Aspirin intervention, inflammation and the oral microbiome

A Thesis

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Abstract

Specific pro-inflammatory oral taxa have been shown to be increased in the gut microbiome of individuals with chronic inflammatory disorders of the intestine, including inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC), as well as colorectal cancer (CRC). Aspirin is associated with decreased risk of colorectal cancer, potentially by modulating the gut and the oral microbiome. However, it remains unclear how these pro-inflammatory oral taxa would respond to anti-inflammatory agents such as aspirin. In this dissertation, we aimed to evaluate the effect of aspirin intervention on the relative abundance of specific pro-inflammatory oral taxa and the relative abundance of inferred functional traits linked to inflammation in a double-blind placebo-controlled trial.

In the first manuscript, we evaluated the effect of a 6-week aspirin intervention on the relative abundance of pro-inflammatory oral taxa in a randomized placebo-controlled trial. We found that the change over-time in the relative abundance of 9 out of the 12 pre-specified taxa at the genus level, and 1 out of 2 pre-specified taxa at the family level differed between the aspirin and placebo groups. These preliminary findings suggest that aspirin may change the relative abundance of oral taxa associated with oral dysbiosis and inflammation.

In manuscript 2, we evaluated the effect of a 6-week aspirin intervention on the relative abundance of inferred functional traits linked to a key bacterial metabolite with inflammatory properties, lipopolysaccharide (LPS). We did not find an association between aspirin intervention and the change in relative abundance of a priori selected

inferred functional traits for LPS. However, we observed a positive correlation between the relative abundance of oral taxa associated with CRC risk and inflammation, and the relative abundance of inferred functional traits of LPS, a bacterial metabolite associated with inflammation. These findings are in line with the current literature on bacterial virulence factors.

Lastly, we investigated whether inflammation-related oral taxa and inflammation-related gut taxa are correlated and whether oral and gut microbiome communities respond similarly to anti-inflammatory agents such as aspirin in a double-blind placebo-controlled trial. Our results show that aspirin may induce changes in oral and gut alpha diversity in a similar fashion. In addition, our findings of an inverse correlation between SCFA-producing gut taxa and pro-inflammatory oral taxa suggest that the study of pro-inflammatory oral taxa may be important for understanding the link between inflammation and the gut microbiome.

Overall, these findings are in line with a growing body of evidence highlighting the role of the oral microbiome in chronic inflammatory disorders of the intestine, including CRC.

Table of Contents

List of Tables

List of Figures

Chapter 1. INTRODUCTION

Although the overall rates of colorectal cancer (CRC) incidence and mortality have decreased during the past 20 years [1], CRC remains a leading cause of cancer related deaths in the United States. The progression of adenomatous polyps (adenomas) in the colon into invasive and metastatic cancerous tumors (carcinomas) is largely preventable if adenomas are detected through screening and removed, with a CRC 5-year survival rate of 90% if the diagnosis occurs while the disease is still localized [2]. However, the 5-year survival rate for CRC sharply declines to under 15%, if invasion or metastasis are detected at diagnosis [1].

Colonoscopy is considered the gold standard for CRC screening, and has been effective at improving CRC related outcomes[2]. However, it is an invasive and expensive procedure, which are barriers to its effective implementation in the general population [3]. Home-based fecal occult blood tests (FOBTs) are another option for CRC screening, but these tests used less frequently, likely due perceptions that they are not effective in reducing cancer-associated mortality. The shortcomings of current screening methods highlight the need for a sensitive, non-invasive diagnostic test for CRC and precancerous lesions[3]. Such a test might increase patient screening rates, as more than 30% of individuals from high-risk groups (ie, age ≥50 years) report never having been screened for CRC[4].

The potential use of aspirin (acetylsalicylic acid) as a chemopreventive agent has been a promising venue in CRC research[5]. Aspirin is thought to affect the development of

tumors through the cyclooxygenase (COX)-dependent pathway[6,7], which perpetuates pro-inflammatory signals by promoting cellular proliferation, angiogenesis and apoptotic resistance, as well as through COX-independent pathways[8]. However, the potential harms associated with regular aspirin use, including greater gastrointestinal (GI) toxicity with advancing patient age[7,9], highlight the need to clarify the mechanisms by which aspirin prevents the development of colorectal neoplasia. Furthermore, these inflammatory pathways could be leveraged to develop mechanistic biomarkers for a personalized risk stratification approach that is crucial to the future of aspirin chemoprevention. A better understanding of the mechanisms underlying the anti-inflammatory effects of aspirin could help optimize chemopreventive CRC approaches. In the future, this could provide a platform to identify those most likely to benefit from aspirin chemoprevention. In the absence of such knowledge, the development of an effective prophylactic approach in patients most likely to benefit from aspirin treatment will likely remain difficult.

Recently, there has been an increasing appreciation for the human microbiome in CRC research as a potential tool for screening. The human microbiome includes the collective genome of all bacteria, archaea, fungi, protists, and viruses found in and on the human body[10]. This collection of symbiotic microbes inhabits the oral, tissue, and gastrointestinal microenvironment, and is associated with many diseases such as obesity, periodontal disease, inflammatory bowel disease, cardiovascular disease, and cancer[10]. Previous studies on the gut microbiome and CRC found that the relative abundance of certain gut taxa (such as *Fusobacterium*, *Peptostreptococcus*, and *Porphyromonas*) was

consistently increased in CRC cases, while the relative abundance of other gut taxa (such as *Bifidobacterium, Ruminococcaceae, Lachnospiraceae, Faecalibacterium and Roseburia*) was consistently decreased in CRC cases, compared to controls[11–14]. In addition, these taxa are also involved in chronic inflammatory disorders of the intestine such as inflammatory bowel diseases (IBDs)[15,16], Crohn's disease (CD)[15,16] and ulcerative colitis (UC)[17–19], where they have similar associations to those observed in CRC cases compared to controls.

Interestingly, the relative abundance of specific oral taxa (such as *Fusobacterium, Peptostreptococcus,* and *Porphyromonas*) has also been found to be increased in oral microbiome of CRC cases, compared to healthy controls[20,21]. The relative abundance of these oral taxa is also increased in periodontitis[20–23], suggesting that these oral taxa have a pro-inflammatory role within of the oral cavity. Moreover, these pro-inflammatory oral taxa can be found in the gut microbiome of CRC cases[3,24,25], as well as the gut microbiome of IBD, CD and UCD cases[15–19]. However, it is unclear whether these oral taxa traveled down the GI track from the oral cavity after periodontitis, or if inflammation in the gut facilitated their translocation from the oral to the gut microbiome[10,26,27].

These pro-inflammatory oral taxa are highly adherent to host epithelial cells, can form biofilms, and produce bacterial enzymes which promote bacterial survival in the colon by degrading host proteins and triggering an inflammatory response[28]. Further, prior studies of these bacterial taxa have also identified specific bacterial genes that can

promote colorectal carcinogenesis via inflammation and deoxyribonucleic acid (DNA) damage[29,30]. We proposed that the composition of the oral microbiome and the presence of specific bacterial genes promoting inflammatory signals in the oral bacterial community can contribute to the risk of chronic inflammatory disorders of the intestine, including CRC, and should be investigated to identify potential chemopreventive approaches to CRC.

Our overall objective was to (1) identify the effect of aspirin intervention on the relative abundance of pro-inflammatory oral taxa which are increased in chronic inflammatory disorders of the intestine, including CRC, (2) identify the effect of aspirin intervention on the relative abundance of oral inferred functional traits linked to inflammation and (3) examine the correlation between inflammation-related oral taxa and inflammation-related gut taxa. Our central hypothesis was that the oral microbiome affects chronic inflammatory disorders of the intestine and CRC risk via inflammatory processes, and aspirin treatment would increase the abundance of protective commensal oral taxa, while decreasing the abundance of harmful oral taxa by modulating the inflammatory response. Likewise, we anticipated that aspirin treatment would decrease the abundance of oral inferred functional traits linked to inflammation. In line with this central hypothesis, we hypothesized that the relative abundance of pro-inflammatory oral taxa would be correlated to pro-inflammatory gut taxa, and inversely correlated to anti-inflammatory gut taxa.

The first manuscript evaluated the effect of a 6-week aspirin intervention on the relative abundance of pro-inflammatory oral taxa in a randomized placebo-controlled trial. The second manuscript evaluated the effect of a 6-week aspirin intervention on the relative abundance of inferred functional traits linked to a key bacterial metabolite with inflammatory properties, lipopolysaccharide (LPS). The third manuscript investigated whether inflammation-related oral taxa and inflammation-related gut taxa are correlated and whether oral and gut microbiome communities respond similarly to anti-inflammatory agents such as aspirin. We addressed our hypotheses in the ASMIC study, a randomized placebo-controlled double-blinded study, in which 50 healthy subjects, 50-75 years old, received either aspirin or placebo treatment for 6 weeks. The study collected information about demographic characteristics of participants, as well as multiple blood, urine, saliva and stool specimens.

Chapter 2. BACKGROUND & SIGNIFICANCE

A. Inflammation and colorectal cancer

Approximately 70% of CRC cases are of unknown etiology, and factors associated with increased CRC risk include diet, alcohol, chronic inflammation of the gastrointestinal tract[31] and more recently the gut[3,24,25] and oral microbiome[20,21]. Chronic inflammation has been previously linked to the etiology of CRC, and it has been shown that inflammatory bowel diseases (IBDs), especially ulcerative colitis (UC), increases CRC risk, whereas nonsteroidal anti-inflammatory drugs (NSAIDs) reduce CRC risk [32]. The colon may be especially prone to carcinogenesis via inflammation, because of rapidly dividing cells and the presence of microbial flora causing permanent low-grade inflammation of colon mucosa.

Inflammation is part of the physiological response to tissue injury, and can be also be triggered by infection, autoimmune diseases, tumors and other pathologies[33]. Inflammation is also accompanied by the release of cytokines and cellular mediators, including tumor necrosis factor-α (TNF-α), interleukins 1 and 6(IL-1 and IL-6, respectively), interferon-γ (INF-γ), transforming growth factor-β (TGF-β), and oxidant-generating enzymes[34,35]. Persistent inflammation can lead to chronic inflammation, which is characterized by the secretion of cyclooxygenase (COX, also known as prostaglandin-endoperoxide synthase) from macrophages and epithelial cells, as well as the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[32,36]. Chronic inflammation is a risk factor for many conditions including cardiovascular disease, diabetes, rheumatoid arthritis, inflammatory bowel diseases (IBDs)[15,16] such as Crohn's disease (CD)[15,16] and ulcerative colitis (UC)[17–19] as well as cancer.

Cyclooxygenase is a key enzyme in the prostaglandin biosynthetic pathway that produces prostaglandins, which are lipids that can influence both gastrointestinal inflammation, and colon tumor progression[37]. COX-2, the inducible isoform of COX, has received considerable attention due to its role in human cancers. The activation of COX-2 in tumor tissue leads to the promotion of angiogenesis, resistance to apoptosis (also known as cellular death), localized immune modulation, and an increase of tumor cell invasiveness and metastasis[36]. In CRC, the overexpression of COX-2 in tumor tissues correlates with poor prognosis, while inhibition of COX-2 activity is likely to be the main mechanism through which non-steroidal anti-inflammatory drugs (NSAIDs) exhibit their protective effects on CRC development [32].

Inflammation-driven carcinogenesis also involves the activation of the NF-κB pathway, which can occur in response to stimuli from the human microbiome[37]. The NF-κB pathway activation mediates the production of pro-inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 8 (IL-8), which have a pathogenic role in CRC by allowing survival and proliferation of intestinal epithelial cells. The NF-κB pathway also serves as an important regulator of the genes encoding for tumor necrosis factor (TNF) and cyclooxygenase 2 (COX-2). These cytokines are often overexpressed in inflammatory bowel disease as well as in colorectal adenoma, a precursor condition to CRC[37]. Ultimately, the NF-κB pathway plays a key role in inflammation-driven carcinogenesis by generating an environment that promotes mutations, and simultaneously prevents damaged cells from undergoing apoptosis, both key features of cancerous cells[28].

B. Inflammation and the gut microbiome

The gut microbiome, either as individual microbes or as a microbial community exerting a collective effect, may potentiate or mitigate colorectal cancer (CRC) risk[10]. However, the mechanisms by which microbes influence tumorigenesis in the intestine, a particularly microbially rich and immunologically complex environment in the human body, remain to be fully clarified. The composition of the gut microbiome is closely tied to host inflammation, and likely plays a role in CRC carcinogenesis by creating a feedback loop of events in the gut. Dysbiotic bacteria can disrupt or damage the intestinal epithelium, triggering an inflammatory response such as the activation of the nuclear factor Kappa B (NF)-κB pathway[33]**.** The activation of the (NF)-κB pathway plays a role in CRC tumor initiation by enhancing cytokines and other signaling pathways, such as the adenomatous polyposis colin (APC) / Beta-catenin pathway (leading to chromosomal instability), or the microsatellite instability pathway (associated with DNA mismatch repair genes)[38,39] which can convert intestinal epithelial cells into tumor-initiating cells. In addition, the inflammatory response can promote shifts in the gut microbial community towards a greater abundance of pro-inflammatory bacteria that cause the continued breakdown of host proteins, stimulating an immune response that then provides more nutrients for the bacteria to subsist on, and thus completing the feedback loop[11].

Several products of the gut microbiome directly interact with the NF-κB pathway, highlighting the interdependence of inflammatory pathways and the human microbiome in shaping colorectal carcinogenesis. For example, reactive nitrogen species (RNS) and reactive oxygen species (ROS) are generated and influenced by microbes or microbial products from the human microbiome, and accumulate within epithelial cells in the colon

[28]. These compounds play important roles in the initiation and progression of inflammation-driven carcinogenesis by activating the NF-κB pathway, by directing cell proliferation and inhibiting apoptosis (also known as cellular death). In addition to activating the NF-κB pathway, these compounds can also alter DNA, leading to mutations, deletions and chromosomal instability; if left unrepaired, these changes can lead to carcinogenesis[28].

However, gut bacteria have also been associated with mechanisms linked to reduced CRC risk. Some of the gut bacterial taxa, particularly from the *Bifidobacterium*[40], *Coprococcus*[41], *Faecalibacterium*, *Eubacterium* and *Roseburia*[42] genera, are well known producers of short chain fatty acids (SCFAs)[43,44]. SCFAs are important microbial metabolites that supply help maintain epithelial health and homeostasis. Specifically, the SCFA, butyrate, has been shown to have substantial anti-tumorigenenic properties, including the ability to inhibit tumor cell proliferation, initiate apoptosis in tumor cells, and mediate T-regulatory cell homeostasis[45]. The loss of these important bacterial populations, in concert with an enrichment of pathogenic bacterial populations, likely plays a synergistic role in potentiating tumorigenesis in colorectal cancer carcinogenesis.

C. Inflammation and the oral microbiome

The human oral microbiome comprises more than 700 bacterial taxa, and is considered to be the second most diverse microbiome community in the body, after the gut microbiome[12]. The oral microbiome includes a large number of opportunistic

pathogens that are involved in periodontal, respiratory and cardiovascular diseases, as well as chronic inflammatory disorders of the intestine, including CRC [12,46,47].

Several recent studies also suggest that oral pathogens associated with periodontal disease, such as *Fusobacterium*, *Peptostreptococcus*, and *Porphyromonas*, may also be involved in pathogenesis of not only oral cancers, but also cancers in digestive tract, including CRC[12,20,21]. In addition, the relative abundance of these oral taxa is also increased in in IBDs[48]. The mouth is a reservoir for these pathogens, allowing for colonization of the gastrointestinal tract, which may be exposed to the oral microbiome through saliva or the blood stream under abnormal environmental conditions. However, it is unclear whether these oral taxa traveled down the GI track from the oral cavity after periodontitis, or if inflammation in the gut facilitated their translocation from the oral to the gut microbiome[10,26,27].

Oral taxa are also adept at coaggregation with epithelial cells and each other using adhesins to form biofilms in the oral cavity, and thus facilitating their survival in the colon environment as well[11]. Many oral taxa, including *Fusobacterium* and *Porphyromonas*, possess virulence factors allowing them to adhere to other bacteria and create a microenvironment suitable for biofilm formation. Oral bacterial taxa such as *Fusobacterium* and *Porphyromonas* are often found in biofilms, and their ability to create a polymicrobial oral community can further damage the colon by producing specific metabolites such as polyamines and genotoxic reactive oxygen species which both protect the microbiota present and promote tumorigenesis by creating a pro-inflammatory state in the gut[11,20]. Furthermore, polyamines are known to be essential for biofilm formation and promote cancer cell proliferation. Thus, polymicrobial oral communities

produce inflammatory metabolites that disrupt healthy colonic metabolism, promoting tumor cell proliferation while protecting the stability of their biofilms[11].

Furthermore, biofilms containing oral taxa have been isolated from both healthy colonic mucosa and CRC tumors, indicating their ability to invade the gut microenrivronment[26]. Thus, cancer-associated biofilms found in the colonic mucosa are similar in both community membership and invasiveness to those of a dysbiotic oral microbiome[11]. However, the oral microbiome has not been extensively explored as a potential marker in CRC carcinogenesis, and we hypothesized that the oral microbiome influences the composition of the gut microbiome and CRC carcinogenesis.

Chapter 3. STUDY DESIGN: Effect of Aspirin on the Gut Microbiome (ASMIC)

A. Study Design and Population

The "Effect of Aspirin on the Gut Microbiome (ASMIC)" study was a randomized clinical trial that aimed to determine whether treatment with aspirin results in favorable shift in the composition of gut microbiome[49]. This randomized, placebo-controlled, double-blinded study targeted 50 healthy subjects, between 50 and 75 years old who lived in the greater Twin Cities area.

Participants were recruited from 1056 individuals who had previously given consent to be contacted for future studies after participating in two CRC-related studies: Evaluation of SEPT9 Biomarker Performance for Colorectal Cancer Screening (PreSEPT, NCT00855348) and Validation and Comparison of Biomarkers for the Early Detection of Colorectal Adenocarcinoma (BCCD, NCT01511653). Of these, 350 were willing to participate, but only 50 met eligibility criteria **(Figure 3-1)**. Exclusion criteria for this study included: use of any antibiotic prescription in the last 3 months; use of any NSAIDs >2 times a week in the last 3 months; use of antiplatelet or anticoagulant medication, medications for diabetes, or hypertension within the past 30 days; gastrointestinal (GI) cancer or any serious GI condition or surgery within 6 months; any serious active medical (cancer, CVD) or psychiatric illness; BMI ≥ 40 or ≤ 17 kg/m^2; unexplained change in weight of >4.5 kg within the past 6 months; or major changes in eating habits within the past 3 months. Eligibility was further confirmed at baseline visit (Visit 1).

B. Baseline data collection

At Visit 1, 50 eligible subjects signed a consent form and were randomized to the aspirin (N=30) or placebo (N=20) arm according to a 5-block randomization scheme. We used unequal between-arm allocation to increase precision in the aspirin arm, because within-arm changes over time were deemed more important in aspirin than in the placebo arm. The duration of treatment (6 weeks) was based on trial of mesalazine for irritable bowel syndrome (IBS), in which mesalazine taken for 4 weeks altered microbiome composition, with this effect being reversed after a 4-week washout[50]. Given that the relatively healthy ASMIC participants may have had a microbial community more resistant to change than those with IBS, the duration of treatment (including placebo) and washout was extended to 6 weeks.

Additionally, at baseline (Visit 1), a trained technician measured subjects' height and weight and asked a series of questions about health, medication use, and diet. These questions were similar to the brief dietary questionnaire used in the Human Microbiome Project[51].

C. Data collection at follow-up

After the 6-week treatment intervention (Visit 2), participants answered the same series of questions about health, medication use, and diet they completed at baseline. Visits were followed up with five phone calls at 3-week intervals to ask about changes in health status and possible adverse events and to discuss subjects' upcoming stool collection. Each participant collected 5 stool samples (every 3 weeks) and two urine samples, one before (baseline) and one after treatment (Week 6). Forty-nine subjects

completed the study and provided five stool samples; one subject quit after completing the treatment phase and providing three stool samples. Study participants and all study staff (except the study statistician and pharmacist) were blinded to the treatment given; the pharmacist and the statistician knew the contents of medication bottles through a blind code.

D. Sample collection and laboratory analysis

Stool samples were collected at home by study participants in stool collection kits containing 95% ethanol. After collection, stool samples were put on ice and transported by local courier within 72 hours, before being put into -80º C freezer until needed for analysis. For oral samples, tongue swabs were collected by trained study staff, and were frozen at -20 ℃ after collection. Blood and urine samples were collected during clinic visits by trained study staff, aliquoted after collection, and then frozen at -20 ℃. All samples were transferred to -80 ℃ within 24 hours of sample collection **(Figure 3-2)**.

Urine samples were collected to assess the effectiveness of aspirin treatment using the urinary metabolite of prostaglandin E2, PGE-M, adjusted for creatinine levels. Prostaglandins are a product of COX-2 activity[7,9], and urinary PGE-M levels are reduced in aspirin users because PGE-M reflects the production of systemic PGE2, which is suppressed by aspirin [52,53]. Pre-post treatment change in PGE-M levels allowed the study investigators to check for compliance with aspirin intake. PGE-M was analyzed using high-performance liquid chromatography/mass spectrometry (HPLC/MS)[54] and normalized for creatinine levels measured using a test kit from Enzo Life Sciences, thus

expressed in ng/mg creatinine. Both PGE-M and urinary creatinine were measured in the Eicosanoid Core Laboratory at Vanderbilt University Medical Center.

E. Medication preparation and treatment compliance

Aspirin and placebo (lactose) capsules were compounded and packaged by Fairview Investigational Drug Services (IDS), according to Good Manufacturing Practices. Blind-coded study bottles of 50 pills containing either aspirin or placebo were prepared for a six-week daily treatment according to the randomization scheme. Compliance was assessed in two ways: (1) The study investigators compared the number of pills returned after treatment completion to the expected number based on treatment duration, and (2) the study investigators assessed between-arm difference in urinary PGE-M levels and their change after treatment.

F. DNA extraction

Bacterial DNA extraction, sequencing and amplification were conducted at the University of Minnesota Genomics Center (UMGC)[55]. To characterize the stool and oral microbiome of our study participants, bacterial DNA was extracted from tongue swab samples and stool samples using the MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA) according to the manufacturer's instructions[56].

Quality control on the final DNA product was performed using a spectrophotometer reading. DNA purity was assessed using the Qubit dsDNA High Sensitivity Kit to measure the A260/280 DNA Yield test. The A260/280 DNA

absorbance method is a simple and well-validated quality control procedure for evaluating DNA purity, and only requires commonly available laboratory equipment[57]. The extraction process yielded approximately 100 µl DNA per sample, of which 25 µl was used by UMGC for PCR amplification, and the leftover DNA was stored at -20°C.

G. DNA amplification and sequencing

The V4 variable region of the 16S rRNA gene from each DNA sample was amplified and sequenced, using validated DNA probes and the Illumina 454 MiSeq Personal Sequencing platform[58]. During the DNA amplification steps, the UMGC facility used sterile water controls for each batch. In addition, two wells on each 96-well sample plate were reserved for positive and negative controls. Sequence data were deposited in the Sequence Reach Archive of the National Center for Biotechnology Information under BioProject accession number SRP127801.

A8. Taxonomy assignment

Sequence processing and analysis were performed using the DADA2 bioinformatics software (version 1.6.0). Forward and reverse reads were trimmed to 200 nt to remove low-quality reads and merged. High quality sequences were aligned against the SILVA database version 132.

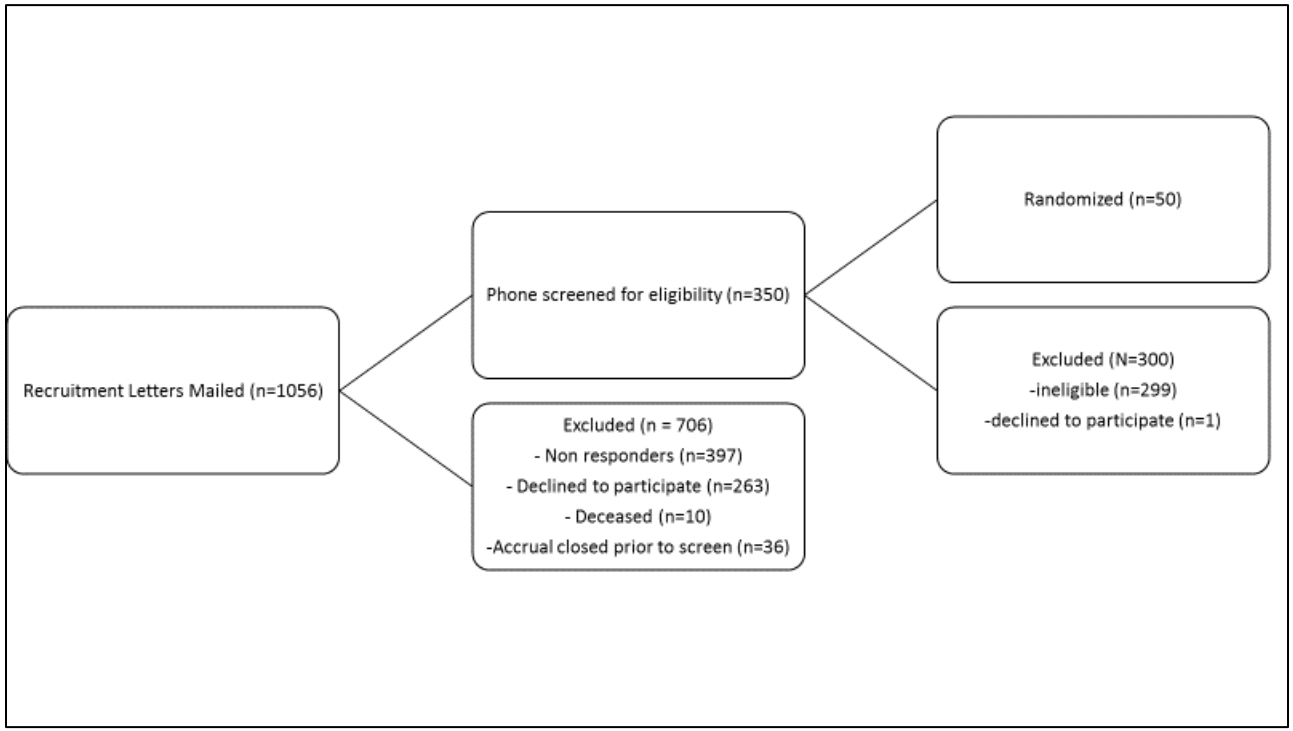


Figure 3-1. Diagram of the ASMIC trial recruitment

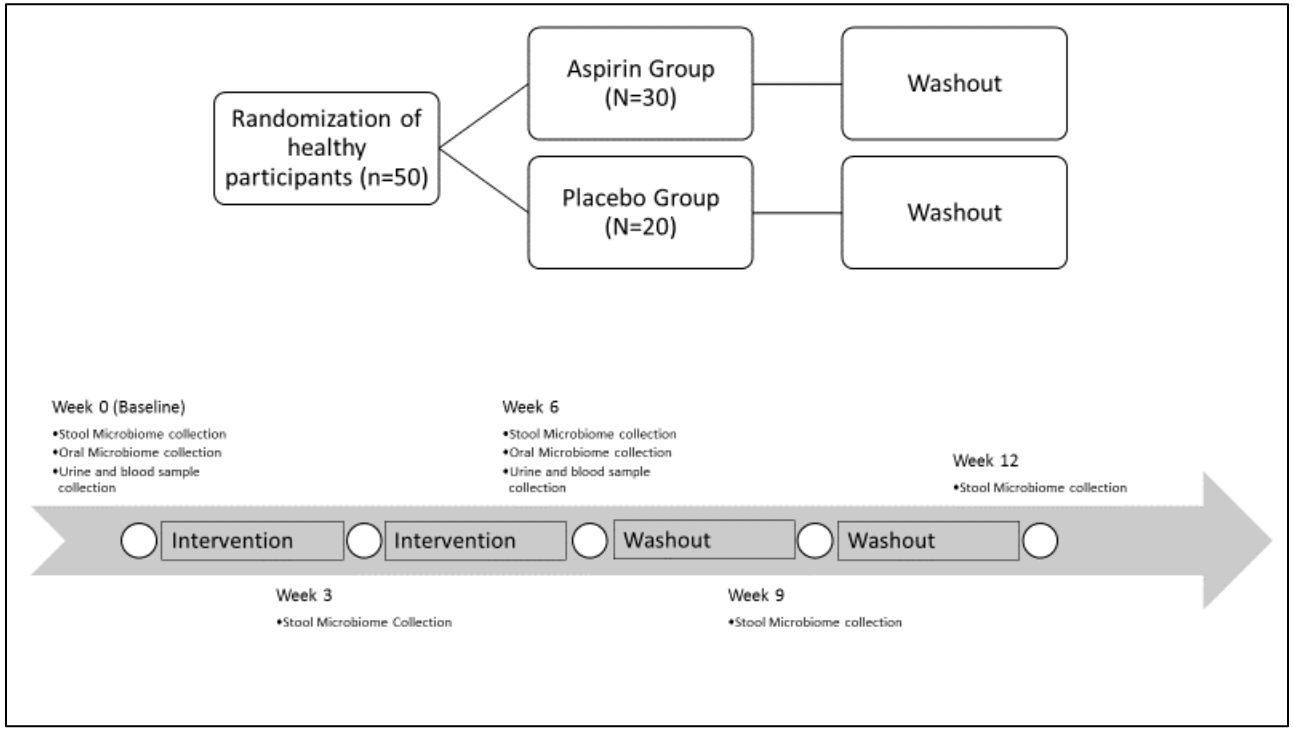


Figure 3-2. Diagram of the ASMIC trial intervention

Chapter 4. MANUSCRIPT I – EFFECTS OF ASPIRIN INTERVENTION ON INFLAMMATION- ASSOCIATED ORAL BACTERIAL TAXA

A. Synopsis

Background: Several bacterial taxa that are consistently enriched in the gut microbiome of inflammatory bowel diseases (IBDs) cases, as well as colorectal cancer (CRC) cases are also found in the oral cavity. These oral taxa may play a role in both oral dysbiosis and colorectal carcinogenesis by stimulating an inflammatory response. We evaluated the effect of a 6-week aspirin intervention on the relative abundance of oral bacterial taxa in a randomized placebo-controlled trial.

Methods: Fifty healthy subjects, 50-75 years old, were randomized to receive either 325 mg aspirin (N=30) or placebo (N=20) orally once per day for 6 weeks. Oral samples were collected at baseline and after treatment (week 6), and the V4 region of the 16S rRNA gene was sequenced using Illumina MiSeq technology. The data were analyzed using the standard DADA2 workflow. We tested the association of intervention assignment with alpha-diversity using linear mixed effect models, and beta-diversity using PERMANOVA (Adonis function). In addition, aspirin treatment was hypothesized to influence specific bacterial taxa selected based on the published literature. The following taxa were included in the analysis: *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Gemella*, *Neisseria*, *Streptococcus*, *Haemophilus*, *Campylobacter*, *Veillonella*, *Capnocytophaga*, *Granullicatella*, *Rothia*, *Actinomyces* (at the genus level), *Ruminococcaceae* and *Lachnospiraceae* (at the family level). In our main analysis, we estimated the association between aspirin use and the changes in the relative abundance of the specified taxa from pre- to post-treatment (baseline to week 6) using a mixed effect

regression model (lme4 package) with a binomial distribution. The log of odds ratio (β estimate) for the interaction term was used to compare aspirin to placebo intervention for post- versus pre-treatment. Finally, in an exploratory analysis, we estimated the change in the pre-specified taxa of interest, relative to other taxa in the microbial community.

Results: Aspirin treatment was not associated with alpha or beta diversity. However, we found that the change over-time in relative abundance of 9 out of the 12 pre-specified taxa at the genus level, and 1 out of 2 pre-specified taxa at the species level differed between the aspirin and placebo groups. In the aspirin group, there were greater increases in the relative abundances of *Neisseria, Streptococcus, Actinomyces, Rothia*, and greater decreases in the relative abundance of *Prevotella, Veillonella, Fusobacterium, Porphyromonas* at the genus level, and *Lachnospiraceae* at the family level compared to the placebo group. These changes were confirmed using the balance approach.

Conclusions: These preliminary findings suggest that aspirin may change the relative abundance of oral taxa associated with oral dysbiosis or CRC. Further studies are needed to understand the impact of the duration and dosage of the aspirin intervention on the oral microbiome.

B. Introduction

Many oral pathogens linked to chronic inflammation thrive under conditions associated with dysbiosis -- an imbalance in the human microbiome linked to a disease, where the ecological balance of the oral cavity is disrupted [10,20,21,26,59–61]. In turn, this inflammation leads to the bacterial plaque and gum infection that lead to the development of periodontitis, which could serve as an indicator of inflammation in the oral cavity[20–23].

Recent studies have linked oral dysbiosis, and more specifically, periodontitis to colorectal cancer (CRC) risk[20,21]. However, research on a direct association between the oral microbiome and CRC risk has been limited[10], although there are several potential mechanisms explaining how oral bacteria can contribute to cancer risk. For example, while ethanol in the alcohol is not strongly carcinogenic, oral bacteria species, such as *Neisseria*, may convert ethanol to acetaldehyde, which is a recognized human carcinogen[62–64]. In addition, oral bacteria activate nitrosamine, a compound found in tobacco smoke, to its carcinogenic form -- nitrosodiethylamine (NDEA), as shown by the International Agency for Research on Cancer (IARC)[65]. This important role for oral bacteria in carcinogen metabolism is further supported by the observation that smoking potentiates the alcohol-related production of acetaldehyde by oral bacteria, potentially contributing to alcohol-tobacco interactions in carcinogenesis[66]. Taken together, these data indicate that the oral microbiome may affect CRC risk by activating alcohol- and smoking-related carcinogens in the oral cavity. Subsequently, those carcinogens may travel along the GI tract and cause local effects such as the stimulation of DNA methyltransferase or 5'-C-phosphate-G-3 (CpG) island methylation in the colon[66,67].

Another important pathway through which the oral microbiome may be associated with CRC development is by promoting local and systemic inflammatory responses, since inflammatory factors are well-documented risk factor for CRC[32,33]. Certain bacteria found in the oral microbiome have also been detected in colorectal tumor tissue, and have been shown to promote inflammation of the intestinal epithelium[11,29]. In addition, these taxa are also involved in chronic inflammatory disorders of the intestine such as inflammatory bowel diseases (IBDs)[15,16], Crohn's disease (CD)[15,16], ulcerative colitis (UC)[17–19], where they have similar associations to those observed in CRC cases compared to controls. In particular, *Fusobacterium* and *Porphyromonas* are oral taxa known to work synergistically, and possess several virulence factors that promote both bacterial survival and CRC developtment[11,27,29,68]. These specific virulence properties include the ability to form biofilms, the production of lipopolysaccharides, and the activation the NF-κB pathway, which facilitate CRC development by creating a pro-inflammatory enviroment[11,29]. This inflammation, in turn could lead to CRC development, by impacting either the APC/Beta-catenin pathway (leading to chromosomal instability), or the microsatellite instability pathway (associated with DNA mismatch repair genes)[38,39] in the host. However, it is unclear how these bacteria observed in the gut microbiome or in the intestinal epithelium traveled down the GI track from the oral cavity[10,26,27].

Given the role of inflammation in CRC development, the potential use of aspirin (acetylsalicylic acid) and other nonsteroidal anti-inflammatory drugs (NSAIDs) as chemopreventive agents has been a promising venue in CRC research. In particular, aspirin irreversibly inhibits cyclooxygenase enzymes (COX)-1[6] and COX-2[7]. COX enzymes normally produce prostaglandins, most of which are pro-inflammatory, and

thromboxanes, which promote clotting[7,9]. However, aspirin-modified COX-2 produces lipoxins, most of which are anti-inflammatory[9]. Aspirin may also exert its anti-inflammatory effect through COX-independent mechanisms, including direct effects on cytokines and transcription factors, modulation of estrogen biosynthesis through the effects on aromatization of androgens, and inhibition of oxidative DNA damage. The effect of aspirin on these pathways may indirectly affect the oral microbiome by counteracting the virulence factors of inflammation-associated taxa[8].

Thus, we hypothesized that aspirin may modulate the oral microbiome through its anti-inflammatory effects. The proposed study estimated the effect of a 6-week aspirin intervention on oral bacteria community using saliva samples from 50 healthy participants within a pilot double-blind randomized placebo-controlled trial.

C. Materials and methods

C1. Parent Study design

Our study was conducted within the parent study: "Effect of Aspirin on the Gut Microbiome (ASMIC)"[49]. ASMIC was a randomized clinical trial that aimed to determine whether treatment with aspirin results in favorable shift in the composition of gut microbiome. The ASMIC study recruited 50 healthy subjects, 50-75 years old, from the PRospective Evaluation of SEPTin 9 (PRESEPT) cohort living in the greater Twin Cities area. Upon confirmation of eligibility, the subjects were asked to refrain from consuming any other NSAIDs and over-the-counter medications containing NSAIDs and from having major changes in their diet for the duration of the study. Participants were randomized into

an aspirin (N=30) and placebo arm (N=20) using a block randomization scheme (N=5 per block). Each of the participant received a 325 mg aspirin pill or placebo (lactose) once a day for 6 weeks, and the 6-week intervention period was followed by 6 weeks of washout **(Figure 4-1)**.

C2. Data Collection

At baseline, a clinic visit to obtain informed consent and collect demographic information and assignment was scheduled for every participant (Visit 1, Week 0). During the initial visit (Visit 1), a brief medical and dietary history was recorded. Participant also attended a clinic visit after the 6-week intervention period (Visit 2).

C3. Sample Collection

The blood, urine and oral samples were collected by trained study staff during the two clinic visits at week 0 (Collection 1 at Visit 1, before the intervention) and week 6 (Collection 2 at Visit 2, after the intervention). Urine samples were collected in the parent study to assess the effectiveness of aspirin treatment using the urinary metabolite of prostaglandin E2, PGE-M, adjusted for creatinine levels. As discussed above, prostaglandins are a product of COX-2 activity[7,9], while their urinary metabolite PGE-M has been shown to be a CRC associated inflammatory biomarker[52,53]. For oral sample, the participants were instructed by the study staff using a tongue swab which was then frozen at -20 ℃ after collection. Blood and urine samples were aliquoted after collection, then frozen at -20 ℃. All samples were transferred to -80 ℃ within 24 hours of sample collection.

C4. DNA extraction

All bacterial DNA extraction, sequencing and amplification was conducted at the University of Minnesota Genomics Center (UMGC). To characterize the oral microbiome of our study participants, bacterial DNA was extracted from tongue swab samples using the MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA) according to the manufacturer's instructions[56]. Quality control on the final DNA product was performed using a spectrophotometer reading. DNA purity was assessed using the Qubit dsDNA High-Sensitivity Kit to measure the A260/280 DNA Yield test. The A260/280 DNA absorbance method is a simple and well-validated quality control procedure for evaluating DNA purity, and only requires commonly available laboratory equipment[57]. The extraction process yielded approximately 100 µl DNA per sample, of which 25 µl was used by UMGC for PCR amplification, and the leftover DNA was stored at -20°C.

To characterize the oral microbiome of our study participants, we sequenced a single common gene across many bacteria known as the 16S rRNA gene. The 16S rRNA gene is an ubiquitous bacterial gene, which codes for ribosomal RNA, an essential component of all bacterial genomes. Ribosomal RNA has regions that are highly conserved (essential for ribosome function), as well as regions that vary across bacteria. These variable regions are mutated but still functional , and this variability allows for differentiation among oral bacteria[10].

C5. DNA amplification and sequencing

The V4 variable region of the 16S rRNA gene from each DNA sample was amplified and sequenced, using validated DNA probes and the Illumina 454 MiSeq Personal Sequencing platform[58]. During the DNA amplification steps, the UMGC facility used sterile water controls for each batch. In addition, two wells on each 96-well sample plate were reserved for positive and negative controls. Quality control on the final DNA product was performed using the qubit dsDNA HiSensitivity Kit for DNA yield to measure the A260/280 DNA Yield test for DNA purity[57]. At the completion of the analysis, the sequenced genetic data was archived in the Sequence Read Archive at the National Center for Biotechnology Information[69].

C6. Taxonomy assignment

Sequence processing and analysis were performed using the DADA2 bioinformatics software (version 1.6.0). Forward and reverse reads were trimmed to 200 nt to remove low-quality reads and merged. High quality sequences were aligned against the SILVA database version 132. After our taxonomy assignment, all subsequent analyses were restricted to bacterial groups that represent at least 1% of total reads classifiable at each taxonomic level.

C7. Statistical analysis

We tested for differences in the demographic characteristics of the ASMIC participants using t-tests for continuous variables, and chi-square tests for categorical variables. In addition, we evaluated pre-post treatment change in PGE-M levels, adjusted for baseline PGE levels, to check for compliance with aspirin intake, as PGE-M is

suppressed by aspirin intake and served as a marker of treatment compliance for our study.

The bioinformatics analysis described in the "Taxonomy" section generated an Operational Taxonomic Unit (OTU) table with features (i.e. bacterial taxa) for the oral microbiome samples. This OTU table was used to determine the number of species in the bacterial community (alpha diversity) and changes in the population structure of the bacterial community (beta diversity). In our analysis, we generated various alpha diversity metrics including the Chao1, ACE (abundance-based coverage estimators), Fisher, Shannon Index, and Simpson metrics for richness. However, Shannon indices were the main metric derived to estimate α-diversity[70–72]. The change in alpha diversity measures between the aspirin and placebo groups from baseline and post intervention (after 6 weeks) was assessed using linear mixed effect regression models. These models were further adjusted for age, gender, BMI and library size.

Beta diversity was assessed by first calculating the Bray-Curtis metric as a distance measure on the rarefied and log-transformed abundance data. Then, the Bray-Curtis values for the aspirin and placebo groups were summarized using principal coordinates analysis (PCoA). Finally, we used Permutational multivariate analysis of variance (PERMANOVA), as implemented by the Adonis function in the vegan package was used to test the differences in beta diversity measures between the aspirin and placebo groups post intervention (after 6 weeks).

C8. Main analyses

The outcomes in the main analysis were (1) the change in the microbial community composition and abundance (alpha and beta-diversity) before and after the 6-week treatment, and (2) the change in relative abundance of a priori chosen major pro-inflammatory oral bacterial taxa. These bacterial taxa were selected based on the literature. Because this was a pilot study and hence of small size, and because we expected a modest effect, we decided to focus on a small number of individual bacterial taxa identified in previous studies. We selected oral bacterial taxa that were either a) enriched in oral dysbiosis and CRC, or b) had biological functions relevant to oral dysbiosis or colorectal carcinogenesis[11–14]. This resulted in selecting 12 taxa at the genus level, and 2 taxa at the family level.

First, we calculated the prevalence (presence vs. absence) and average relative abundance of each of our a priori oral taxa of interest. Then, we estimated the association between aspirin use and the changes in the relative abundance of pre-specified taxa from pre- to post-treatment (baseline to week 6) using a mixed effect regression model with a binomial distribution in which the logarithm of odds ratio (β estimate) for the interaction term compared aspirin to placebo intervention for week 6 vs the baseline.

Characterizing changes in the relative abundance of a priori taxa can be challenging, as estimating changes in relative abundance relies on the assumption that the increase or decrease in relative abundance of an individual taxon is driven by changes in this taxon ant not the changes in relative abundance of unmeasured taxa. The changes in unmeasured taxa arise due to interactions in the microbial community such as competition or mutualism[73]. Computationally, this is reflected by the relative abundances of all taxa in the sample adding up to 1.

Given that our main analysis used relative abundances of pre-specified taxa, we performed an exploratory analysis of microbial balances to confirm the findings from the main analysis.

C9. Exploratory analyses

In this analysis, we applied two concepts: the concept of "microbial balances" and the concept of "reference frames". These concepts were introduced by Morton et al.[73,74], and are described in detail below.

C9.a. Microbial Balances: Microbial balances is a term used to describe the log-transformed ratios (log ratio) of the relative abundances between two taxa. When using these log ratio values, a log ratio of 0 indicates that the two taxa of interest are roughly in the same proportion in the sample. The use of microbial balances may circumvent biases introduced by unobserved or unknown taxa when relative abundances are analyzed, because these unobserved taxa can affect the relative abundances of the taxa of interest. When we calculate the log ratio between two taxa of interest, the bias due to unobserved taxa is diminished, because any unobserved taxa affecting the relative abundance values will be present in the numerator and denominator, and thus the bias will be cancelled out[73].

C9.b. Reference frames: In order to compute the log ratios described above, we first identified one taxon which served as the common denominator in all of ratios, or in other words, served as "reference frame". In order to select our reference frame, we implemented a differential ranking analysis[74] by comparing the logarithm of the fold

change in relative abundance of all taxa at week 6 between the aspirin and placebo arm to generate differentials. All taxa were analyzed simultaneously using multinomial regression to generate differentials, and these differentials were reported on a logarithmic scale to base 2 (i.e. a log2 fold change of 1.5 means that the taxon's relative abundance is increased by a multiplicative factor of 2^1.5 between the two arms in our multinomial regression model).

From the output of our multinomial regression, we ranked the beta estimates for the differentials in each taxon between arms, and found that most of the genera in the *Ruminoccocaceae* family changed more in the aspirin vs the placebo group after the 6 week intervention (i.e., ranked higher in the aspirin group compared to the placebo group). Thus, we selected the *Ruminococcaceae* family as a reference -- the denominator value for log ratios -- against which we compared our other taxa of interest.

This analysis was executed using the gneiss and songbird packages in QIIME2[73], and visualized using the QURRO package in QIIME2[74].

C9.c. Implementation of microbial balances: Once we selected the *Ruminococcaceae* family as a reference, we computed the log ratio values for all our taxa of interest at week 0 and at week 6 separately. Of note, each taxon was a numerator and *Ruminoccocaceae* was a denominator for all the taxa. Finally, to compare the change over-time in the log ratio for each taxon across arms, we used linear mixed effect regression, in which log ratio values at week 0 and week 6 were entered as repeated values. Significant balances for the change over time in the aspirin vs placebo group were determined with a p-value cutoff at 0.05, after controlling for the expected rate of false positive using a False Discovery Rate (FDR) Adjustment [3].

***C9.d. Differential abundance analysis*:** In addition to our main and exploratory analyses, we also ran a differential abundance analysis using the DESeq2 package to simultaneously test multiple bacterial taxa for the comparison of post-intervention oral samples between the aspirin and placebo treatment arm. The DESSeq2 package also generates results as log fold changes (the effect size estimate is reported on a logarithmic scale to base 2, and i.e. a log2 fold change of 1.5 means that the taxon's relative abundance is increased by a multiplicative factor of 2^1.5 between the two arms), and allowed us to control for the expected rate of false positive using a False Discovery Rate Adjustment[3].

The analyses were conducted using the DADA2, EdgeR, vegan, phyloseq and DESeq2 packages in the R Statistical Analysis software package, Version 3.4 (CRAN) (2-sided tests, $\alpha = 0.05$), and the songbird as well as the QURRO packages in QIIME2.

D. Results

D1. Parent Study cohort

The study cohort included 50 participants who were randomized to the aspirin group (N = 30) and placebo group (N=20). Participant demographics were balanced for age (mean age in the aspirin group = 62.2 years, mean age in the placebo group = 61.2, p-value for difference = 0.5649), BMI (mean BMI in the aspirin group = 27.3, mean BMI in the placebo group = 28.2, p-value for difference = 0.49), and for baseline PGE-M levels (mean PGE-M levels in the aspirin group =11.82 ng/mg creatinine, mean PGE-M levels in the placebo group = 12.94 ng/mg creatinine, p-value for the difference = 0.739) (**Table 4-1**). After 6 weeks of intervention, 47 participants (94%) had at least 90% pill

compliance, and changes in urinary PGE-M indicated high treatment compliance as well. After adjustment for baseline PGE-M, the change in PGE-M was -6.17 (95% CI: -9.16; -3.18) mg/dL for aspirin vs. placebo ($p < 0.001$) (**Table 4-1**).

D2. Analyses of Alpha and Beta diversity

When the analysis was conducted in each individual arm, we found that α-diversity was decreased at week 6 compared to baseline, in both the aspirin and placebo arms (Shannon index change of -0.206 in the aspirin arm, and -0.108 in the placebo arm) (**Figure 4-2**).The change in alpha diversity (Shannon index) between baseline and week 6 was statistically significant in the aspirin but not in the placebo arm (**Figure 4-3**). However, when the change over-time in α-diversity were compared across arms, the difference in the changes was not statistically significant (p-value > 0.05) (**Table 4-2**). Overall, we did not find an association between the composition of oral microbiome samples and aspirin intervention at week 6 (using weighted UniFrac β-diversity index, permutational multivariate analysis of variance [PERMANOVA] p-value > 0.05; **Figure 4-4 and Table 4-3)**.

D3. Analyses of the change in relative abundance of a priori selected taxa

D3.a. Main analysis: At baseline, the prevalence for our a priori selected oral taxa ranged from 95% to 100%, but their relative abundance was low (1.45 – 18.44% of all oral taxa at baseline) **(Figure 4-5).** In the analysis of the change in individual taxa, we found that an over-time change in relative abundance differed between the aspirin and placebo groups in 9 out of the 12 pre-specified taxa at the genus level, and 1 out of the 2 pre-specified taxa at the family level, after adjusting for age, gender and BMI. Compared

to placebo, in the aspirin group, there were greater increases in the relative abundances of *Neisseria, Streptococcus, Actinomyces*, *Rothia* and greater decreases in the relative abundance of *Prevotella, Veillonella, Fusobacterium*, *Porphyromonas* at the genus level **(Table 4-4 and Figures 4-6, 4-7)** and *Lachnospiraceae* at the family level (**Table 4-5 and Figure 4-8).**

D3.b. Exploratory analysis of log ratios: We assessed the abundance of log ratios (henceforth referred to as balances) at week 6 and the change in the relative abundance of all a priori taxa relative to Ruminococcaceae in response to the treatment. We observed a larger increase in the relative abundance of *Aggregatibacter, Bifidobacterium* and *Corynebacterium* in the aspirin group than in the placebo group, and a smaller increase in the proportions of *Actinomyces, Campylobacter, Gemella, Granullicatela, Lachnospiraceae, Neisseria, Prevotella, Rothia, Streptococcus* and *Veillonella* in the aspirin than in the placebo group, between baseline and week 6 (**Table 4-6 and Figure 4-9**).

D3.c. Differential analysis based on DESeq2: In our differential analysis, we did not find an association between the fold change in relative abundance of oral taxa and aspirin intervention at week 6 (**Supplemental tables 4-1 and 4-2**).

E. Discussion

E1. Main Findings

This pilot longitudinal study found that the over-time changes in the post-treatment relative abundance of a priori selected bacterial taxa differed between the aspirin and placebo groups, suggesting that aspirin was associated with the change over

time in the relative abundance of specific taxa in the oral microbiome. In the aspirin group, there were greater increases in the relative abundances of *Neisseria, Streptococcus, Actinomyces, Rothia* and greater decreases in the relative abundance of *Prevotella, Veillonella, Fusobacterium, Porphyromonas,* and *Lachnospiraceae,* compared to the placebo group. These findings are in agreement with our hypothesis that aspirin may modulate the abundance of specific bacteria, which may be of importance in the development of CRC. While the associations with specific bacterial groups were rather modest and may represent chance findings, the changes in microbial composition were reproducible using a novel balance tree approach. However, these findings lacked statistical significance after Bonferroni adjustment for multiple comparisons in this relatively small sample and require confirmation in larger studies.

Although the oral taxa are primarily involved in upper gastrointestinal malignancies, periodontal, respiratory and cardiovascular and other systemic diseases[12,46,47], these bacteria also have a role in local and systemic chronic inflammation. Previous studies have found that circulating levels of inflammatory markers, such as C-reactive protein (CRP)[75] and phospholipase A2(PLA2)[76] levels are associated with specific members of the oral microbiota. Our results are in line with these findings, and suggest aspirin may influence the oral microbiome in the direction consistent with decreasing inflammation.

Our findings of significant inverse associations between aspirin treatment and *Prevotella, Veillonella, Fusobacterium, Porphyromonas,* and *Lachnospiraceae* are consistent with their understood role in the CRC in previous studies, which showed that these taxa to be more abundant in the oral microbiome[11,21,26] and gut microbiome[3,11–

[14,26,28,31,60] of CRC cases compared to cancer-free individuals. In the present study, we were unable to directly evaluate the association between these taxa and CRC given that our study enrolled healthy participants, but these findings are in line with the role of these bacteria in CRC and the protective association between aspirin treatment and CRC risk[32,36,37]. In particular, the present study also found a decrease in the relative abundance of *Fusobacterium* and *Porphyromonas* in the aspirin arm, and the proinflammatory properties of these bacteria were reported in animal models[68]. Our findings of a positive association between aspirin treatment and *Neisseria*, *Streptococcus*, *Actinomyces* is also consistent with their understood role in the oral microbiome, as these taxa are often associated with oral health or are considered commensals in the oral cavity.

Although the exact mechanisms behind the translocation of oral bacteria to the gut microbiome remains to be understood, a "driver-passenger" model has been proposed[60], and the biological traits of several oral taxa of interest, including biofilm formation[28] and adhesion factors[11,76], make it plausible for oral bacteria to survive the transfer from the oral cavity to the colon, and adhere to the colonic mucosa. Further, the oral microbiome has been associated with various cancers along the gastrointestinal tract, including head and neck cancer[77], esophageal cancer[10], lung cancer[78], pancreatic cancer[10] and CRC[11,21,26]. Taken together, these findings suggest that the oral microbiome may be a risk factor for CRC and should be further investigated along the gut microbiome in order to understand the role of the microbiome in CRC.

E2. Explanation of our results in context of CRC risk.

Few studies have examined the association between oral bacterial taxa and inflammation in the context of CRC risk [12]. One study among 99 CRC subjects found that *Haemophilus, Parvimonas, Prevotella, Alloprevotella, Lachnoanaerobaculum, Neisseria* and *Streptococcus* were less abundant in individuals with CRC than in healthy individuals[26]. These findings are in agreement with our finding for *Neisseria* and *Streptococcus,* and in contrast to our findings for *Prevotella*. However, findings for *Prevotella* are inconsistent across studies. A nested case placebo study of 231 cases and 432 placebos in the Southern Community Cohort Study found that *Treponema denticola* and *Prevotella intermedia* were associated with increased CRC risk[79], which is in agreement with our findings for *Prevotella*. Another study conducted among 190 participants in a population based case placebo study found that in age- and batch-adjusted models, CRC history was associated with increase abundance of the oral genus *Rothia*[12], which was not in agreement with our findings of greater relative abundance of *Rothia* in the aspirin group post intervention. These differences could be explained by the high genomic diversity and functionality of taxa at the species and strain taxonomic level. It is also possible that the inconsistent associations in various studies may reflect the complex nature of the microbiome in which the detected changes in certain bacterial taxa may reflect changes in the whole system that we are unable to assess. However, our exploratory analyses using balances was designed to account for changes in the relative abundance of unobserved oral taxa, and the findings from these exploratory analyses confirmed the findings of our main analysis.

E3. Explanation of our results in context of inflammation (Oral microbiome and inflammation)

Aspirin intervention was not related to α-diversity, β-diversity, or overall differential abundance, suggesting that aspirin treatment has only a limited effect on variation among core taxa. Aspirin could influence bacterial taxa via either systemic or local mechanisms. A systemic mechanism of aspirin might involve the inactivation of cyclooxygenase enzymes, the subsequent suppression of prostaglandins, and the production of anti-inflammatory lipoxins[9], leading to the clearance of inflammatory bacteria by macrophages and other immune cells. A local mechanism may be explained by the formation of salicylic acid from aspirin in the liver, and its permeation into human gastrointestinal tissue[6,8], where salicylic acid may come in contact with enteric bacteria after ingestion. Most likely, aspirin-induced changes in oral microbiome would influence the GI tract via s systemic inflammation[80].

The absence of association between oral microbiota and aspirin at the community level was unexpected, but given the proposed mechanisms of action of aspirin on the microbiome via suppressing the systemic inflammation, it is plausible that the effect of aspirin on oral bacterial taxa would be modest and not reflected by the changes in overall composition. Although the current literature largely assumes that gut dysbiosis is most important in the development of an inflammatory phenotype, our results indicate that aspirin treatment was associated with a decrease in relative abundance of several pro-inflammatory a priori selected oral taxa. This highlights the importance of studying microbial communities in various sites along the digestive tract for a more comprehensive understanding of dysbiotic states and their impact on inflammation.

E4. Strengths and Limitations

A major strength of our study is its novelty and robust design. We provide evidence of aspirin-induced changes in the oral microbiome in a randomized study of healthy individuals with repeated measures of microbial composition. Although we did not collect information about oral hygiene practices, dental disease history, or smoking, which are likely to be critical determinants of oral microbiome, the inclusion of a placebo arm and randomized allocations allowed us to limit the influence of unmeasured confounders. Due to the longitudinal nature of the microbial dataset, we selected a novel method based on balances in abundance of specific taxa and were able to confirm changes in relative abundance of our a priori selected taxa. However, the findings from the individual associations should not be over-interpreted, and further studies are needed to confirm the findings.

The main limitations of our study are the limited number of subjects and modest duration. In addition, given our healthy sample, we were unable to evaluate certain taxa that are overrepresented in CRC patients, but rarely observed in heathy people. However, our main objective was to evaluate the influence of aspirin on bacteria predisposing to inflammation and CRC, and we found that aspirin changed several bacterial taxa in a direction consistent with reduced CRC risk. Finally, the results presented here are likely influenced by the methods employed to collect and measure oral microbes. Variability occurs even within oral cavity, partly due to spatial variations in the availability of oxygen[51]. The oral samples collected as tongue swabs in this study are likely to over-represent microbes present in the surface of the oral cavity as well as saliva, and less

likely to include microbes from dental plaques[51], which may have different role in dysbiotic oral conditions[81].

E5. Conclusions

In conclusion, this double-blind, randomized, placebo-controlled pilot trial suggests that aspirin induces changes in the oral microbiome, including changes in several bacteria previously shown to be associated with CRC or inflammation. We identified changes in the relative abundance of a priori selected taxa that are in agreement with an inverse association between aspirin use and CRC risk. We observed a greater increase in commensal and health-associated taxa, and a greater decrease in pro-inflammatory taxa in the aspirin group, compared to the placebo group.

Although our findings for aspirin-induced changes in individual bacteria are preliminary, our study may inform the design of a future larger clinical trial that will examine the effect of different doses of aspirin on oral microbiome in individuals at high risk for CRC. In these future studies, the adequate dosage and duration of aspirin treatment or other prophylactic treatment needed to affect the microbiome may be determined accordingly.

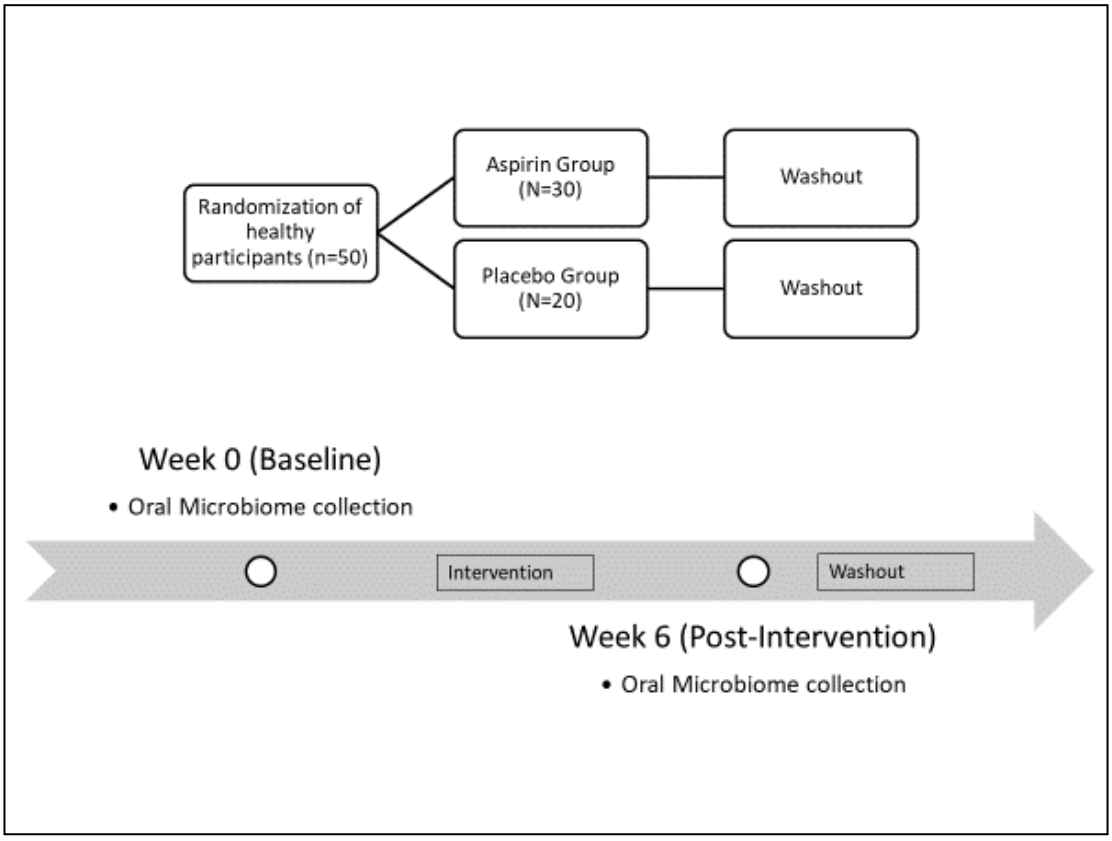


Figure 4-1. Diagram of the ASMIC trial and intervention

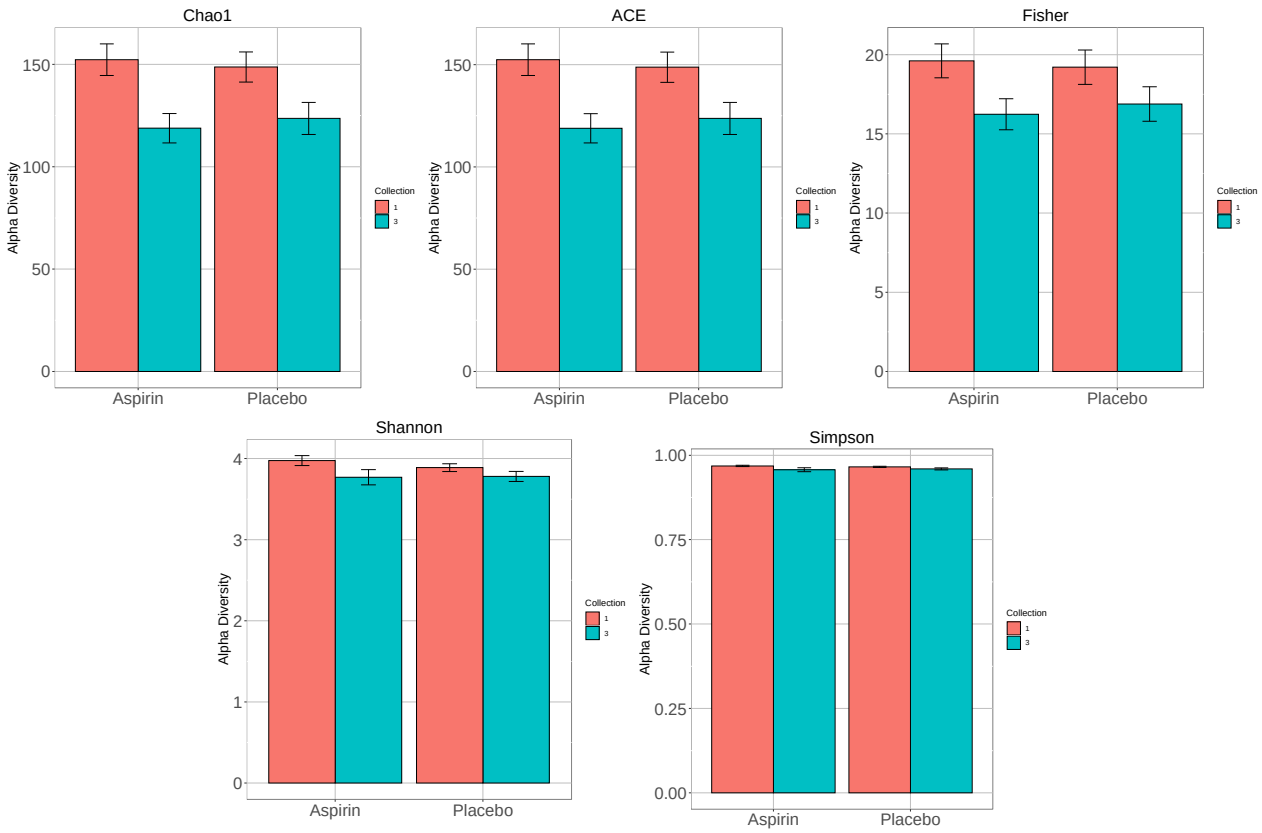


Figure 4-2. Alpha diversity metrics in the aspirin and placebo groups at Week 0 and Week 6.

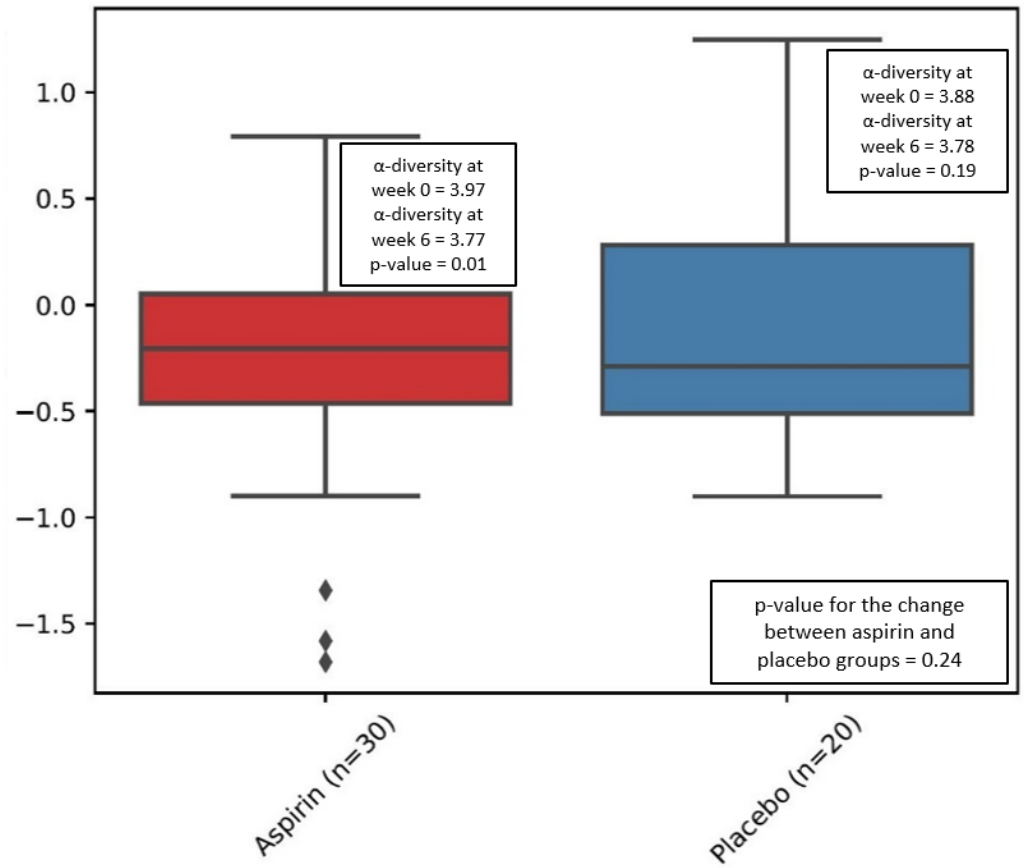


Figure 4-3. Pairwise test for the change in Shannon alpha-diversity within the aspirin and placebo arm.

*The Wilcoxon signed rank test was used to test for significance within each arm, and the Krustal Willis test was used to test for overall significance.

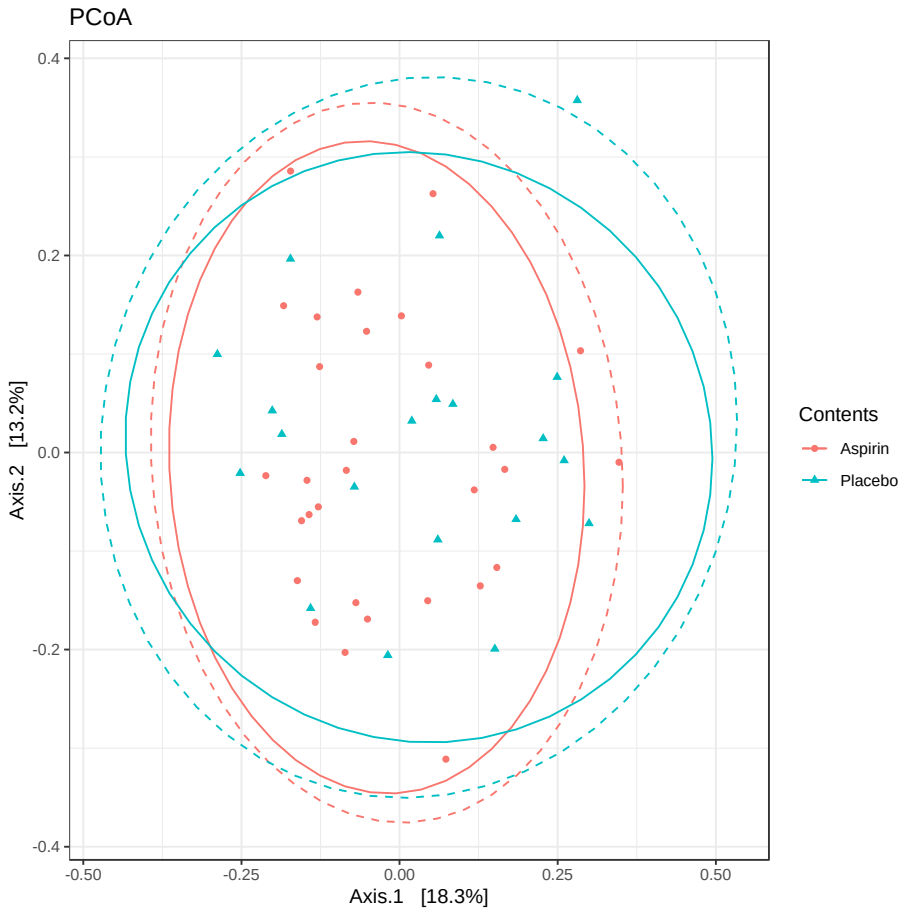


Figure 4-4. Principal Coordinates Analysis with Multidimensional scaling (PCOA) plot for the Beta diversity in the oral microbiome samples at in the aspirin and placebo treatment arms at week 6.

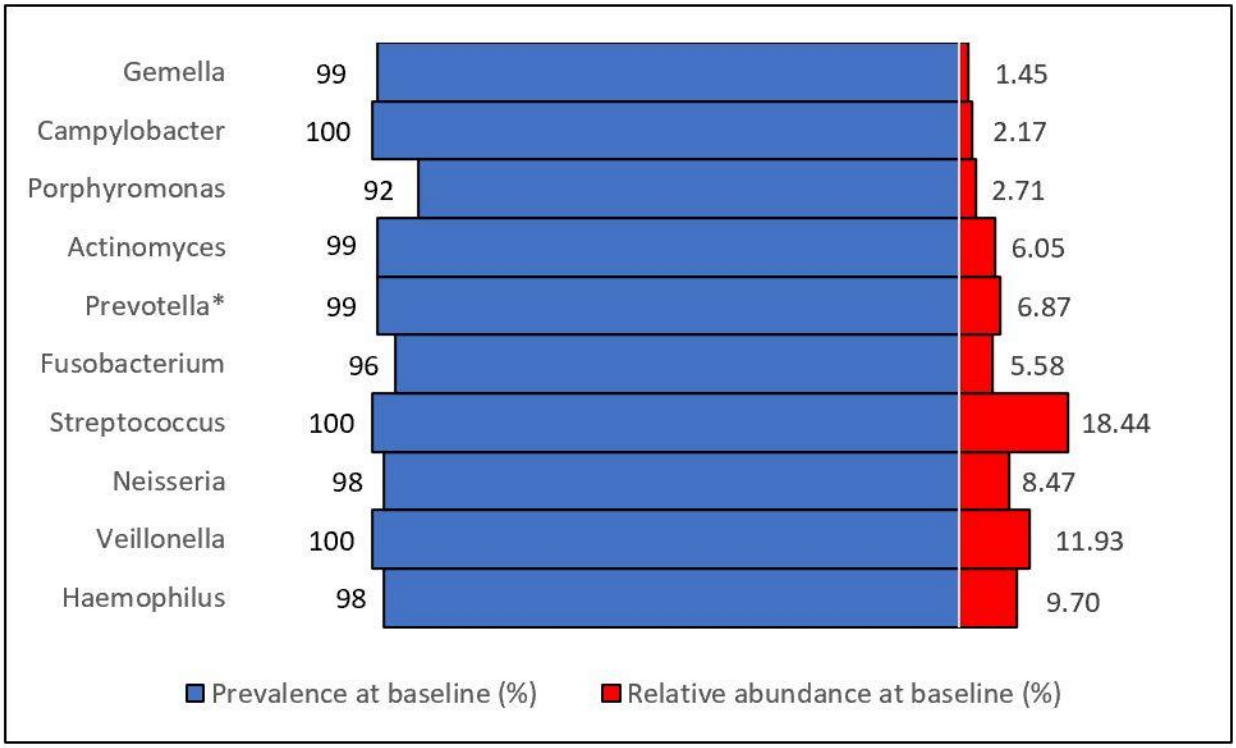


Figure 4-5. Distribution of pre-specified oral taxa (genus-level) in the ASMIC trial.

Prevalence represents the detection prevalence (Presence vs. Absence of taxa, %)

Abundance represents the relative abundance (%)

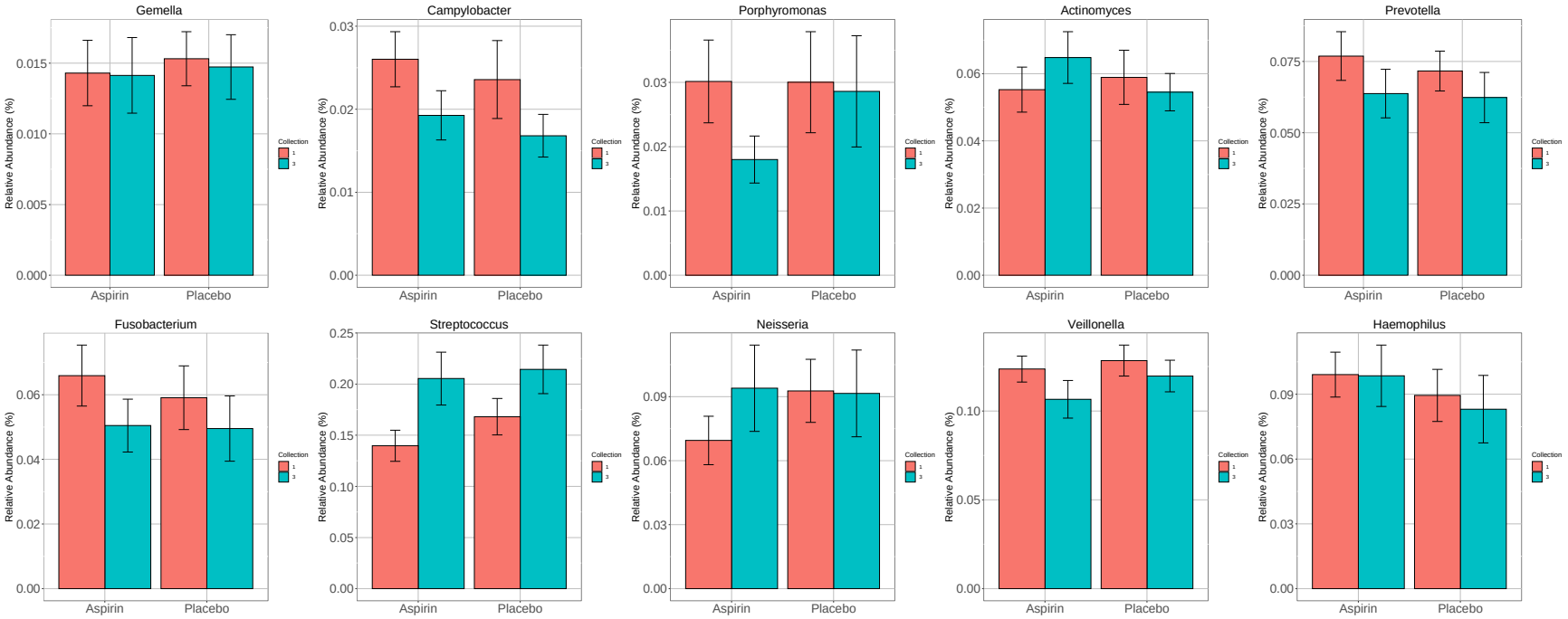


Figure 4-6. Change in relative abundance of pre-specified taxa in the aspirin and placebo group from baseline (week 0) to post intervention (week 6)

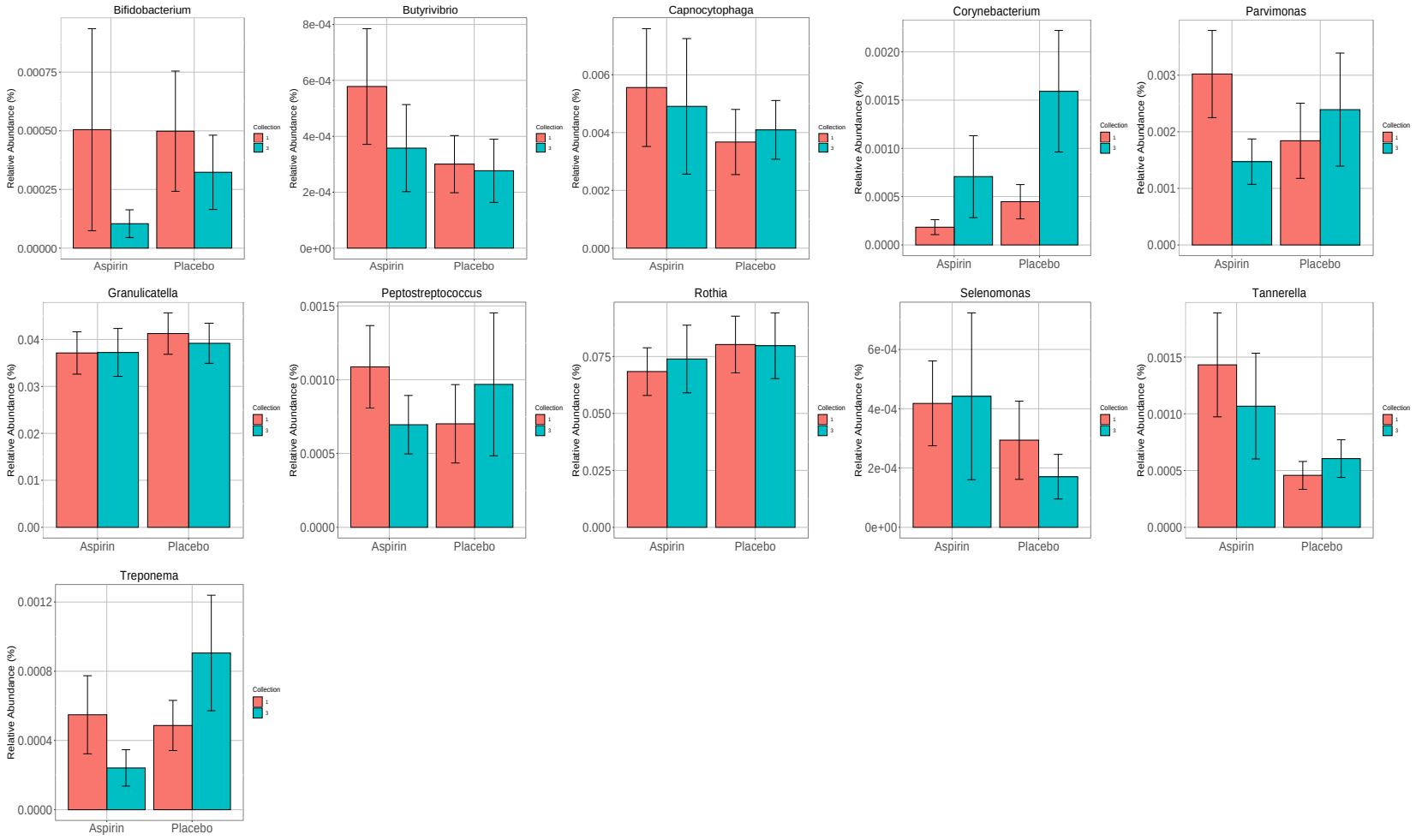


Figure 4-7. Change in relative abundance of pre-specified bacterial taxa in the aspirin and placebo group from baseline (week 0) to post intervention (week 6)

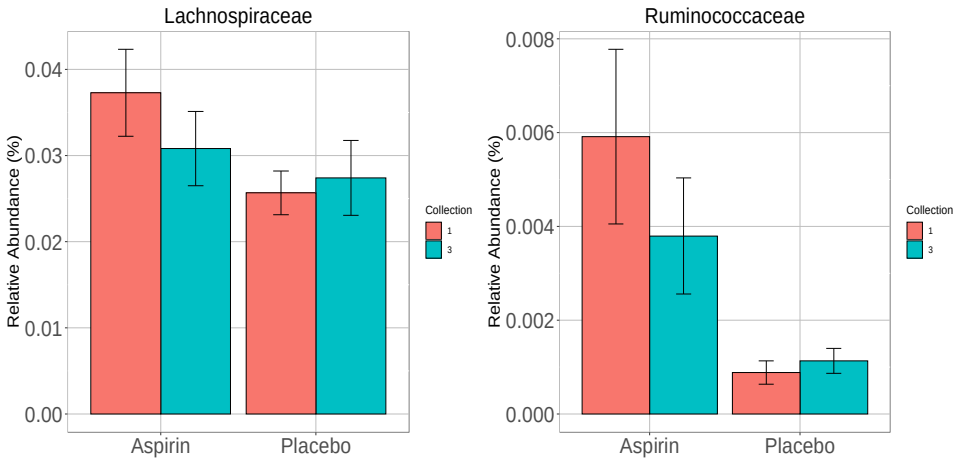


Figure 4-8. Change in relative abundance of pre-specified taxa at the family level in the aspirin and placebo group from baseline (week 0) to post intervention (week 6)

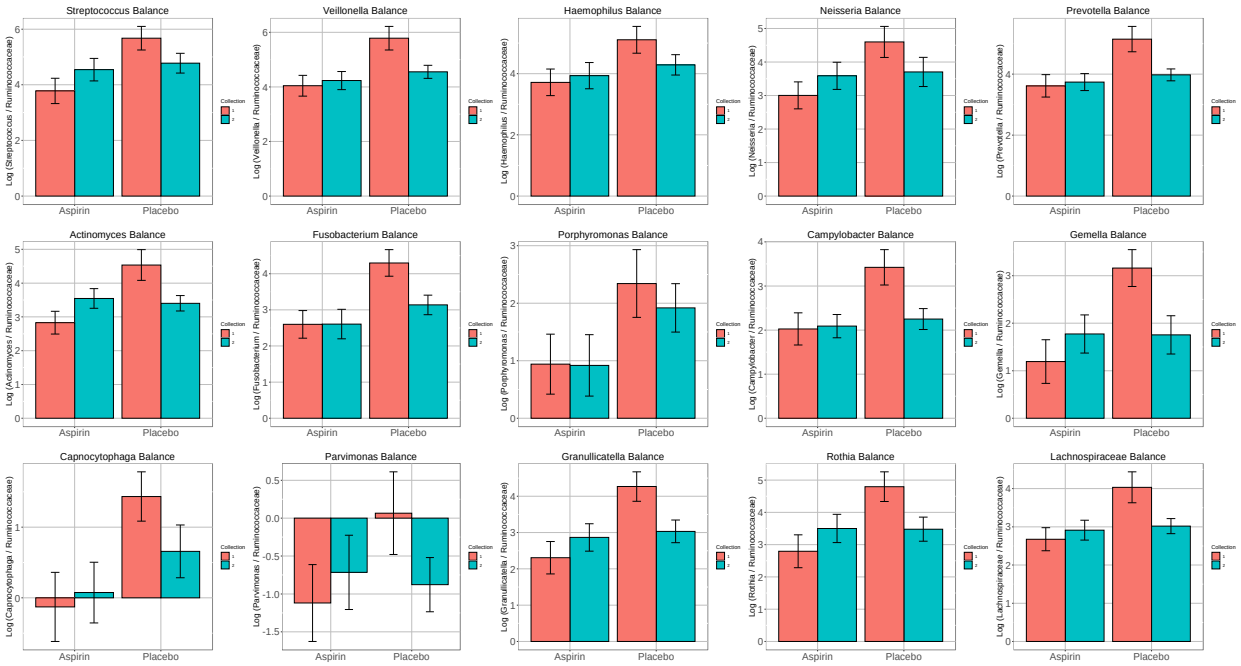


Figure 4-9. Change in the balance of pre-specified taxa relative to the Ruminococcaceae taxa (at the family level) in the aspirin and placebo group from baseline (week 0) to post intervention (week 6)

Table 4-1: ASMIC study descriptive statistics

Characteristics	Aspirin Group	Placebo Group	p-value
N (%)	30 (60.0%)	20 (40.0%)	
Age, Mean (SD) y	62.2 (5.1)	61.2 (5.2)	0.5649
Sex, Female%	23 (76.7%)	9(45.0%)	0.02
BMI, Mean (SD), kg/m2	27.3 (4.3)	28.2 (4.8)	0.4995
Baseline Urinary PGE-M (adjusted for creatinine, mg/dL)	11.82 (13.59)	12.94 (7.39)	0.739
Change in Urinary PGE-M at 6 weeks (adjusted for creatinine, mg/dL)	-5.31 (0.96)	0.86 (1.18)	<0.001

Table 4-2: Association between aspirin treatment and post-intervention alpha diversity (week 6)*

			Adjusted Models*			
Alpha Diversity Measure	Change in the ASA group	Change in the Placebo group	Estimate	Std. Error	t-value	Pr(>|t|)
Shannon	-0.206	-0.108	-0.018	0.133	-0.133	0.895

* **Adjusted models post intervention.** The adjusted model was adjusted for, age, gender, and BMI

Table 4-3: Association between treatment and post-intervention beta diversity (week 6)

Time Point	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)**
Post intervention crude model	1	0.1374	0.13744	0.91695	0.01954	0.41
Post intervention adjusted model*	1	0.1554	0.155428	1.03715	0.0221	0.32

* Crude and adjusted models and included. The adjusted model was adjusted for, age, gender, and BMI

** The presented p-value is for the PERMANOVA F test.

Table 4-4: Effect of aspirin treatment on the change over-time in abundance of pre-specified bacterial taxa at the genus level using linear mixed effect models

Taxa	Predictor	Average change in relative abundance pre to post intervention (% Change, Aspirin Arm)	Average change in relative abundance pre to post intervention (% Change, Placebo Arm)	Crude Models			Multivariate Models		
				Estimate	Std. Error	Pr(>|z|)	Estimate	Std. Error	Pr(>|z|)
Streptococcus	Placebo (vs. Aspirin)	6.498	4.704	0.219	0.19	0.248	0.238	0.202	0.237
	Collection 3 (vs. Collection 1)			0.473	0.009	<2e-16	0.429	0.009	<2e-16
	Intervention * Collection Interaction			-0.152	0.013	<2e-16	-0.109	0.013	4.01E-16
Veillonella	Placebo (vs. Aspirin)	-1.653	-0.995	0.014	0.105	0.894	0.016	0.112	0.8888
	Collection 3 (vs. Collection 1)			0.004	0.013	0.782	0.003	0.013	0.8247
	Intervention * Collection Interaction			-0.095	0.02	1.79E-06	-0.094	0.02	2.64E-06
Haemophilus	Placebo (vs. Aspirin)	-0.018	-0.556	-0.157	0.199	0.4314	-0.033	0.192	0.8616
	Collection 3 (vs. Collection 1)			0.068	0.015	5.97E-06	0.075	0.015	6.08E-07
	Intervention * Collection Interaction			0.046	0.023	0.0495	0.039	0.024	0.1014
Neisseria	Placebo (vs. Aspirin)	2.409	-0.033	0.37	0.333	0.268	0.267	0.341	0.433
	Collection 3 (vs. Collection 1)			0.461	0.016	<2e-16	0.461	0.016	<2e-16
	Intervention * Collection Interaction			-0.294	0.024	<2e-16	-0.294	0.024	<2e-16
Prevotella_7	Placebo (vs. Aspirin)	-1.191	-1.135	-0.116	0.17	0.4944	-0.093	0.181	0.60725
	Collection 3 (vs. Collection 1)			0.035	0.02	0.0867	0.05	0.021	0.01525
	Intervention * Collection Interaction			-0.074	0.032	0.0204	-0.089	0.032	0.00535
Actinomyces	Placebo (vs. Aspirin)	0.981	-0.554	0.175	0.177	0.323	0.131	0.186	0.483
	Collection 3 (vs. Collection 1)			0.241	0.023	<2e-16	0.244	0.023	<2e-16
	Intervention * Collection Interaction			-0.468	0.036	<2e-16	-0.471	0.036	<2e-16
Fusobacterium	Placebo (vs. Aspirin)	-1.58	-0.854	-0.133	0.28	0.636	-0.155	0.3	0.605
	Collection 3 (vs. Collection 1)			-0.21	0.023	<2e-16	-0.203	0.023	<2e-16
	Intervention * Collection Interaction			0.167	0.036	2.62E-06	0.16	0.036	6.80E-06
Porphyromonas	Placebo (vs. Aspirin)	-1.252	-0.193	0.01	0.312	0.974	0.156	0.321	0.6272
	Collection 3 (vs. Collection 1)			-0.414	0.05	<2e-16	-0.413	0.05	<2e-16

	Intervention * Collection Interaction			0.403	0.065	4.66E-10	0.403	0.065	4.67E-10
Campylobacter	Placebo (vs. Aspirin)	0.019	0.017	-0.099	0.183	0.58804	-0.161	0.176	0.36083
	Collection 3 (vs. Collection 1)			-0.191	0.059	0.00117	-0.19	0.059	0.00129
	Intervention * Collection Interaction			-0.197	0.099	0.04644	-0.197	0.099	0.04678
Gemella	Placebo (vs. Aspirin)	0.014	0.015	0.129	0.191	0.499	0.16	0.204	0.434
	Collection 3 (vs. Collection 1)			0.126	0.084	0.134	0.129	0.084	0.125
	Intervention * Collection Interaction			-0.048	0.134	0.718	-0.052	0.134	0.7
Capnocytophaga	Placebo (vs. Aspirin)	-0.065	0.042	-0.26	0.421	0.54	-0.397	0.473	0.401
	Week 6 (vs. Week 0)			0.01	0.147	0.928	0.012	0.147	0.935
	Treatment * Collection Interaction			-0.17	0.392	0.659	-0.161	0.393	0.682
Parvimonas	Placebo (vs. Aspirin)	-0.155	0.055	0	0.34	0.996	0.112	0.353	0.751
	Week 6 (vs. Week 0)			-0.71	0.482	0.138	-0.686	0.483	0.155
	Treatment * Collection Interaction			1.2	0.606	0.048	1.107	0.616	0.072
Granullicatella	Placebo (vs. Aspirin)	0.012	-0.206	0.23	0.155	0.134	0.355	0.156	0.023
	Week 6 (vs. Week 0)			0.07	0.036	0.045	0.083	0.037	0.024
	Treatment * Collection Interaction			-0.1	0.055	0.082	-0.106	0.055	0.056
Rothia	Placebo (vs. Aspirin)	0.553	-0.054	0.3	0.249	0.23	0.459	0.257	0.074
	Week 6 (vs. Week 0)			0.34	0.018	<2e-16	0.354	0.018	<2e-16
	Treatment * Collection Interaction			-0.23	0.026	<2e-16	-0.238	0.026	<2e-16

***Multivariate models were adjusted for age, gender, and BMI**

Table 4-5: Effect of aspirin treatment on the change over-time in abundance of pre-specified bacterial taxa at the family level using linear mixed effect models.

				Crude Model			Adjusted Model		
Bacterial Taxa	Covariate	Change in Aspirin Arm	Change in Placebo Arm	Estimate	Std Error	p-value	Estimate	Std Error	p-value
Lachnospiraceae	Placebo (vs. Aspirin)			-0.424	0.172	0.014	-0.395	0.182	0.030
	Week 6 (vs. Week 0)	-0.646586	0.173493	-0.084	0.040	0.033	-0.077	0.040	0.053
	Treatment * Collection Interaction			0.349	0.070	<0.001	0.342	0.070	<0.001
Ruminococcaceae	Placebo (vs. Aspirin)			-2.137	1.079	0.048	-2.383	1.122	0.034
	Week 6 (vs. Week 0)	-0.21178394	0.02479694	-0.306	0.195	0.117	-0.306	0.195	0.116
	Treatment * Collection Interaction			0.614	1.473	0.677	0.788	1.501	0.600

***Multivariate models were adjusted for age, gender, and BMI.**

Table 4-6: Effect of aspirin treatment on the change over time in the balance of pre-specified taxa relative to the Ruminococcaceae (at the family level) in the aspirin and placebo group.

Balance	Covariate	Change in Balance in ASA Group	Change in Balance in PCB Group	Estimate	Std Error	Pr(>|t|)	FDR adjusted p-values
Actinomyces / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.707	0.485	0.000716	
	Week 6 (vs. Week 0)	0.716	-1.133	0.716	0.323	0.031463	
	Treatment * Collection Interaction			-1.849	0.511	0.000708	0.0163
Aggregatibacter / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			2.286	0.705	0.00167	
	Week 6 (vs. Week 0)	1.289	-0.419	1.289	0.537	0.02026	
	Treatment * Collection Interaction			-1.708	0.848	0.0498	0.0822
Bifidobacterium / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			2.004	0.709	0.00579	
	Week 6 (vs. Week 0)	1.073	-0.757	1.073	0.56	0.06143	
	Treatment * Collection Interaction			-1.83	0.885	0.04417	0.0822
Butyrivibrio / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.453	0.484	0.00344	
	Week 6 (vs. Week 0)	0.427	-0.757	0.427	0.373	0.25773	
	Treatment * Collection Interaction			-1.184	0.589	0.05004	0.0822
Campylobacter / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.398	0.471	0.00384	
	Week 6 (vs. Week 0)	0.064	-1.174	0.064	0.359	0.85822	
	Treatment * Collection Interaction			-1.238	0.568	0.03413	0.0822
Capnocytophaga / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.561	0.618	0.0135	
	Week 6 (vs. Week 0)	0.203	-0.775	0.203	0.401	0.615	
	Treatment * Collection Interaction			-0.978	0.634	0.1293	0.1565
Corynebacterium / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			2.002	0.733	0.00761	
	Week 6 (vs. Week 0)	1.382	-0.846	1.382	0.549	0.01518	
	Treatment * Collection Interaction			-2.228	0.868	0.01342	0.0617
Fusobacterium / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.699	0.537	0.00216	
	Week 6 (vs. Week 0)	0.007	-1.161	0.007	0.381	0.98456	
	Treatment * Collection Interaction			-1.168	0.602	0.05823	0.0893
Gemella / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.966	0.608	0.00178	
	Week 6 (vs. Week 0)	0.58	-1.405	0.58	0.413	0.16656	
	Treatment * Collection Interaction			-1.985	0.653	0.00383	0.044
Granullicatella / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.96	0.573	0.000951	
	Week 6 (vs. Week 0)	0.559	-1.234	0.559	0.429	0.19914	
	Treatment * Collection Interaction			-1.793	0.679	0.011069	0.0617
Haemophilus / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.391	0.606	0.0244	
	Week 6 (vs. Week 0)	0.218	-0.82	0.218	0.404	0.5916	
	Treatment * Collection Interaction			-1.038	0.638	0.1106	0.1496
Lachnospiraceae / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.358	0.433	0.00232	
	Week 6 (vs. Week 0)	0.237	-1.014	0.237	0.325	0.46923	
	Treatment * Collection Interaction			-1.251	0.514	0.01862	0.0667
Neisseria / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.594	0.616	0.0113	
	Week 6 (vs. Week 0)	0.627	-0.842	0.584	0.463	0.2127	
	Treatment * Collection Interaction			-1.478	0.731	0.0489	0.0822
Parvimonas / Ruminococcaceae Ratio	Placebo (vs. Aspirin)	0.405	-0.944	1.186	0.706	0.0969	
	Week 6 (vs. Week 0)			0.405	0.457	0.3802	

	Treatment * Collection Interaction			-1.349	0.722	0.068	0.0978
Peptostreptococcus / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			0.92	0.586	0.121	
	Week 6 (vs. Week 0)	0.197	-0.644	0.198	0.413	0.635	
	Treatment * Collection Interaction			-0.842	0.654	0.204	0.2133
Porphyromonas / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.402	0.76	0.0686	
	Week 6 (vs. Week 0)	-0.021	-0.423	-0.021	0.558	0.9696	
	Treatment * Collection Interaction			-0.402	0.882	0.6507	0.6507
Prevotela / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.536	0.475	0.00172	
	Week 6 (vs. Week 0)	0.126	-1.173	0.126	0.366	0.7318	
	Treatment * Collection Interaction			-1.299	0.579	0.02942	0.0822
Rothia / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			2	0.661	0.00326	
	Week 6 (vs. Week 0)	0.706	-1.316	0.706	0.484	0.15139	
	Treatment * Collection Interaction			-2.022	0.766	0.01114	0.0617
Selenomonas / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.1	0.468	0.0207	
	Week 6 (vs. Week 0)	0.098	-0.825	0.098	0.399	0.8062	
	Treatment * Collection Interaction			-0.924	0.631	0.1497	0.1722
Streptococcus / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.896	0.606	0.0024	
	Week 6 (vs. Week 0)	0.763	-0.898	0.763	0.438	0.0875	
	Treatment * Collection Interaction			-1.661	0.692	0.0203	0.0667
Tannerella / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			0.929	0.604	0.128	
	Week 6 (vs. Week 0)	0.28	-0.686	0.28	0.46	0.545	
	Treatment * Collection Interaction			-0.966	0.728	0.19	0.2081
Treponema / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.331	0.596	0.0282	
	Week 6 (vs. Week 0)	0.261	-0.828	0.261	0.432	0.5481	
	Treatment * Collection Interaction			-1.09	0.683	0.1172	0.1498
Veillonella / Ruminococcaceae Ratio	Placebo (vs. Aspirin)			1.741	0.516	0.00109	
	Week 6 (vs. Week 0)	0.189	-1.232	0.189	0.428	0.66116	
	Treatment * Collection Interaction			-1.421	0.676	0.04083	0.0822

Supplemental Table 4-1: Differential abundance (Genus level agglomeration) between the Aspirin and Placebo groups post intervention (crude model)

Taxa	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Bacteroides	9.24	-4.715	1.756	-2.684	0.007	0.429
Johnsonella	5.46	5.179	2.275	2.277	0.023	0.672
Haemophilus	2679.93	-0.732	0.557	-1.316	0.188	0.999
Veillonella	2772.40	0.304	0.291	1.046	0.296	0.999
Neisseria	2331.23	-0.303	0.503	-0.603	0.546	0.999
Streptococcus	6313.31	-0.186	0.391	-0.475	0.635	0.999
Fusobacterium	1094.39	-0.093	0.453	-0.205	0.838	0.999
Prevotella_7	1467.83	0.217	0.309	0.703	0.482	0.999
Granulicatella	1046.33	0.039	0.363	0.109	0.913	0.999
Actinomyces	1467.06	-0.089	0.304	-0.293	0.769	0.999
Rothia	2806.87	-0.365	0.487	-0.75	0.453	0.999
Porphyromonas	511.51	0.433	0.651	0.665	0.506	0.999
Campylobacter	398.74	-0.026	0.303	-0.085	0.933	0.999
Leptotrichia	709.42	-0.516	0.396	-1.303	0.193	0.999
Gemella	376.00	-0.164	0.425	-0.386	0.700	0.999
Atopobium	172.64	0.529	0.44	1.203	0.229	0.999
Megasphaera	129.60	0.237	0.552	0.429	0.668	0.999
Moryella	59.24	-0.082	1.002	-0.082	0.935	0.999
Prevotella_6	132.37	0.287	0.463	0.621	0.534	0.999

*All log fold change values for the post intervention (week 6) DESeq2 analysis are for the abundance in the placebo group vs. the aspirin group.

Supplemental Table 4-2: Differential abundance (Genus level agglomeration) between the Aspirin and Placebo groups post-intervention (adjusted model)

Taxa	baseMean	log2FoldChange**	lfcSE	stat	pvalue	padj
Bacteroides	9.24	-6.568	2.089	-3.144	0.002	0.098
Johnsonella	11.44	5.979	2.423	2.468	0.014	0.401
Haemophilus	2679.93	-0.223	0.597	-0.375	0.708	0.991
Veillonella	2772.40	0.358	0.29	1.236	0.216	0.991
Neisseria	2331.23	-0.215	0.54	-0.398	0.691	0.991
Streptococcus	6313.31	-0.203	0.42	-0.483	0.629	0.991
Fusobacterium	1094.39	-0.052	0.489	-0.107	0.915	0.991
Prevotella_7	1467.83	0.118	0.322	0.366	0.714	0.991
Granulicatella	1046.33	0.055	0.381	0.143	0.886	0.991
Actinomyces	1467.06	-0.228	0.315	-0.722	0.470	0.991
Rothia	2806.87	-0.173	0.509	-0.34	0.734	0.991
Porphyromonas	511.51	0.428	0.712	0.6	0.548	0.991
Campylobacter	398.74	-0.076	0.309	-0.247	0.805	0.991
Leptotrichia	709.42	-0.607	0.414	-1.468	0.142	0.991
Gemella	376.00	0.026	0.449	0.058	0.954	0.991
Atopobium	172.64	0.363	0.47	0.773	0.440	0.991
Megasphaera	129.60	0.152	0.6	0.254	0.800	0.991
Moryella	59.24	-0.838	3.113	-0.269	0.788	0.991
Prevotella_6	132.37	0.354	0.489	0.724	0.469	0.991

* The negative binomial model was adjusted for age, gender, and BMI

**All log fold change values for the post intervention (week 6) DESeq2 analysis are for the abundance in the placebo group vs. the aspirin group.

Chapter 5. MANUSCRIPT II – ORAL TAXA AND INFERRED FUNCTIONAL TRAITS FOR ORAL TAXA IN A RANDOMIZED PILOT TRIAL OF ASPIRIN.

A. Synopsis

Background: Certain oral taxa harbor functional traits which play a role in both oral disease and may be important to the development of inflammatory bowel diseases (IBDs), as well as colorectal cancer (CRC). In a randomized double-blind placebo-controlled trial, we evaluated the effect of a 6-week aspirin intervention on the relative abundance of inferred functional traits linked to a key microbial metabolite, lipopolysaccharides (LPS) which have inflammatory properties. The relative abundance of these functional traits was inferred using the previously validated "PICRUSt" computational tool.

Methods: Fifty healthy subjects, 50-75 years old, were randomized to receive either aspirin (N=30) or placebo (N=20) for 6 weeks. Oral samples were collected at baseline and after treatment (week 6), and the V4 region of the 16S rRNA gene was sequenced using Illumina MiSeq technology. The Operational Taxonomic Unit (OTU) table was generated using the standard QIIME1.9.1 workflow, and then processed using PICRUSt 1.14 to generate a metagenome table of inferred functional traits. Based on a literature review, we selected inferred functional traits for bacterial metabolites associated with CRC risk: short-chain fatty acids (SCFA) pathways (butanoate, propanoate and pyruvate pathways); lipopolysaccharide (LPS) biosynthesis pathways and LPS biosynthesis proteins, as well as oral taxa associated with CRC risk and inflammation (*Fusobacterium, Porphyromonas, Prevotella, Gemella, Neisseria, Streptococcus, Haemophilus, Campylobacter, and Veillonella*). We used Pearson correlation coefficients to correlate

the relative abundance of oral taxa with the relative abundance of inferred functional traits. Then, we estimated the association between treatment intervention and changes in the relative abundance of inferred functional traits from pre- to post-treatment (baseline to week 6) using a mixed effect regression model with a binomial distribution. In this analysis, the log of odds ratio (β estimate) for the interaction term (treatment*time) was used to compare the aspirin to placebo arm for week 6 vs the baseline.

Results: At baseline, we observed correlations between two inferred functional traits linked to a bacterial metabolite associated with increased inflammation (LPS, LPS biosynthesis proteins) and several oral taxa. Positive correlations were observed for three oral taxa (*Campylobacter*, *Porphyromonas* and *Fusobacterium*) ($P<0.05$) associated with periodontitis, and inverse correlations with three other commensal oral taxa (*Rothia, Granulicatella, Streptococcus*). However, we did not find an association between the aspirin intervention and the relative abundance of the a priori selected inferred functional traits.

Conclusions: These preliminary findings of a positive correlation between the relative abundance of oral taxa associated with CRC risk and inflammation, and the relative abundance of inferred functional traits of LPS, a bacterial metabolite associated with inflammation, are in line with the current literature on bacterial virulence factors.

B. Introduction

Recent studies of the oral microbiome and colorectal cancer (CRC) have found significant differences in the relative abundances of specific oral taxa in the oral microbiomes of CRC cases, compared to controls[20,21]. More specifically, these studies have reported that the relative abundance of certain oral taxa (such as *Fusobacterium, Peptostreptococcus,* and *Porphyromonas*) were increased in CRC cases, compared to controls[20,21]. Further, the relative abundance of these oral taxa is also increased in periodontitis, suggesting that these oral taxa also play a role in the inflammation of the oral cavity[20–23]. These bacterial taxa can also be found in the gut microbiome and intestinal epithelium of individuals with chronic inflammatory disorders of the intestine such as inflammatory bowel diseases (IBDs)[15,16] as well as CRC cases[3,24,25], although it is unclear whether those bacteria traveled down the GI track from the oral cavity after periodontitis, or if inflammation in the gut facilitated their translocation from the oral to the gut microbiome[10,26,27].

Most community-profiling efforts on the oral microbiome in relation to CRC have relied on molecular markers (such as the 16S rRNA gene) which characterize the oral microbiome at the taxonomic level, and information on the association between functional trait-based patterns in the oral microbiome and inflammation and CRC remain limited. Several studies showed that oral taxa associated with periodontitis (including *Fusobacterium, Peptostreptococcus,* and *Porphyromonas*) exhibit traits involved with bacterial functions such as adherence to host epithelial cells, mucus degradation, and biofilm formation. These traits promote bacterial survival in the colon and may stimulate an inflammatory response[11,27,29,68]. These taxa are also involved in lipopolysaccharide

(LPS) production[82,83], a key bacterial metabolite also associated with inflammation. The inflammation, in turn, could lead to CRC development, by impacting either the APC/Beta-catenin pathway or the microsatellite instability pathway in the host[38,39]. Further, studies of the gut microbiome and CRC found associations between pro-inflammatory bacterial metabolites, such as LPS, [82,83] and anti-inflammatory bacterial metabolites, such as the short chain fatty acids (SCFA, butyrate and propionate) and CRC risk[25,84]. Taken together, these findings suggest that the association between the oral microbiome and inflammation occur at the level of the oral bacterial community's function, rather than at the taxonomic level alone. While a 16S rRNA bacterial community profile can reveal the presence of specific bacterial taxa, it was not originally designed to provide information on their functional traits[85].

A computational approach (PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states) was devised to predict community functionality using 16S rRNA data and a reference database, as the phylogenies of bacterial taxa constructed from core genes are similar to those constructed from taxonomic marker genes (e.g.,16S rRNA)[86,87]. Although using phylogenetic marker genes such as the 16S rRNA gene to create a profile of bacterial community does not provide direct evidence of a community's functional capabilities, PICRUSt metagenome predictions were strongly correlated with metagenome data from the Human Microbiome Project (HMP) (Spearman r = 0.82) [86].

Given the role of oral bacteria in inflammation and CRC development, it is possible that the relative abundance of these bacteria and of their functional traits would change under the effect of aspirin (acetylsalicylic acid) since aspirin has anti-

inflammatory properties, and has been associated with decreased CRC risk. In particular, aspirin irreversibly inhibits cyclooxygenase enzymes (COX)-1[6] and COX-2[7]. COX enzymes normally produce prostaglandins, most of which are pro-inflammatory, and thromboxanes, which promote clotting[7,9]. However, aspirin-modified COX-2 produces lipoxins, most of which are anti-inflammatory[9]. Aspirin may also exert its anti-inflammatory effect through COX-independent mechanisms, including direct effects on cytokines and transcription factors, modulation of estrogen biosynthesis through the effects on aromatization of androgens, and inhibition of oxidative DNA damage[29]. The effect of aspirin on these pathways may indirectly affect the oral microbiome by counteracting the virulence factors of inflammation-associated taxa[8], and a functional trait based analysis of the oral microbiome after aspirin treatment could provide valuable insight on the effects of aspirin on the oral microbiome.

Therefore, the aim of this study was to infer functional traits of oral taxa from 16S rRNA using PICRUSt, clarify how the inferred functional pathways were related to the oral taxonomic data, and evaluate the effect aspirin intervention on the relative abundance of a priori selected functional traits in a randomized double-blind placebo-controlled trial. We focused specifically on inferred functional traits linked to LPS production, a key microbial metabolite with a pro-inflammatory capacity[84], because it is active in both the oral and gut microbiome, whereas the production of SCFAs (such as acetate, propionate and butyrate), key anti-inflammatory bacterial metabolites, occurs after fermentation in the gut and thus it is relevant to the gut microbiome[84,88–90]. Therefore, we studied the association between a 6-week aspirin intervention and the relative abundance of inferred functional traits related to LPS in the oral microbiome.

C. Materials and Methods

C1. Parent Study design

Our study was conducted within the parent study: "Effect of Aspirin on the Gut Microbiome (ASMIC)"[49]. ASMIC was a randomized clinical trial that aimed to determine whether treatment with aspirin results in favorable shift in the composition of gut microbiome. The ASMIC study recruited 50 healthy subjects, 50-75 years old, from the Evaluation of SEPT9 Biomarker Performance for Colorectal Cancer Screening (PreSEPT) cohort living in the greater Twin Cities area. Upon confirmation of eligibility, the subjects were asked to refrain from consuming any other NSAIDs and over-the-counter medications containing NSAIDs and from having major changes in their diet for the duration of the study. Participants were randomized into an aspirin (N=30) and placebo arm (N=20) using a block randomization scheme (N=5 per block). Each of the participant received a 325 mg aspirin pill or placebo (lactose) once a day for 6 weeks, and the 6-week intervention period was followed by 6 weeks of washout.

C2. Data Collection

At baseline, a clinic visit to obtain informed consent and collect demographic information and assignment was scheduled for every participant (Visit 1, Week 0). During the initial visit (Visit 1), a brief medical and dietary history was recorded. Participant also attended a clinic visit after the 6-week intervention period (Visit 2) (**Figure 5-1**).

C3. Sample Collection

The blood, urine and oral samples were collected by trained study staff during the two clinic visits at week 0 (Collection 1 at Visit 1, before the intervention) and week 6 (Collection 2 at Visit 2, after the intervention). Urine samples were collected in the parent study to assess the effectiveness of aspirin treatment using the urinary metabolite of prostaglandin E2, PGE-M, adjusted for creatinine levels. As discussed above, prostaglandins are a product of COX-2 activity[7,9], while their urinary metabolite PGE-M has been shown to be a an inflammatory biomarker for CRC[52,53]. For oral samples, tongue swabs were collected by the trained study staff, and were frozen at -20 ℃ after collection. Blood and urine samples were aliquoted after collection, and then frozen at -20 ℃. All samples were transferred to -80 ℃ within 24 hours of sample collection.

C4. DNA extraction

Bacterial DNA extraction, sequencing and amplification were conducted at the University of Minnesota Genomics Center (UMGC). To characterize the oral microbiome of our study participants, bacterial DNA was extracted from tongue swab samples using the MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA) according to the manufacturer's instructions[56]. Quality control on the final DNA product was performed using a spectrophotometer reading. DNA purity was assessed using the Qubit dsDNA High Sensitivity Kit to measure the A260/280 DNA Yield test. The A260/280 DNA absorbance method is a simple and well-validated quality control procedure for

evaluating DNA purity, and only requires commonly available laboratory equipment[57]. The extraction process yielded approximately 100 µl DNA per sample, of which 25 µl was used by UMGC for PCR amplification, and the leftover DNA was stored at -20°C.

To characterize the oral microbiome of our study participants, we sequenced a single common gene across many bacteria known as the 16S rRNA gene. The 16S rRNA gene is an ubiquitous bacterial gene, which codes for ribosomal RNA, an essential component of all bacterial genomes. Ribosomal RNA has regions that are highly conserved (essential for ribosome function), as well as regions that vary across bacteria. These variable regions are mutated but still functional, and this variability allows for differentiation among oral bacteria[10].

C5. DNA amplification and sequencing

The V4 variable region of the 16S rRNA gene from each DNA sample was amplified and sequenced, using validated DNA probes and the Illumina 454 MiSeq Personal Sequencing platform[58]. During the DNA amplification steps, the UMGC facility used sterile water controls for each batch. In addition, two wells on each 96-well sample plate were reserved for positive and negative controls. At the completion of the analysis, the sequenced genetic data will be archived in the Sequence Read Archive at the National Center for Biotechnology Information[69].

C6. Taxonomy assignment

The 16S rRNA data were analyzed as indicated by the PICRUSt genome prediction software[http://picrust.github.io/picrust/]. For this functional analysis, the sequence data was processed using the Quantitative Insights for Molecular Ecology (QIIME) pipeline (Version 1.9.1) with Operational taxonomic units (OTUs) assigned at 97% identity using a closed reference approach and the taxonomic classification was performed against the Greengenes database (Version 13.5).

C7. Inferred Functional Trait Assignment

We analyzed 16S rRNA gene sequence data from our oral samples studies using the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) pipeline, to predict the functional composition of a microbial metagenome (referred to as inferred functional traits) using marker gene data and a database of reference genomes. This computational approach uses an extended ancestral-state reconstruction algorithm that predicts which gene families are present and then combines gene families to estimate the composite metagenome using 16S information.

The PICRUSt pipeline is a two-step process developed to predict the functional composition of a microbial community's metagenome from its 16S profile. In the first step, we downloaded a table of inferred functional traits for each organism designed for PICRUSt based on the Greengenes reference database version 13.5. This table listed all the expected functional traits for each taxa in our sample (i.e., LPS biosynthesis for the *Fusobacterium* taxon). In the second step, the table of inferred functional traits for all microbial taxa was (1) combined with the relative abundance of 16S rRNA genes in our

samples and (2) corrected for expected 16S rRNA gene copy number, to generate the expected abundances of inferred functional traits in the entire community **(Figure 5-2)**.

Hierarchical functional predictions were performed using the KEGG Orthology (KO) database and default settings. The KO assignments were made in three tiers, where each tier corresponds to a specific functional assignment, with KO tier 1 being least specific (i.e. broadest functional trait groups, such as "metabolism") and KO tier 3 being most specific (i.e. most specific functional trait, such as "LPS biosynthesis"). In this analysis, functional predictions were assigned up to KO tier 3 for all genes.

Based on a literature review, we selected functional traits linked to pathways associated with an increase in CRC risk through LPS production (LPS biosynthesis and LPS biosynthesis proteins). In addition, to examine how the inferred functional pathways were related to the taxonomic data, we tested the correlation between a priori selected oral bacterial taxa and the inferred functional pathways generated as described above. The oral taxa were selected based on an enriched abundance or biological functions in oral dysbiosis or colorectal carcinogenesis, as shown in literature. The following oral taxa were included in the analysis: *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Gemella*, *Neisseria, Streptococcus, Haemophilus, Campylobacter, Veillonella*, *Capnocytophaga, Granullicatella, Rothia*, and *Actinomyces* [11–14].

C8. Statistical analysis

C8.a. Correlation analysis: To clarify how the inferred functional pathways were related to the oral taxonomic data in this analysis, we first used Spearman correlation coefficients to relate the relative abundance of the a priori selected inferred functional

traits at KO tier 3 to the relative abundance of our a priori selected oral bacterial taxa at baseline (**Table 5-2**). In addition, we also computed the change over-time in relative abundance in both the inferred functional traits and the oral bacterial taxa and tested the correlation in these changes in each treatment arm. Spearman correlation coefficients were considered significant at a p-value cutoff at 0.05, after controlling for the expected rate of false positive using a False Discovery Rate (FDR) adjustment[3].

C8.b. Linear mixed effect analysis: To estimate the association between treatment intervention and changes in the relative abundance of inferred functional traits from pre- to post-treatment (baseline to week 6), we used a mixed effect regression model with a binomial distribution. In this analysis, the log of odds ratio (β estimate) for the interaction term (treatment*time) was used to compare the aspirin to placebo arm for week 6 vs the baseline.

C8.c. Differential abundance analysis: Using the DESeq2 package, we ran a differential abundance analysis to simultaneously test multiple inferred functional traits in post-intervention oral samples and compare them across arms. The DESeq2 package allowed us to control for the expected rate of false positive using a False Discovery Rate Adjustment[3].

The analyses were conducted using the DADA2, phyloseq and DESeq2 packages in the R Statistical Analysis software package, Version 3.4 (CRAN) (2-sided tests, $\alpha = 0.05$).

D. Results

D1. Parent Study cohort

The study cohort included 50 participants who were randomized to the aspirin group (N = 30) and placebo group (N=20). Participant demographics were balanced for age (mean age in the aspirin group = 62.2 years, mean age in the placebo group = 61.2, p-value for difference = 0.5649), BMI (mean BMI in the aspirin group = 27.3, mean BMI in the placebo group = 28.2, p-value for difference = 0.49), and for baseline PGE-M levels (mean PGE-M levels in the aspirin group =11.82 ng/mg creatinine, mean PGE-M levels in the placebo group = 12.94 ng/mg creatinine, p-value for the difference = 0.739). After 6 weeks of intervention, 47 participants (94%) had at least 90% pill compliance. The high compliance to treatment intervention in the study was also confirmed by larger changes in urinary PGE-M in the aspirin arm. After adjustment for baseline PGE-M, the change in PGE-M was -6.17 (95% CI: -9.16; -3.18) mg/dL for aspirin vs. placebo (p <0.001) (**Table 5-1**).

D2. Correlation between oral taxa and oral functional traits at baseline

The a priori selected inferred functional pathways for LPS were present in all participants at baseline at baseline (100% prevalence) but had relatively low abundance (0.48 – 1.09% of all inferred functional traits) **(Figure 5-3).** Our main interest was in inferred functional pathways related to LPS, as this bacterial metabolite is active in the oral microbiome and associated with inflammation. To determine how our a priori selected oral taxa (*Fusobacterium, Porphyromonas, Prevotella, Gemella, Neisseria, Streptococcus, Haemophilus, Campylobacter, Veillonella, Capnocytophaga, Granullicatella, Rothia, Actinomyces)* may contribute to differences in the relative abundance of functional traits in the aspirin and placebo arms at baseline, we performed

correlation analyses using the inferred functional pathways of interest (LPS biosynthesis, LPS Biosynthesis proteins). We observed a positive correlation between pathways linked to bacterial virulence gene (LPS biosynthesis, LPS biosynthesis proteins) and several pro-inflammatory oral taxa (*Campylobacter*, *Porphyromonas* and *Fusobacterium*) ($P<0.05$) and an inverse correlation with several other oral taxa (*Rothia, Granulicatella, Streptococcus*) which are commensal members of the oral cavity, after adjustment for false discovery rate (**Table 5-3**).

D3. Correlation between the over-time changes in relative abundance of oral taxa and the changes in relative abundance of oral functional traits (from baseline to Week 6)

Compared to the placebo arm, in the aspirin arm, we observed larger positive correlation coefficients for correlations between the changes in relative abundance of inferred functional pathways linked to the pro-inflammatory bacterial metabolite LPS (LPS biosynthesis, LPS biosynthesis proteins), and the changes in relative abundance of *Fusobacterium* and *Neisseria* (**Table 5-4).** Also, we observed a stronger inverse correlation between the changes in relative abundances of inferred functional traits linked to LPS, and a change in relative abundance of *Streptococcus* in the aspirin vs. placebo arm (**Table 5-4**).

D4. Association between aspirin treatment and relative abundances of oral functional traits

We did not find any associations between aspirin treatment and the over-time changes in relative abundances of a priori selected inferred functional traits (**Table 5-5**). Likewise, when we compared the distribution of inferred functional traits at week 6 (post-intervention) between aspirin and placebo arm with DESeq2, the relative abundance of inferred functional traits of interest did not differ across arms after accounting for multiple comparisons **(Supplemental table 5-1).**

E. Discussion

E1. Description of the study findings

In this pilot longitudinal randomized double-blind placebo-controlled study, we found some correlations between the abundance of a priori specified oral bacterial taxa and the relative abundance of inferred functional traits for the LPS bacterial metabolite, which suggests that these taxa may contribute to these traits. However, the results of these descriptive analyses should be interpreted with caution, as functional traits were predicted from taxonomic information, and may not accurately represent the actual metagenomic functions of the samples. We were unable to find any evidence of an association between the change in the relative abundance of inferred functional traits linked to LPS and aspirin treatment after a 6-week intervention. It is possible that a 6-week aspirin intervention was too short to generate a detectable effect size for the change in relative abundance of inferred functional traits in this study. Given that some oral taxa share functional traits, changes at the taxonomic level of the oral microbiome would be more easily detectable than changes at the functional level.

It has been established that bacterial traits such as LPS production promotes the colonization of pathogenic strains of oral bacteria such as *Fusobacterium* and *Porphyromonas*[91,92]. However, the distribution and frequency of most of these pro-inflammatory traits in the oral microbiome are not known, and the development of methods for the quick and direct detection of these traits is an active area of research. In addition to the known pro-inflammatory and anti-inflammatory oral bacterial taxa, there are the typically non-pathogenic members of the oral microbiome, which may harbor genes coding for functional traits associated with inflammation, or genes that promote a healthy microbiome community[93,94]. In our study, we showed that PICRUSt-reconstructed (i.e. inferred) functional traits were present in 100% of our samples, which is in line with the capacity of the oral microbiome as a community to harbor important genes for health and disease, even in the absence of specific taxa of interest.

E2. Importance of PICRUSt inferred functional traits and related metabolites in context of inflammation

Among the bacterial metabolites produced from the functional traits of interest in this study, LPSs are potent endotoxins present in the outer membrane of Gram-negative bacteria, which causes chronic immune responses associated with inflammation. LPS levels have been correlated with increased levels of TNF-α, IL6 and CRP in previous studies[95]. In this study, we observed a positive correlation between the relative abundance of several pre-specified gram-negative oral taxa, such as *Porphyromonas* and *Fusobacterium*, and the predicted relative abundance of inferred functional traits linked to LPS biosynthesis, which is in line with the biological characteristics of these oral taxa.

In addition to LPS, the human gut microbiota also produces another important bacterial metabolite related to inflammation from the fermentation of dietary non-digestible carbohydrates -- SCFAs. These metabolites have been reported to have strong immunomodulatory effects on the host[84,88–90]. In particular, butyrate is a known energy source for colonocytes, while both butyrate and propionate play a role in epigenetic changes in the colon[90]. Butyrate and propionate can act as histone deacetylase (HDAC) inhibitors, which silence the transcription of specific inflammatory genes[42,88,89]. In addition, the SCFA propionate has been shown to be a key regulator of LPS responses capable of blocking inflammatory responses, in in vitro experiments[88,89]. Finally, a previous study found that high propionate-producing bacteria (Lachnospiraceae) were significantly enriched in mice that exhibited lower levels of inflammation[84].

In line with their anti-inflammatory effects, SCFAs are produced by gut taxa which are decreased in the gut microbiome of CRC cases. Bacterial groups contributing to butyrate formation are predominantly found in *Firmicutes*, *Ruminococcaceae* and *Lachnospiraceae* families. *Faecalibacterium prausnitzii* (from the *Ruminococcaceae* Family) is one of the most abundant gut taxa present in the healthy human microbiota which produces butyrate[42], but *Eubacterium* and *Roseburia* (from the *Lachnospiraceae* family[42]) are also butyrate-producing bacteria. A few other key gut taxa involved in butyrate production include *Bifidobacterium*[40], *and Coprococcus* species[41]. *Roseburia* and *Blautia* from the *Lachnospiraceae* family, as well as the *Veillonella* taxa from the *Veillonellaceae* family, are known producers of propionate in the gut[42]. Although SCFAs and pathways linked to SCFA production have an anti-inflammatory function, these metabolites are produced after fermentation of non-dietary carbohydrates in the gut, and

thus were not considered as a priori inferred functional traits of interest in the oral microbiome.

E3. Strengths and Limitations

Several factors may explain the lack of association observed in this study. First, this study had a relatively small sample size, and the intervention was only over a short duration and likely lacked power to find a statistically significant association. Second, PICRUSt only provides information on inferred functional traits, which does not translate to biological activity, and may fail to capture the true effect of aspirin on the oral microbiome. Lastly, we conducted our study in healthy individuals in whom the inflammation-associated inferred functional traits would be less abundant. For instance, LPS is expected to be more abundant in those with CRC or other diseases associated with inflammation, whereas SCFA-associated pathways, are more relevant to the gut microbiome where fermentation and SCFA production occurs, and this would not be reflected in the oral microbiome. Thus, examining oral samples in healthy individuals is not the best option to examine the effect of aspirin on the functional contents of the oral microbiome.

Nonetheless, there could be value in replicating the use of PICRUSt to generate inferred functional traits from 16S rRNA datasets to identify changes in pro-inflammatory and anti-inflammatory pathways linked to key bacterial metabolites in a larger study. PICRUSt provides an inexpensive approach to metagenomic exploration of the microbiome, while being strongly correlated to metagenomic data, according to previous work on the HMP dataset (Spearman r = 0.82)[86]. In addition, PICRUSt could

provide some insight into the core functions of lower abundance taxa, which play a larger contribution in taxonomic variation from sample to sample in the oral and gut microbiome. This approach could help investigators identify core functions which are unique to those lower abundance taxa and potentially drive their role in health and disease[86].

E4. Conclusions

In this report, instead of focusing on the presence of oral taxa, we used the PICRUSt tool to indirectly detect inferred functional traits that could be associated with inflammation in the oral samples. We found that each oral sample had functional traits linked to inflammation in our study. Although we did not find an association between the inferred functional traits of interest and aspirin intervention in this study, the inferred functional traits approach described in this analysis could provide a quick method for screening microbiome samples to ascertain the presence of pro-inflammatory and protective functional traits in studies involving individuals with CRC.

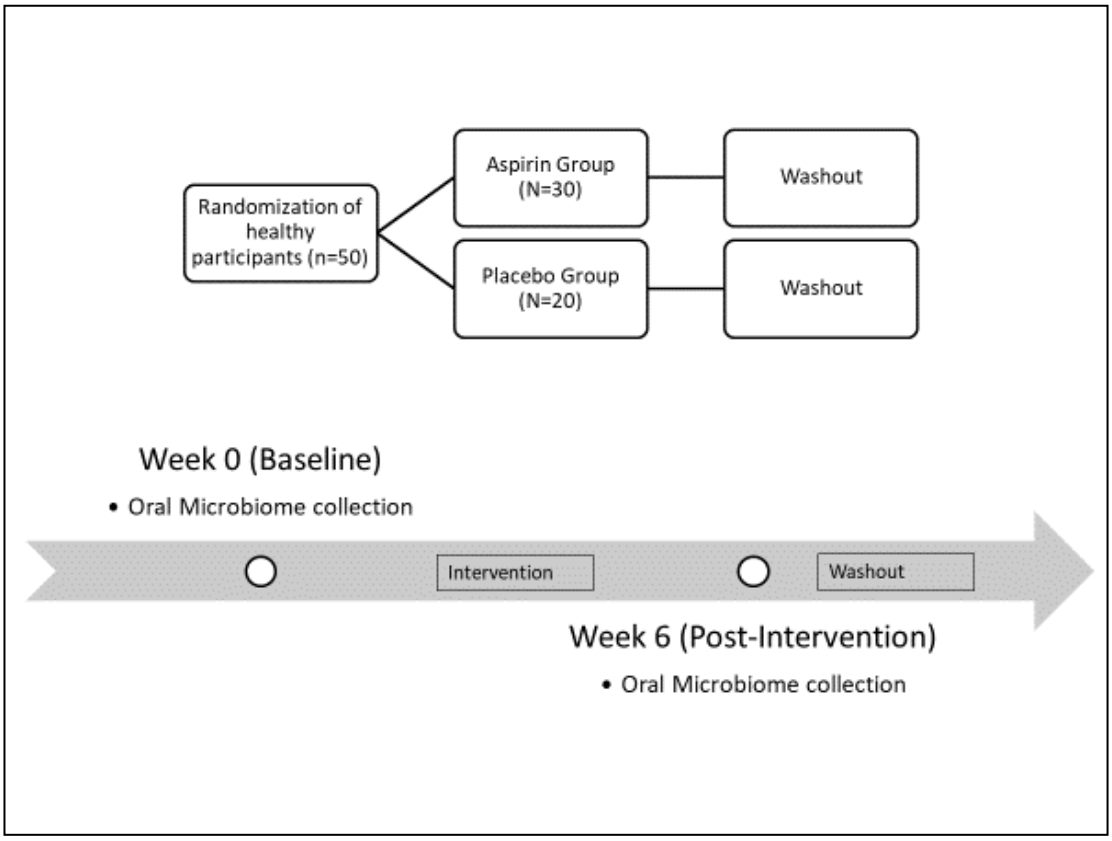


Figure 5-1. Diagram of the ASMIC trial intervention

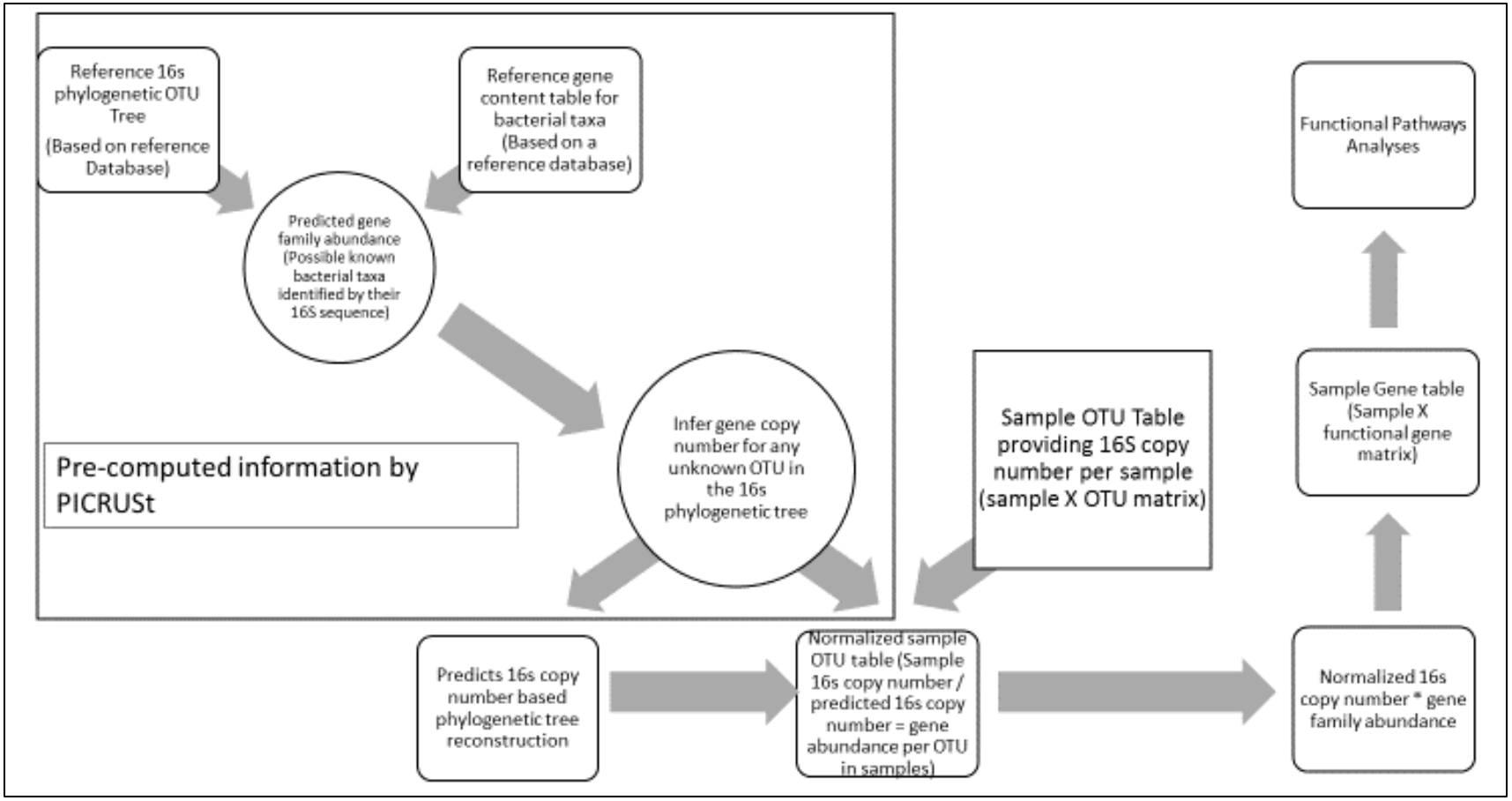


Figure 5-2. PICRUSt Schematic

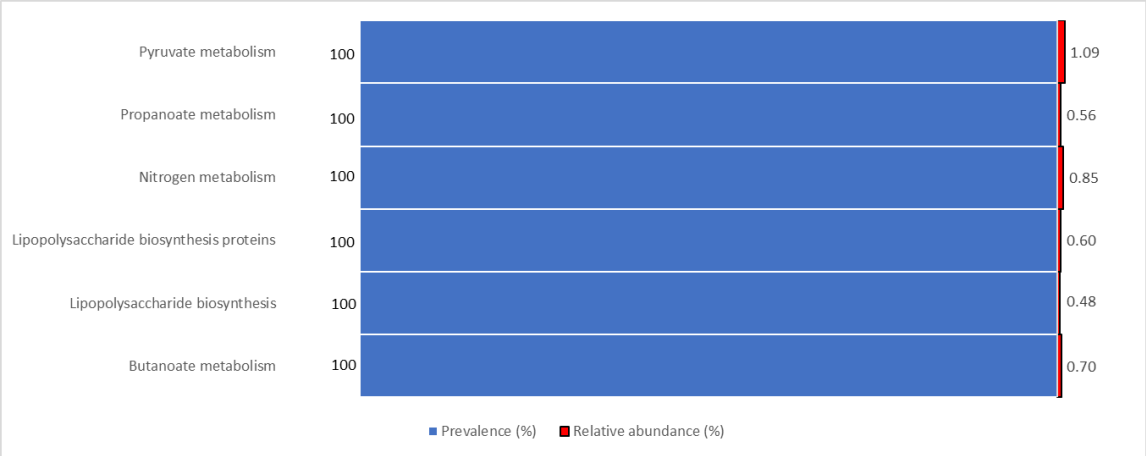


Figure 5-3. Prevalence and abundance of pre-specified inferred functional traits (PICRUSt pathways) at baseline.

Table 5-1: ASMIC study descriptive statistics

Characteristics	Aspirin Group	Placebo Group	p-value
N (%)	30 (60.0%)	20 (40.0%)	
Age, Mean (SD) y	62.2 (5.1)	61.2 (5.2)	0.56
Sex, Female%	23 (76.7%)	9 (45.0%)	0.02
BMI, Mean (SD), kg/m2	27.3 (4.3)	28.2 (4.8)	0.49
Baseline Urinary PGE-M (adjusted for creatinine, mg/dL)	11.82 (13.59)	12.94 (7.39)	0.74
Change in Urinary PGE-M at 6 weeks (adjusted for creatinine, mg/dL)	-5.31 (0.96)	0.86 (1.18)	<0.001

Table 5-2. List of pre-specified inferred functional traits (PICRUSt pathways) and their hypothesized effect on inflammation / CRC risk.

Pathway function	Association with CRC risk	Description
Lipopolysaccharide (LPS) biosynthesis	↑	LPS is a gram negative bacteria cell wall endotoxin which increases inflammation[94,97]
Lipopolysaccharide (LPS) biosynthesis proteins	↑	LPS is a gram negative bacteria cell wall endotoxin which increases inflammation[94,97]

Table 5-3. Correlation between pre-specified inferred functional traits (PICRUSt pathways), and oral taxa associated with CRC risk and inflammation at baseline (Week 0).

A priori selected PICRUSt pathways		Gemella	Campylobacter	Porphyromonas	Actynomyces	Prevotella	Fusobacterium	Streptococcus	Neisseria	Veillonella	Tannerella	Rothia	Parvimonas	Lactobacillus	Granulicatella	Eikenella	Capnocytophaga
Lipopolysaccharide biosynthesis	PCC	-0.122	**0.369**	**0.432**	0.137	-0.190	**0.597**	**-0.822**	**0.435**	-0.201	**0.321**	**-0.826**	*0.293*	**-0.349**	**-0.346**	0.199	**0.352**
	p-value	0.398	**0.008**	**0.002**	0.343	0.186	**<.0001**	**<.0001**	**0.002**	0.161	**0.023**	**<.0001**	*0.039*	**0.013**	**0.014**	0.167	**0.012**
	FDR p-value	0.398	**0.0213**	**0.0064**	0.3659	0.2126	**<.0001**	**<.0001**	**0.0064**	0.2055	**0.0368**	**<.0001**	*0.0567*	**0.0249**	**0.0249**	0.2055	**0.0249**
Lipopolysaccharide biosynthesis proteins	PCC	-0.150	**0.416**	**0.401**	0.166	-0.155	**0.557**	**-0.787**	**0.411**	-0.166	**0.305**	**-0.836**	0.245	**-0.314**	**-0.374**	0.200	**0.352**
	p-value	0.298	**0.003**	**0.004**	0.250	0.284	**<.0001**	**<.0001**	**0.003**	0.250	**0.031**	**<.0001**	0.087	**0.026**	**0.007**	0.163	**0.012**
	FDR p-value	0.298	**0.0096**	**0.0107**	0.2857	0.298	**<.0001**	**<.0001**	**0.0096**	0.2857	**0.0496**	**<.0001**	0.1265	**0.0462**	**0.016**	0.2173	**0.024**

*Pearson correlation coefficient with an FDR p-value <0.05 are in bold.

**Pearson correlation coefficient with a p-value <0.05 are in italics.

***The full SILVA taxonomy for the *Prevotella* genus presented was (Kingdom: *Bacteria*, Phylum: *Bacteroidetes*, Class: *Bacteroidetes*, Order: *Bacteroidales*, Family: *Prevotellaceae*, Genus: *Prevotella_7).*

Table 5-4. Correlation between the changes in relative abundance of pre-specified inferred functional traits (PICRUSt pathways), and the changes in relative abundance of oral taxa associated with CRC risk and inflammation from Week 0 to week 6.

Aspirin Arm																	
A priori selected PICRUSt pathways		Gemella	Campylobacter	Porphyromonas	Actynomyces	Prevotella	Fusobacterium	Streptococcus	Neisseria	Veillonella	Tannerella	Rothia	Parvimonas	Lactobacillus	Granulicatella	Eikenella	Capnocytophaga
Lipopolysaccharide biosynthesis	PCC	-0.082	0.301	0.241	0.265	0.072	**0.562**	**-0.851**	**0.471**	-0.248	-0.041	**-0.410**	-0.100	-0.069	-0.348	-0.123	0.017
	p-value	0.665	0.106	0.199	0.157	0.705	**0.001**	**0.000**	**0.009**	0.186	0.830	**0.025**	0.601	0.718	0.060	0.517	0.928
	FDR p-value	0.724	0.008	0.195	0.430	0.928	**0.001**	**0.001**	**0.008**	0.324	0.724	**0.001**	0.724	0.623	0.008	0.928	0.368
Lipopolysaccharide biosynthesis proteins	PCC	-0.097	0.324	0.214	0.289	0.082	**0.568**	**-0.807**	**0.467**	-0.183	-0.034	-0.490	-0.112	-0.030	*-0.385*	-0.126	0.024
	p-value	0.608	0.080	0.257	0.122	0.666	**0.001**	**0.000**	**0.009**	0.332	0.859	0.006	0.557	0.875	*0.036*	0.507	0.900
	FDR p-value	0.811	0.214	0.514	0.279	0.819	**0.009**	**0.002**	**0.037**	0.591	0.900	0.032	0.811	0.900	*0.114*	0.811	0.900
Placebo Arm																	
A priori selected PICRUSt pathways		Gemella	Campylobacter	Porphyromonas	Actynomyces	Prevotella	Fusobacterium	Streptococcus	Neisseria	Veillonella	Tannerella	Rothia	Parvimonas	Lactobacillus	Granulicatella	Eikenella	Capnocytophaga
Lipopolysaccharide biosynthesis	PCC	-0.022	*0.577*	0.318	-0.096	-0.278	0.405	*-0.465*	0.363	-0.340	0.352	*-0.521*	0.327	0.126	-0.430	0.406	*0.601*
	p-value	0.926	*0.008*	0.171	0.688	0.235	0.076	*0.039*	0.116	0.143	0.128	*0.019*	0.159	0.596	0.058	0.076	*0.005*
	FDR p-value	0.926	*0.062*	0.229	0.734	0.289	0.175	*0.156*	0.228	0.229	0.228	*0.099*	0.229	0.681	0.175	0.175	*0.062*
Lipopolysaccharide biosynthesis proteins	PCC	-0.056	*0.533*	0.283	-0.030	-0.260	0.366	-0.412	0.360	-0.304	0.335	*-0.582*	0.319	0.200	*-0.463*	0.361	*0.571*
	p-value	0.814	*0.016*	0.226	0.902	0.268	0.113	0.071	0.119	0.192	0.149	*0.007*	0.170	0.398	*0.040*	0.118	*0.009*
	FDR p-value	0.868	*0.083*	0.302	0.902	0.330	0.237	0.227	0.237	0.279	0.264	*0.068*	0.272	0.455	*0.160*	0.237	*0.068*

*Pearson correlation coefficient with an FDR p-value <0.05 are in bold.

**Pearson correlation coefficient with a p-value <0.05 are in italics.

***The full SILVA taxonomy for the *Prevotella* genus presented was (Kingdom: *Bacteria*, Phylum: *Bacteroidetes*, Class: *Bacteroidetes*, Order: *Bacteroidales*, Family: *Prevotellaceae*, Genus: *Prevotella_7)*

Table 5-5. Effect of aspirin treatment on the change over-time in relative abundances of pre-specified PICRUSt functional pathways using a linear mixed effect model.

Inferred functional pathway function	Association with CRC risk	Change in the Aspirin Group	Change in the Placebo Group	Predictor	Beta estimate	std error	z-value	p-value
Lipopolysaccharide biosynthesis				Placebo (vs. Aspirin)	-0.061	0.077	-0.797	0.426
	↑	-0.0263	-0.0139	Week 6 (vs. Week 0)	-0.028	0.023	-1.202	0.229
				Intervention * Collection Interaction	0.003	0.036	0.086	0.932
Lipopolysaccharide biosynthesis proteins				Placebo (vs. Aspirin)	-0.042	0.066	-0.638	0.523
	↑	-0.0222	-0.0135	Week 6 (vs. Week 0)	-0.020	0.019	-1.085	0.278
				Intervention * Collection Interaction	-0.002	0.029	-0.075	0.940

*The Lipopolysaccharide pathways were selected due to their pro-inflammatory role.

Supplemental table 5-1. Differential abundance for PICRUSt pathways for the placebo group vs. the aspirin group post intervention (Week 6).

Pathways	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Biotin metabolism	6550.939	0.111	0.042	2.603	0.009	0.988
Prion diseases	245.825	0.626	0.264	2.368	0.018	0.988
Phosphonate and phosphinate metabolism	2745.603	0.166	0.072	2.306	0.021	0.988
Transcription machinery	42931.007	-0.087	0.040	-2.187	0.029	0.988
Nitrotoluene degradation	3759.790	0.206	0.097	2.125	0.034	0.988
ABC transporters	141786.212	0.085	0.041	2.060	0.039	0.988
Other transporters	12143.523	0.058	0.029	2.025	0.043	0.988
Prenyltransferases	14069.085	-0.086	0.043	-1.987	0.047	0.988
Nitrogen metabolism	30530.888	0.069	0.035	1.973	0.048	0.988
Glutamatergic synapse	4637.966	-0.078	0.040	-1.972	0.049	0.988
Steroid hormone biosynthesis	1133.087	-0.514	0.273	-1.881	0.060	0.988
Glycan biosynthesis and metabolism	1750.800	0.306	0.168	1.817	0.069	0.988
Lysosome	4715.927	-0.240	0.132	-1.812	0.070	0.988
Cyanoamino acid metabolism	11938.962	0.099	0.056	1.777	0.076	0.988
Other ion-coupled transporters	55246.546	0.078	0.044	1.758	0.079	0.988
Two-component system	65924.512	0.087	0.050	1.753	0.080	0.988
Glycosphingolipid biosynthesis - ganglio series	1896.626	-0.265	0.153	-1.733	0.083	0.988
Electron transfer carriers	1439.530	0.412	0.239	1.725	0.084	0.988
Protein processing in endoplasmic reticulum	2881.978	-0.110	0.065	-1.696	0.090	0.988
Glycosaminoglycan degradation	3273.186	-0.265	0.160	-1.654	0.098	0.988
One carbon pool by folate	26515.310	-0.061	0.037	-1.649	0.099	0.988

*The differential abundance results are presented for the Placebo group vs. the aspirin group

***Base Mean** represents the average number of reads per pathway at week 6

***Log2FoldChange** represents the fold change of abundance on the log 2 scale between the placebo group and the aspirin group

***lfcSE** represents the standard error for the Log2FoldChange metric

***stat** represent the Wald statistic for placebo vs. aspirin

Chapter 6: MANUSCRIPT III – ASSOCIATIONS BETWEEN THE ORAL AND GUT MICROBIOME

A. Synopsis

Background: The gut microbiome has been shown to be associated with chronic inflammatory disorders of the intestine, including inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and ulcerative colitis (UC), as well as colorectal cancer (CRC). In addition, certain pro-inflammatory oral taxa have been also be found in the gut microbiome of individuals with CD, UC and CRC. However, it is not clear whether inflammation-related oral taxa and inflammation-related gut taxa are correlated and whether oral and gut microbiome communities respond similarly to anti-inflammatory agents such as aspirin. To address these gaps, we examined these questions in a double-blind placebo-controlled trial.

Methods: Fifty healthy subjects, 50-75 years old, were randomized to receive either aspirin (N=30) or placebo (N=20) for 6 weeks. Fecal and oral samples were collected at baseline and after treatment (week 6), and the V4 region of the 16S rRNA gene in oral and gut microbiome samples was sequenced using Illumina MiSeq. The sequenced data were processed using the standard DADA2 workflow. We examined the correlation between alpha diversity (assessed as a Shannon index) in the oral and gut microbiome at baseline, and after the 6-week treatment intervention in each arm. We also tested the association between the change in oral alpha diversity and the change in gut alpha diversity using linear mixed effect models. We tested the association between oral beta diversity and gut beta diversity (assessed using Bray-Curtis distances) at baseline

and after the 6-week treatment intervention in each arm using PERMANOVA (Adonis function).

In the analysis of individual taxa, we selected the following taxa: (A) pro-inflammatory gut taxa (*Parabacteroides, Alistipes, Blautia, Escherichia/Shigella, Dialister, Bacteroides, Streptococcus, Dorea, Veillonella, Slackia, Actinomyces,* and *Prevotella*); (B) anti-inflammatory gut taxa (i.e. taxa producing short-chain fatty acids (SCFA), such as *Faecalibacterium, Roseburia, Bifidobacterium* and *Coprococcus*), and (C) pro-inflammatory oral taxa also found in the gut microbiome of CRC cases vs controls (*Veillonella, Streptococcus, Fusobacterium, Porphyromonas, Parvimonas,* and *Prevotella*). We conducted two analyses by examining Pearson correlations at baseline between (1) taxa in group A and C; and (2) taxa B and C. The relative abundance of each taxon was standardized using arcsine square root transformation, so that a one unit of each taxon was equal to one standard deviation. Of note, we did not examine the changes over-time in individual taxa because of the modest aspirin-induced changes in the relative abundance of pre-specified oral and gut taxa.

Results: Alpha diversity in the oral microbiome was not correlated with alpha diversity in the gut microbiome at baseline. However, at week 6, after treatment intervention, alpha diversity in the oral microbiome was correlated with alpha diversity in the gut microbiome in the aspirin arm ($r=0.37$ & p-value = 0.04) but not in the placebo arm ($r = 0.28$ & p-value = 0.24). Further, we found that a 1 unit increase in the change over time of the oral Shannon index was associated with a 0.43 unit increase in the change over time of the gut Shannon index (p-value = 0.01) in the aspirin arm, but the change in the oral Shannon index was not associated with the change in the gut Shannon

index in the placebo arm (p-value = 0.95). Beta diversity in the oral microbiome was distinct from beta diversity in the gut microbiome at baseline (PERMANOVA p-value <0.001). At week 6 (post-treatment), beta diversity in the oral microbiome remained distinct from beta diversity in the gut microbiome in the aspirin arm (PERMANOVA p-value <0.001), as well as in the placebo arm (PERMANOVA p-value <0.001).

In the correlation analyses of individual taxa at baseline, we found that *Bifidobacterium*, a SCFA-producing gut taxon, was inversely correlated with three pro-inflammatory oral taxa (*Fusobacterium:* r = -0.27 & p-value = 0.06, *Porphyromonas:* r = -0.29 & p-value = 0.04 and *Parviromonas:* r = -0.30 & p-value = 0.03). Another SCFA-producing gut taxon, *Coprococcus*, showed similar trends (*Fusobacterium*: r = -0.29 & p-value = 0.04, *Porphyromonas:* r = -0.24 & p = 0.10 and *Parviromonas*: r = -0.29 & p-value = 0.04). A pro-inflammatory gut taxon, *Streptococcus*, was positively correlated with the pro-inflammatory oral taxon *Prevotella* (r = 0.29 & p-value = 0.04). However, unexpectedly, another pro-inflammatory gut taxon, *Blautia*, was inversely correlated with the pro-inflammatory oral taxon *Prevotella* (r = -0.33 & p-value = 0.02).

Conclusions: Our results indicate that aspirin may induce changes in oral and gut alpha diversity in a similar fashion. In addition, our findings of an inverse correlation between SCFA-producing gut taxa and pro-inflammatory oral taxa suggest that the study of pro-inflammatory oral taxa may be important for understanding the link between inflammation and the gut microbiome. If replicated in larger studies, these findings may help inform interventions using the oral microbiome to mitigate chronic inflammatory disorders of the intestine.

B. Introduction

Previous studies have reported that the relative abundance of certain gut taxa (such as *Fusobacterium, Peptostreptococcus,* and *Porphyromonas*) was consistently increased in CRC cases, while the relative abundance of other gut taxa (such as *Bifidobacterium, Ruminococcaceae, Lachnospiraceae, Faecalibacterium and Roseburia*) was consistently decreased in CRC cases, compared to controls[3,24,25]. In addition, these taxa are also involved in chronic inflammatory disorders of the intestine such as inflammatory bowel diseases (IBDs)[15,16], Crohn's disease (CD)[15,16], ulcerative colitis (UC)[17–19], where they have similar associations to those observed in CRC cases compared to controls. Further, gut taxa consistently increased in CRC cases also exhibit traits involved with bacterial functions such as adherence to host epithelial cells, mucus degradation, and biofilm formation. These traits promote bacterial survival in the colon and may stimulate an inflammatory response[11,27,29,68]. The inflammation, in turn, could lead to CRC development, by impacting either the APC/Beta-catenin pathway or the microsatellite instability pathway in the host[38,39].

In addition to the existing literature on the gut microbiome and CRC, recent studies have also found an association between the oral microbiome and CRC risk[20,21]. Specifically, these studies found that the relative abundance of specific oral taxa (such as *Fusobacterium, Peptostreptococcus,* and *Porphyromonas*) was increased in oral microbiome of CRC cases, compared to healthy controls[20,21]. The relative abundance of these oral taxa is also increased in periodontitis[20–23], and in IBDs[48], suggesting that these oral taxa have a pro-inflammatory role within of the oral cavity.

Along with their pro-inflammatory role in the oral cavity, oral taxa such as *Fusobacterium, Peptostreptococcus,* and *Porphyromonas* can also be found in the gut microbiome of CRC cases**(Figure 6-1)**[3,24,25], as well as the gut microbiome of IBD, CD and UCD cases[15–19]. However, it is unclear whether these oral taxa traveled down the GI track from the oral cavity after periodontitis, or if inflammation in the gut facilitated their translocation from the oral to the gut microbiome[10,26,27]. To our knowledge, one of the mechanisms underlying the association between the oral microbiome, the gut microbiome and inflammation is through bacteremia and the systemic diffusion of inflammatory mediators (such as Lipopolysaccharides, a key group of bacterial metabolites linked to inflammation[84]) from oral lesions caused by specific pro-inflammatory oral taxa[96,97]. Pro-inflammatory oral taxa such as *Fusobacterium* and *Porphyromonas* are key members of oral plaque, which protects these organisms form the immune system as the plaque extends and propagates below the gingival menbrane. Once the plaque breach the gingival membrane, pro-inflammatory oral taxa can either enter the blood stream, or release inflammatory mediators such as LPS into the blood stream, which could affect inflammation in the gut microbiome[97]. Thus oral plaques which play a key role in the development of periodontitis, can promote chronic inflammation, not only within the oral cavity, but also throughout the human body[97].

Another mechanism underlying the association between the oral microbiome, the gut microbiome and inflammation could involve oral taxa reaching the gut microbiome through swallowed saliva, as saliva production ranges from 0.75 to 1.5 L/day, and contains oral bacteria[98]. Although there are natural barriers along the gastroinstestinal tract such as increased PH and a mucosal barrier in the colon[98], several of the oral taxa

which are associated with periodontitis (including *Fusobacterium*, *Peptostreptococcus*, *Aggregatibacter* and *Porphyromonas*) exhibit traits such as adherence to host epithelial cells, mucus degradation, biofilm formation, and these traits promote bacterial survival in the colon[11,27,29,68]. Once translocated from the oral microbiome to the gut microbiome, these oral taxa could cause impairment of the gut barrier function and promote inflammation in the colon[11,27,29,68]. A previous study by Flemer et al.[26] profiled the microbiota in oral swabs and stools of patients with colorectal cancer (CRC) and controls, and found that several operational taxonomic units (OTUs) such as *Fusobacterium* and *Peptostreptococcus* were shared between oral swabs and stool samples.

The currently proposed mechanisms rely on the presence of dysbiosis due to inflammation related taxa in both the oral and gut microbiome. However, few epidemiologic studies have examined the correlation between pro-inflammatory oral taxa and inflammation related (pro or anti-inflammatory) gut taxa. Further, the diversity and composition of the oral and gut microbiomes could respond to intervention with aspirin (acetylsalicylic acid) in a similar. Aspirin irreversibly inhibits cyclooxygenase enzymes (COX)-1[6] and COX-2[7], and the resulting aspirin-modified COX-2 enzyme produces lipoxins, most of which are anti-inflammatory[9]. Aspirin may also exert its anti-inflammatory effect through COX-independent mechanisms, including direct effects on cytokines and transcription factors, modulation of estrogen biosynthesis through the effects on aromatization of androgens, and inhibition of oxidative DNA damage. The effect of aspirin on these pathways may indirectly affect the oral microbiome and the gut microbiome by counteracting the virulence factors of inflammation-associated taxa, and altering microbial diversity and composition[8].

Therefore, in the present study we hypothesized that relative abundance of pro-inflammatory oral taxa would be correlated to the relative abundance of pro-inflammatory and anti-inflammatory gut taxa at a cross-sectional level. We further hypothesized that given the interplay between bacterial dysbiosis and inflammation, community shifts in the oral microbiome in response to aspirin intervention would be correlated with community shifts in the gut microbiome in response to aspirin intervention. To test these hypotheses, we examined the correlation between oral alpha diversity and gut alpha diversity, as well as the correlation between inflammation related oral taxa and inflammation related gut taxa in the ASMIC study.

C. Materials and Methods

C1. Parent Study design

Our study was conducted within the parent study: "Effect of Aspirin on the Gut Microbiome (ASMIC)"[49]. ASMIC was a randomized clinical trial that aimed to determine whether treatment with aspirin results in favorable shift in the composition of gut microbiome. The ASMIC study recruited 50 healthy subjects, 50-75 years old, from the Evaluation of SEPT9 Biomarker Performance for Colorectal Cancer Screening (PreSEPT) cohort living in the greater Twin Cities area. Upon confirmation of eligibility, the subjects were asked to refrain from consuming any other NSAIDs and over-the-counter medications containing NSAIDs and from having major changes in their diet for the duration of the study. Participants were randomized into an aspirin (N=30) and placebo arm (N=20) using a block randomization scheme (N=5 per block). Each of the participant received a 325 mg

aspirin pill or placebo (lactose) once a day for 6 weeks, and the 6-week intervention period was followed by 6 weeks of washout.

C2. Data Collection

At baseline, a clinic visit to obtain informed consent and collect demographic information and assignment was scheduled for every participant (Visit 1, Week 0). During the initial visit (Visit 1), a brief medical and dietary history was recorded. Participant also attended a clinic visit after the 6-week intervention period (Visit 2) (**Figure 6-1**).

C3. Sample Collection

Participants underwent a 6-week intervention (N=30 in the aspirin arm, and N=20 in the placebo arm), and provided stool samples baseline (at week 0, Day 1), after completing intervention (at week 6), and after a washout period (at week 12) for 16s rRNA sequencing. Stool samples were collected using home sample collection kits. For each collection, participants were provided with four collection tubes (2 with ethanol, 2 with no preservative), a stool collection kit, a water-soluble bag, and a biohazard bag. After the collection, samples were stored at -20 ℃ by the participant, shipped on ice to the study center via courier services, and placed at -20 ℃ upon receipt. The stool samples were then transferred to long term, -80 ℃ storage within 24 hours of reception.

The blood, urine and oral samples were collected by trained study staff during the two clinic visits at week 0 (Collection 1 at Visit 1, before the intervention) and week 6

(Collection 2 at Visit 2, after the intervention). Urine samples were collected in the parent study to assess the effectiveness of aspirin treatment using the urinary metabolite of prostaglandin E2, PGE-M, adjusted for creatinine levels. As discussed above, prostaglandins are a product of COX-2 activity[7,9], while their urinary metabolite PGE-M has been shown to be a an inflammatory biomarker for CRC[52,53]. For oral samples, tongue swabs were collected by trained study staff, and were frozen at -20 ℃ after collection. Blood and urine samples were aliquoted after collection, and then frozen at -20 ℃. All samples were transferred to -80 ℃ within 24 hours of sample collection.

C4. DNA extraction

Bacterial DNA extraction, sequencing and amplification were conducted at the University of Minnesota Genomics Center (UMGC). To characterize the oral microbiome of our study participants, bacterial DNA was extracted from tongue swab samples using the MoBio Powersoil DNA extraction kits (MoBio, Carlsbad, CA) according to the manufacturer's instructions[56]. Quality control on the final DNA product was performed using a spectrophotometer reading. DNA purity was assessed using the Qubit dsDNA High Sensitivity Kit to measure the A260/280 DNA Yield test. The A260/280 DNA absorbance method is a simple and well-validated quality control procedure for evaluating DNA purity, and only requires commonly available laboratory equipment[57]. The extraction process yielded approximately 100 µl DNA per sample, of which 25 µl was used by UMGC for PCR amplification, and the leftover DNA was stored at -20°C.

To characterize the stool and oral microbiome of our study participants, we sequenced a single common gene across many bacteria known as the 16S rRNA gene. The 16S rRNA gene is an ubiquitous bacterial gene, which codes for ribosomal RNA, an essential component of all bacterial genomes. Ribosomal RNA has regions that are highly conserved (essential for ribosome function), as well as regions that vary across bacteria. These variable regions are mutated but still functional, and this variability allows for differentiation among oral bacteria[10].

C5. DNA amplification and sequencing

The V4 variable region of the 16S rRNA gene from each DNA sample was amplified and sequenced, using validated DNA probes and the Illumina 454 MiSeq Personal Sequencing platform[58]. During the DNA amplification steps, the UMGC facility used sterile water controls for each batch. In addition, two wells on each 96-well sample plate were reserved for positive and negative controls. At the completion of the analysis, the sequenced genetic data will be archived in the Sequence Read Archive at the National Center for Biotechnology Information[69].

C6. Taxonomy assignment

Sequence processing and analysis were performed using the DADA2 bioinformatics software (version 1.6.0). Forward and reverse reads were trimmed to 200 nt to remove low-quality reads and merged. High quality sequences were aligned against the SILVA database version 132.

C7. Statistical analysis

We tested for differences in the demographics characteristics of our participants using t-tests for continuous variables, and chi-square tests for our categorical variables. In addition, to check for compliance with aspirin intake, we evaluated pre-post treatment change in PGE-M levels, adjusted for baseline PGE-M levels, because PGE-M is suppressed by aspirin intake and it may serve as a marker of treatment compliance for our study.

The bioinformatics analysis described in the "Taxonomy" section generated an Operational Taxonomic Unit (OTU) table with features (i.e. bacterial taxa) for the oral microbiome samples. This OTU table was also used to determine the number of taxa in the bacterial community (alpha diversity) and the overall population structure of the bacterial community (beta diversity).

C8. Analyses of alpha and beta diversity

We generated the Shannon Index as a metric for alpha diversity in oral samples and in fecal samples [70–72]. We selected the Shannon index as our quantitative metric for alpha diversity, which allowed us to evaluate both taxonomic richness (i.e. the number of OTUs) and evenness (i.e. the abundance of different OTUs) in samples. In the first analysis of alpha diversity we examined the correlation between the oral Shannon index and the gut Shannon index at baseline (Week 0) in all participants. Then, in the second analysis of alpha diversity, we examined the correlation between the oral Shannon index and the gut Shannon index post intervention (Week 6) in each arm separately. Finally, we

used linear mixed effect regression to test the association between the change in oral Shannon index and the change in gut Shannon index in the aspirin and placebo arms, separately. Of note, we expected to see changes in the Shannon index in the aspirin arm but not in the placebo arm, because we hypothesized that aspirin would induce changes in microbiome diversity by affecting pro-inflammatory oral and gut taxa in a similar fashion.

We assessed beta diversity using Bray-Curtis as a distance measure for the abundance data after the data was rarefied and log transformed. We selected Bray-Curtis as a quantitative distance metric to evaluate changes in beta diversity composition, which allowed us to evaluate microbial community composition by considering both the presence of taxa (yes/no) from any given pairwise comparison, while also weighting each comparison by taxon abundance[99]. After generating our Bray-Curtis distance measures, we used Permutational multivariate analysis of variance (PERMANOVA), as implemented by the Adonis function in the vegan package, to test the differences between oral beta diversity and gut beta diversity. In the first beta diversity analysis, we tested for the difference between oral and gut beta diversity at baseline (Week 0). In the second beta diversity analysis, we tested for the difference between oral and gut beta diversity post intervention (Week 6) in each arm separately. Of note, we were unable to test for the change over time in oral beta diversity and gut beta diversity, as the PERMANOVA procedure is based on all pairwise Bray-Curtis distance comparisons, and calculating a change in all pairwise distances could not be implemented with only 2 time points.

C9. Pearson correlation analyses of individual oral and gut taxa

We selected the following a priori oral and gut taxa of interest based on literature. We included (A) pro-inflammatory gut taxa (*Parabacteroides, Alistipes, Blautia, Escherichia/Shigella, Dialister, Bacteroides, Streptococcus, Dorea, Veillonella, Slackia, Haemophilus, Actinomyces,* and *Prevotella)*[3,10–12,14,25,26], (B) anti-inflammatory, i.e. SCFA-producing gut taxa (*Faecalibacterium, Roseburia, Bifidobacterium* and *Coprococcus)*[3,25,100], and (C) pro-inflammatory oral taxa found in the gut microbiome of CRC cases, and whose relative abundances were increased in the gut microbiome of CRC cases, compared to controls (*Veillonella, Streptococcus, Fusobacterium, Porphyromonas, Parvimonas,* and *Prevotella)*[10,11,26,47,101,102]. All correlation analyses were done at the genus level for the oral and gut taxa.

First, we calculated the prevalence and relative abundance of the oral and gut taxa of interest. Then, we applied a variance-stabilizing arcsine square root transformation to the relative abundances of our taxa of interest. The arcsine square root transformed relative abundance of each taxon and then standardized each taxon by dividing the arcsine square root transformed value by its standard deviation, so that a 1 unit change was equivalent to a 1 standard deviation change in the relative abundance of each taxon[103]. We conducted correlation analyses (the Pearson correlation coefficients and p-value) between (1) A and C and (2) B and C for the standardized relative abundances in oral and stool samples at baseline.

The taxonomic assignment, alpha diversity and beta diversity metrics were generated using the R Statistical Analysis software package, Version 3.3 (CRAN) using the QIIME, EdgeR, vegan, and phyloseq (2-sided tests, $\alpha = 0.05$). Bray-Curtis beta diversity distance metrics were generated using the q2 longitudinal plugin in QIIME2

(version 2019.08). All correlation analyses were conducted using the SAS software (version 9.3; SAS Institute, Cary, NC).

D. Results

D1. Study cohort

The study cohort included 50 participants (**Table 6-1**) who were randomized to the aspirin group (N = 30) and placebo group (N=20). Participant demographics were balanced for age (mean age in the aspirin group = 62.2 years, mean age in the placebo group = 61.2, p-value for difference = 0.5649), BMI (mean BMI in the aspirin group = 27.3, mean BMI in the placebo group = 28.2, p-value for difference = 0.49), and for baseline PGE-M levels (mean PGE-M levels in the aspirin group =11.82 ng/mg creatinine, mean PGE-M levels in the placebo group = 12.94 ng/mg creatinine, p-value for the difference = 0.739). After 6 weeks of intervention, 47 participants (94%) had at least 90% pill compliance, and changes in urinary PGE-M indicated high treatment compliance as well. After adjustment for baseline PGE-M, the change in PGE-M was -6.17 (95% CI: -9.16; -3.18) mg/dL for aspirin vs. placebo (p <0.001).

D2. Alpha and Beta diversity analyses

At baseline, we found that alpha-diversity was increased in the gut microbiome samples (Shannon index = 5.97) compared to the oral microbiome samples (Shannon index = 5.00), but oral alpha diversity was not correlated with gut alpha diversity (r=0.06

& p-value = 0.63) **(Figure 6-2)**. However, after the 6 week treatment intervention, alpha diversity in the oral microbiome was correlated with alpha diversity in the gut microbiome in the aspirin arm (r=0.37 & p-value = 0.04) but not in the placebo arm (r = 0.28 & p-value = 0.24) **(Figure 6-3)**. Moreover, we found that a 1 unit increase in the change over time of the oral Shannon index was associated with a 0.43 unit increase in the change over time of the gut Shannon index (p-value = 0.01) in the aspirin arm, but the oral Shannon index was not associated with the gut Shannon index in the placebo arm (p-value = 0.95) **(Table 6-2)**. In the analysis of oral and gut beta diversity matrices defined as Bray-Curtis distances, beta diversity in the oral microbiome was distinct from beta diversity in the gut microbiome at baseline ($P < 0.001$ in permutational multivariate analysis of variance, PERMANOVA; **Table 6-3**). After the 6 week treatment intervention, beta diversity in the oral microbiome remained distinct from beta diversity in the gut microbiome in the aspirin arm post intervention ($P < 0.001$, PERMANOVA; **Table 6-4**), as well as in the placebo arm post intervention ($P < 0.001$, PERMANOVA; **Table 6-4**).

D3. Correlation analyses for individual oral and gut taxa

At baseline, the stool samples contained large proportions of *Bacteroides* (33%), followed by *Alistipes* (4.28%), (4.22%) and *Faecalibacterium* (4.1%) (**Figure 6-5**), which were in line with the composition of the gut microbiome of healthy participants in the Human Microbiome Project(HMP)[51]. The oral samples at baseline contained large proportions of *Streptococcus* (15.1%), *Veillonella* (12.5%), *Haemophilus* (9.53%) and *Neisseria* (7.9%) (**Figure 6-5**) and these proportions are similar to the healthy buccal

microbiome in the HMP, where oral samples were dominated by *Streptococcus* and *Haemophilus*[51].

D3.a. Correlation analysis between pro-inflammatory oral taxa and pro-inflammatory gut taxa at baseline (Figure 6-6)

We found that the standardized relative abundance of the pro-inflammatory gut taxon, *Streptococcus*, was positively correlated with the standardized relative abundance of the pro-inflammatory oral taxon *Prevotella* (r = 0.29 & p-value = 0.04), while the standardized relative abundance of the pro-inflammatory gut taxon, *Blautia*, was inversely correlated with the standardized relative abundance of the pro-inflammatory oral taxon *Prevotella* (r = -0.33 & p-value = 0.02) (**Table 6-5**).

D3.b. Correlation analysis between pro-inflammatory oral taxa and anti-inflammatory gut taxa (Figure 6-7)

The standardized relative abundance of *Bifidobacterium*, a SCFA-producing gut taxon, was negatively correlated with the standardized relative abundance of three disease associated oral bacterial taxa (*Fusobacterium*: r = -0.27 & p = 0.06, *Porphyromonas*: r = -0.29 & p = 0.04 and *Parviromonas*: r = -0.30 & p = 0.03) **(Table 6-6)**. Another SCFA-producing gut taxon, *Coprococcus*, showed similar trends (*Fusobacterium*: r = -0.29 & p = 0.04, *Porphyromonas*: r = -0.24 & p = 0.10 and *Parviromonas*: r = -0.29 & p = 0.04) **(Table 6-6)**.

E. Discussion

E1. Description of the study findings for alpha diversity

In this longitudinal placebo-controlled study of a 6-week aspirin intervention, we found that stool samples had a greater alpha diversity than oral samples. This is in line with comparisons of alpha diversity across various body sites among healthy participants from the Human Microbiome Project[51]. In addition, we found that, after treatment intervention, there was a statistically significant correlation in alpha diversity between the oral and fecal samples in the aspirin arm, but not in the placebo arm. Likewise, changes in alpha diversity in the oral samples were associated with changes in the alpha diversity of the fecal samples in the aspirin arm, compared to the placebo arm. Of note, there was no correlation between diversity in the oral microbiome and the gut microbiome at baseline. These results suggest that aspirin may induce changes in both oral and gut alpha diversity in a similar fashion. Given that pro-inflammatory oral taxa have been found in the gut microbiome of individuals with chronic inflammatory disorders[3,15–19,24,25], it is plausible that the oral and gut microbiome would undergo similar changes in response to a pro-inflammatory environment, and thus would respond in a similar fashion to aspirin intervention.

E2. Description of the study findings for beta diversity

Our results for beta diversity showed that oral and gut microbial community composition were distinct from each other at baseline, as well as after the 6-week

treatment intervention within the aspirin and placebo arms. These findings are in line with previous findings from the Human Microbiome Project[51] on microbial community composition across the gastrointestinal tract. In this study, we used Bray-Curtis as a quantitative distance metric to evaluate changes in beta diversity composition, which allowed us to evaluate microbial community composition by considering both the presence of taxa (yes/no) from any given pairwise comparison, while also weighting each comparison by taxon abundance. It is not surprising that the oral and gut community composition was distinct at baseline and post intervention, as fecal microbiome samples are dominated by taxa in the *Bacteroides* and *Prevotella* genera[51,104,105], while oral microbiome samples are dominated by taxa in *Streptococcocus, Haemophilus, Prevotella* and *Veillonella* genera[51,106]. In addition, the abundance of these taxa differs between oral and fecal samples, which would lead to distinct community compositions, as reflected by the beta diversity.

E3. Description of the study findings for correlations between a priori selected oral and gut taxa

In addition to our findings for community diversity and composition, we found inverse correlations between the relative abundance of three pre-selected pro-inflammatory oral taxa and two pre-selected anti-inflammatory SCFA-producing gut taxa at baseline, which is in line with the roles of these taxa with regards to inflammation. However, the direction of correlations between the relative abundance of pro-inflammatory gut and oral taxa was unexpected for some other pro-inflammatory taxa in this study.

Several biological mechanisms may explain how pro-inflammatory oral taxa play a role in inflammation at various parts of the gastrointestinal tract. Previous studies have shown an association between oral taxa such as *Porphyromonas* and *Fusobacterium*, inflammation and periodontal disease[20–23]. The gastrointestinal tract may be exposed to colonization from the oral microbiome through saliva or the blood stream, under abnormal environmental conditions[11,31,107]. Cooperation between these oral species likely equips them well for a life in the colon, as periodontal species are adept at coaggregation using adhesins, and biofilms containing oral microbes have been isolated from the colonic mucosa of patients with CRC[11], as well as inflammatory bowel disease[7,8] indicating the ability of these oral taxa to survive the natural barriers in the gastrointestinal tract and invade the gut microenvironment[26,108]. Thus, inflammation-associated biofilms found in the colonic mucosa are similar in both community membership and invasiveness to those found in the oral cavity of a host with an enriched pro-inflammatory oral microbiome [11].

Our findings of an inverse correlation between these pro-inflammatory oral taxa, and SCFA- producing taxa such as *Bifidobacterium* and *Coprococcus*, are in line with the reported role of SCFAs in health and disease due to their impact on systemic inflammation. LPS are one of the major disease-associated bacterial metabolites present in the outer membrane of Gram-negative bacteria such as *Porphyromonas* and *Fusobacterium*, which cause chronic immune responses associated with inflammation, and have been previously associated with increased levels of TNF-α, IL6 and CRP[95]. *Bifidobacterium* and *Coprococcus* are two of many gut microbiome species known to be producers of SCFAs fatty acids, specifically Butyrate[40,41]. These SCFAs are microbial

products which have been reported to have strong immunomodulatory effects on the host, including the inhibition of LPS production in in vitro studies[84,88–90].

E4. Strengths and Limitations

A strength of our study is that we used a validated method to standardize the relative abundance of bacterial taxa of interest across samples sites, which allowed us to examine the correlation between the oral microbiome and the gut microbiome in healthy participants. In addition, we used the longitudinal nature of the study to examine the change in community diversity between the oral and fecal microbiome samples. However, the findings from the community diversity analyses should not be over-interpreted, as the directionality of the association between oral alpha diversity and gut alpha diversity could not be determined within this study design.

This study also had several limitations, and these may explain the inconsistent correlations between pro-inflammatory oral taxa and pro-inflammatory gut taxa. First, this pilot study had a relatively small sample size, and the intervention was only over a short duration and likely lacked power to find a statistically significant association. Because of the short duration of the intervention and modest aspirin-induced changes in both oral and gut microbiome, we focused on correlations between oral and gut taxa of interest at baseline and did not study changes over time for individual taxa. Second, the relative abundances of pro-inflammatory taxa such as *Fusobacterium* and *Porphyromonas* are much lower in the gut microbiome of healthy participants compared to those with chronic inflammatory disorders of the intestine (including CRC), making the evaluation of their correlation with their oral microbiome counterparts challenging.

However, these taxa are present in higher proportions in the oral microbiome compared to the gut microbiome, and growing evidence suggests that pro-inflammatory oral taxa have the ability to transition to the gut microbiome[10,26,27]. Moreover, the pro-inflammatory oral taxa under study were shown to be enriched in the gut microbiome of individuals with chronic inflammatory diseases (such as CD, UC and CRC) compared to those without the diseases[20–23]. Thus, understanding the relationship between pro-inflammatory oral taxa in the oral cavity and inflammation associated gut taxa may provide valuable insight into ways to mitigate the invasion of the gut microbiome by pro-inflammatory oral taxa.

E5. Conclusions

In this report, we found that aspirin may induce changes in oral and gut alpha diversity in a similar fashion. These changes in alpha diversity in response to aspirin are in line with the current literature on changes to microbiome community diversity in response to a pro-inflammatory environment. In addition, we found an inverse association between the standardized relative abundance of pro-inflammatory oral taxa and the standardized relative abundance of anti-inflammatory gut taxa with properties. If replicated in larger studies, these findings may help inform interventions using the oral microbiome to mitigate chronic inflammatory disorders of the intestine.

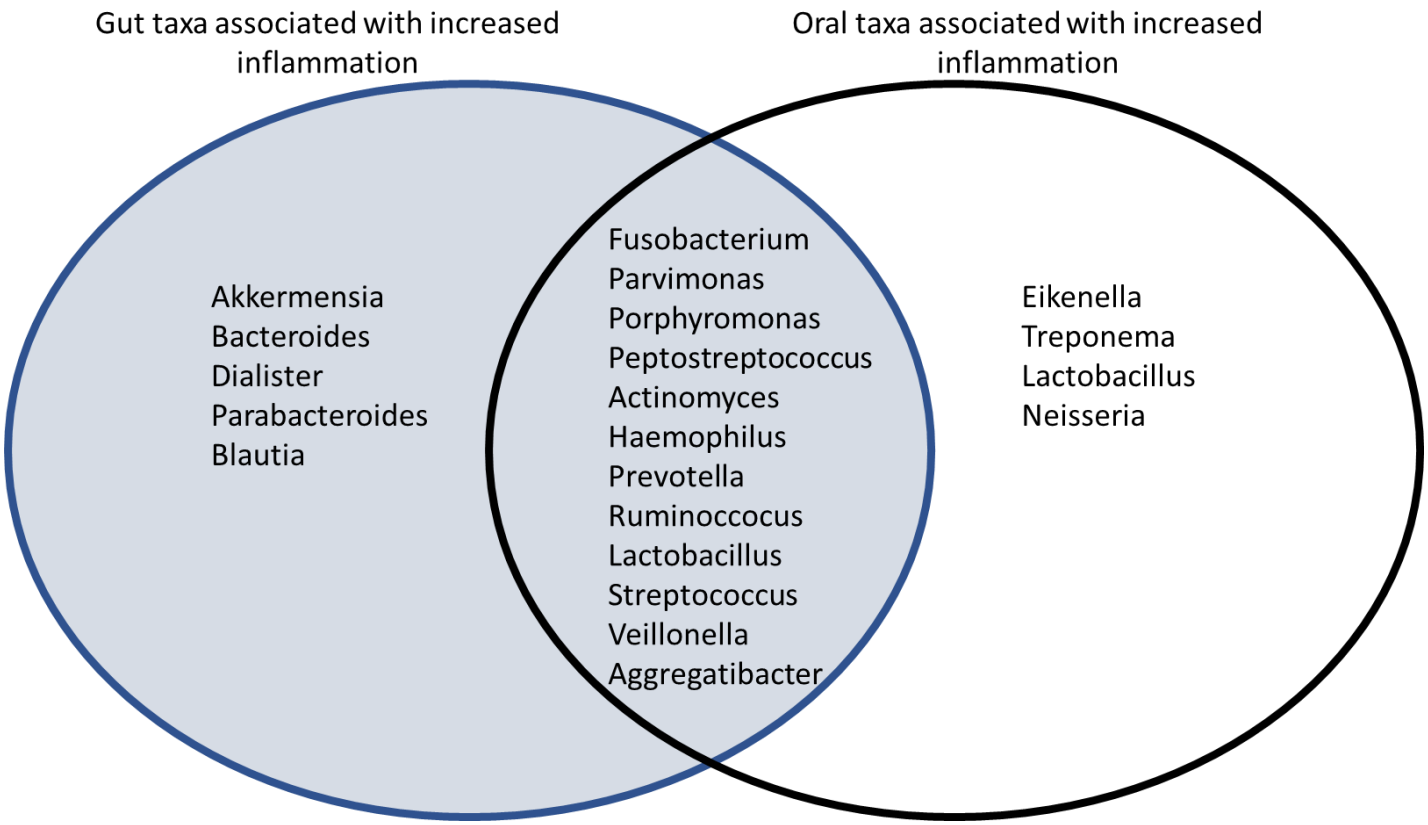


Figure 6-1. Diagram of the overlap between pro-inflammatory oral taxa and pro-inflammatory gut taxa.

The gut taxa associated with inflammation were selected based on studies by Shah et al[3], Borges Canha et al[25], Vogtmann et al[10] and Flemmer et al[26].

The oral taxa associated with inflammation were selected based on studies by Boillot et al[101], Flemmer et al[26], Vogtmann et al[10], Demmer et al[47], Flynn et al[11] and Gao et al[102].

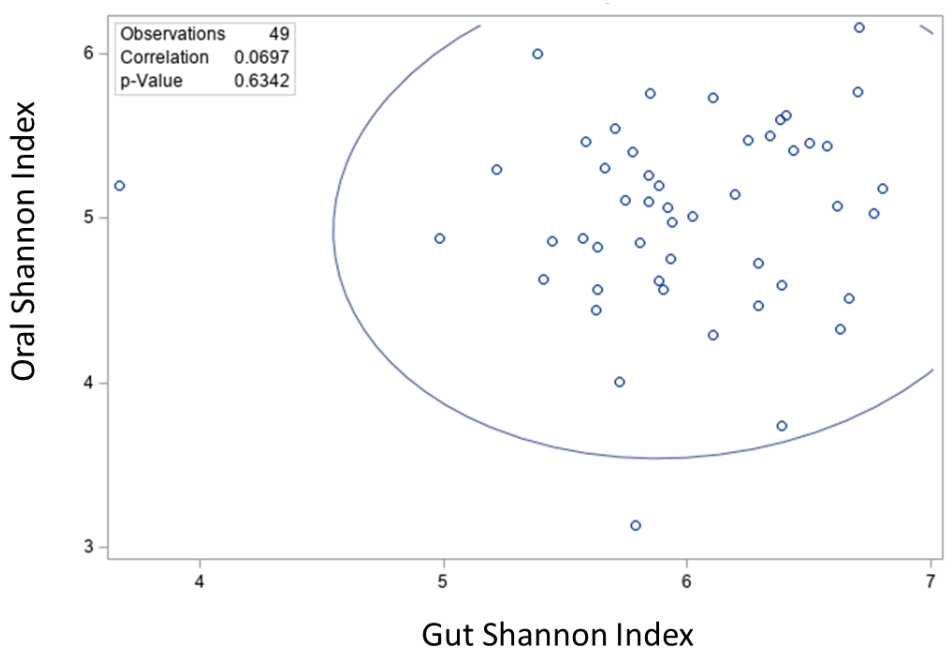


Figure 6-2. Scatterplot for the Pearson correlation between the oral Shannon index and the gut Shannon index at baseline (week 0).

The Shannon index was generated for the oral and gut microbiome samples separately as a metric for alpha diversity.

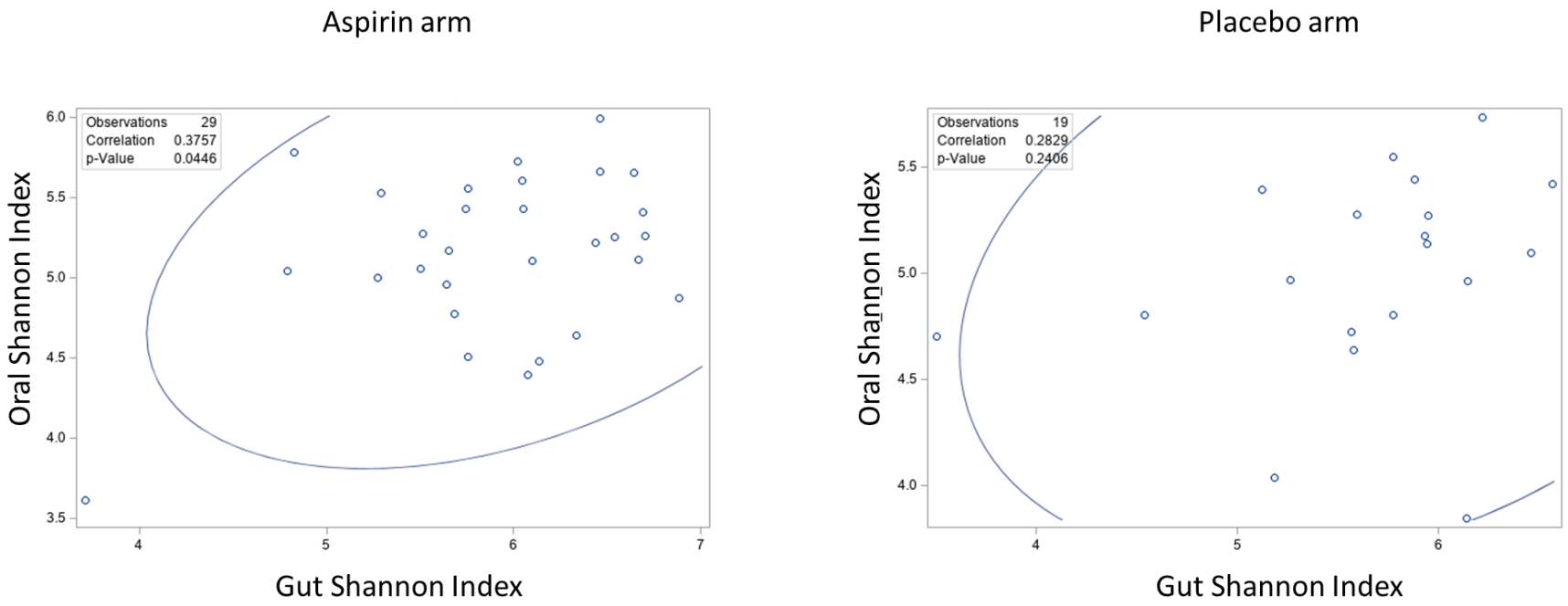


Figure 6-3. Scatterplot for the Pearson correlation between the oral Shannon index and the gut Shannon index post-intervention (week 6).

The Shannon index was generated for the oral and gut microbiome samples separately as a metric for alpha diversity.

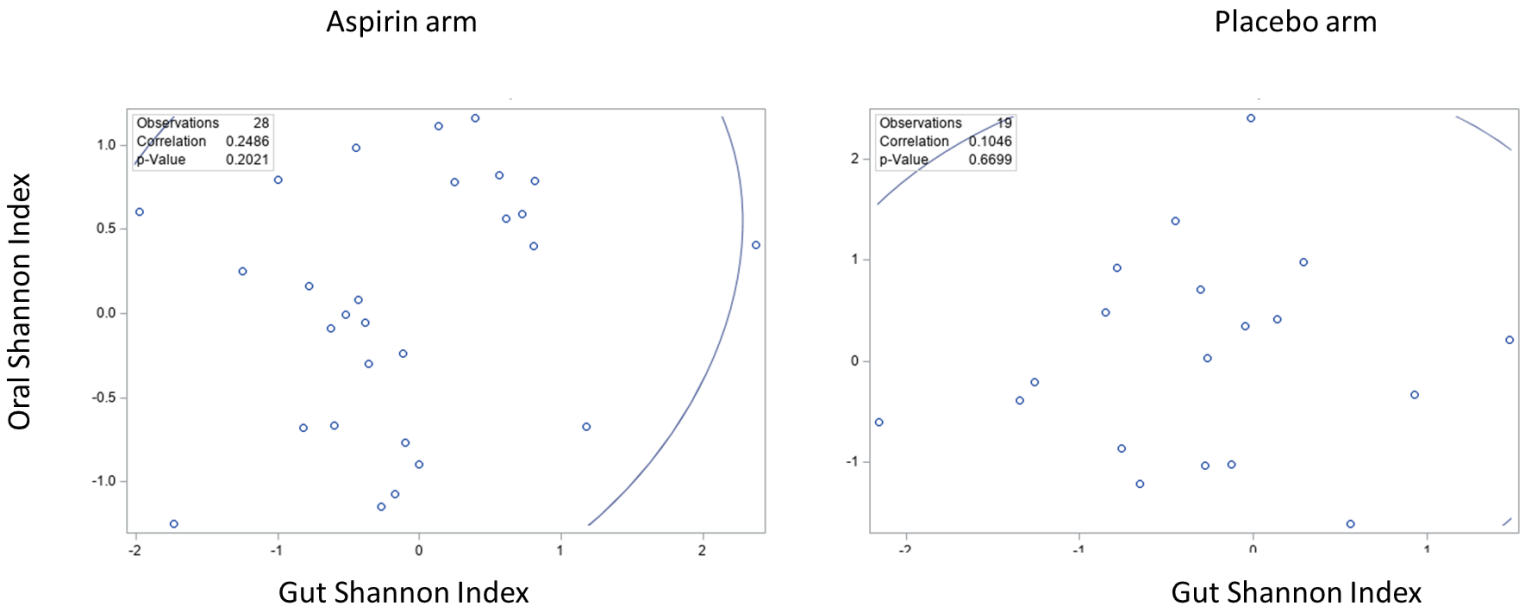
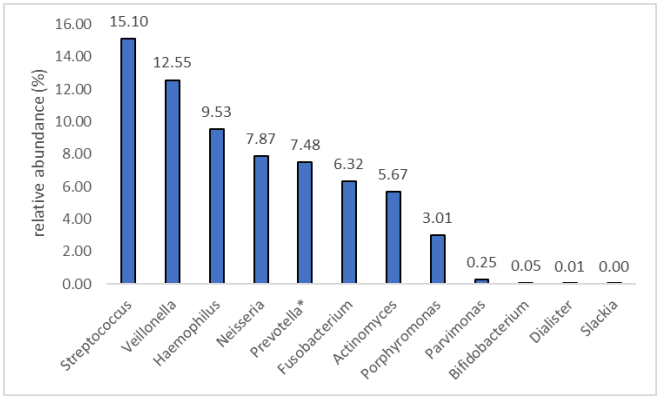
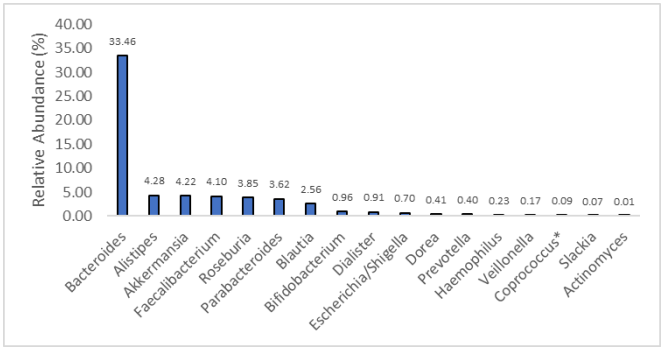


Figure 6-4. Scatterplot for the Pearson correlation between the change in oral Shannon index and the change in gut Shannon index between baseline (week 0) and post-intervention (week 6).

The Shannon index was generated for the oral and gut microbiome samples separately as a metric for alpha diversity.



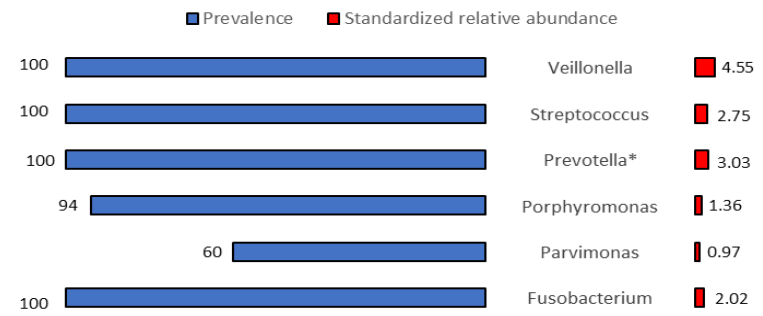
Oral Taxa



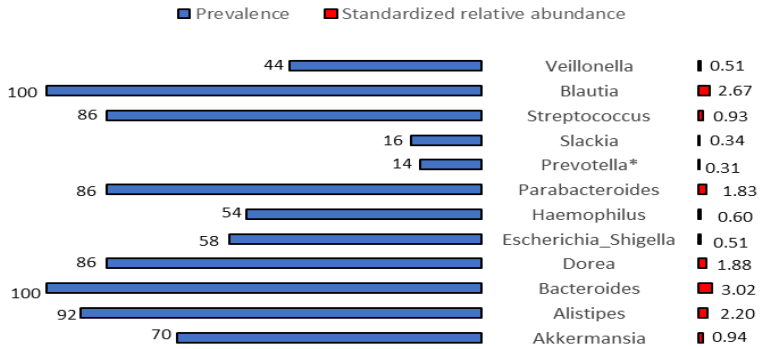
Gut Taxa

Figure 6-5. Relative abundance of oral and gut taxa associated with inflammation at baseline.

Average relative abundance of a priori selected oral and gut taxa at the genus level
*The full SILVA taxonomy for the Coprococus genus presented was (Domain: *Bacteria*, Phylum: *Firmicutes*, Class: *Clostridia*, Order: *Clostridiales*, Family: *Lachnospiraceae*, Genus: *Coprococcus_2)*



Oral taxa associated with increased inflammation



Gut taxa associated with increased gut dysbiosis

Figure 6-6. Prevalence and standardized abundance of a priori selected oral taxa associated with inflammation, and gut taxa associated with gut dysbiosis at baseline (Week 0).

*The full SILVA taxonomy for the *Prevotella* genus presented was (Kingdom: *Bacteria*, Phylum: *Bacteroidetes*, Class: *Bacteroidetes*, Order: *Bacteroidales*, Family: *Prevotellaceae*, Genus: *Prevotella_7)*

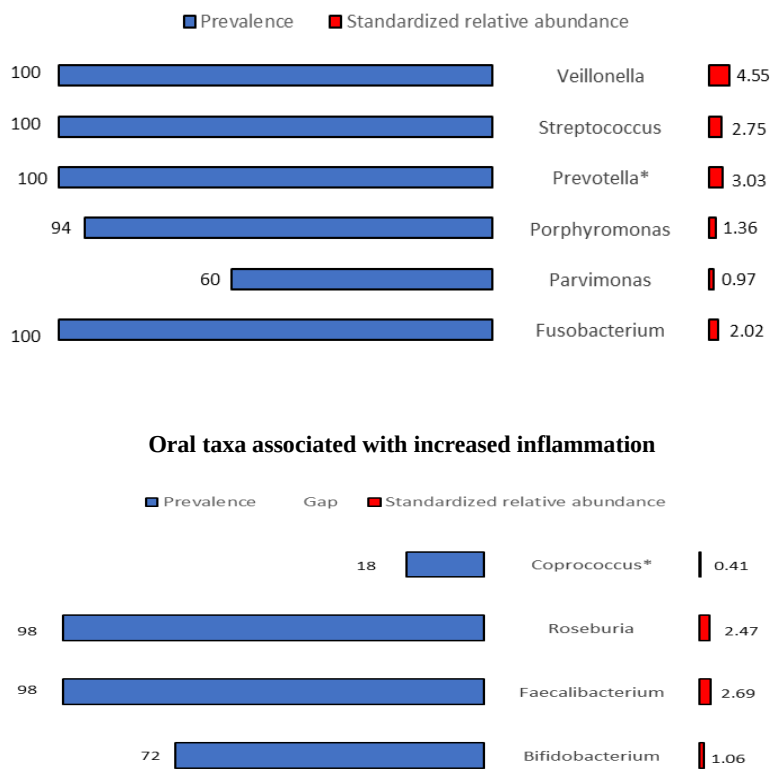


Figure 6-7. Prevalence and standardized abundance of a priori selected oral taxa associated with inflammation, and gut taxa associated with short chain fatty acid production at baseline (Week 0).

*The full SILVA taxonomy for the Coprococus genus presented was (Domain: *Bacteria*, Phylum: *Firmicutes*, Class: *Clostridia*, Order: *Clostridiales*, Family: *Lachnospiraceae*, Genus: *Coprococcus_2).*

*The full SILVA taxonomy for the *Prevotella* genus presented was (Kingdom: *Bacteria*, Phylum: *Bacteroidetes*, Class: *Bacteroidetes*, Order: *Bacteroidales*, Family: *Prevotellaceae*, Genus: *Prevotella_7)*

Table 6-1. Descriptive statistics for the ASMIC participants

Characteristics	Aspirin Group	Placebo Group	p-value
N (%)	30 (60.0%)	20 (40.0%)	
Age, Mean (SD) y	62.2 (5.1)	61.2 (5.2)	0.5649
Sex, Female%	23 (76.7%)	9(45.0%)	0.02
BMI, Mean (SD), kg/m2	27.3 (4.3)	28.2 (4.8)	0.4995
Baseline Urinary PGE-M (adjusted for creatinine)	11.82 (13.59)	12.94 (7.39)	0.739

Table 6-2. Mixed effect regression models for the association between the change in oral alpha diversity and the change in gut alpha diversity from Week 0 to week 6.

Aspirin Arm						
	Change in gut alpha diversity	Change in oral alpha diversity	Estimate	Std. Error	t-value	Pr(>|t|)
Oral Shannon Index	-0.09	0.13	0.43	0.16	2.58	0.01
Placebo Arm						
	Change in gut alpha diversity	Change in oral alpha diversity	Estimate	Std. Error	t-value	Pr(>|t|)
Oral Shannon Index	-0.30	0.04	0.01	0.17	0.07	0.95

Table 6-3. PERMANOVA model for the association between oral beta diversity and gut beta diversity in the ASMIC study at baseline (Week 0).

Comparison of Beta diversity in the oral vs gut microbiome at baseline				
Group Comparison	Sample size	Permutations	pseudo-F	p-value
Oral (vs. gut)	100	999	59.19	0.001

** The presented p-value is for the PERMANOVA F test.

Table 6-4. PERMANOVA model for the association between oral beta diversity and gut beta diversity in the aspirin and placebo arms in the ASMIC study post intervention (Week 6).

Comparison of Beta diversity in the oral vs gut microbiome in the aspirin arm at week 6				
Group Comparison	Sample size	Permutations	pseudo-F	p-value
Oral (vs. gut)	60	999	33.49	0.001
Comparison of Beta diversity in the oral vs gut microbiome in the placebo arm at week 6				
Group Comparison	Sample size	Permutations	pseudo-F	p-value
Oral (vs. gut)	40	999	19.31	0.001

** The presented p-value is for the PERMANOVA F test.

Table 6-5. Pearson correlations between the standardized relative abundance of pro-inflammatory oral taxa and the standardized relative abundance of pro-inflammatory gut taxa at baseline (Week 0).

Oral taxon / Gut taxon	*Fusobacterium*	*Parvimonas*	*Porphyromonas*	*Prevotella****	*Streptococcus*	*Veillonella*
Akkermansia	0.05	0.05	**0.27***	**-0.28***	0.03	-0.17
Alistipes	0.11	0.17	0.17	-0.06	**-0.26***	-0.03
Bacteroides	-0.02	0.03	-0.03	0.06	-0.06	0.01
Dorea	0.10	0.10	0.12	-0.11	0.01	-0.13
Escherichia_Shigella	-0.16	-0.13	-0.11	0.10	0.02	0.08
Haemophilus	-0.02	-0.22	-0.20	0.05	0.03	0.09
Parabacteroides	-0.13	-0.01	-0.10	0.06	0.16	0.09
Slackia	-0.05	-0.14	-0.04	0.09	-0.15	0.18
Streptococcus	-0.14	-0.23	-0.17	**0.29***	0.06	0.25
Blautia	0.23	0.07	0.18	**-0.33***	-0.03	-0.18
Veillonella	-0.03	-0.22	-0.17	0.08	0.03	**0.28***

*Pearson correlation coefficient with a p-value <0.10

**Pearson correlation coefficient with a p-value <0.05

***The full SILVA taxonomy for the *Prevotella* genus presented was (Kingdom: *Bacteria*, Phylum: *Bacteroidetes*, Class: *Bacteroidetes*, Order: *Bacteroidales*, Family: *Prevotellaceae*, Genus: *Prevotella_7)*

Table 6-6. Pearson correlations between the standardized relative abundance of pro-inflammatory oral taxa and the standardized relative abundance of gut taxa associated with short chain fatty acid production at baseline (Week 0).

Oral taxon / Gut taxon	*Fusobacterium*	*Parvimonas*	*Porphyromonas*	*Prevotella***	*Streptococcus*	*Veillonella*
Bifidobacterium	-0.27*	-0.30**	-0.29**	0.12	0.03	0.17
Faecalibacterium	0.17	0.13	0.13	-0.16	-0.32**	0.12
Roseburia	-0.03	-0.02	-0.17	0.27*	-0.05	0.05
*Coprococcus****	-0.29**	-0.29	-0.24*	0.22	0.05	0.27*

*Pearson correlation coefficient with a p-value <0.10

**Pearson correlation coefficient with a p-value <0.05

***The full SILVA taxonomy for the *Prevotella* genus presented was (Kingdom: *Bacteria*, Phylum: *Bacteroidetes*, Class: *Bacteroidetes*, Order: *Bacteroidales*, Family: *Prevotellaceae*, Genus: *Prevotella_7)*. The full SILVA taxonomy for the Coprococcus genus presented was (Domain: *Bacteria*, Phylum: *Firmicutes*, Class: *Clostridia*, Order: *Clostridiales*, Family: *Lachnospiraceae*, Genus: *Coprococcus_2)*.

Chapter 7. SUMMARY

A. Summary of results

An association between chronic inflammation and CRC has been investigated extensively over recent decades, and the potential use of aspirin (acetylsalicylic acid) as a chemopreventive agent has been a promising venue in CRC research. Although daily aspirin intake has been associated with a reduction in one's risk of developing CRC[5], the mechanism behind this association has not been firmly established. Recent studies have found an association between the gut[3,15–19,24,25] and oral[20,21,48] microbiome and chronic inflammatory disorders of the intestine, including CRC, but it is not known if aspirin exerts its chemo preventive effects in CRC through the gut and the oral microbiome. To address these questions, we examined three hypotheses about effect of aspirin on the relative abundance of pro-inflammatory oral taxa and inferred functional traits, as well as the correlation between inflammation associated oral and gut taxa in a longitudinal placebo-controlled study of a 6-week aspirin intervention.

In the first manuscript, we hypothesized that aspirin may modulate the oral microbiome through its anti-inflammatory effects. We found that that the over-time changes in the post-treatment relative abundance of a priori selected bacterial taxa differed between the aspirin and placebo groups, suggesting that aspirin was associated with the change over time in the relative abundance of specific taxa in the oral microbiome. In the aspirin group, there were greater increases in the relative abundances of *Neisseria, Streptococcus, Actinomyces, Rothia* and greater decreases in the relative abundance of *Prevotella, Veillonella, Fusobacterium*, *Porphyromonas,* and *Lachnospiraceae,* compared to the placebo group. These findings are in agreement with

our hypothesis that aspirin may modulate the abundance of specific bacteria, which may be of importance in the development of CRC. While the associations with specific bacterial groups were rather modest and may represent chance findings, the changes in microbial composition were reproducible using a novel balance tree approach. However, these findings lacked statistical significance after Bonferroni adjustment for multiple comparisons in this relatively small sample and require confirmation in larger studies.

The second manuscript hypothesized that inferred functional traits linked to LPS production, a key microbial metabolite with a pro-inflammatory capacity[84]. Although we were unable to find any evidence of an association between the change in the relative abundance of inferred functional traits for LPS and aspirin treatment after a 6-week intervention, we found some correlations between the abundance of a priori specified oral bacterial taxa and the relative abundance of inferred functional traits for the LPS bacterial metabolite, which suggests that these taxa may contribute to these traits. However, the results of these descriptive analyses should be interpreted with caution, as functional traits were predicted from taxonomic information, and may not accurately represent the actual metagenomic functions of the samples.

The third manuscript examined whether the relative abundance of pro-inflammatory oral taxa would be correlated to the relative abundance of pro-inflammatory and anti-inflammatory gut taxa at a cross-sectional level. We further hypothesized that community shifts in the oral microbiome in response to aspirin intervention would be correlated with community shifts in the gut microbiome in response to aspirin intervention. We found that changes in alpha diversity in the oral samples were associated with changes in the alpha diversity of the fecal samples in the

aspirin arm, compared to the placebo arm, suggesting that aspirin may induce changes in both oral and gut alpha diversity in a similar fashion. In addition to our findings for community diversity and composition, we found inverse correlations between the relative abundance of three pre-selected pro-inflammatory oral taxa and two pre-selected anti-inflammatory SCFA-producing gut taxa at baseline, which is in line with the roles of these taxa with regards to inflammation. However, the direction of correlations between the relative abundance of pro-inflammatory gut and oral taxa was unexpected for some other pro-inflammatory taxa in this study.

B. Main strengths and limitations

The three studies that we conducted have common strengths. First, all three studies were conducted within a longitudinal, placebo-controlled trial. This robust study design allowed us to examine the oral and gut microbiome in a randomized study of healthy individuals with repeated measures of microbial composition. Although we did not collect information about oral hygiene practices, dental disease history, or smoking, which are likely to be critical determinants of oral microbiome, the inclusion of a placebo arm and randomized allocations allowed us to limit the influence of unmeasured confounders. In addition, we used validated analytic methods in each study to evaluate our hypotheses, ranging from the use of linear mixed effect regression models to using the using arcsine square root transformation to standardize the relative abundances of pre-specified taxa of interest.

However, there were also some important limitations in our studies. One common limitation across all three studies was that due to its nature as a pilot study, and hence of

small size, and because we expected a modest effect for our treatment intervention, we focused on a small number of individual oral and gut taxa identified in previous studies, as well as a small subset of inferred functional traits linked to LPS. This limitation reduced the generalizability of our findings and highlights a need for the replication of these findings in a larger study setting. A second important limitation to consider is the impact of diet on the composition of both the oral and gut microbiome, which we were unable to assess in this study, due to limited dietary information collected. Finally, our findings are also likely influenced by the methods used to collect and measure oral microbes. Variability occurs even within oral cavity, partly due to spatial variations in the availability of oxygen[51]. The oral samples collected as tongue swabs in this study are likely to over-represent microbes present in the surface of the oral cavity as well as saliva, and less likely to include microbes from dental plaques[51], which may have different role in periodontitis and chronic inflammatory disorders of the intestine, including CRC[81].

C. Future Directions

Although our findings of aspirin-induced changes in pre-specified oral bacteria, on the correlation between pre-specified oral taxa and pro-inflammatory inferred functional traits as well as the inverse correlation between pro-inflammatory oral taxa and anti-inflammatory gut taxa are preliminary, our studies may inform the design of a future larger clinical trial.

Ideally, a large long-term randomized study is necessary to further investigate the effect of aspirin and other NSAIDs on the microbiome in chronic inflammatory disorders

of the intestine, including CRC, but such a trial is unlikely to be mounted due to issues of cost and feasibility. Therefore, it is essential to examine the association between aspirin or NSAID use and CRC risk in large cohort studies with detailed information about frequency, dosage and duration of NSAIDs use collected at several follow-ups. Although such studies are unlikely to have oral and fecal samples collected at several follow follow-ups, interest in microbiome research is growing, allowing investigators to study the long-term effect of NSAID use on the oral and gut microbiome in cohort studies. If the association between the oral or the gut microbiome and NSAID use is confirmed in cohort studies, it will be of benefit to conduct clinical trials of shorter duration, using change in appropriate biomarkers for inflammation or CRC risk such as PGE-M as surrogate endpoints.

Another important avenue of research that could benefit from our findings is research on the link between inflammation of the oral microbiome and inflammation of the gut microbiome. Along with their pro-inflammatory role in the oral cavity, oral taxa such as *Fusobacterium*, *Peptostreptococcus,* and *Porphyromonas* can also be found in the gut microbiome of IBD, CD, UCD and CRC cases[3,15–19,24,25]. However, it is unclear whether these oral taxa traveled down the GI track from the oral cavity after periodontitis, or if inflammation in the gut facilitated their translocation from the oral to the gut microbiome[10,26,27]. Our findings suggest that considering the microbiome at multiple sites along the gastrointestinal tract can improve our understanding of the role of the microbiome in chronic inflammation of the intestine, particularly CRC. Ideally, our findings should be tested in other large prospective cohort studies that have repeated measurements of inflammatory biomarkers such as PGE-M, C-reactive protein (CRP)

and inflammatory cytokines IL-1, IL-6 and TNF-α, which regulate the inflammatory response. Further studies on the association between oral and gut microbial diversity and composition, with an emphasis on inflammatory bacterial metabolites and pre-specified oral and gut taxa of interest, could improve our understanding of this complex relationship between the oral microbiome, the gut microbiome and host inflammation.

Findings from our studies do not have immediate clinical implications. They provide evidence that the oral microbiome may play a role in inflammation, which in turn could increase risk for CRC. This thesis is a step towards understanding the role of the oral microbiome in inflammation, which is important for identifying high-risk groups and creating strategies for cancer prevention in the future.

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Appendix: Dictionary of Abbreviations

5'-C-phosphate-G-3 (CpG)

Adenomatous popyposis colin (APC)

Body mass index (BMI)

C-reactive protein (CRP)

Colorectal cancer (CRC)

Crohn's disease (CD)

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase

Deoxyribonucleic acid (DNA)

Evaluation of SEPT9 Biomarker Performance for Colorectal Cancer Screening (PreSEPT)

Fairview Investigational Drug Services (IDS)

False Discovery Rate (FDR)

Fecal occult blood tests (FOBTs)

Gastrointestinal (GI)

High-performance liquid chromatography/mass spectrometry (HPLC/MS)

Human Microbiome Project (HMP)

Inflammatory bowel diseases (IBDs)

Interferon-γ (INF-γ)

Interleukin 1 (IL-1)

Interleukin 6 (IL-6)

International Agency for Research on Cancer (IARC)

Lipopolysaccharide (LPS)

Nitrosodiethylamine (NDEA)

Nonsteroidal anti-inflammatory drugs (NSAIDs)

Nuclear factor Kappa B (NF)-κB

Operational Taxonomic Unit (OTU)

Permutational multivariate analysis of variance (PERMANOVA)

Phospholipase A2(PLA2)

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)

Reactive nitrogen species (RNS)

Reactive oxygen species (ROS)

Short chain fatty acids (SCFAs)

Transforming growth factor-β (TGF-β)

Tumor necrosis factor-α (TNF-α)

Ulcerative colitis (UC)

University of Minnesota Genomics Center (UMGC)