Biology Honours Proposal

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Title

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

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 reductions in the abundance of *P. gingivalis* in the oral microbiome which subsequently reduces gingipain production. 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 nitrate reductase 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.

Objectives

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 nitrate reductase genes in human oral microbiomes. The main objectives are to design and validate primers and probes for RT-qPCR (reverse transcriptase quantitative polymerase chain reaction) to target four genes, 16SrRNA, *hmuY, kgp* and nitrate reductase, and to determine if nitrate supplementation impacts *P. gingivalis* abundance and the expression levels of the four genes in human oral microbiome samples.

There are two specific conceptual objectives that will be addressed within the study:

Objective 1: The first objective of this study is to assess how dietary nitrate supplementation modifies the oral microbial community by analyzing the overall abundance of *P. gingivalis* and the expression levels of 16SrRNA, *hmuY, kgp*, and nitrate reductase genes.

Objective 2: The second objective of this study is to assess the relationship between microbial community modifications and secondary physiological outcomes post-nitrate supplementation.

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 using 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.

Objective 1: Primers and probes will be designed for RT-qPCR to target 16SrRNA, *hmuY, kgp,* and nitrate reductase 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. RNA that was extracted from the samples 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 nitrate reductase 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. Experimentation for this portion of the project will take place in the TRUGen Lab. 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*.

Data analysis
Objective 1: Rstudio will be used to assess microbial diversity. Paired T-tests will be used to assess normalized shifts in microbial communities of *P. gingivalis* and gingipains pre- and post-nitrate supplement intervention.

Objective 2: Linear regression will be used to assess shifts in microbial communities compared to shifts in physiological outcomes including blood pressure and FMD. Depending on the nature of results, two or three linear regressions may be assessed to compare various factors.

Expected Results
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 nitrate reductase genes 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 a dietary nitrate supplement does have an effect on *P. gingivalis* and gingipain abundance in the oral cavity, as well as on secondary physiological responses.

Timeline

Timeline	Task
May–August 2022	Objective 1 Data Collection
September–October 2022	Objective 1 Data Analysis
November–December 2022	Objective 2 Correlational Analyses
January–March 2023	Thesis Writing

Late January	Update Presentation
Late March – Early April	TRU Poster Conference
April	Thesis Defence

Budget

Item	Cost
Taqman MGB Probes	$1,200
Taqman Multiplex Master Mix	$160
SSIV Vilo Master Mix	$700
Custom TQMN Gene Ex Assays (small)	$300
Custom TQMN Gene Ex Assays (medium)	$600
Custom TQMN GX Assay (small)	$300
Custom TQMN GX Assay (medium)	$2,000
Tips, tubes, qPCR plates	$250
TOTAL COST	**$5,510**

The above expenses for the Honours project will be split between MR's Research Acceleration Grant and JV's NSERC Discovery Grant.

Literature Sources

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7. Singhrao SK, Harding A, Poole S, Kesavalu L, Crean S. 2015. *Porphyromonas gingivalis* periodontal infection and its putative links with Alzheimer's disease. Mediators Inflamm. [accessed 2022 May 1]; 137357. doi: 10.1155/2015/137357