The Effects of Nitrate Supplementation on *Porphyromonas gingivalis* Abundance and the Expression Levels of *hmuY*, *kgp,* and *NarG* Genes in the Human Oral Microbiome

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Introduction

Periodontal disease is one of the most widespread diseases in westernized countries, including North America, and is thought to be the leading cause of tooth loss in older populations.[2] *Porphyromonas gingivalis* is the keystone pathogen found in patients with periodontitis. It is a gram-negative, anaerobic bacterium and produces toxic cysteine proteases, called gingipains, that include lysine-gingipain (Kgp), arginine-gingipain A (RgpA), and arginine-gingipain B (RgpB).[1] Kgp and RgpA/B are crucial to the pathogenicity of *P. gingivalis* and are involved in host colonization, suppression of host defenses, nutrient acquisition, and tissue destruction.[1]

Levels of this bacterium have also been found in the brains of patients with Alzheimer's disease suggesting that *P. gingivalis*, and the gingipains it produces, is involved in the pathophysiology that leads to cognitive decline in this disease.[1,2,3,7] Gingipains that are released in the brain can damage Tau proteins which mediate neuronal functioning.[1] Gingipains are characterized as narrow-spectrum virulence targets; broad-spectrum antibiotics do not eliminate *P. gingivalis* and, instead, add to its resistance. Dominy et al.[1] found that directly inhibiting gingipain production using short peptide analogs decreases colonization of the brain by P. gingivalis, therefore, reducing neurodegeneration in Alzheimer's disease. Kgp inhibitors have shown promise in reducing the amount and persistence of P. gingivalis present in the brains of mice.[1] The Kgp inhibitors also block the acquisition of host heme by P. gingivalis – a process mediated by a unique hemophore, hmuY, that acts as a biomarker for P. gingivalis.[5]

Another mechanism that may provide similar effects to that of gingipain inhibitors is the administration of a dietary nitrate supplement. It is suggested that the oral microbiome is influenced by nitrate, and the salivary composition can be altered via dietary nitrate supplementation.[6] The nitrate-nitrite-nitric oxide pathway relies on bacteria within the oral microbiome to convert nitrate to nitrite, and this pathway is thought to be an important contributing factor to overall body health.[6] Nitric oxide is a vasodilator that is retrieved by the microbes in the oral cavity.[4] The production of nitric oxide via nitrate supplementation is known to cause systemic benefits including decreased blood pressure and arterial stiffness, improved endothelial function, and reversed metabolic syndrome[4,6] Likewise, Rosier et al.[4] suggested that nitrate could be used as a dietary intervention to promote eubiosis in the oral microbiome, and significantly decrease the abundance of *P. gingivalis* post nitrate supplementation.[4]

Previously conducted studies have yielded promising data in reducing the abundance of *P. gingivalis* present in the oral microbiome which subsequently reduces the production of gingipains. This project builds on a larger study that analyzes the effect of nitrate supplementation on the abundance of *P. gingivalis* in the oral microbiome as well as the effect on secondary physiological responses such as blood pressure and flow mediated dilation (FMD). To my knowledge, no other studies have specifically analyzed the16SrRNA, *hmuY*, *kgp*, and *Nar*G genes as well as the absolute abundance of *P. gingivalis* in response to in vivo nitrate supplementation. It is hypothesized that a reduction of *P. gingivalis* in the oral microbiome could lower the risk of periodontitis and Alzheimer's disease and improve various cardiovascular and physiological responses. Future studies can be conducted to assess the effects of nitrate supplementation on those with Alzheimer's disease and to see if there is a link between oral microbiome dysbiosis and cognitive impairment.

Objective

This project builds on a study currently being conducted by Dr. Rakobowchuk, Dr. Bottos, and Dr. Van Hamme that analyzes the effect of nitrate supplementation on the abundance of *P. gingivalis* in the oral microbiome. The results of this study will be compiled with data from other aspects of the study previously conducted by one Honours and one Directed Study student, to provide supporting data for a grant proposal resubmission in 2022. If the proposal is successful, then future studies can be conducted with participants from older age groups to assess the effects of nitrate supplementation on those more susceptible to cognitive decline and Alzheimer's disease and to see if there is a link between oral microbiome dysbiosis and cognitive impairment.

The central question being asked is how does ten-day nitrate supplementation affect the absolute abundance of *P. gingivalis* and expression of gingipains, *hmuY*, and *NarG* genes in human oral microbiomes. The main objectives are to design and validate primers and probes for RT-qPCR to target four genes, 16SrRNA, *hmuY*, *kgp*, and *NarG*, and to determine if nitrate supplementation impacts P. gingivalis abundance and the expression levels of the four genes in human oral microbiome samples.

Materials and Methods

Experimental design

Ten healthy participants, including seven males and three females between 20-49 years of age, were recruited to participate in the study. Participants underwent a flow mediated dilation (FMD) protocol to measure endothelial dependent dilation of the brachial artery. Following this, blood was drawn from an antecubital vein and oral samples of saliva, floss and a tongue swab were taken. Participants were then given twenty 400 mg potassium nitrate oral supplements to be taken twice a day for ten days before returning for the aforementioned sampling post-supplementation.

Methods

Oral samples were subjected to an RNA extraction protocol by an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), and DNase was used to remove the DNA from these samples. All RNA extracts were subsequently stored at -80°C. Primers and probes will be designed for RT-qPCR to target 16SrRNA, *hmuY*, *kgp*, and *NarG* using Geneious Prime software and publicly available sequence data from NCBI and the Human Oral Microbiome Database. RNA has been previously extracted from the nitrate treatment clinical trial oral microbiome and stored at -80°C. Sampling of the oral microbiome was done pre- and post- nitrate supplementation. Subsequently, RNA was extracted from the samples and will be subjected to RT-qPCR analysis using a Quant Studio 3 Real-Time PCR system to quantify the abundance of *P. gingivalis* and the expression levels of the 16SrRNA, *hmuY*, *kgp*, and *NarG* genes. Standard curves for each gene will be prepared using known quantities of plasmid carrying each of the genes. These plasmids will be purchased. All RT-qPCR analyses will include triplicate standard curves, RNA-free

controls to check for contamination, and triplicate samples to verify reproducibility. Results will be compared pre- and post-nitrate supplementation using paired-T tests to see if there are significant differences in gene expression levels, and in absolute abundance of *P. gingivalis*.

Results

Table 1. Primer and probe sequences designed to target *hmuY*, *kgp*, *NarG*, and 16SrRNA genes.

Name of Primer/Probe	Sequence
HmuY_384F	CCACTTTCGCCACAATTG
HmuY_416P	GCGGTGAAGAGCCATGTCCCA
HmuY_513R	AATACGAAACGTGGCAGT
HmuY_496F	ACTGCCACGTTTCGTATT
HmuY_556P	TGGAGGGTTGGTTCGGCTCGT
HmuY_627R	TCTGTGCATTGCCATTGA
HmuY_536F	TTTGGTTACTGCTTCGGG
HmuY_556P	TGGAGGGTTGGTTCGGCTCGT
HmuY_639R	TTTTCTCCGCACTCTGTG
Kgp_80F	ATGCTCCGACTACTCGAA
Kgp_123P	GCAGTTCGATGCAAGCTTTTCGTTCA
Kgp_183R	ACCTTTGGTCTCCACCTT
Kgp_362F	TGCCACATCAACCCTCTA
Kgp_390P	TGATGATCCCGAAAAGGTTCCCTTCGT
Kgp_437R	GCATAAGCAGCAGCATTG
Kgp_2436F	AGGACAGGGTGAAGTTGT
Kgp_2457P	CCCCGGTGGTGTTTACGACTATTGCA
Kgp_2516R	CACATCTTTCCGGATGCA
Pg16S_226F	TAAGATAGGCATGCGTCC
Pg16S_276P	AGGCGACGATGGGTAGGGGAA
Pg16S_319R	AGTGTGGGGGATAAACCT
Pg16S_582F	GTTGTTCGGTAAGTCAGC
Pg16S_643P	CCGGGCTTGAGTTCAGCGGC
Pg16S_721R	AATCGGAGTTCCTCGTGA
Pg16S_1177F	GGTGTGGATGACGTCAAT
Pg16S_1236P	TGGGAGGGACAATGGGCAGCT
Pg16S 1294R	TGGGGAAGGGTTTAGAGA
NarG_2383F	CTGTATGCCGACGTGATT
NarG_2438P	ACACGTCCGACATGCACCCGT

Table 1. Primer and probe sequences designed to target *hmuY*, *kgp*, *NarG*, and 16SrRNA genes.

NarG_2515R	CCCAGTCGGATTTGCTTT
NarG_2433F	CTTGAACACGTCCGACAT
NarG_2451P	GCACCCGTTCATCCACCCGT
NarG_2513R	CAGTCGGATTTGCTTTGC
NarG_3251F	CCGCCAGCATTTCTATCA
NarG_3308P	TGCCTACCGTCCCGCAGTCG
NarG_3361R	TTCATGCCCAGCAGTTTT

Discussion

In this study, the first round of primers and probes were designed for RT-qPCR to target the 16SrRNA, *hmuY*, *kgp*, and *NarG* genes using Geneious Prime software and publicly available sequence data from NCBI and the Human Oral Microbiome Database. Three sets of primer sequences were designed for each of the genes of interest. In early trials, the 384F and 513R primer set for the *hmuY* gene is showing promising results after PCR when tested with the stock *P. gingivalis* positive control. Furthermore, method validation of the reverse transcription protocol has taken place, showing positive results with the conversion of RNA to cDNA.

It is expected that the abundance of *P. gingivalis* and gingipains in the oral cavity will decrease from pre to post nitrate supplement intervention as well as the expression of the *hmuY* gene. It is also expected that the expression of the *NarG* gene will increase pre- to post-nitrate supplementation. Additionally, it is expected that arterial blood pressure and stiffness will decrease, and endothelial function will improve post-intervention. The results of the study will indicate if dietary nitrate supplementation does have an effect on *P. gingivalis* and gingipain abundance in the oral microbial community, as well as on secondary physiological responses.

Literature Sources

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