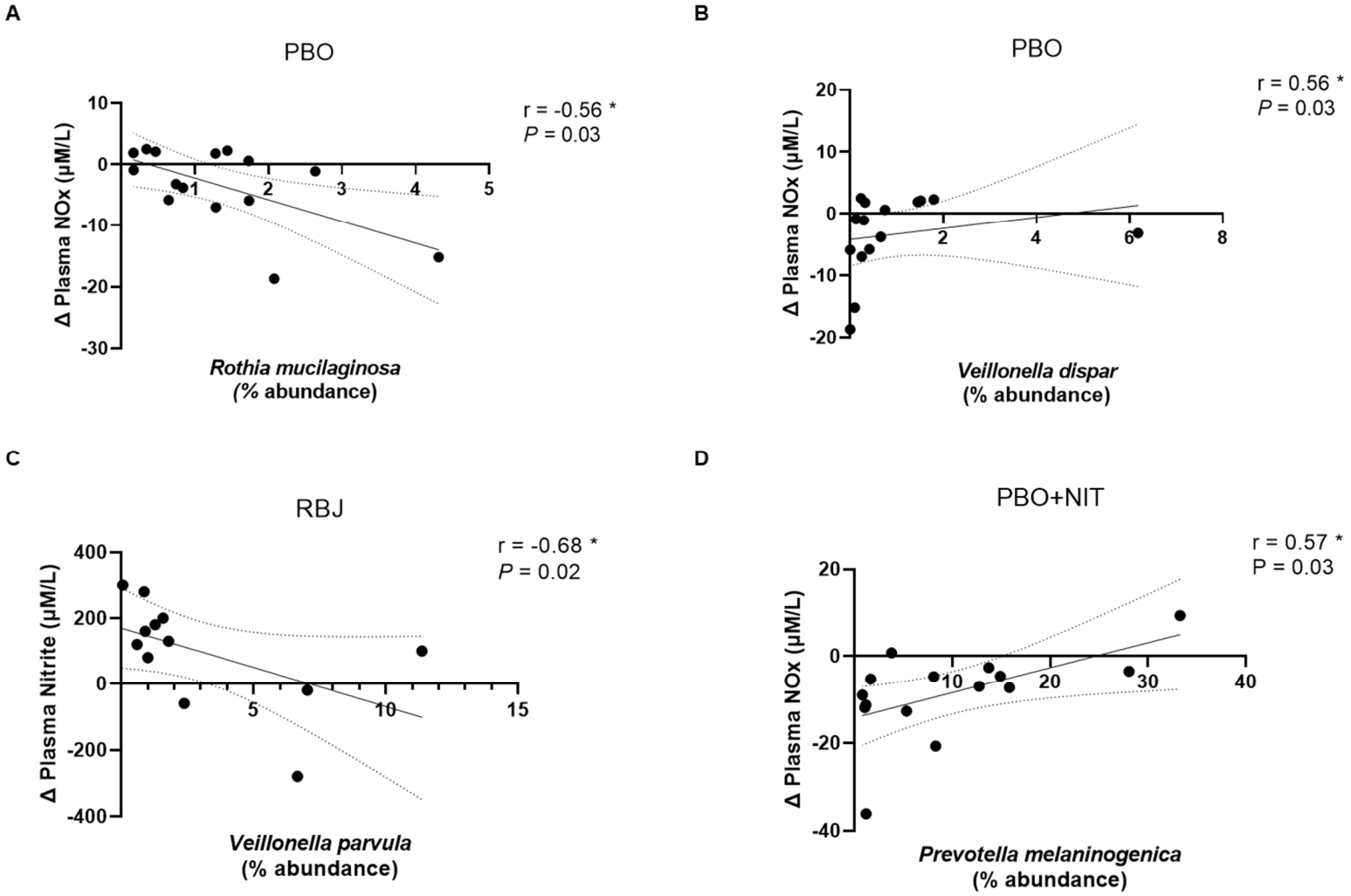


Figure 4.7 Correlations between *Rothia mucilaginosa* (A) and *Veillonella dispar* (B) with peak change in plasma NOx in the PBO group, *Veillonella parvula* with peak change in plasma nitrite in the RBJ group (C), *Prevotella melaninogenica* with peak change in plasma NOx in the PBO+NIT group (D) at the chronic visit, n = 15. *denotes statistical significance, $P < 0.05$. Only significant correlations between individual species and change in plasma NOx are shown.

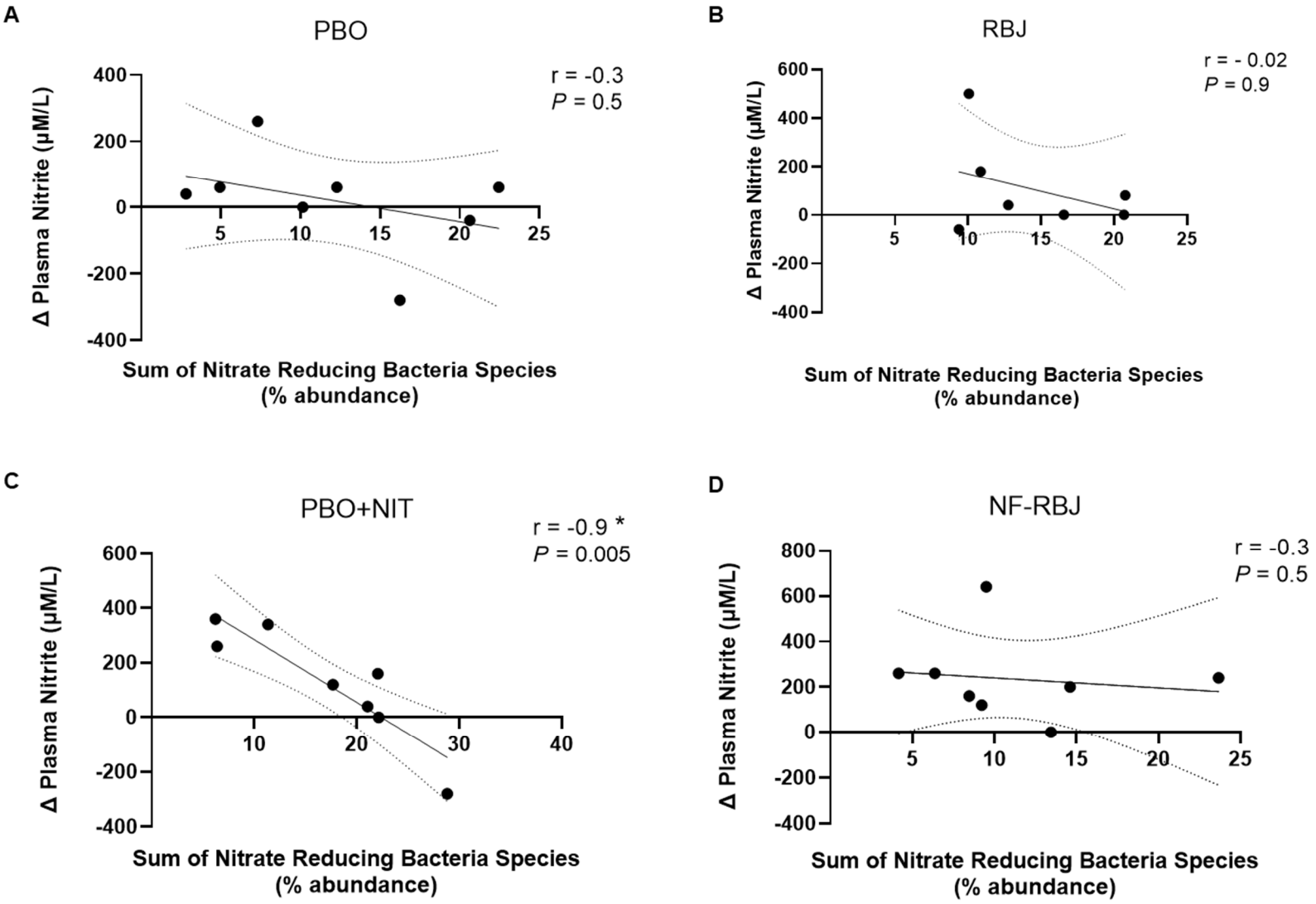


Figure 4.8 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma nitrite from baseline per treatment group in females only at the acute visit, n = 8. *denotes statistical significance, $P < 0.05$.

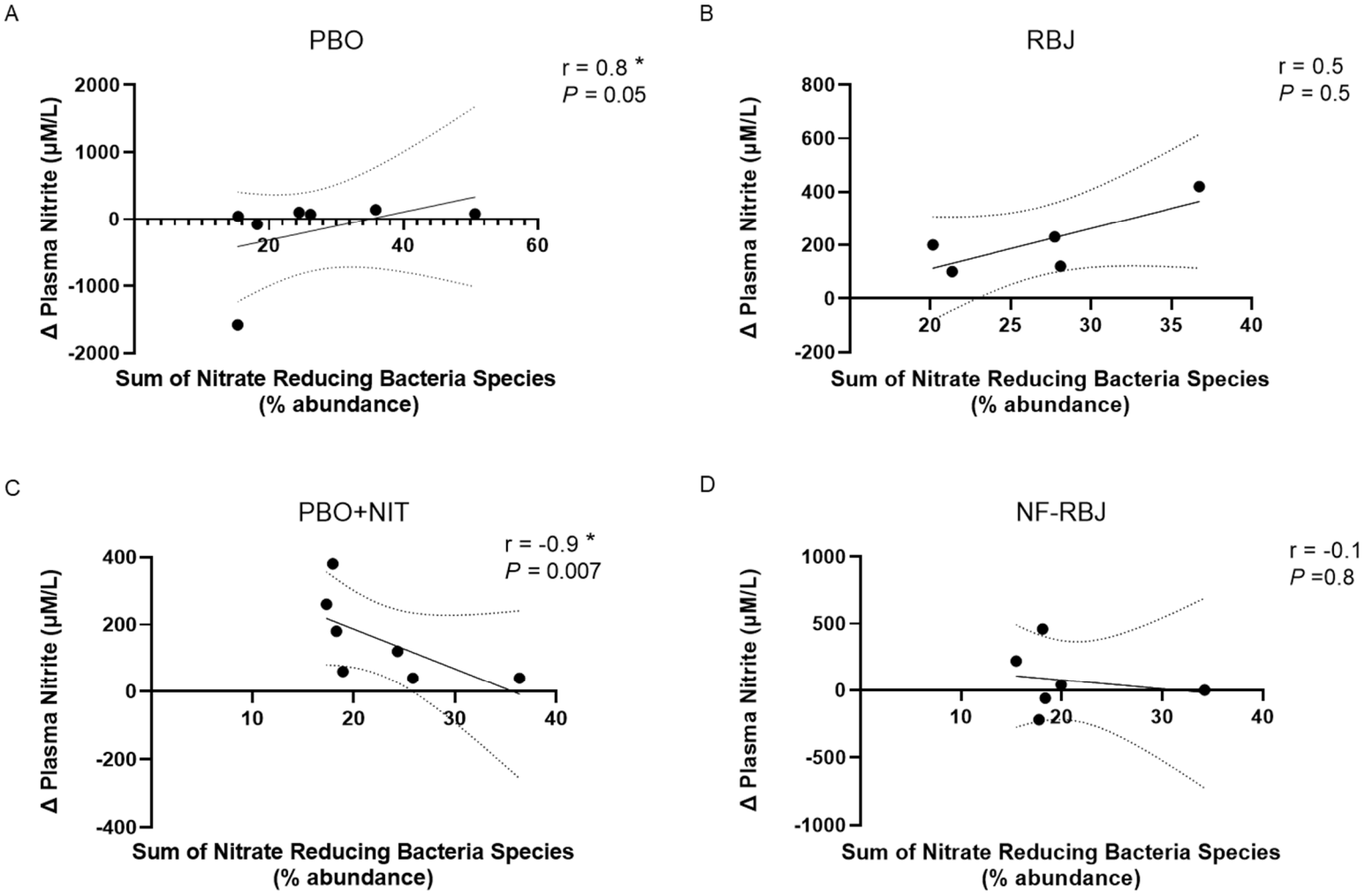


Figure 4.9 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma nitrite from baseline per treatment group in males only at the acute visit, n = 8. *denotes statistical significance, $P < 0.05$.

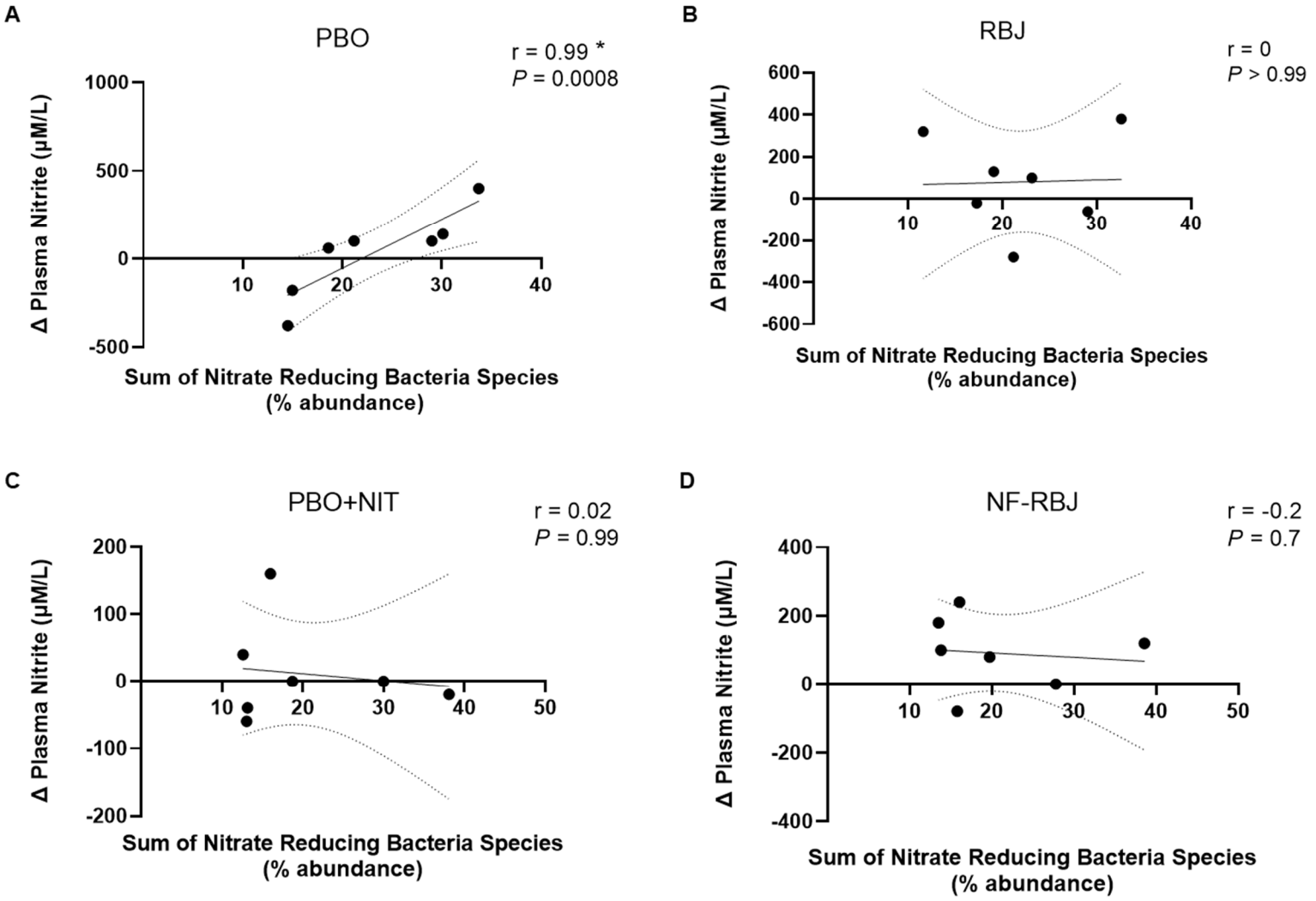


Figure 4.10 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma nitrite from baseline per treatment group in males only at the chronic visit, n = 8. *denotes statistical significance, $P < 0.05$.

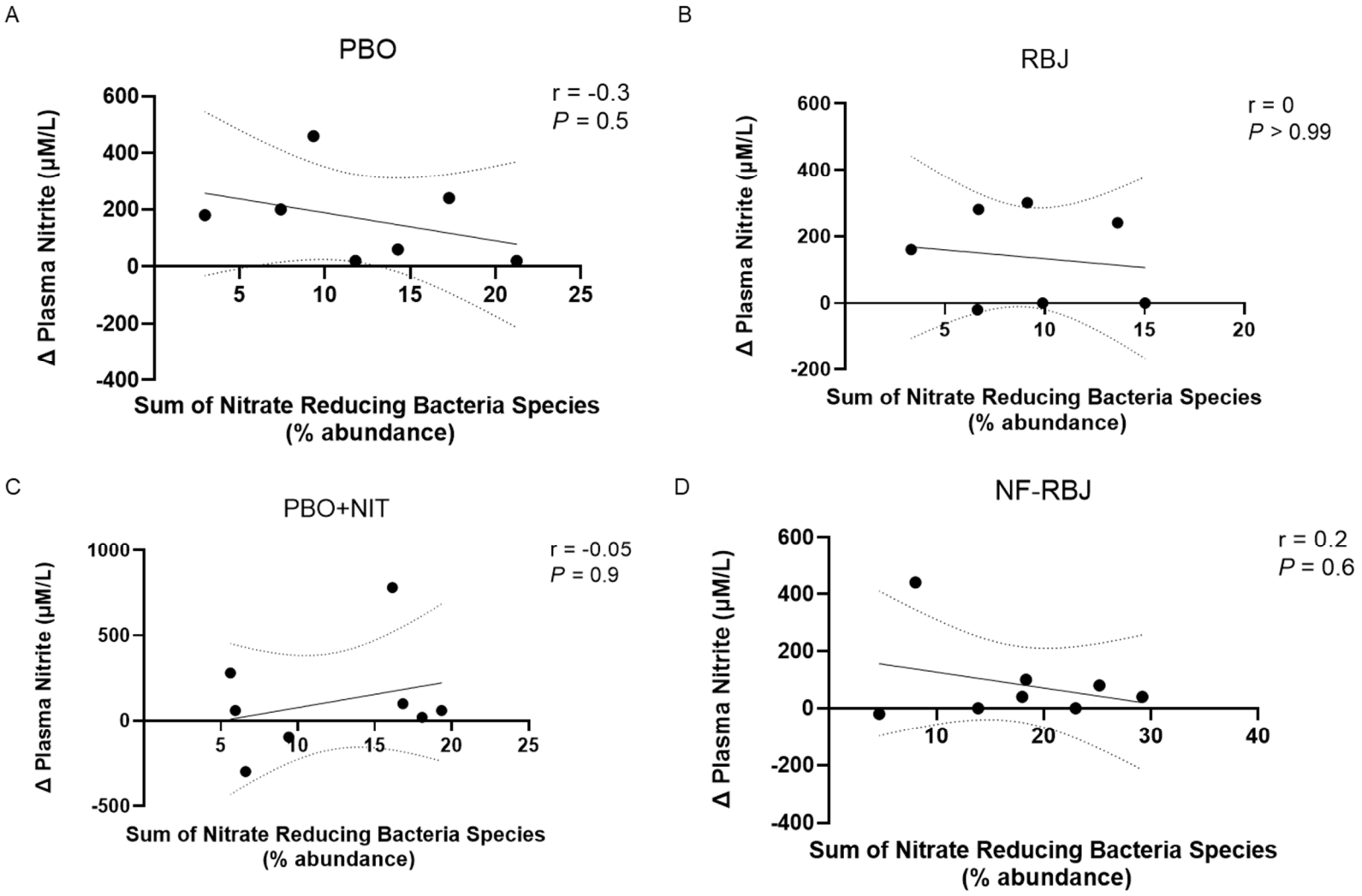


Figure 4.11 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma nitrite from baseline per treatment group in females only at the chronic visit, n = 8. *denotes statistical significance, $P < 0.05$.

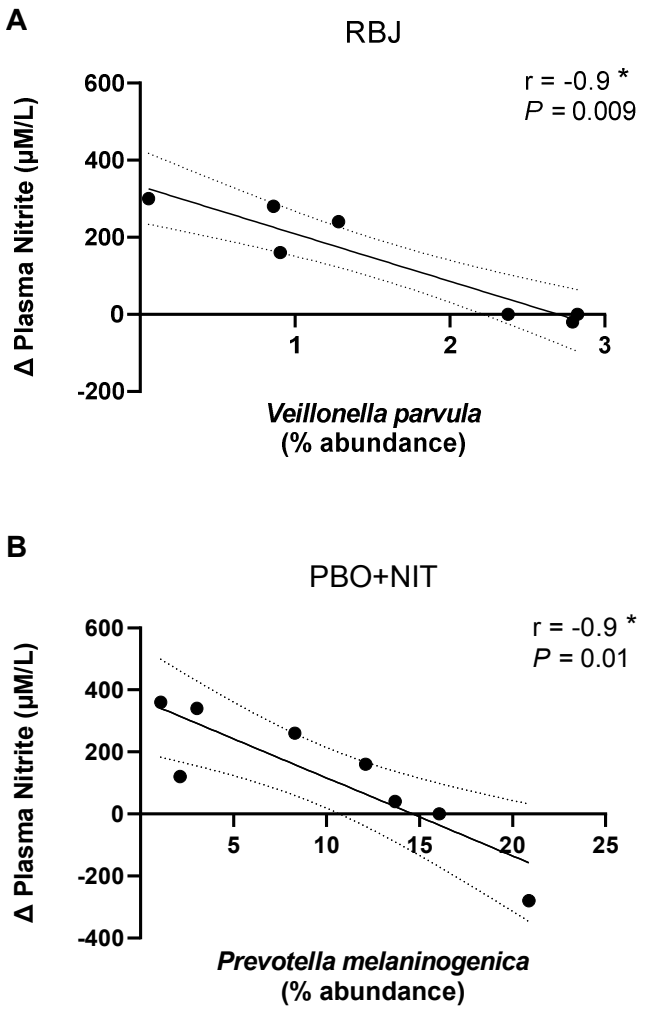


Figure 4.12 (A) Correlation between *Prevotella melaninogenica* and peak change in plasma nitrite in females in the RBJ group at the acute visit, n= 8. (B) Correlation between *Veillonella parvula* and peak change in plasma nitrite in females in the RBJ group at the chronic visit, n = 7. *denotes statistical significance, $P < 0.05$. Only significant correlations between individual species and change in plasma nitrite are shown.

A

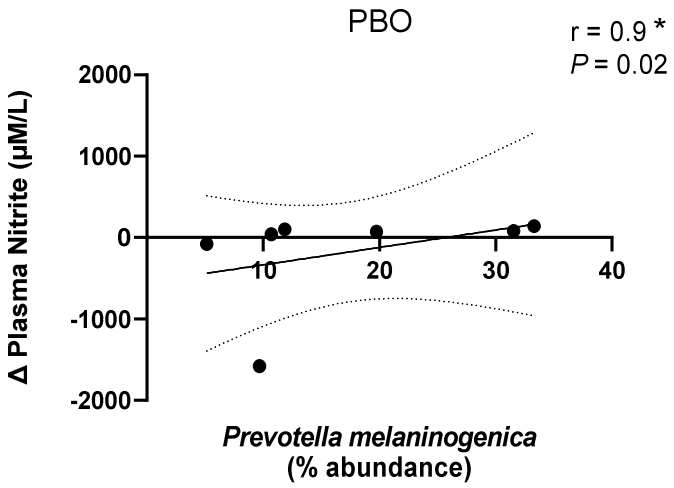


Figure 4.13 Correlations between abundance of *Prevotella melaninogenica* and peak change in plasma nitrite in males only in the PBO group at the acute visit, n= 7. *denotes statistical significance, $P < 0.05$. Only significant correlations between individual species and change in plasma nitrite are shown.

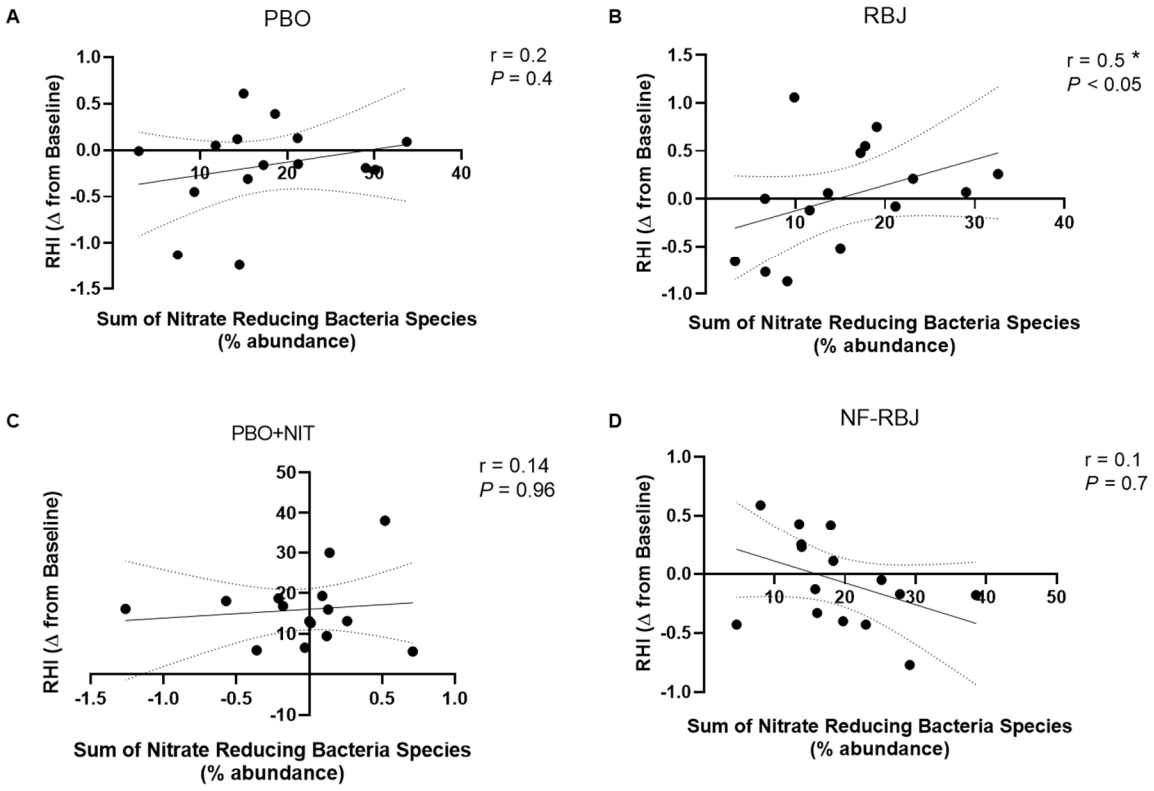


Figure 4.14 Correlations between total (sum of) nitrate-reducing bacteria species and RHI change from baseline per treatment group at the chronic visit. n = 15. *denotes statistical significance, $P < 0.05$.

REFERENCES

1. Luiking, Y. C., Engelen, M. P. K. J. & Deutz, N. E. P. Regulation of nitric oxide production in health and disease. *Curr. Opin. Clin. Nutr. Metab. Care* **13**, 97–104 (2010).
2. Naseem, K. M. The role of nitric oxide in cardiovascular diseases. *Mol. Aspects Med.* **26**, 33–65 (2005).
3. Donato Anthony J., Machin Daniel R. & Lesniewski Lisa A. Mechanisms of Dysfunction in the Aging Vasculature and Role in Age-Related Disease. *Circ. Res.* **123**, 825–848 (2018).
4. Johnson, S. A., Litwin, N. S. & Seals, D. R. Age-Related Vascular Dysfunction: What Registered Dietitian Nutritionists Need to Know. *J. Acad. Nutr. Diet.* (2019). doi:10.1016/j.jand.2019.03.016
5. Lundberg, J. O., Weitzberg, E. & Gladwin, M. T. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Discov.* **7**, 156–167 (2008).
6. Qin, L. *et al.* Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13434–13439 (2012).
7. Lundberg, J. O. Nitrate transport in salivary glands with implications for NO homeostasis. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 13144–13145 (2012).
8. Kapil, V., Weitzberg, E., Lundberg, J. O. & Ahluwalia, A. Clinical evidence demonstrating the utility of inorganic nitrate in cardiovascular health. *Nitric Oxide* **38**, 45–57 (2014).
9. Lidder, S. & Webb, A. J. Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway: Vascular effects of dietary nitrate. *Br. J. Clin. Pharmacol.* **75**, 677–696 (2013).
10. Vanhatalo, A. *et al.* Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. *Free Radic. Biol. Med.* **124**, 21–30 (2018).

11. Burleigh, M. *et al.* Dietary nitrate supplementation alters the oral microbiome but does not improve the vascular responses to an acute nitrate dose. *Nitric Oxide Biol. Chem.* **89**, 54–63 (2019).
12. Burleigh, M. C. *et al.* Salivary nitrite production is elevated in individuals with a higher abundance of oral nitrate-reducing bacteria. *Free Radic. Biol. Med.* **120**, 80–88 (2018).
13. Litwin, Nicole S. *The Efficacy of Red Beetroot Supplementation on Parameters of Cardiometabolic Health in Overweight and Obese Middle-Aged/Older Adults*. (Colorado State University, 2019).
14. Calvert, J. W. & Lefer, D. J. Clinical translation of nitrite therapy for cardiovascular diseases. *Nitric Oxide Biol. Chem.* **22**, 91–97 (2010).
15. Hord, N. G., Tang, Y. & Bryan, N. S. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am. J. Clin. Nutr.* **90**, 1–10 (2009).
16. Bondonno, C. P., Croft, K. D. & Hodgson, J. M. Dietary Nitrate, Nitric Oxide, and Cardiovascular Health. *Crit. Rev. Food Sci. Nutr.* **56**, 2036–2052 (2016).
17. Nohria, A. *et al.* Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. *J. Appl. Physiol. Bethesda Md 1985* **101**, 545–548 (2006).
18. Doel, J. J., Benjamin, N., Hector, M. P., Rogers, M. & Allaker, R. P. Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur. J. Oral Sci.* **113**, 14–19 (2005).
19. Hyde, E. R. *et al.* Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: implications for nitric oxide homeostasis. *PloS One* **9**, e88645 (2014).
20. Lauer, T. *et al.* Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc. Natl. Acad. Sci.* **98**, 12814–12819 (2001).
21. Liddle, L. *et al.* Variability in nitrate-reducing oral bacteria and nitric oxide metabolites in biological fluids following dietary nitrate administration: An assessment of the critical difference. *Nitric Oxide Biol. Chem.* **83**, 1–10 (2019).

22. Velmurugan, S. *et al.* Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double-blind, placebo-controlled study123. *Am. J. Clin. Nutr.* **103**, 25–38 (2016).
23. Wade, W. G. The oral microbiome in health and disease. *Pharmacol. Res.* **69**, 137–143 (2013).
24. Silva Mendez, L. S., Allaker, R. P., Hardie, J. M. & Benjamin, N. Antimicrobial effect of acidified nitrite on cariogenic bacteria. *Oral Microbiol. Immunol.* **14**, 391–392 (1999).
25. Hohensinn, B. *et al.* Sustaining elevated levels of nitrite in the oral cavity through consumption of nitrate-rich beetroot juice in young healthy adults reduces salivary pH. *Nitric Oxide* **60**, 10–15 (2016).
26. Kianoush, N. *et al.* Bacterial Profile of Dentine Caries and the Impact of pH on Bacterial Population Diversity. *PLoS ONE* **9**, (2014).
27. Hall, M. W. *et al.* Inter-personal diversity and temporal dynamics of dental, tongue, and salivary microbiota in the healthy oral cavity. *Npj Biofilms Microbiomes* **3**, 1–7 (2017).
28. Larsen, T. & Fiehn, N.-E. Dental biofilm infections – an update. *APMIS* **125**, 376–384 (2017).
29. Webb Andrew J. *et al.* Acute Blood Pressure Lowering, Vasoprotective, and Antiplatelet Properties of Dietary Nitrate via Bioconversion to Nitrite. *Hypertension* **51**, 784–790 (2008).
30. Kapil, V. *et al.* Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertens. Dallas Tex 1979* **56**, 274–281 (2010).
31. Vanhatalo, A. *et al.* Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **299**, R1121-1131 (2010).
32. Gilchrist, M., Shore, A. C. & Benjamin, N. Inorganic nitrate and nitrite and control of blood pressure. *Cardiovasc. Res.* **89**, 492–498 (2011).

33. Kenjale, A. A. *et al.* Dietary nitrate supplementation enhances exercise performance in peripheral arterial disease. *J. Appl. Physiol. Bethesda Md 1985* **110**, 1582–1591 (2011).
34. Siervo, M. *et al.* Ageing modifies the effects of beetroot juice supplementation on 24-hour blood pressure variability: An individual participant meta-analysis. *Nitric Oxide Biol. Chem.* **47**, 97–105 (2015).
35. Zhurakivska, K. *et al.* Do Changes in Oral Microbiota Correlate With Plasma Nitrite Response? A Systematic Review. *Front. Physiol.* **10**, (2019).
36. Lundberg, J. O., Weitzberg, E., Lundberg, J. M. & Alving, K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* **35**, 1543–1546 (1994).
37. Montenegro, M. F. *et al.* Blood Pressure-Lowering Effect of Orally Ingested Nitrite Is Abolished by a Proton Pump Inhibitor. *Hypertens. Dallas Tex 1979* **69**, 23–31 (2017).
38. Lara, J. *et al.* Effects of inorganic nitrate and beetroot supplementation on endothelial function: a systematic review and meta-analysis. *Eur. J. Nutr.* **55**, 451–459 (2016).

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

The goal of this dissertation was to test the hypothesis that acute and chronic red beetroot juice supplementation would attenuate impairments in postprandial vascular endothelial function in middle-aged/older men and postmenopausal women with overweight or obesity, and to investigate the nitrate-dependent and -independent effects of red beetroot juice contributing to vascular and clinical responses. We postulated that these responses would be associated with increased nitric oxide (NO) metabolites and reduced oxidative stress and inflammation. A secondary hypothesis was that chronic red beetroot juice supplementation would influence the oral microbiota, and that changes would be associated with NO metabolites.

Contrary to our hypothesis, acute and chronic (4-week daily) supplementation with red beetroot juice did not alter postprandial vascular endothelial function after consumption of a single high-fat meal, despite increases in circulating NO metabolites. Except for NO metabolites, there were no consistent nitrate-dependent and/or -independent effects of red beetroot juice on postprandial vascular endothelial function or other measured parameters, ruling out any individual contributions of inorganic nitrate or other bioactive compounds in red beetroot juice in this study. Additionally, consumption of a high-fat meal did not significantly impair postprandial vascular endothelial function even though it led to significant alterations in several hemodynamic parameters and plasma insulin and triglyceride concentrations. No changes in biomarkers of oxidative stress or inflammation were observed.

Regarding the secondary hypothesis, it was observed that following chronic consumption of inorganic nitrate, alpha-diversity of the oral microbiota (specifically species evenness) was significantly lower than baseline indicating that inorganic nitrate may alter the oral microbiota. Similar trends, albeit non-significant, were seen in alpha-diversity indices following chronic red beetroot juice supplementation suggesting a potential influence of red

beetroot juice on the oral microbiota. Sex differences in the oral microbiota were also explored and revealed that men responded differently to chronic inorganic nitrate supplementation and experienced changes in alpha-diversity of the oral microbiota, compared to females. Contrarily, no significant differences in oral nitrate-reducing bacteria were observed after chronic red beetroot juice or inorganic nitrate supplementation. Additionally, no significant correlations were observed with total oral nitrate-reducing bacteria and changes in circulating NO metabolites in plasma and saliva, while some individual species were correlated with changes in NO metabolites. Total oral nitrate-reducing bacteria was positively correlated with reactive hyperemia index, a measure of vascular endothelial function, following chronic consumption of RBJ.

The results of this dissertation research suggest that red beetroot juice may not be an effective therapeutic strategy for improving postprandial vascular endothelial function in this population; however, this cannot be fully determined at this time due to the negligible effects of the high-fat meal on postprandial vascular endothelial function in this study. Thus, more research is needed investigating the therapeutic effects of red beetroot juice in this population. Future studies that can induce a greater insult to the vascular endothelium following consumption of a high-fat meal is warranted and may yield different results than that obtained in this research. It should be mentioned that the dose of red beetroot juice used may have been ineffective at attenuating postprandial impairments in vascular endothelial function as other research has shown that larger doses of red beetroot juice containing higher amounts of inorganic nitrate counteracts postprandial impairments in vascular endothelial function. Additionally, the population studied in this research may not be as responsive to red beetroot juice as other populations that have been studied. Our population was of healthy status in which their metabolic flexibility could have prevented or minimized the effects of the high-fat meal. Since this is an aging population with increased cardiometabolic risk, it is conceivable that these factors may have lessened the efficacy of the red beetroot juice and an individual's ability to

convert dietary inorganic nitrate into NO. Whether larger and/or more frequent doses of red beetroot juice in middle-aged and older individuals are required to enhance dietary nitrate conversion and subsequently increase NO bioavailability is unknown. It could be possible that longer durations of red beetroot juice supplementation may also be needed in middle-aged and older adults to obtain sustained optimal levels of NO bioavailability needed to induce beneficial vascular effects.

Based on this data, it appears that chronic red beetroot juice supplementation may alter the oral microbiota likely due to the nitrate-dependent effects on bacterial nitrate reduction. However, this cannot be determined at this time as alterations in bacterial abundance following chronic supplementation were non-significant. Additionally, the nitrate-free red beetroot juice placebo conferred alterations to the abundance of oral nitrate-reducing bacteria suggesting that this placebo is not biologically inert and that the other bioactive compounds in red beetroot juice may also influence the oral microbiota. Future studies should consider this in their experimental design. Inter-individual variation in oral bacterial communities and in bacterial nitrate reduction may contribute to our non-significant results. Additionally, aging may also influence the efficiency of dietary nitrate metabolism to nitrite and NO. Aging is associated with reduced salivary flow rate and oral dysbiosis, which may have also influenced our results; however, these parameters were not assessed in our study, nor was salivary nitrite measured, limiting our ability to determine whether nitrate reduction to nitrite occurred. Future studies should include baseline oral health assessment and monitor salivary pH and flow rate in addition to quantifying nitrite in saliva when investigating the influence of red beetroot juice on the enterosalivary circulation of dietary nitrate and the oral microbiota. Additionally, aging may also be associated with changes in acid production and the gut microbiota, which can also play a role in nitrate metabolism and influence the efficiency of the conversion of nitrate to NO. Therefore, future studies should include analysis of the gut microbiota and whether it plays a role in NO bioavailability as well as NO-dependent vascular endothelial function.

Taken together, these data indicate that the dietary nitrate-nitrite-NO pathway is intact in middle-aged/older adults with overweight and obesity as evidenced by increased circulating NO metabolites (plasma and saliva nitrate/nitrite). Although the high-fat meal challenge used in the present study led to postprandial alterations, in some, but not all cardiometabolic parameters measured, acute nor chronic red beetroot juice supplementation modulated these cardiometabolic responses to the high-fat meal. On the other hand, chronic red beetroot juice supplementation may alter the oral microbiota in this population, possibly due to nitrate-dependent effects on bacterial nitrate reduction. Further research is needed in this population, as well as other populations of increased cardiovascular risk, to determine whether red beetroot juice can be used as a potential oral-microbiota targeted therapy for improving NO bioavailability and postprandial vascular endothelial function.

APPENDIX 1: SUPPLEMENTAL DATA FOR CHAPTER 3

SUPPLEMENTAL TABLES

Supplemental Table 3S.1

Nutrient composition of the treatments.[1]

	PBO	RBJ	PBO+NIT	NF-RBJ
Calories, kcal	70	100	100	70
Total fat, g	<0.1	0	0	<0.1
Total carbohydrates, g	25	25	15	15
Protein, g	3	<0.1	<0.1	3
Sodium, mg	200	3	3	200
Potassium, mg	630	1	190	1
Inorganic nitrate, mg	0	250-300	300	0

Abbreviations: NF-RBJ, nitrate-free red beetroot juice; PBO, placebo; PBO+NIT, placebo plus potassium nitrate; RBJ, red beetroot juice.

[1]Values are per 70 mL.

Supplemental Table 3S.2

Nutrient composition of test meal.

Calories, kcal	868
Calories from fat, kcal	441
Calories from saturated fat	198
Total fat, g	49
Saturated fat, g	22
Cholesterol, mg	440
Total carbohydrate, g	74
Fiber, g	3
Protein, g	29

Supplemental Table 3S.3

Gene specific primers used in qRT-PCR.

Common Name	Name	Primer Sequence
p47phox	Neutrophil cytosol factor 1	s: 5'ACC TCC TCG ACT TCT TCA AG-3' as: 5'CAT CTT TGG GCA TCA AGT ATG-3'
NFκB-p65	Nuclear factor kappa B-p65 subunit	s: 5'-CGA GTG AAC CGA AAC TCT GG-3' as: 5'-GGT CCC GTG AAA TAC ACC TC-3'
TNF-α	Tumor necrosis factor-alpha	s: 5'-CTG TGA GGA GGA CGA ACA TC-3' as: 5'-TGA GCC AGA AGA GGT TGA GG-3'
TLR-4	Toll-like receptor-4	s: 5'-GCC TGT GCT GAG TTT GAA TAT-3' as: 5'-CCA GAA CTG CTA CAA CAG ATA C-3'
GADD34	Growth arrest and DNA damage inducible protein 34	s: 5'-GAA GAG GGA GTT GCT GAA GAG G-3' as: 5'-GGA GAC AAG GCA GAA GTA GAG G-3'
XBP1s	Spliced X box binding protein-1	s: 5'TTG TCT CAG TGA AGG AAG AAC-3' as: 5'-TAG GCA GGA AGA TGG CTT TGG-3'

Abbreviations: s, sense; as, antisense.

Supplemental Table 3S.4

Acute effects of PBO, RBJ, PBO+NIT, and NF-RBJ on postprandial blood pressure and hemodynamic parameters.

	PBO	RBJ	PBO+NIT	NF-RBJ
bSBP				
0 Hours	122 ± 2	124 ± 2	125 ± 2	123 ± 2
1 Hours	122 ± 2[ab]	122 ± 2[ab]	125 ± 2[a]	120 ± 2[b]
2 Hours	120 ± 2	121 ± 2	120 ± 2	122 ± 2
4 Hours	121 ± 2	121 ± 2	124 ± 2	122 ± 2
bDBP				
0 Hours	76 ± 2	75 ± 2	75 ± 2	75 ± 2
1 Hours	69 ± 2	69 ± 2	71 ± 2	70 ± 2
2 Hours	71 ± 2	70 ± 2	70 ± 2	71 ± 2
4 Hours	72 ± 2	71 ± 2	73 ± 2	75 ± 2
bPP				
0 Hours	46 ± 2	48 ± 2	49 ± 2	48 ± 2
1 Hours	54 ± 2*,[a]	53 ± 2*,[a]	54 ± 2*,[a]	49 ± 2[b]
2 Hours	49 ± 2	51 ± 2	50 ± 2	51 ± 2*
4 Hours	48 ± 2	50 ± 2	51 ± 2	49 ± 2
aSBP				
0 Hours	113 ± 2	115 ± 2	116 ± 2	115 ± 2
1 Hours	108 ± 2*	109 ± 2*	110 ± 2*	108 ± 2*
2 Hours	108 ± 2*	109 ± 2*	108 ± 2*	110 ± 2*
4 Hours	111 ± 2	110 ± 2*	112 ± 2	112 ± 2
aDBP				
0 Hours	77 ± 2	76 ± 2	76 ± 2	76 ± 2
1 Hours	69 ± 2*	70 ± 2*	72 ± 2*	71 ± 2*
2 Hours	72 ± 2*	72 ± 2*	71 ± 2*	72 ± 2*
4 Hours	73 ± 2*	73 ± 2*	74 ± 2	74 ± 2
aHR				
0 Hours	58 ± 2	59 ± 2	58 ± 2	57 ± 2
1 Hours	66 ± 2*	66 ± 2*	65 ± 2*	67 ± 2*
2 Hours	64 ± 2*	63 ± 2*	62 ± 2*	61 ± 2*
4 Hours	62 ± 2	62 ± 2	61 ± 2	60 ± 2
aPP[§]				
0 Hours	36 ± 1	39 ± 1	39 ± 1	38 ± 1
1 Hours	38 ± 1	38 ± 1	38 ± 1	35 ± 1
2 Hours	36 ± 1	37 ± 1	36 ± 1	38 ± 1
4 Hours	37 ± 1[a]	37 ± 1[a]	32 ± 1*,[b]	38 ± 1[a]
aMAP				
0 Hours	90 ± 2	90 ± 2	91 ± 2	90 ± 2
1 Hours	84 ± 2*	85 ± 2*	86 ± 2*	85 ± 2*
2 Hours	85 ± 2*	86 ± 2*	85 ± 2*	85 ± 2*

4 Hours	87 ± 2	86 ± 2*	88 ± 2	87 ± 2
AP				
0 Hours	11 ± 1	14 ± 1	13 ± 1	12 ± 1
1 Hours	7 ± 1*	7 ± 1*	8 ± 1*	7 ± 1*
2 Hours	7 ± 1*	7 ± 1*	9 ± 1*	8 ± 1*
4 Hours	9 ± 1*	10 ± 1*	11 ± 1	10 ± 1

Data are presented as least square means ± SEM, n = 15. Data are presented as untransformed unless otherwise indicated. Values were compared with the use of the PROC MIXED procedure in SAS version 9.4. There were significant main effects of time for bDBP, bPP, aSBP, aDBP, aHR, aMAP and AP (all $P < 0.0001$). There were no significant main effects of treatment on brachial or aortic blood pressure parameters. Values in a row without a common superscript letter differ, $P < 0.05$. *Denotes values significantly different from baseline within a treatment group, $P < 0.05$. §Denotes natural log-transformation. Abbreviations: bSBP, brachial systolic blood pressure; bDBP, brachial diastolic blood pressure; bPP, brachial pulse pressure; aSBP, aortic systolic blood pressure; aDBP, aortic diastolic blood pressure; aPP, aortic pulse pressure; aHR, aortic heart rate; aMAP, aortic mean arterial pressure; AP, augmented pressure.

Supplemental Table 3S.5

Chronic effects of PBO, RBJ, PBO+NIT, and NF-RBJ on postprandial blood pressure and hemodynamic parameters.

	PBO	RBJ	PBO+NIT	NF-RBJ
bSBP				
0 Hours	124 ± 2	121 ± 2	122 ± 2	122 ± 2
1 Hours	120 ± 2[a]	120 ± 2[a]	122 ± 2[a]	116 ± 2[b]
2 Hours	121 ± 2	123 ± 2	121 ± 2	119 ± 2
4 Hours	122 ± 2[a]	122 ± 2[a]	127 ± 2*,[b]	119 ± 2[a]
bDBP				
0 Hours	75 ± 1	74 ± 1	74 ± 1	74 ± 1
1 Hours	69 ± 1*	69 ± 1*	70 ± 1*	67 ± 1*
2 Hours	71 ±1*,[a]	72 ± 1[a]	71 ± 1*,[a]	68 ± 1*,[b]
4 Hours	72 ± 2*,[a]	72 ± 1[a]	75 ± 2[b]	71 ± 1[c]
bPP				
0 Hours	49 ± 2	47 ± 2	48 ± 2	48 ± 2
1 Hours	51 ± 2	51 ± 2*	52 ± 2*	48 ± 2
2 Hours	50 ± 2	51 ± 2*	50 ± 2	51 ± 2
4 Hours	49 ± 2[ab]	50 ± 2[ab]	52 ± 2*,[a]	47 ± 2[b]
aSBP				
0 Hours	115 ± 2	112 ± 2	113 ± 2	113 ± 2
1 Hours	108 ± 2*	108 ± 2*	109 ± 2*,[a]	104 ± 2*,[b]
2 Hours	109 ± 2*, [ab]	111 ± 2[a]	110 ± 2*, [ab]	107 ± 2*,[b]
4 Hours	111 ± 2*,[a]	111 ± 2[a]	116 ± 2[b]	110 ± 2[a]
aDBP				
0 Hours	77 ± 1	75 ± 1	76 ± 1	75 ± 1
1 Hours	70 ± 1*	70 ± 1*	71 ± 1*	69 ± 1*
2 Hours	72 ± 1*,[a]	73 ± 1	73 ± 1*,[a]	69 ± 1*,[b]
4 Hours	75 ± 1*,[a]	73 ± 1[a]	76 ± 1[b]	71 ± 1
aPP[§]				
0 Hours	38 ± 1	37 ± 1	37 ± 1	37 ± 1
1 Hours	37 ± 1	37 ± 1	37 ± 1	35 ± 1
2 Hours	37 ± 1	38 ± 1	37 ± 1	38 ± 1
4 Hours	37 ± 1[ab]	38 ± 1[ab]	39 ± 1[a]	35 ± 1[b]
aHR				
0 Hours	60 ± 2	58 ± 2	59 ± 2	57 ± 2
1 Hours	64 ± 2*	64 ± 2*	63 ± 2*	63 ± 2*
2 Hours	62 ± 2	61 ± 2	62 ± 2	59 ± 2
4 Hours	60 ± 2[ab]	61 ± 2[ab]	59 ± 2[a]	62 ± 2*,[b]
aMAP				
0 Hours	90 ± 1	89 ± 1	89 ± 1	88 ± 1
1 Hours	84 ± 1*	84 ± 1*	85 ± 1*	82 ± 1*
2 Hours	85 ± 1*,[ab]	87 ± 1[a]	86 ± 1[a]	83 ± 1*,[b]

4 Hours	87 ± 1*,[a]	87 ± 1[a]	91 ± 1[b]	87 ± 1[a]
AP				
0 Hours	12 ± 1	11 ± 1	12 ± 1	11 ± 1
1 Hours	7 ± 1*,[ab]	8 ± 1*,[ab]	6 ± 1*,[a]	8 ± 1*,[b]
2 Hours	8 ± 1*	9 ± 1*	8 ± 1*	8 ± 1*
4 Hours	10 ± 1*	10 ± 1	9 ± 1*	11 ± 1

Data are presented as least square means ± SEM, *n* = 15. Data are presented as untransformed unless otherwise indicated. Values were compared with the use of the PROC MIXED procedure in SAS version 9.4. There were significant main effects of time for bSBP, bDBP, bPP, aSBP, aDBP, aHR, aMAP, and AP (bPP: $P = 0.009$, all others $P < 0.0001$). There were significant main effects of treatment for bSBP ($P = 0.0002$), bDBPe ($P = 0.0027$), aSBP ($P = 0.0037$), aDBP ($P = 0.0128$), and aMAP ($P = 0.0041$). Values in a row without a common superscript letter differ, $P < 0.05$. *Denotes values significantly different from baseline within a treatment group, $P < 0.05$. §Denotes natural log-transformation. Abbreviations: Abbreviations: bSBP, brachial systolic blood pressure; bDBP, brachial diastolic blood pressure; bPP, brachial pulse pressure; aSBP, aortic systolic blood pressure; aDBP, aortic diastolic blood pressure; aPP, aortic pulse pressure; aHR, aortic heart rate; aMAP, aortic mean arterial pressure; AP, augmented pressure.

Supplemental Table 3S.6

Fold changes in gene expression relative to baseline (Acute Visit, 0 Hours) in PBMCs after acute and chronic treatment exposure.

	PBO	RBJ	PBO+NIT	NF-RBJ
p47phox				
Acute, 0 hrs	1.0	1.0[a]	1.0	1.0
Acute, 4 hrs	1.3 ± 0.3	1.5 ± 0.2[b]	1.2 ± 0.3	1.0 ± 0.2
Chronic, 0 hrs	1.6 ± 0.3	1.0 ± 0.2[ab]	1.4 ± 0.3	1.1 ± 0.2
Chronic, 4 hrs	1.3 ± 0.3	1.2 ± 0.2[ab]	1.6 ± 0.3	1.4 ± 0.2
NFκB				
Acute, 0 hrs	1.0	1.0	1.0	1.0
Acute, 4 hrs	1.3 ± 0.3	1.5 ± 0.2	1.4 ± 0.4	1.1 ± 0.2
Chronic, 0 hrs	1.2 ± 0.3	1.0 ± 0.2	1.2 ± 0.4	1.0 ± 0.2
Chronic, 4 hrs	1.4 ± 0.3	1.2 ± 0.2	1.7 ± 0.4	1.4 ± 0.2
TNF-α				
Acute, 0 hrs	1.0[ab]	1.0	1.0[a]	1.0[a]
Acute, 4 hrs	0.9 ± 0.2[ab]	0.7 ± 0.1	0.7 ± 0.1[ab]	0.6 ± 0.1[b]
Chronic, 0 hrs	1.2 ± 0.2[a]	1.0 ± 0.1	1.0 ± 0.1[ab]	1.1 ± 0.1[ac]
Chronic, 4 hrs	0.6 ± 0.2[b]	0.7 ± 0.1	0.6 ± 0.1[b]	0.8 ± 0.1[abc]
TLR-4				
Acute, 0 hrs	1.0[a]	1.0[a]	1.0	1.0[a]
Acute, 4 hrs	1.9 ± 0.2[b]	1.7 ± 0.2[bd]	1.3 ± 0.2	1.5 ± 0.4[a]
Chronic, 0 hrs	1.3 ± 0.2[ab]	1.0 ± 0.2[ac]	1.0 ± 0.2	1.1 ± 0.4[ac]
Chronic, 4 hrs	1.5 ± 0.2[ab]	1.9 ± 0.2[bd]	1.3 ± 0.2	2.4 ± 0.4[b]
GADD34				
Acute, 0 hrs	1.0[a]	1.0[a]	1.0[ab]	1.0[ab]
Acute, 4 hrs	0.57 ± 0.3[a]	0.6 ± 0.1[bc]	0.8 ± 0.3[a]	0.6 ± 0.1[b]
Chronic, 0 hrs	1.7 ± 0.3[b]	0.8± 0.1[ab]	1.7 ± 0.3[b]	1.1 ± 0.1[a]
Chronic, 4 hrs	0.4 ± 0.3[ac]	0.4 ± 0.1[c]	0.7 ± 0.3[a]	0.7 ± 0.1[b]
XBP1s				
Acute, 0 hrs	1.0[a]	1.0	1.0	1.0
Acute, 4 hrs	0.7 ± 0.1[ab]	0.9 ± 0.1	1.5 ± 0.3	1.0 ± 0.2
Chronic, 0 hrs	0.8 ± 0.1[ab]	0.9 ± 0.1	1.5 ± 0.3	1.1 ± 0.2
Chronic, 4 hrs	0.5 ± 0.1[b]	0.9 ± 0.1	1.1 ± 0.3	1.0 ± 0.2

Data are presented as least square means ± SEM, *n* = 15. Values represent untransformed fold changes in expression and were compared with use of the PROC MIXED procedure in SAS version 9.4. Values in a column without a common superscript letter differ, $P < 0.05$. There were no significant main effects of time in the models for *XBP1s*, *NFκB* and *p47phox* for any treatment group. There was a significant main effect of time for *GADD34* in the PBO, RBJ and NF-RBJ groups ($P < 0.05$). There was only a significant main effect of time for *TNF-α* in the NF-RBJ group ($P = 0.0223$), and a significant main effect of time for *TLR-4* in the RBJ group ($P = 0.004$). Abbreviations: NFκB, nuclear factor kappa B; TNF-α; tumor necrosis factor-alpha; TLR-4, toll-like receptor-4; GADD34, growth arrest and DNA damage inducible protein 34; XBP1s, spliced X box binding protein-1.

APPENDIX 2: SUPPLEMENTAL DATA FOR CHAPTER 4

SUPPLEMENTAL FIGURES

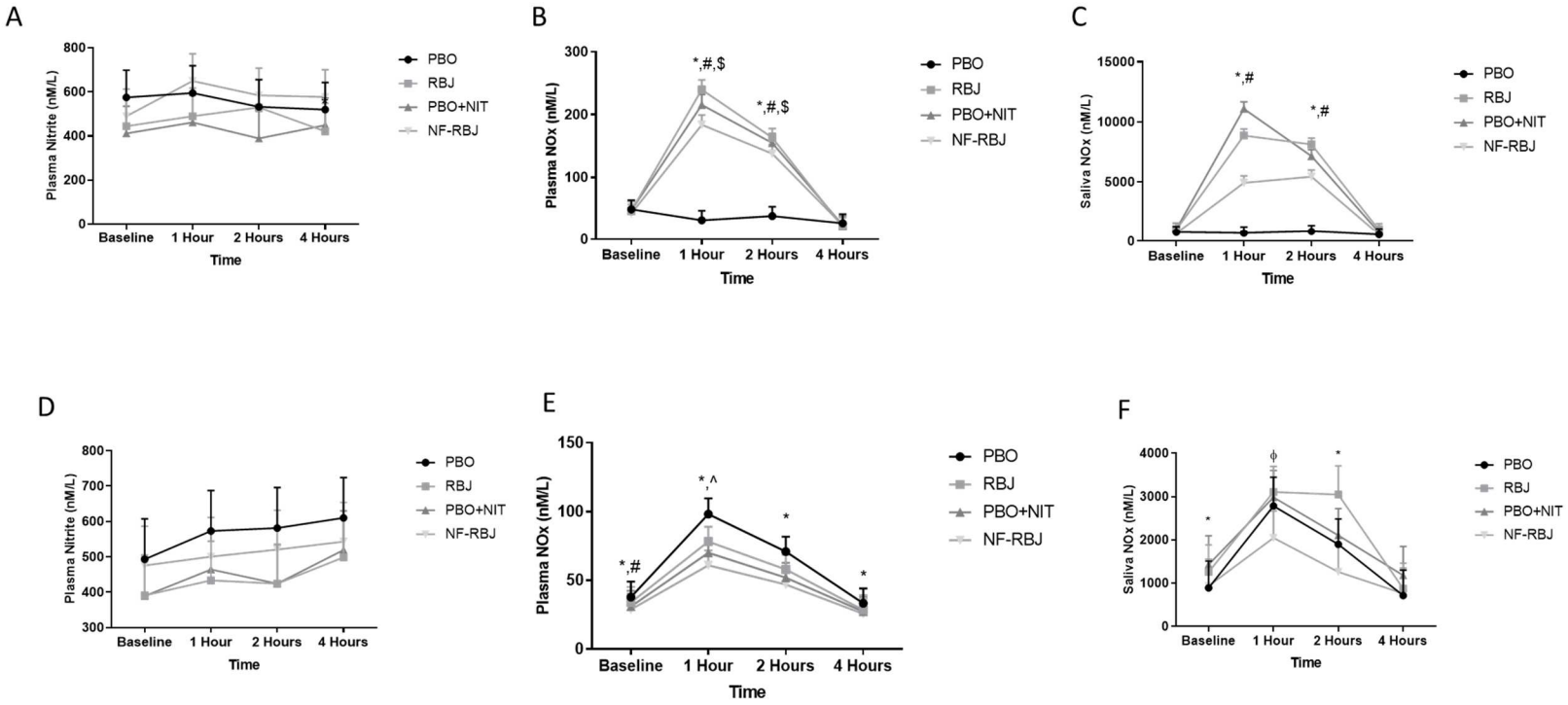


Figure S4.1 Plasma nitrite, plasma NOx and saliva NOx in females at the acute visit (A-C, respectively) and at the chronic visit (D-F, respectively). Time points annotated with symbols represent significant time*treatment interactions between treatment groups. *RBJ significantly different than PBO and NF-RBJ, $P < 0.01$. #PBO+NIT significantly different than PBO and NF-RBJ, $P < 0.01$. $NF-RBJ significantly different than PBO, $P < 0.05$. ^PBO+NIT significantly different than NF-RBJ, $P < 0.01$. φRBJ and PBO+NIT significantly different than NF-RBJ, $P < 0.01$.

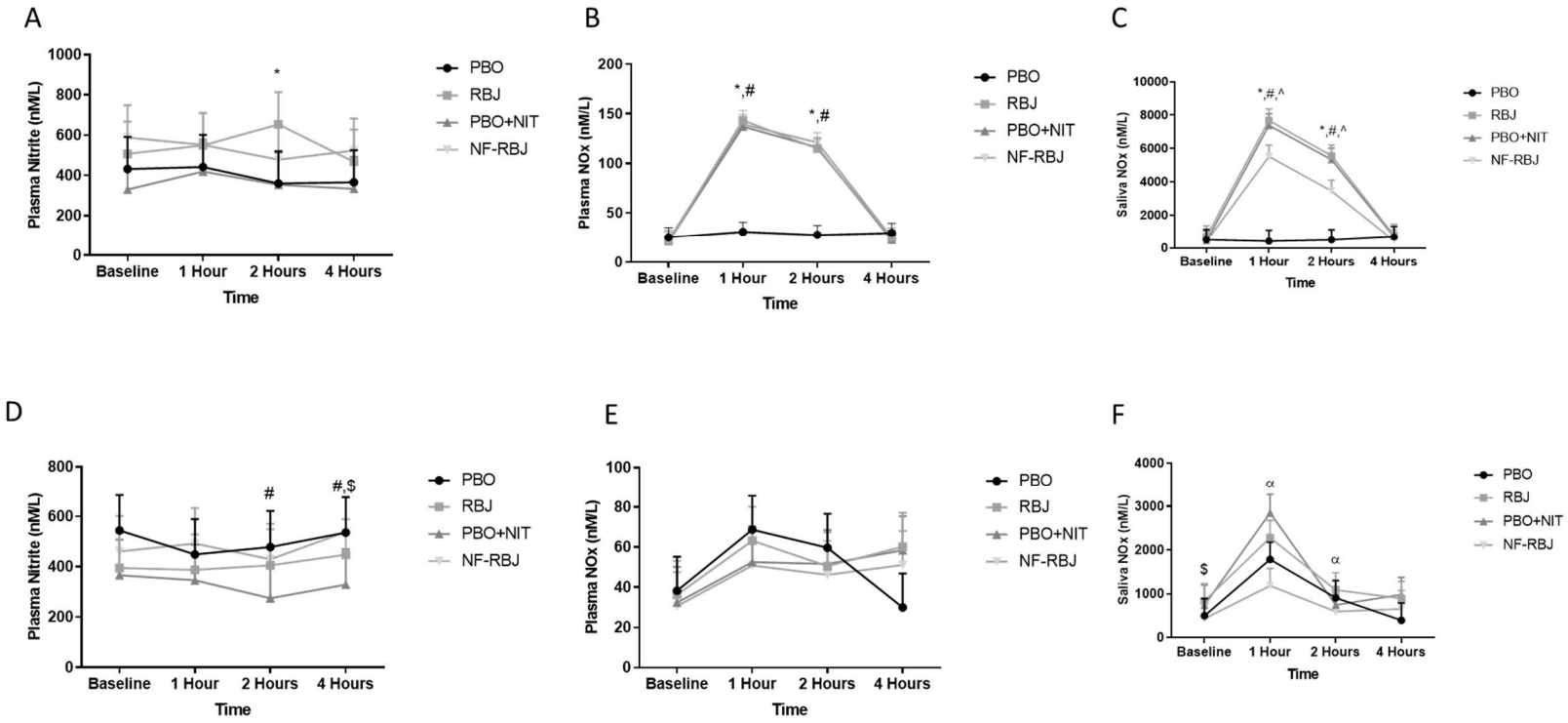


Figure S4.2 Plasma nitrite, plasma NOx and saliva NOx in males at the acute visit (A-C, respectively) and at the chronic visit (D-F, respectively). Time points annotated with symbols represent significant time*treatment interactions between treatment groups. *RBJ significantly different than PBO and NF-RBJ, $P < 0.01$. #PBO+NIT significantly different than PBO and NF-RBJ, $P < 0.01$. $NF-RBJ significantly different than PBO, $P < 0.05$. ^PBO+NIT significantly different than NF-RBJ, $P < 0.01$. φRBJ and PBO+NIT significantly different than NF-RBJ, $P < 0.01$. αRBJ significantly different than PBO, PBO+NIT and NF-RBJ, $P < 0.05$.

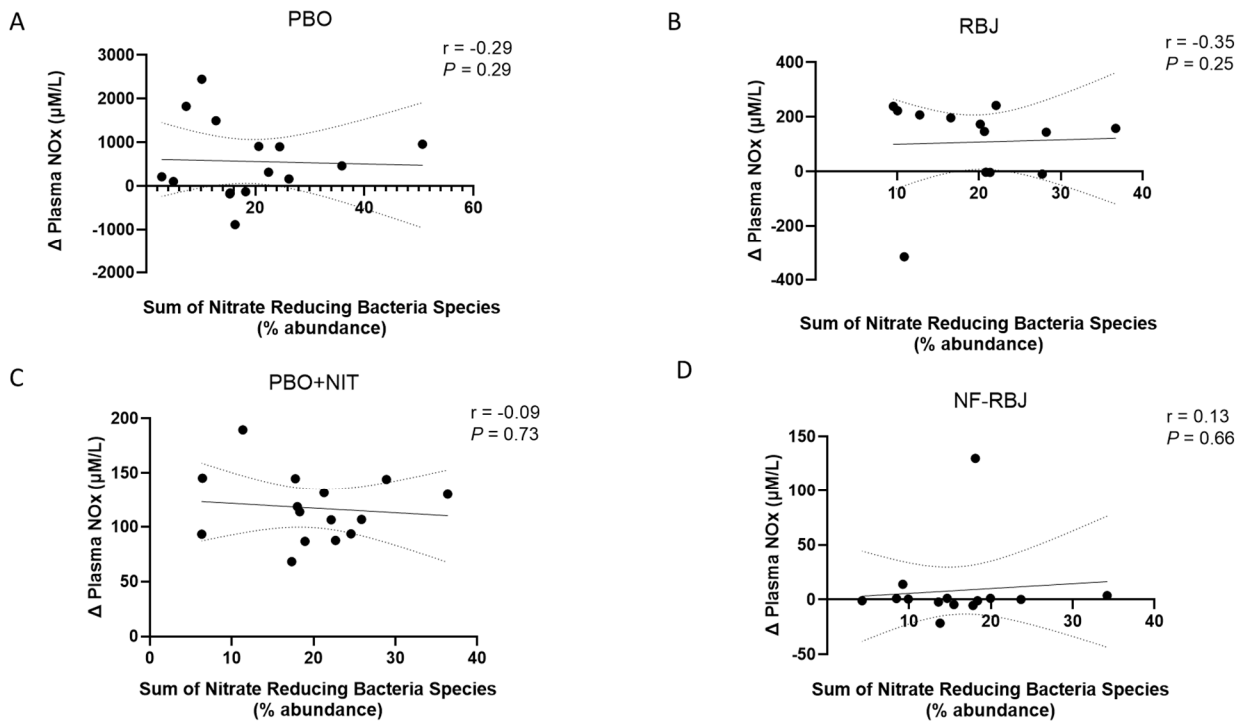


Figure 4S.3 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma NOx at the acute visit per treatment group, n = 15.

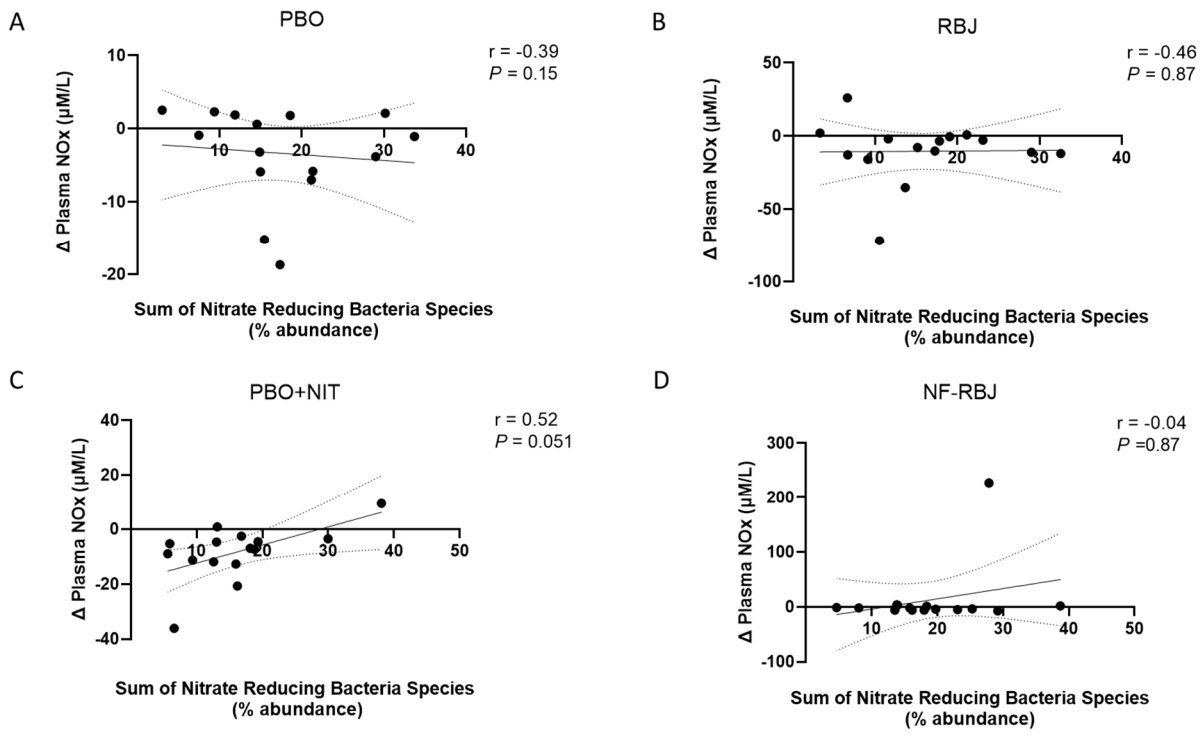


Figure 4S.4 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma NOx at the chronic visit per treatment group, n = 15.

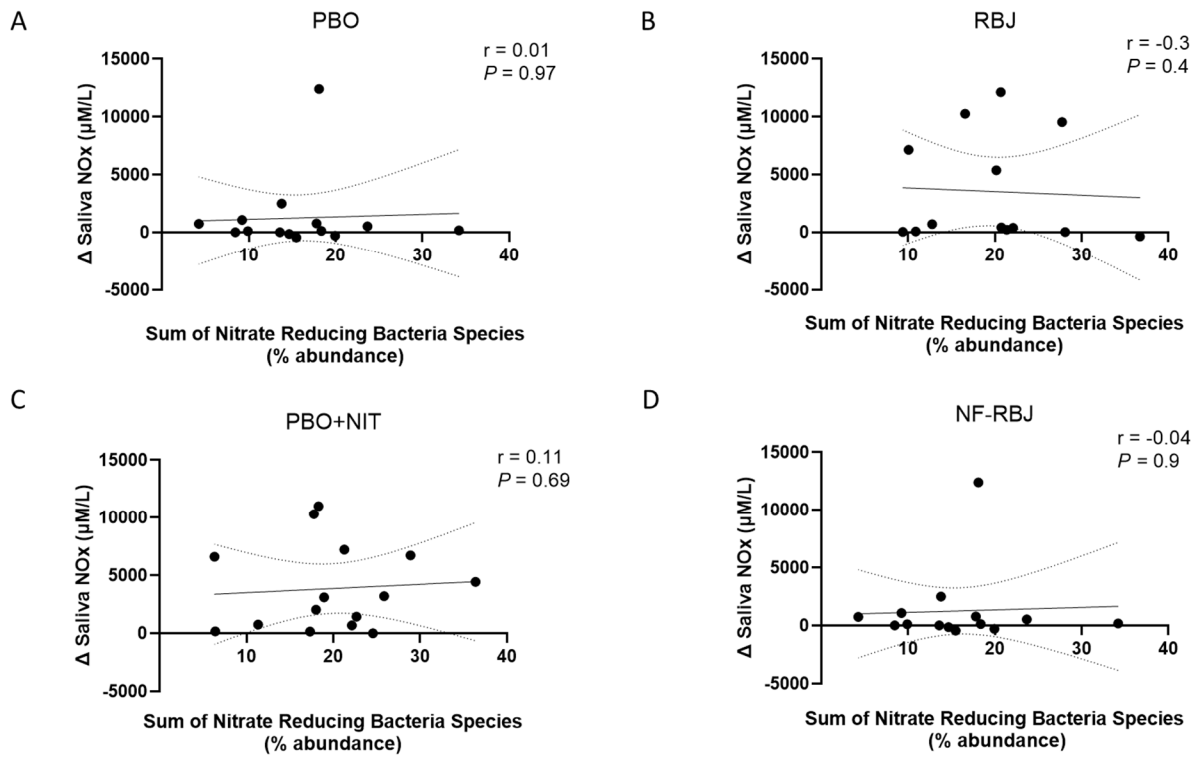


Figure 4S.5 Correlations between the sum of nitrate-reducing bacteria species and peak change in saliva NOx at the acute visit per treatment group, n = 15.

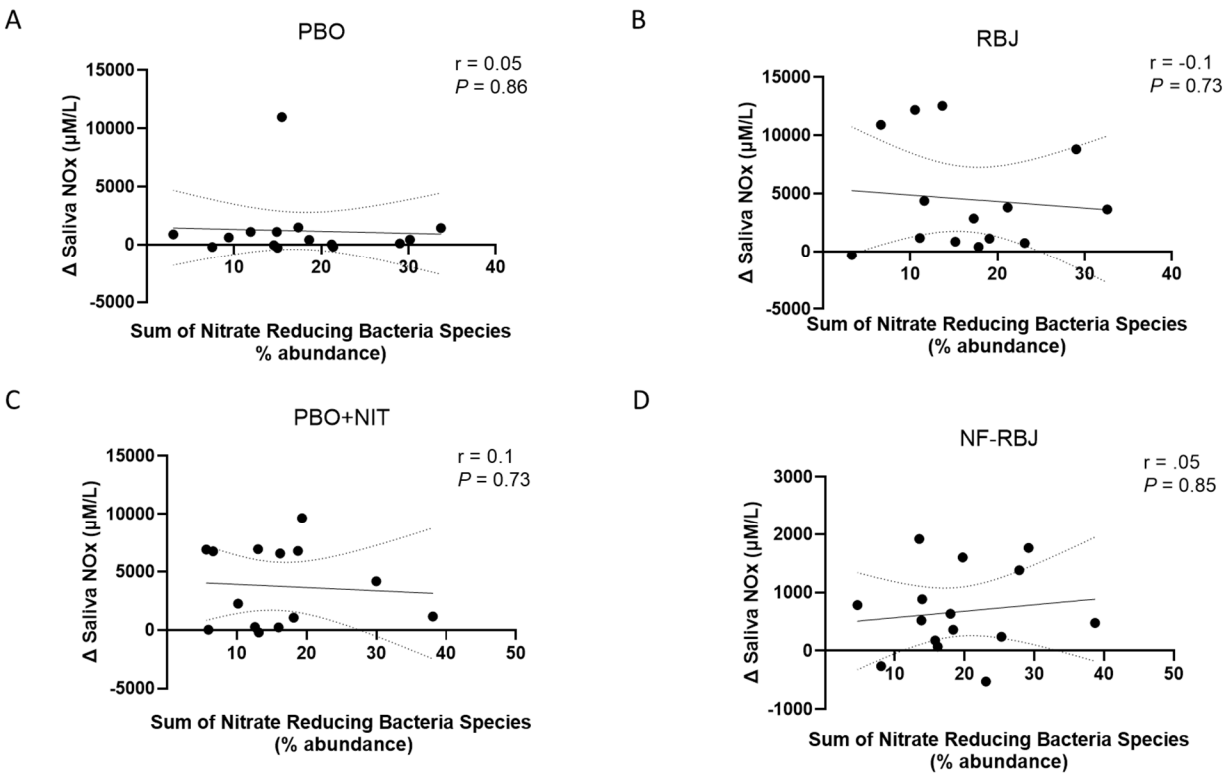


Figure 4S.6 Correlations between the sum of nitrate-reducing bacteria species and peak change in saliva NOx at the chronic visit per treatment group, n = 15.

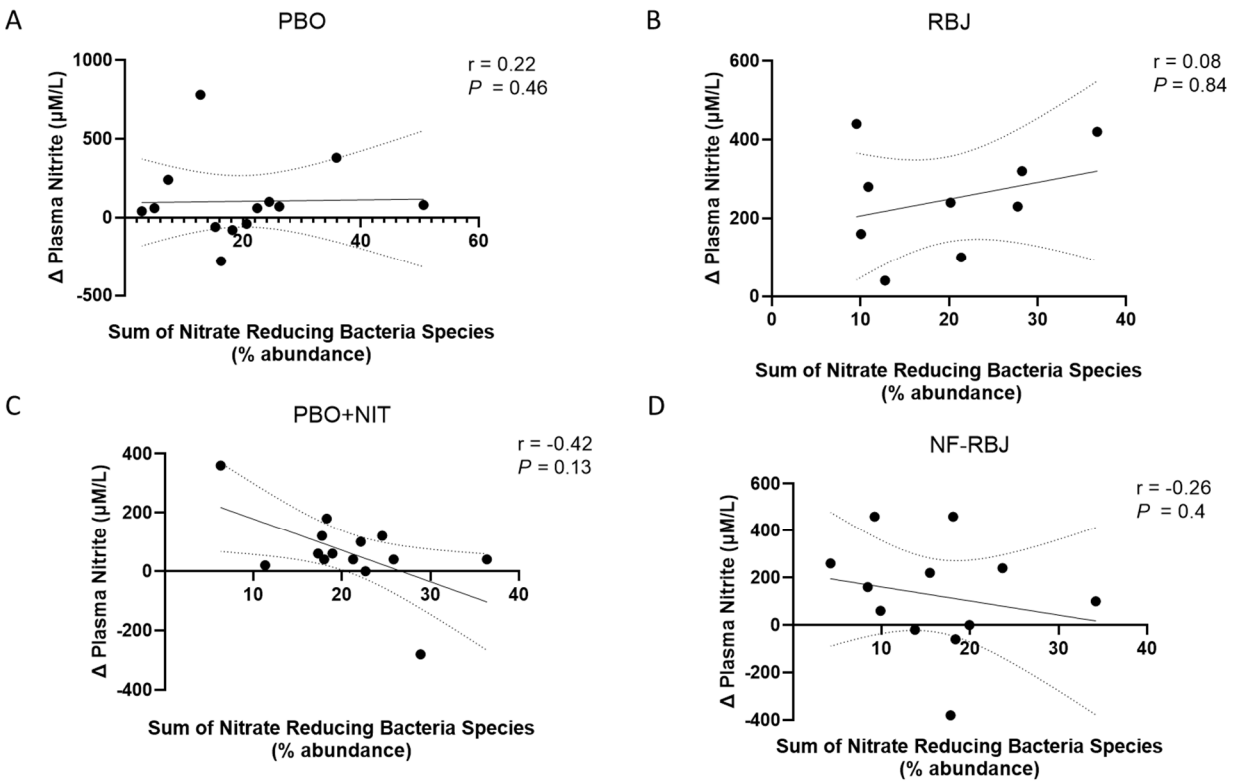


Figure 4S.7 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma nitrite at the acute visit per treatment group, n = 15.

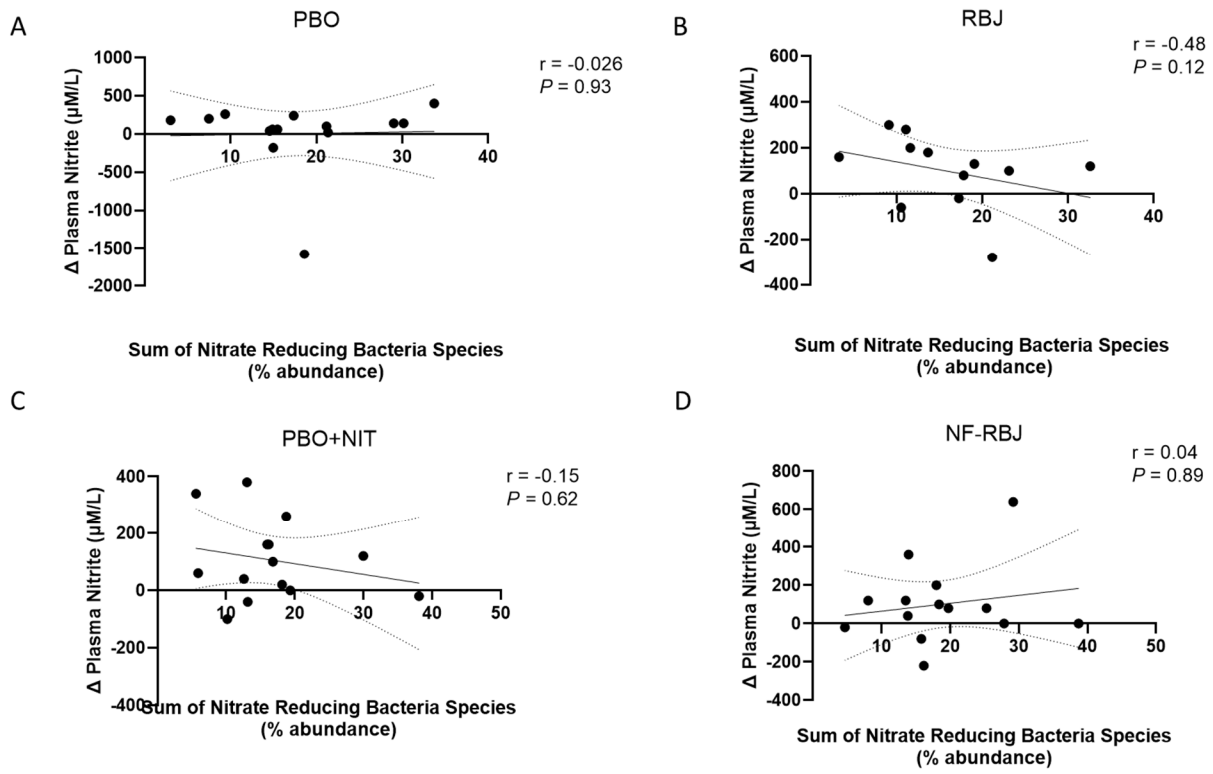


Figure 4S.8 Correlations between the sum of nitrate-reducing bacteria species and peak change in plasma nitrite at the chronic visit per treatment group, n = 15.

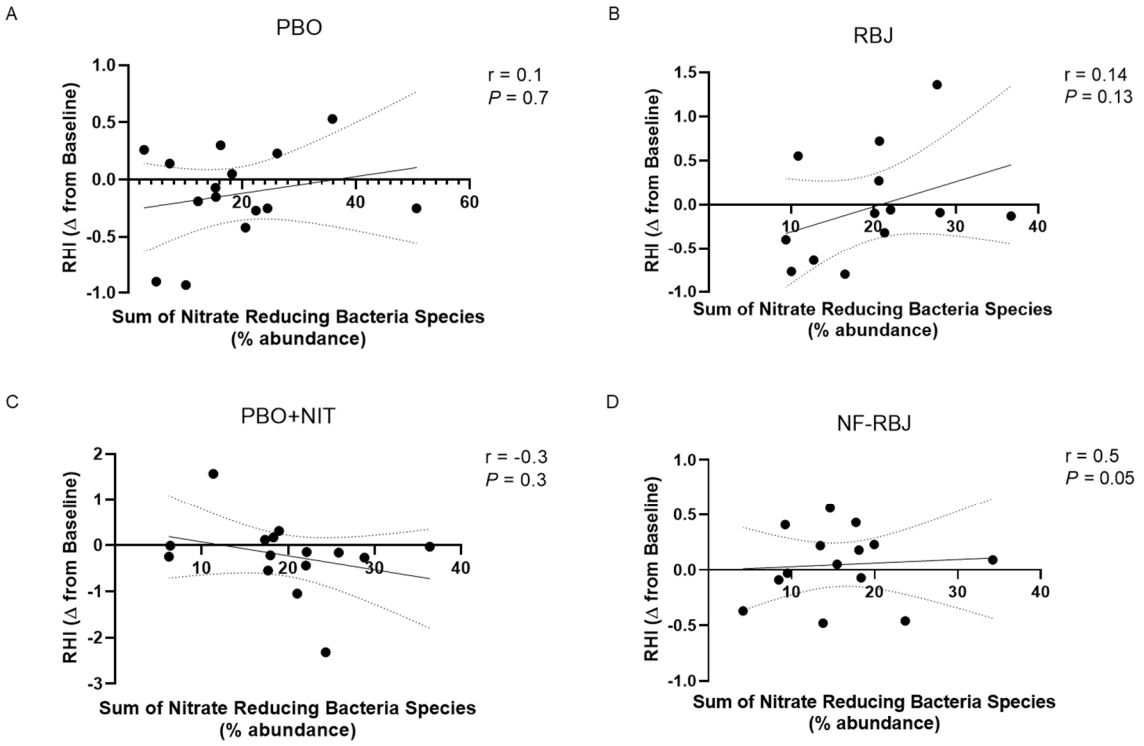


Figure 4S.9 Correlations between the sum of nitrate-reducing bacteria species and RHI change from baseline at the acute visit per treatment group, n = 15.