INVESTIGATING THE INTERACTIONS BETWEEN THE GUT MICROBIOME AND INFLAMMATION DURING PREGNANCY IN A MULTIETHNIC COHORT

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DEDICATION PAGE

I dedicate this thesis to my many families, which include my parents, dear friends and supportive lab colleagues who all have been incremental in my career endeavors and success in my life. I can't begin to explain how the endless encouragement has allowed me to persevere in times of self-doubt and through hardships of a global pandemic, all while providing me continued inspiration to reach for my dreams.

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ABSTRACT

Preterm birth rates are decreasing across the United States; however, the state of Hawai‘i continues to experience higher risks of adverse pregnancy outcomes. Research is limited within this diverse population and therefore exemplifies a lack of understanding in pathologies related to Indigenous communities. This study aims to investigate dietary influences on the labile maternal microbiome which may contribute to ethnic health disparities. Dietary factors are a significant modulator of microbiota composition and can induce microbial shifts causing changes in metabolite production and cytokine regulation. This interaction subsequently alters systemic immune responses that contribute to many metabolic pathologies which may be implicated in adverse pregnancy outcomes.

Forty-one pregnant women were recruited from the four most prominent ethnic groups in Hawai‘i, i.e., Native Hawaiian, Filipino, Japanese, and non-Hispanic White. Rectal microbial swabs were collected during each trimester (12 weeks, 20 weeks, and 34-36 weeks), followed by DNA extraction for 16s RNA sequencing to assess changes in microbiome composition and butyrate production capacity. Blood specimen were collected at two time points (12 weeks and within 24 hours of labor) for inflammatory cytokine profiling using Luminex technology.

During the first trimester, inflammatory markers correlated with one another while microbiome characteristics, such as F:B ratio and alpha diversity, were weakly correlated with cytokines. Progression into the third trimester altered correlations of immune response, in that inflammatory markers were no longer strongly associated and there was a shift to slight positive correlations among inflammation states and microbiome characteristics. While there were no ethnic differences observed in cytokine profiles or microbiome characteristics and composition in the first trimester, progression to the third trimester reported differences in physiological

responses to pregnancy in an ethnicity dependent manner. Unlike the other ethnic groups, Japanese women did not experience a reduction in F:B ratios over time. Similarly, cytokines IL-6, IL-8 and chemokine MCP-1 and VEGF-A experienced ethnic-specific differences in the third trimester.

In the early stages of pregnancy, there was an observed relationship between the gut microbiome and immune responses indicating ethnicity did not impact baseline characteristics. However, the loss of cytokine relationships over the course of pregnancy is likely due to the observed ethnic differences in third trimester immune responses, suggesting ethnicity may be a variable in pregnancy trajectories. Therefore, characterization of ethnic differences in the physiological responses to pregnancy offers novel insight that may help explain the increased prevalence of adverse pregnancy outcomes in Hawai‘i.

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AF	amniotic fluid
BA	bile acid
CRP	c-reactive protein
DC	dendritic cell
F:B	Firmicutes to Bacteroidetes
FFAR2/3	free fatty acid receptors 2 and 3
GPCR	g-coupled protein receptor
HDAC	histone deacetylase
IFN-g	interferon gamma
IL-1b	interleukin 1b
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
I3A	indole-3-acetate
JABSOM	John A. Burns School of Medicine
NHPI	Native Hawaiian and Pacific Islander
NK	natural killer cell
PBMC	peripheral mononuclear blood cells
PE	preeclampsia
PAI-1	plasminogen activator inhibitor 1
PTB	preterm birth

SCFA	short chain fatty acid
LPS	lipopolysaccharides
MCP-1	monocyte chemoattractant protein-1
OTU	operational taxonomic unit
QIIME	quantitative insights into microbiology ecology
TNF-a	tumor necrosis factor alpha
T2D	type 2 diabetes
VEGF-A	vascular endothelial growth factor A

CHAPTER 1. INTRODUCTION

The importance of the symbiotic relationship between humans and the metagenome of their microbiome is inherent in the role of disease susceptibility.[1] Dysbiosis of these microbial communities have been implicated in multiple metabolic pathophysiologies and diseases, however distinguishing between a healthy and dysbiotic microbiome in diseased states is still in its infancy. There are many complexities when considering a healthy microbiome as microbial communities shift across age, ethnicity, pregnancy, geography and lifestyle factors.[2] Common features have been proposed to define a healthy microbiome such as prevalent organisms and metabolic pathways, diversity, stability, resistance and resilience of the microbial community.[3-6] These features represent the richness of the ecosystem, its responsiveness to perturbations and the ability to return to its pre-perturbed state.[7,8] Classical definitions of dysbiosis can be considered as three entities: bloom of pathobionts, loss of commensal species and loss of diversity.[9]

In the absence of disease, the microbiome displays a significant degree of interpersonal diversity, therefore elucidating the complex nature of characterizing a healthy microbiome composition.[10] Understanding the mechanisms that contribute to development and maintenance of dysbiotic states is crucial to ascertain therapeutic interventions to avoid disease susceptibility. The most prevailing factors influencing intestinal microbial composition include infection, inflammation, diet and pregnancy.[11] However, inter-individual variability of the microbiome is modulated by two key factors: genetic and environmental variability.[12] Moreover, dietary patterns are thought to be a focal contributor of environmental variation within an individual's lifestyle.

The primary inhabitant of the large intestine is anaerobic bacteria such as *Bacteroides* spp., *Bifidobacterium* spp., *Lactobacilli*, *Streptococci*, and eubacteria and these bacteria represent 30% of the entire bacterial population abundance.[13] This population increases short chain fatty acid (SCFA) production which decreases the pH levels within the large intestine; elucidating protective properties against overgrowth of pH-sensitive pathogenic bacteria.[14] The gut microbiome is comparative to a "metabolic organ" in which SCFAs can influence homeostasis and in turn play a vital role in the occurrence of metabolic dysfunction during gestation.[15] This perturbed state of homeostasis puts pregnant women at particular risk for a variety of metabolic disorders due to the exceptional physiological state of gestation.[16]

Additionally, over the course of pregnancy, alterations to the gut microbiome facilitate changes in the synthesis of SCFAs and contribute to adaptions in metabolic functions.[17] Fluctuating SCFA levels contribute to changes in carbohydrate and glucose metabolism normally regulated by butyrate and propionate, which stimulate intestinal glucogenesis.[13] In healthy individuals, a reduction of propionic acid is observed throughout pregnancy, while in obese women its production characteristically increases and is associated with many metabolic adaptions. Additionally, propionic and linear caproic acid levels play an important role in maintaining lower anthropometric parameters, such as weight, during pregnancy.[13]

Pregnancy is a highly dynamic process which influences inflammation states as well as microbiome composition as a result of hormone fluctuations.[18] Alterations in microbiota compositions induce differential immune responses that may cause perturbations to appropriate inflammatory states resulting in adverse pregnancy outcomes.[18] The scope of this thesis is to characterize the dynamics between the microbiome and inflammation to gauge the normal trajectory during pregnancy in a multi-ethnic cohort to identify potential variability between

ethnic groups for which differences in risk of certain pregnancy outcomes are known. Results of this study will offer future insight into potential diagnostic biomarkers to identify and reverse, respectively, adverse pregnancy outcomes in such health disparate populations.

This thesis investigates the influence of the gut microbiome on systemic inflammatory responses via the immunoepigenetic-microbiome axis during pregnancy. Currently, microbial constitutions that impact immune responses during gestation are not clearly understood. Therefore, further investigation to characterize and relate the progressive shifts of the microbiome and inflammatory states in healthy pregnancies is necessary to ascertain relevant biomarkers of adverse pregnancy outcomes for future clinical applications.

1.1 SYSTEMIC INFLAMMATION

Inflammation is a natural and healthy process that maintains a homeostatic balance within the body.[19] A healthy immune system identifies and removes microorganisms and other exogenous material that enters the body, while also regulating endogenous materials such as waste products or diseased cells.[20] Functions of the immune system are separated into two conventions: adaptive immunity and innate immunity.[21] Adaptive immunity is achieved through long-term epigenetic, metabolic, and transcriptional reprogramming of the immune system involving monocytes, macrophages, dendritic cells (DCs), and natural killer (NK) cells in response to prolonged stimuli.[22] The monocyte-macrophage complex is the most important composition of the innate immune system, yet much remains unknown regarding the epigenetic regulation that contributes to lineage differentiation resulting in chronic pro-inflammatory states.[23]

Low grade inflammation is characteristic of metabolic diseases giving rise to the term metabolic inflammation.[24] The prevalence of systemic inflammation is associated with increased

circulating levels of acute phase proteins, such as cytokines, chemokines, and C-reactive protein (CRP).[25,26] Metabolic inflammation is a sterile process that is fueled by adipose and liver tissue with causative factors pointing to nutrients and dietary lipid species. This phenomenon of lipotoxicity affecting organs involved in lipid metabolism is the driving force of metabolic dysfunction.[27]

Healthy pregnancy relies on maintaining a careful balance between immune tolerance and suppression of the maternal immune system.[28] Occurrence of imbalances between proinflammatory and anti-inflammatory cytokines and chemokines can result in aberrant inflammation leading to pregnancy complications directly associated with increased mortality and morbidity of mother and offspring. However, lack of appropriate responses could increase vulnerability to infection.[28,29] Metabolic syndromes are associated with adverse outcomes due to the presence of metabolic imbalances.[30] Both preterm birth and spontaneous abortion have confirmed pathological processes with causal link and defined molecular pathophysiology with infection and inflammation.[31,32] However, the scope of this thesis is not targeted towards infection, but rather focuses on the dynamics of natural inflammation states during pregnancy.

Cytokines are immune mediators that play a pivotal role not only in signaling within the immune system but also in ovulation, implantation, placentation and parturition. Parturition is an inflammatory process where inflammation can be detected in the cervix, myometrium, chorioamniotic membranes and amniotic cavity of women in labor. The infiltration of inflammatory cells in the mentioned tissues along with increased production of pro-inflammatory cytokines and chemokines; such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-a (TNF-a) and interleukin-8 (IL-8), are associated with healthy spontaneous labor at term.[33,34] Recent studies demonstrated upregulation of genes involved in the regulation of

inflammation within the chorioamniotic membranes not experiencing histological chorioamnionitis. This suggests that the lack of inflammatory signatures in maternal circulation indicates a localized inflammatory process in these membranes under normal pregnancy conditions.[35,34] Therefore, healthy pregnancy is proposed to involve physiological activation of the innate immune response associated with phenotypic and metabolic changes in maternal monocytes and granulocytes consistent with intravascular maternal inflammation.[36]

Current knowledge of inflammation during pregnancy includes inflammatory profiles for C-reactive protein (CRP), IL-1, TNF-a, and interleukin 10 (IL-10). During the course of pregnancy CRP levels are elevated and act as opsonin's to neutralize and clear pathogens.[37] Additionally, participation of IL-1 is involved in pregnancy physiology to stimulate myometrial contractions and is produced by the human decidua in response to bacterial products. Increased concentrations and bioactivity of IL-1 has been observed in the amniotic fluid (AF) of women experiencing infection and subsequently preterm labor.[33] In addition, TNF-a has a supporting role in mechanisms of preterm partition through stimulation of prostaglandin production in the amnion, myometrium and decidua. This cytokine induces labor in pregnant animals upon systemic administration, indicating it is a key player in cervical ripening.[39] However, elevated TNF-a occurs in response to bacterial products and increased bioactivity and immunoreactive concentrations have been observed in the AF of women with PTL and intraamniotic infection.[38]

Anti-inflammatory cytokine IL-10 is also understood to be an important signaler for healthy maintenance of pregnancy. During the third trimester, but not in a state of labor, IL-10 is significantly reduced in the placenta compared to first and second trimester. Downregulated IL-10 seems to be a physiological event favoring inflammation before labor is induced and has been implicated in preterm birth (PTB) associated with inflammation.[40,41] As microbiota are able to

facilitate the modulation of inflammation, dietary intervention has the potential to aid in regulation of these processes during pregnancy. However, current research has not yet established its therapeutic potential for maintenance of innate inflammatory responses during gestation.

1.2 DIETARY IMPACT ON MICROBIOME COMPOSITION

Current knowledge indicates a healthy gut microbiome composition will be dominated by four bacterial phyla including Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria, but most endemic bacteria in the gut will be accounted for by Bacteroidetes and Firmicutes. Firmicutes residing in the gut can be classified as two major classes of Gram-positive bacteria, which include Bacilli and Clostridia. However, Gram-negative bacteria such as Bacteroidetes, including *Bacteroides*, represent the most abundant genera in the gut.[42] These represent the common core subsets within the microbiome that are relatively stable and persistent throughout a lifetime.[5]

Variability in dietary habits may contribute to microbiome differences between ethnicities and mediate health disparities. A retrospective study on American data sets demonstrated ethnicity consistently captured gut microbiota as a moderate predicator variable for microbiome differences with stronger effect size than variables such as BMI, age, and sex.[43] It was reported most of the abundance of 12 heritable taxa associated with human genetic variation recurrently varied between ethnicities and may be a product of socioeconomic, dietary healthcare, genetics, or other ethnic-related factors. Many ethnicity-associated taxa were primary gut anaerobic fermenters and methanogens that associate with lower BMI and blood triglyceride levels. Generally, it was found Asian-Pacific Islanders had constrained microbiomes resulting in

reduced butyrate metabolism compared to Caucasian and Hispanic counterparts. About 70% of Asian Americans were born overseas and assimilation into Western lifestyles may cause substantial changes to microbiome composition that could indicate incompatibilities between traditionally harbored microbiota and Western diets.[43]

Carbohydrate consumption may contribute to the large variation observed in gut microbiota composition with respect to consuming Westernized or traditional diets.[44] Imbalanced consumption of carbohydrates is associated with gut dysbiosis and excess intake of processed starchy foods, mono- and disaccharide sweeteners, and artificial sweeteners, is associated with increased risk of metabolic disorders in individuals with higher glycemic index and load.[45,46] Westernized diets are also classified as being high fat which fosters the acquisition of pro-inflammatory gut microbiota.[47] This consequently increases intestinal permeability leading to higher levels of circulating levels of lipopolysaccharides (LPS), a Gram-negative outer membrane, further inducing cytokine and chemokine production regulating inflammatory and innate adaptive immune responses.[48]

In contrast, long term Agarian diets, also known as traditional diets, contain high amounts of starch, fiber, and non-digestible carbohydrates associated with increased microbial richness, diversity and increased production of SCFAs; ascertaining a protective effect of this diet.[49,45] Non-digestible fibers undergo carbohydrate fermentation in the colon into SCFAs, such as acetic, propionic and butyric acid with the facilitation of commensal gut microbes. These common SCFAs account for more than 95% of the total SCFA pool and play a regulatory role in carbohydrate metabolism. The importance of SCFAs is their ability to mediate transmission of signals between the immune system and microbiome while maintaining homeostasis of

inflammatory responses.[13] Hence, a diet lacking in fiber promotes growth of specific bacterial strains that constrain the host's ability to ferment and metabolize nutrients with direct effects on intestinal pH leading to the colonization of pathogenic flora resulting in dysbiosis.

1.3 MICROBIOTA MODULATION OF THE IMMUNE SYSTEM

The gut microbiome modulates global immune responses via various mechanisms. Colonized microbiota can regulate development and activity of immune responses for both epithelial and systemic pathways through metabolite production facilitated by gut microbiota that are absorbed via breaks in the gut epithelial barrier and enter circulation.[50] Perturbations of gut microbiota compositions disrupt the gut mucosa and immune systemic responses via leaky gut syndrome that increases gut permeability, causes microbial imbalances and impairs mucosal immunity.[51] Intestinal permeability resulting from disease or dysbiosis allows for bacterial translocation that largely contributes to chronic and systemic inflammation, and if not repaired can promote continuous inflammatory stimulus leading to autoimmune triggering.[52]

Established dysbiotic landscapes can lead to substantial alterations in both the local mucosal and systemic interactions of immune cells.[53] Aberrant microbial states are maintained via exploitation of the feedback loop between the host immune system and microbiota cross-regulation by pathobionts that flourish under inflammatory conditions. This maintains perpetuation of inflammation states by preserving favorable conditions for pathogenic establishment. In some cases, the perturbed microbiota can be dominantly transferred through immune system hijacking which alters new host microbial colonization niches.[53] This bidirectional relationship between the gut microbiome and host immunity reflects gut immune homeostasis between pro- and anti-inflammatory mechanisms directed by commensal microbiota.[51] An example is colonization of segmented filamentous bacteria in mice that promotes the accumulation of pro-inflammatory T helper 1 (Th1) and T helper 17 (Th17) cells

within the small intestine cells, while anti-inflammatory responses are facilitated by regulatory T cells (Treg cells) via SCFAs.[54,55] Another study indicates that bacteria of the *Clostridium* cluster IV and XIVa, *Bacteroides fragilis* and *Faecalibacterium prausnitzii* promote accumulation of T-reg cells.[51]

Another mechanism in which microbiota regulate both innate and adaptive immunity is by direct contact to immune cells via induction of epigenetic modifications and production of signaling molecules. Clinical evidence has proved that SCFAs directly affect colon function and modulate the intestinal immune system and metabolic processes that ultimately impact systemic immune responses. Microbial-derived SCFAs and bio-transformed bile acid (BA) can influence the immune system by acting on ligand specific cell signaling receptors such as G-coupled protein receptors (GPCRs), TGR5 and FXR, or epigenetic processes.[50] Free fatty acid receptors 2 and 3 (FFAR2/3), which are GPCRs, have widespread expression on many cell receptors including those in the intestines, bone marrow, human immune cells such as peripheral blood mononuclear cells (PBMCs), neutrophils, DCs and the fetal/maternal membrane and placenta.[56] Immune system activation can also occur as a result of epigenetic remodeling and altered gene expression. SCFA-driven histone deacetylase (HDAC) inhibition tends to promote a tolerogenic, anti-inflammatory cell phenotype that is crucial for maintaining immune homeostasis and supports the concept that the microbiota can function as an epigenetic regulator of host physiology.[57]

Diet studies in mouse models provides insight into the relationship between specific microbial metabolites and inflammation. Krishna *et al.* compared metabolite profiles of germ-free mice including control groups fed a low or high fat diet which yielded results supporting the notion of microbiota dependent metabolites. Mice consuming a high fat diet showed depleted metabolite production of tryptamine and indole-3-acetate (I3A), which act as anti-inflammatory

mediators. Both metabolites decreased fatty-acid- and LPS-stimulated production of pro-inflammatory cytokines in macrophages with inhibitory effects on cell migration to a chemokine state. Hepatocytes, which exhibit a large role in metabolic functions, were also influenced by the I3A metabolite. In liver cells, I3A reduced expression of fatty acid synthase and sterol regulatory element-binding protein-1c and attenuated inflammatory responses under lipid loading.[58]

The gut microbiota are shaped by the hormonal milieu governing gender-specific differences in immunity. Bi-directional cross-talk between the endocrine system and microbiota are known to induce bacteria production of hormones, e.g., serotonin, dopamine and somatostatin, while responding to host hormones, e.g., estrogen, and regulating host hormone homeostasis through inhibition of gene prolactin transcription or converting glucocorticoids to androgens.[50] As gut microbiota regulate approximately 10% of host transcriptomes, the intestinal microbiome plays a particularly import role during the course of pregnancy.[59]

1.4 MICROBIOME INTERVENTIONS AND HEALTH OUTCOMES

With the increasing awareness of microbiome associated diseases, there has been renewed attention directed towards therapeutic, diet-based interventions to reverse the onset of pathogenesis. There have been profound advances in microbiome modulation as therapeutic treatment including: antibiotics, pre- and pro-probiotics, dietary interventions, pharmabiotics and fecal microbiota transplantation.[60] Type 2 diabetes (T2D), a metabolic disorder, has reported positive effects from dietary intervention when patients were supplemented with either low fat or low carb diets leading to lower Hb1Ac levels and reduced body weight.[61,62] Furthermore, when intake of carbohydrates were greatly reduced due to a ketonic state, there was a profound response in treatment effectiveness of T2D. Comparison of a traditional low fat diet and a ketogenic diet in overweight and obese patients favored a ketogenic diet due to superior

enhancement of metabolic control in patients resulting in a reduction in antidiabetic therapy.[61] However, this is not an option for all patients, particularly women who are pregnant or lactating due to increased risk of diabetic ketoacidosis, as the dynamic course of pregnancy influences metabolic functions.[63]

Preeclampsia (PE) is a malignant metabolic disease closely associated with other disorders such as obesity, T2D, insulin resistance, atherogenic dyslipidemia, and hyperglycemia commonly observed with gut dysbiosis; however the pathogenesis outside of infection remain unclear.[64] PE is characterized by a new onset of hypertension during the second half of pregnancy with no curative treatment ultimately necessitating delivery of the placenta and fetus to induce preterm birth.[65] Several studies implicate gut dysbiosis as a causative agent of preeclampsia because gut microbiota can modulate the facilitation of insulin resistance and induce chronic inflammation and fat accumulation through gene regulation of energy metabolism.[66] The onset of PE is believed to be a manifestation of placental dysfunction as a result of angiogenic imbalances and inflammatory disturbances.[65]

Microorganisms belonging to the genera *Streptococcus*, *Bifidobacterium*, *Escherichia* and *Lactobacillus* have the capacity to directly affect neurotransmitter synthesis in the nervous system.[67] Imbalances in community abundance may contribute to increased vessel tension and narrowing, increased peripheral resistance, and subsequent arterial hypertension development. Butyrate and its enzyme butyrate kinase have preventative actions against development of arterial hypertension during pregnancy through the intensifying action of plasminogen activator inhibitor 1 (PAI-1), the inflammation marker in overweight and obese women during pregnancy.[67] This is extrapolated due to the number of bacteria responsible for butyrate production and the number of copies of enzyme butyrate kinase being inversely correlated with

systolic and diastolic blood pressure and PAI-1.[59] However, comprehensive maternal microbiome compositions have not yet been associated with the development of PE and other adverse outcomes, which may provide early indications of outcome manifestations and subsequent therapeutic approaches.

Currently it remains uncertain if dietary quality and gut microbiome dysregulation is an influential component in pregnancy-associated outcomes due to SCFA regulation of inflammatory processes. Aberrant immune system responses leading to development of PE is significantly correlated with acetate level reductions in blood serum.[68] This correlation was found to be stronger in obese women, who were more likely to develop PE. A germ-free mice study concluded as much as a 30% increase in acetate contributed to reducing risk of PE most likely due to acetate's influence on blood pressure regulation which can lead to PE clinical features if not maintained. This evidence suggests that SCFAs may partially contribute to pregnancy prognosis and participate in alterations of carbohydrate metabolism. Therefore, microbial compositions which promote synthesis of SCFAs are inherently influential in aiding the maintenance of gestation.[69] Due to the association with the gut microbiome and recent promise in dietary interventions, it is believed that modulating maternal diet could offset or alleviate manifestation of PE.

1.5 HYPOTHESIS AND AIMS

This study aims to characterize the maternal microbiome composition and its relationship with immune modulation during gestation in a multi-ethnic cohort. We hypothesize that microbiome characteristics will associate with immune responses in an ethnic-specific manner

which many contribute to the increased risk of adverse pregnancy outcomes in the diverse population of Hawai‘i. This hypothesis will be tested through two main objectives:

AIM 1:	Assess composition of the maternal microbiome and immune profile in a multi-ethnic cohort over the course of pregnancy.
AIM 2:	Evaluate the relationship between microbial changes and inflammatory markers over the course of pregnancy.

CHAPTER 2. METHODS AND PROCEDURES

2.1 STUDY SUBJECTS AND RECRUITMENT

The Western IRB approved this study in compliance with Hawai‘i Pacific Health IRB protocol. This was a longitudinal cohort pilot study which recruited ten women from the four most prevalent ethnic groups on the Hawaiian islands - Japanese, Filipino, Native Hawaiian and non-Hispanic White.[17] Eligible participants were identified by medical chart review at the Fetal Diagnostic Center at Kapiolani Medical Center for Women and Children while waiting to receive a preliminary ultrasound. Participants were deemed eligible based on inclusion and exclusion criteria stated as the following; Inclusion criteria: women aged 18-45 years, primarily English speaking and literacy, self-identified as Asian, non-Hispanic White, Native Hawaiian and in their first trimester of pregnancy (<14 weeks 0 days gestation) while excluding participants who planned to move out of the area prior to delivery, planning to deliver at another hospital other than Kapiolani Medical Center, multiple gestation, pre-existing diabetes or hypertension, history of bariatric surgery, history of an eating disorder, inflammatory bowel disease or those that are incarcerated. Due to this population having multiethnic backgrounds, ethnicity qualifications required participants to identify one ethnicity as greater than 50% of their ethnicity to participate in the study, while Native Hawaiians of any percent ethnicity were accepted to participate.

2.2 STUDY PROTOCOL

2.2a SAMPLE COLLECTION AND MICROBIOME ANALYSIS

Rectal swabs were collected during first, second, and third trimester visits. A gift card incentive was distributed in portions of $75 at each time point for a total of $225 once all specimens were collected. Specimens were obtained using Copan Diagnostic ESwabs which

maintain bacterial viability for 48 hours. The Institute for Biogenesis Research at the University of Hawai‘i Mānoa, Maunakea lab performed DNA isolation using the Qiagen AllPrep DNA/RNA Extraction Kit. Samples were transferred to the Epigenomics Core at John A. Burns School of Medicine (JABSOM) for further processing and sequencing. The Ion 16s Metagenomics Kit was used to amplify 7 regions in the hypervariable 16s rRNA gene in bacteria with primers V2-4-8, V3-6, V7-9. Amplified fragments underwent multiplex sequencing on the Thermofisher Ion S5 platform and were processed with the compatible Ion Reporter module. These primers offer broad yet high-throughput sequencing capable of bacterial identification at the genus and/or species level with the software output utilizing Greengenes and MicroSEQ ID 16s rRNA reference databases. This allows for classification and relative proportion analysis of present microbes. Bioinformatic assessment performed by the Epigenomics Core grouped microbes in Operational Taxonomic Units (OTUs) and assigned alpha/beta diversity indices using Quantitative Insights into Microbiology Ecology (QIIME) workflow. DNA sequencing results were available for 35 participants from the first trimester, 36 from second trimester, and 30 from the third trimester after filtering DNA quality for samples with greater than 10,000 reads.

2.2b BLOOD PLASMA COLLECTION AND INFLAMMATION MARKER ASSAY

Blood samples were collected from participants during the first trimester ultrasound visit and within 24 hours of delivery at Kapiolani Hospital. The blood was processed within 24 hours by the Epigenomic Core at JABSOM using the Sepmate PBMC Extraction Protocol (StemCell

Technologies). Blood samples were processed into plasma and PBMCs and cryopreserved in cryotubes. Cryopreserved plasma was assessed *ex vivo* for inflammatory activity utilizing Luminex multiplex assays on biomarkers of inflammation: Interferon gamma (IFN-g), IL-1b, IL-6, IL-8, IL-10, monocyte chemoattractant protein 1 (MCP-1), TNF-a, CRP, and vascular endothelial growth factor A (VEGF-A).

2.3 DATA ANALYSIS

Recruitment of 41 participants for this longitudinal cohort was determined by its limited funding and resources as lack of sufficient literature in this field interfered with performing adequate power calculations at the initiation of study. Patient demographics were summarized by median [min, max] for continuous variables and frequencies and percentages for categorical variables. Unpaired two-tailed Mann-Whitney tests were conducted to assess any baseline differences between ethnic groups for demographic data. A Spearman r correlation matrix was created to assess interactions between the microbiome and cytokine profiles for the first and third trimesters.

Microbiome analysis was performed on 131 samples and excluded samples with low yield less than 1000 reads. Alpha diversity scores were characterized with Shannon Index scores that were computed after refraction using the average value of the 10 rarefied values at sequence number 15927. The Firmicutes to Bacteroidetes (F:B) ratio was calculated by dividing total abundance of Firmicutes over total abundance of Bacteroidetes. Microbiome characteristics were summarized by median [min, max] values. Both Shannon index scores and F:B ratios were tested for statistically significant differences between the first and third trimesters using unpaired two-sided Mann-Whitney tests. Additional unpaired two-tailed Mann-Whitney tests were performed to assess differences in microbiome characteristics between ethnicities and weight status groups.

Comparison of presence of the butyrate kinase gene from first to third trimester was also tested using unpaired two-tailed Mann-Whitney tests. The relative abundance of SCFA facilitating microbiota was characterized across trimesters and stratified by ethnic group. Differences in relative abundance within the cohort and by ethnic group was also tested using unpaired two-sided Mann-Whitney tests.

Clinical patient characteristics of cytokine profiles were summarized by median [min, max]. Differences in the change of cytokine levels from first to third trimester were grouped by ethnicity and tested for statistical differences with unpaired two-tailed Mann-Whitney tests. Data analysis was performed on the PRISM platform using two-tailed p-values (p) of less than 0.05 to indicate statistical significance of results.

CHAPTER 3. RESULTS

3.1 PARTICIPANT RECRUITMENT AND FOLLOW UP

From August to November 2019, 504 women were screened for study eligibility. 177 women were deemed eligible, however 67 declined to participate and 69 were missed resulting in 41 participants being enrolled. The remaining 327 women did not meet study eligibility criteria due to: age (6), current medical condition (103), ethnicity (168), non-English speaking (11), not planning on delivering at Kapiolani (20), did not show up to appointment to be approached (13), missed abortion (4), or multiple gestation (2).

3.2 DEMOGRAPHICS

Patient demographics are displayed in Table 1. Initially, there were 10 participants recruited per ethnic status: non-Hispanic White (10), Japanese (10), Filipino (10) and Native Hawaiians (11). The average demographic data for the cohort of 41 women was as follows: age was 29, 56% of participants were nulliparous, BMI was 27.2 kg/m^2 and 17% were obese. However, for the purpose of identifying relationships between the gut microbiome and markers of systemic inflammation, only participants with complete data were utilized in further analysis and represented in the demographics Table 1. In addition, sample processing needed to be optimized and thus five first trimester samples were lost before optimal processing was determined. Two-sided unpaired Mann-Whitney tests of demographic data reported significant baseline differences within the cohort. In the first trimester, Japanese women (p=0.02) and Native Hawaiians (p=0.01) had significantly elevated BMI compared to Filipino women. In fact, within the cohort, Native Hawaiians and Japanese women were the primary constitution of obese women.

Table 1. First trimester participant baseline demographics stratified by ethnicity represented as Median [min, max] in continuous or Freq (%) in categorical. Only participants with recorded microbiome and cytokine clinical data from the first trimester were included.

Characteristic	Non-Hispanic White (n = 8)	Native Hawaiian (n = 10)	Japanese (n = 6)	Filipino (n = 9)
Age	32.50 [20.00, 37.00]	30.00 [23.00, 40.00]	35.00 [27.00, 38.00]	26.00 [22.00, 36.00]
Parity:				
Nulliparous	3 (37.5%)	7 (70%)	4 (66.66%)	5 (55.56%)
Primaparous	4 (50%)	2 (20%)	1 (16.67%)	2 (22.22%)
Multiparous	1 (12.5%)	1 (10%)	1 (16.67%)	2 (22.22%)
BMI	24.30 [21.10, 29.40]	32.50 [17.50, 44.60]	29.15 [23.40, 36.50]	23.10 [20.50, 29.00]
Obesity:				
Normal	4 (50%)	3 (30%)	2 (33.33%)	8 (88.89%)
Overweight	4 (50%)	1 (10%)	1 (16.67%)	1 (11.11%)
Obese	0 (0%)	6 (60%)	3 (50%)	0 (0%)

Table 2. Third trimester participant demographics stratified by ethnicity and represented as Median [min, max] in continuous or Freq (%) in categorical. Only participants with recorded microbiome and cytokine clinical data from the third trimester were included.

Characteristic	Non-Hispanic White (n = 9)	Native Hawaiian (n = 8)	Japanese (n = 7)	Filipino (n = 6)
Age	29.00 [20.00, 37.00]	28.50 [23.00, 40.00]	29.00 [24.00, 38.00]	24.50 [21.00, 31.00]
Parity:				
Nulliparous	5 (55.56%)	5 (62.5%)	4 (57.14%)	3 (50%)
Primaparous	3 (33.33%)	2 (25%)	2 (28.57%)	3 (50%)
Multiparous	1 (11.11%)	1 (12.5%)	1 (14.29%)	0 (0%)
BMI	23.50 [21.10, 29.40]	29.25 [17.50, 42.60]	29.80 [23.40, 36.50]	24.00 [20.50, 32.80]
Obesity:				
Normal	5 (55.56%)	3 (37.5%)	2 (28.57%)	4 (66.66%)
Overweight	4 (44.44%)	1 (12.5%)	2 (28.57%)	1 (16.67%)
Obese	0 (0%)	4 (50%)	3 (42.86%)	1 (16.67%)

3.3 RELATIONSHIP BETWEEN MICROBIAL COMPOSITION AND CYTOKINE PROFILES

To understand the relationship between inflammation and the gut microbiome during the course of pregnancy, we evaluated a panel of cytokines (IL-1b, IL-6, IL-8, IL-10, IFN-g, TNF-a, CRP), a chemokine (MCP-1), and a vascular biomarker (VEGF-1) in the plasma of participants. Many of these cytokines are known biomarkers of systemic inflammation.[70] In addition, using 16S-based sequencing approaches with stool samples of the same participants, we quantified the relative levels of F:B ratio and microbial alpha diversity (using the Shannon Index). Both of which are indicators of homeostatic maintenance of the gut microbiome where a high/low F:B ratio and low diversity score tends to indicate microbiome dysbiosis.[71,72]

Despite an ethnically diverse cohort in this study, we observed significant correlations of biomarker data in the early term of pregnancy overall. Figure 1 shows a correlation matrix of Spearman r values of each biomarker analyzed in relationship to each other and features of the microbiome. In the first trimester we observed a number of significant positive correlations between inflammatory cytokines including IL-1b, IL-6, IL-8, IL-10, IFN-g TNF-a, chemokine MCP-1 and vascular biomarker VEGF-A indicating an elevated and general inflammatory state, which was for the most part negatively correlated with F:B ratio

These results are suggestive of microbial "dysbiosis" in pregnancy that associates with inflammation, consistent with expected metabolic changes that are typically associated with high BMI/obesity. However, unlike the stable relationship observed in obesity, the degree of these associations was markedly reduced in the third trimester of pregnancy to the extent that the strong associations observed in the first trimester disappeared in the third (Figure 2). Weak associations between alpha diversity and F:B ratio became more prominent in the third trimester, particularly switching from mostly negative cytokines correlations to now positive correlations. This change in the strength of these relationships indicate the dynamics in both the levels of cytokine production and microbiome composition over the course of pregnancy and offered a unique opportunity to understand the plasticity of the microbiome in relation to inflammation. In addition, to understand how generalizable these changes were, we stratified the data by ethnic group and weight status.

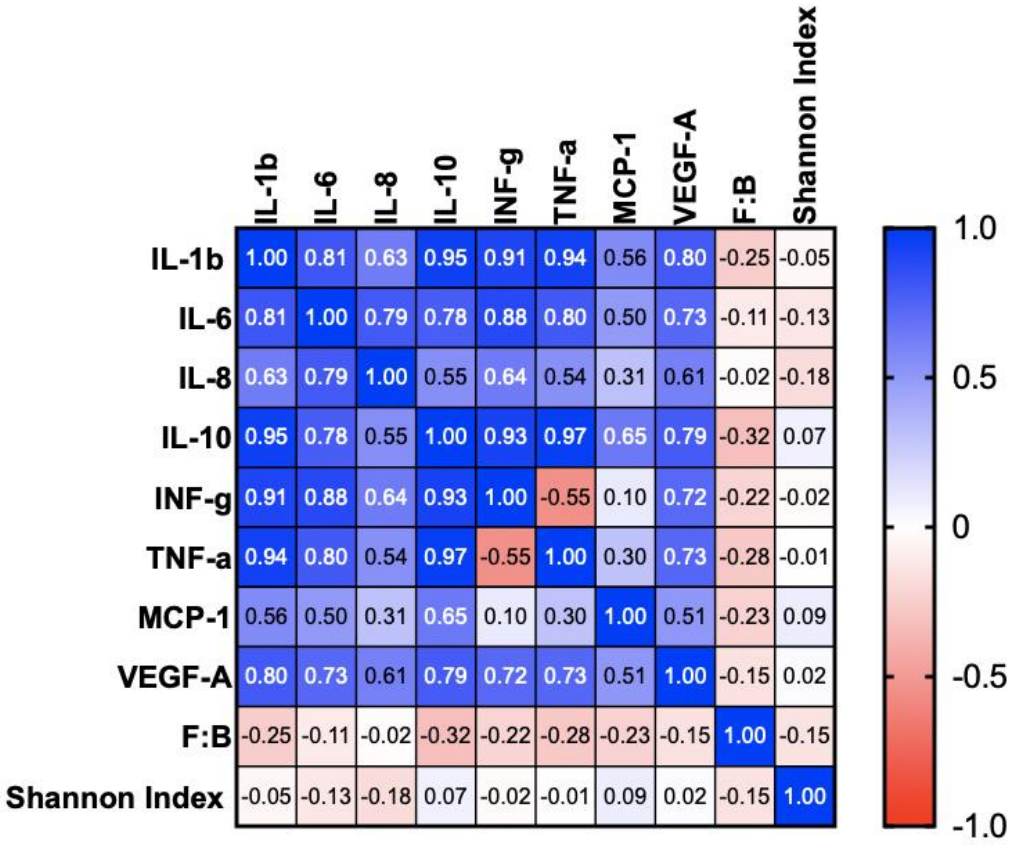


Figure 1. Spearman r correlation matrix of first trimester variables represented as a heat map.

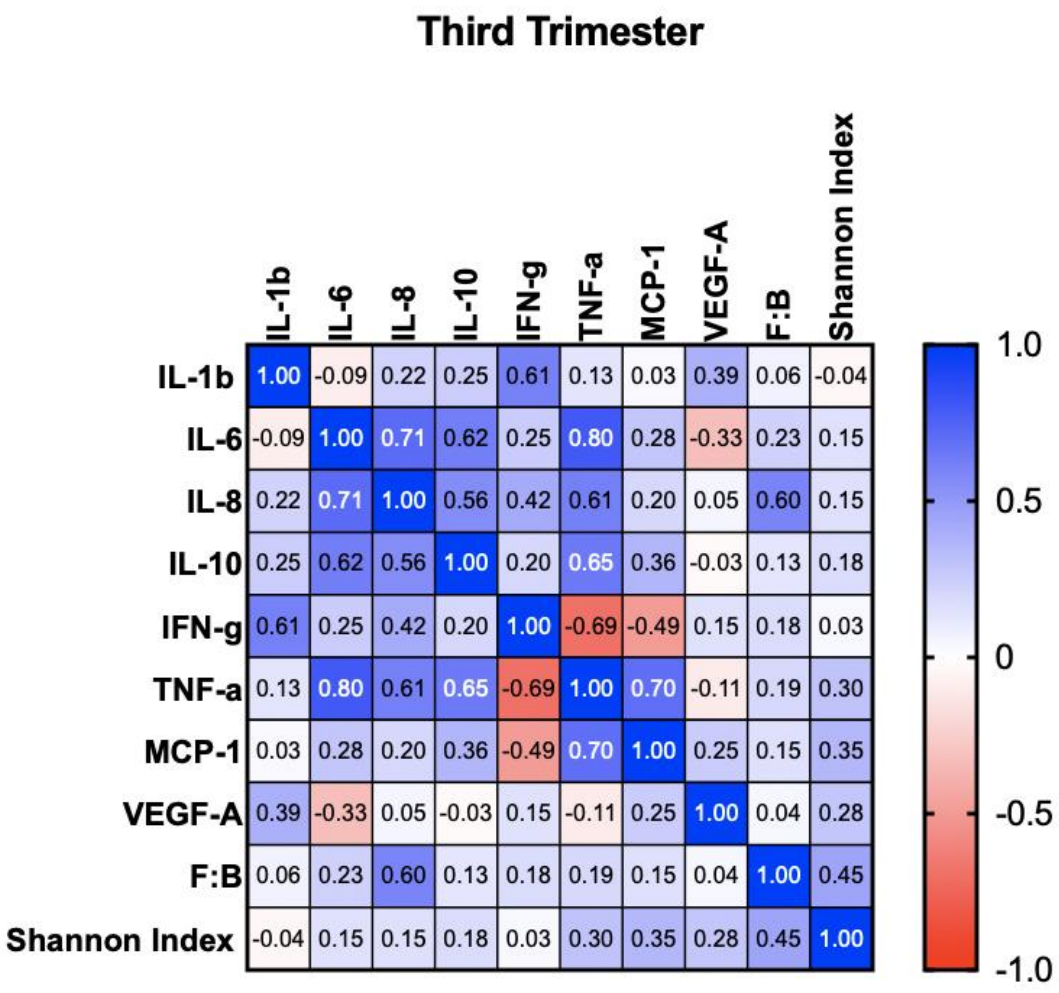


Figure 2. Spearman r correlation matrix of first trimester variables represented as a heat map.

3.4 CHARACTERIZING THE GUT MICROBIOME

Table 3 displays the change over time in alpha diversity and F:B ratio from first to third trimester in our cohort. Figure 3 indicates there were significant reductions in both alpha diversity ($p<0.0001$) and F:B ratio ($p<0.0001$) from first to third trimester. Data was regrouped by ethnicity to investigate the presence of ethnic differences in microbiome characteristic changes during pregnancy from first trimester (Table 4) to third trimester (Table 5). Ethnic grouping of data displayed in Figure 4 revealed non-significant decreases in F:B ratio for Native Hawaiians and Filipinos, yet significant reduction for non-Hispanic Whites ($p<0.005$). However,

the median F:B ratio remained constant in Japanese women who also experienced greater variation indicating increased prevalence of high F:B ratios. Additionally, Shannon Index scores were significantly decreased across trimesters for non-Hispanic Whites ($p<0.05$), Native Hawaiians ($p<0.01$) and Filipinos ($p<0.01$) while Japanese did observe a reduction, although non-significant. To address whether observed shifts in microbiome characteristics were a result of baseline differences in BMI within ethnic groups, microbiome characteristics were regrouped by weight status for first trimester (Table 6) and third trimester (Table 7). Figure 5 shows the changes in F:B ratio and alpha diversity scores across trimesters by weight status. The F:B ratios of the first and third trimesters reported reductions for all weight groups but were significant only in the normal weight ($p<0.01$) and overweight group ($p<0.005$). Similarly, alpha diversity scores were decreased from the first to third trimester for all groups, but only significant for normal weight ($p<0.001$) and obese groups ($p<0.005$).

Table 3. First and third trimester gut microbiome characteristics represented as Median [min, max].

Characteristic	First Trimester	Third Trimester
Shannon Index	4.79 [2.71, 5.53] (n = 33)	3.88 [1.32, 5.03] (n = 30)
F:B ratio	0.54 [0.23, 4.06] (n = 30)	0.13 [0.01, 1.38] (n = 27)

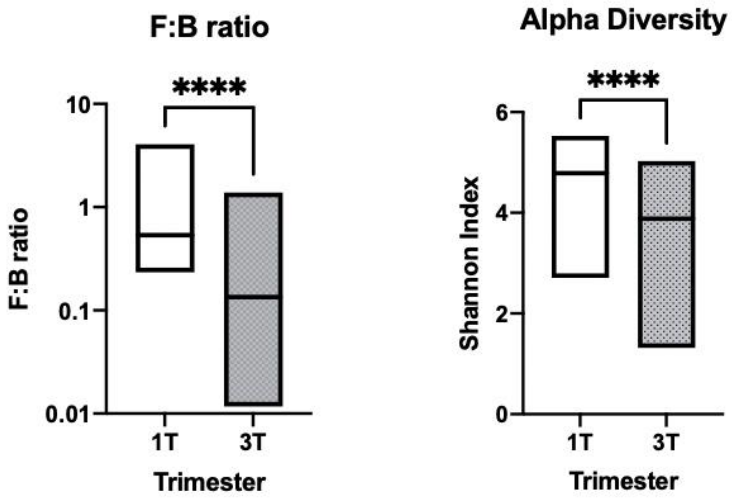


Figure 3. Box plot of log transformed F:B ratio shows differences of gut microbiome characteristics from first to third trimester.

Table 4. Ethnic grouping of first trimester gut microbiome characteristics represented as Median [min, max].

Characteristic	Non-Hispanic White (n = 8)	Native Hawaiian (n = 10)	Japanese (n = 6)	Filipino (n = 9)
Shannon Index	4.74 [3.10, 5.31]	4.89 [4.10, 5.53]	4.79 [2.71, 5.43]	4.75 [3.37, 5.38]
F:B ratio	0.48 [0.33, 12.64]	0.57 [0.47, 2.91]	0.76 [0.34, 28.06]	0.71 [0.23, 193.30]

Table 5. Ethnic grouping of third trimester gut microbiome characteristics represented as Median [min, max].

Characteristic	Non-Hispanic White (n = 9)	Native Hawaiian (n = 8)	Japanese (n = 7)	Filipino
Shannon Index	3.89 [2.97, 5.02]	3.93 [2.66, 4.87]	3.52 [1.82, 5.03]	3.58 [1.32, 4.72] (n = 6)
F:B ratio	0.13 [0.07, 0.61]	0.26 [0.08, 11.14]	0.53 [0.01, 61.05]	0.28 [0.09, 1.19] (n = 5)

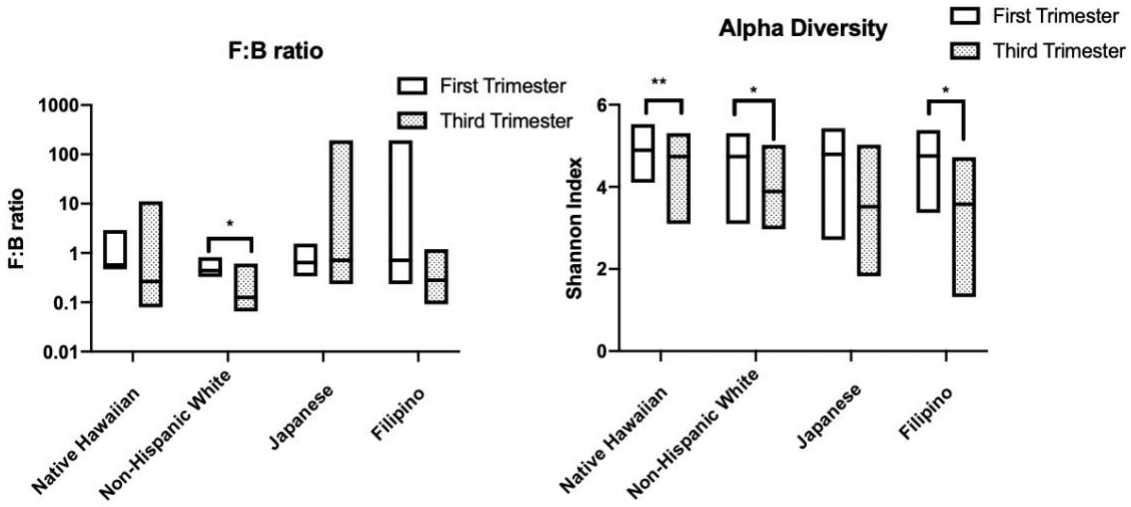


Figure 4. Box plot of log transformed F:B ratio values shows differences of gut microbiome characteristics by ethnicity from first to third trimester.

Table 6. First trimester gut microbiome characteristics grouped by obesity status represented as Median [min, max].

Characteristic	Normal (n = 17)	Overweight (n = 7)	Obese (n = 9)
Shannon Index	4.94 [3.37, 5.38]	4.40 [2.71, 5.53]	4.78 [4.54, 5.43]
F:B ratio	0.58 [0.35, 193.3]	0.53 [0.23, 28.06]	0.88 [0.34, 2.91]

Table 7. Third trimester gut microbiome characteristics grouped by obesity status represented as Median [min, max].

Characteristic	Normal (n = 14)	Overweight (n = 8)	Obese
Shannon Index	3.88 [2.64, 5.02]	3.81 [1.82, 4.75]	3.67 [1.32, 5.03] (n = 8)
F:B ratio	0.24 [0.07, 61.06]	0.11 [0.01, 0.23]	0.53 [0.11, 1.38] (n = 7)

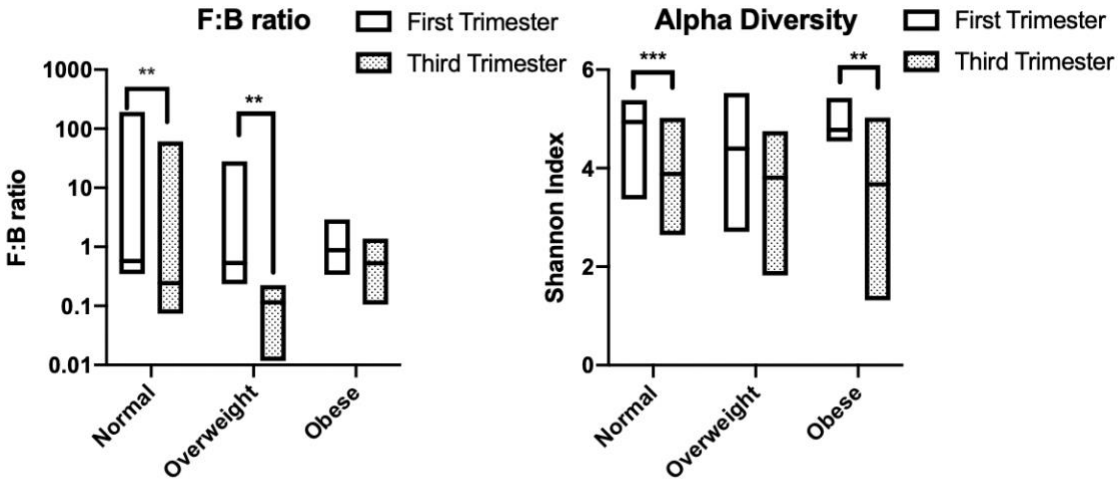


Figure 5. Box plot of log transformed F:B ratio values shows differences of gut microbiome characteristics by weight status from first to third trimester.

3.4a PRESENCE OF BUTYRATE KINASE GENE IN MICROBIAL DNA

Generally, the state of the microbiome is implicated in SCFA production capacity and increased alpha diversity and overall microbial homeostasis typically results in increased SCFA production.[73] Within our multi-ethnic cohort, there is a consensus between ethnic groups that alpha diversity is decreased during pregnancy; therefore, further investigation of butyrate production was evaluated in this cohort. Checking the presence of the Butyrate kinase (*Buk*) gene in bacterial DNA samples offers insight into the metabolic capabilities of the microbiome to convert dietary substrates into butyrate. The median value [min, max] of first and third trimester butyrate kinase presence was 0.01 [0.00, 0.29] and increased to 0.13 [0.00, 7.23] in the third trimester. Unpaired two-sided Mann-Whitney test reported the third trimester microbiome was significantly enriched for the butyrate kinase gene ($p<0.0001$) indicating butyrate production is increased as gestation progresses despite observed decreases in alpha diversity.

3.4b RELATIVE ABUNDANCE OF TAXA ACROSS GESTATION

Although there was a general decrease in alpha diversity and F:B ratio, our cohort expressed increased metabolic capacity to produce a main SCFA named butyrate. Thus, further investigation into the changes in microbiome composition are necessary to understand modulations that conserve this metabolic function despite characteristic changes associated with dysbiotic states. Overall changes in relative abundance of bacteria at the phylum level across the first, second and third trimesters for individuals in our cohort are displayed in Figure 6. The relative abundance of taxa in the first and second trimesters were mostly stable and represented by Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. These 4 phyla are dominate in a healthy microbiome and suggests physiological adaptions in the first and second trimester of pregnancy do not profoundly modulate the microbiome. However, the relative abundance of

microbiota substantially shifted when gestation progressed into the third trimester and was characterized by reductions in the Bacteroidetes, Firmicutes and Actinobacteria while Proteobacteria became enriched. While the 4 dominate phyla in the microbiome remain persistent in the third trimester, differences in the abundance of these taxa suggest a transition to microbial dysbiosis corresponding with observed decreases in alpha diversity scores for the third trimester.

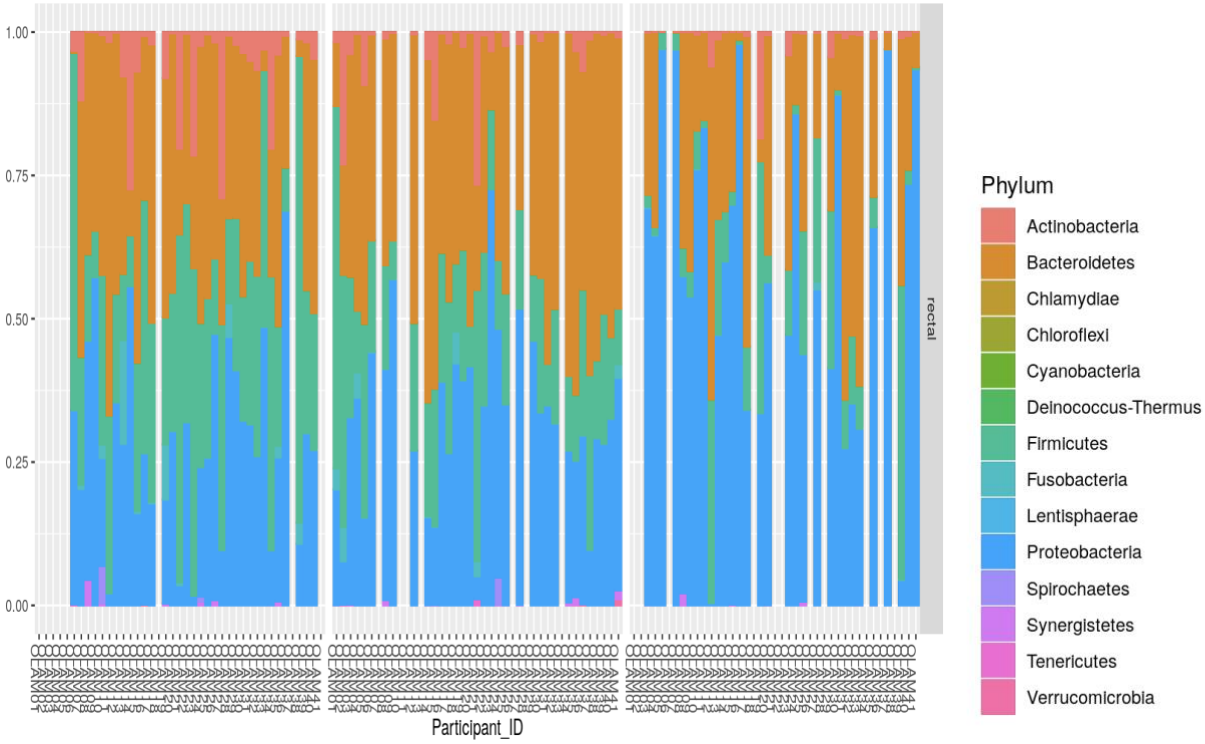


Figure 6. Relative abundance of intestinal microbial phylum across the first, second and third trimesters.

3.4c RELATIVE ABUNDANCE OF MICROBIAL SHORT CHAIN FATTY ACIDS PRODUCERS

To further investigate the effect of microbial shifts that occurr over pregnancy which may affect SCFA production, a selection of 9 genera implicated in the production of acetate, propionate and butyrate were further evaluated. The relative abundance of *Alistipes*, *Bacteroides*,

Bifidobacterium, *Blautia*, *Clostridium*, *Faecalibacterium*, *Lactobacillus*, *Parabacteroides* and *Roseburia* were characterized across the first trimester (Figure 7) and the third trimester (Figure 8). Figure 9 compared the relative abundance of these genera across trimesters for all individuals. The genus *Parabacteroides* ($p<0.05$) exhibited enrichment in the first trimester while *Blautia* ($p=0.0005$), *Faecalibacterium* ($p<0.05$), *Lactobacillus* ($p<0.005$) and *Roseburia* ($p<0.005$) were significantly reduced across trimesters. Figure 10 displays the change in relative abundance of SCFA-producing genera for each ethnic group across pregnancy. Native Hawaiians were the only ethnic group to have a significant reduction of *Blautia* ($p<0.05$) and *Roseburia* ($p<0.01$) and significantly increased *Parabacteroides* ($p<0.05$), while non-Hispanic Whites experienced a significant depletion in *Lactobacillus* ($p<0.01$).

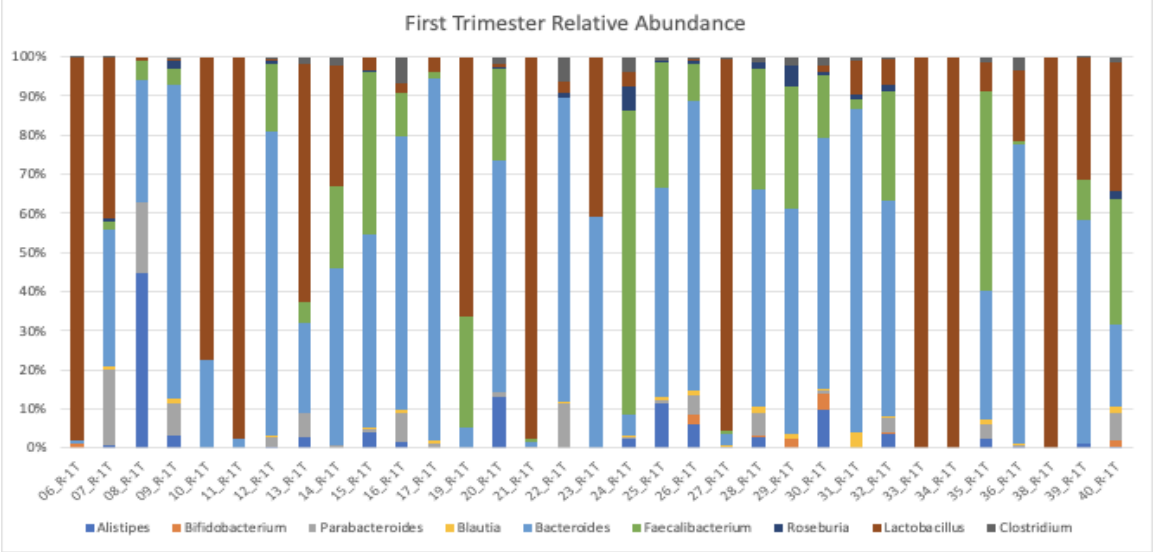


Figure 7. Visual representation of the relative abundance for SCFA-producing genera present in the first trimester across individuals.

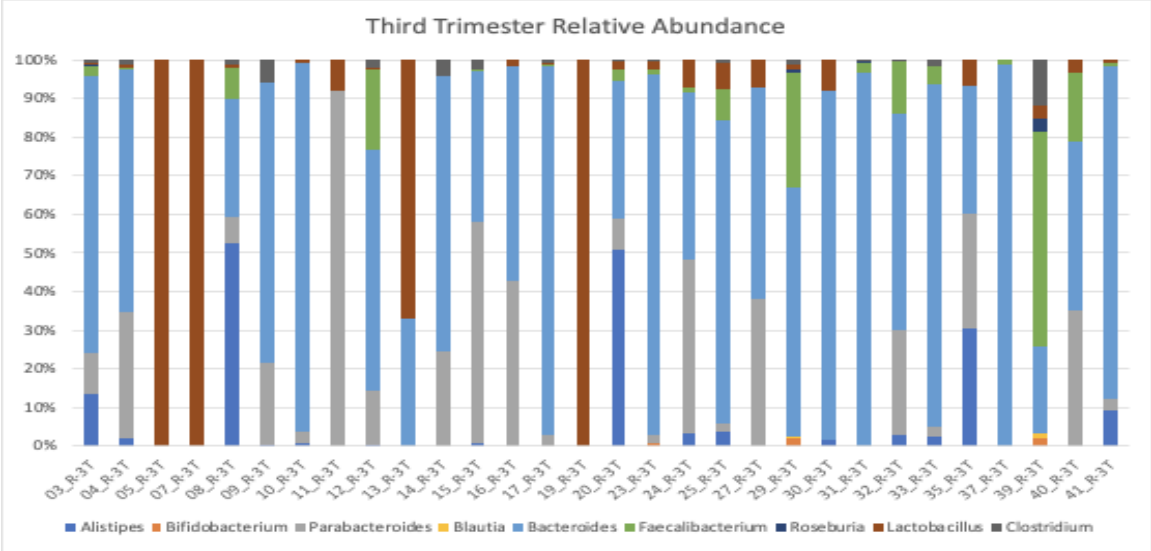


Figure 8. Visual representation of the relative abundance for SCFA-producing genera present in the third trimester across individuals.

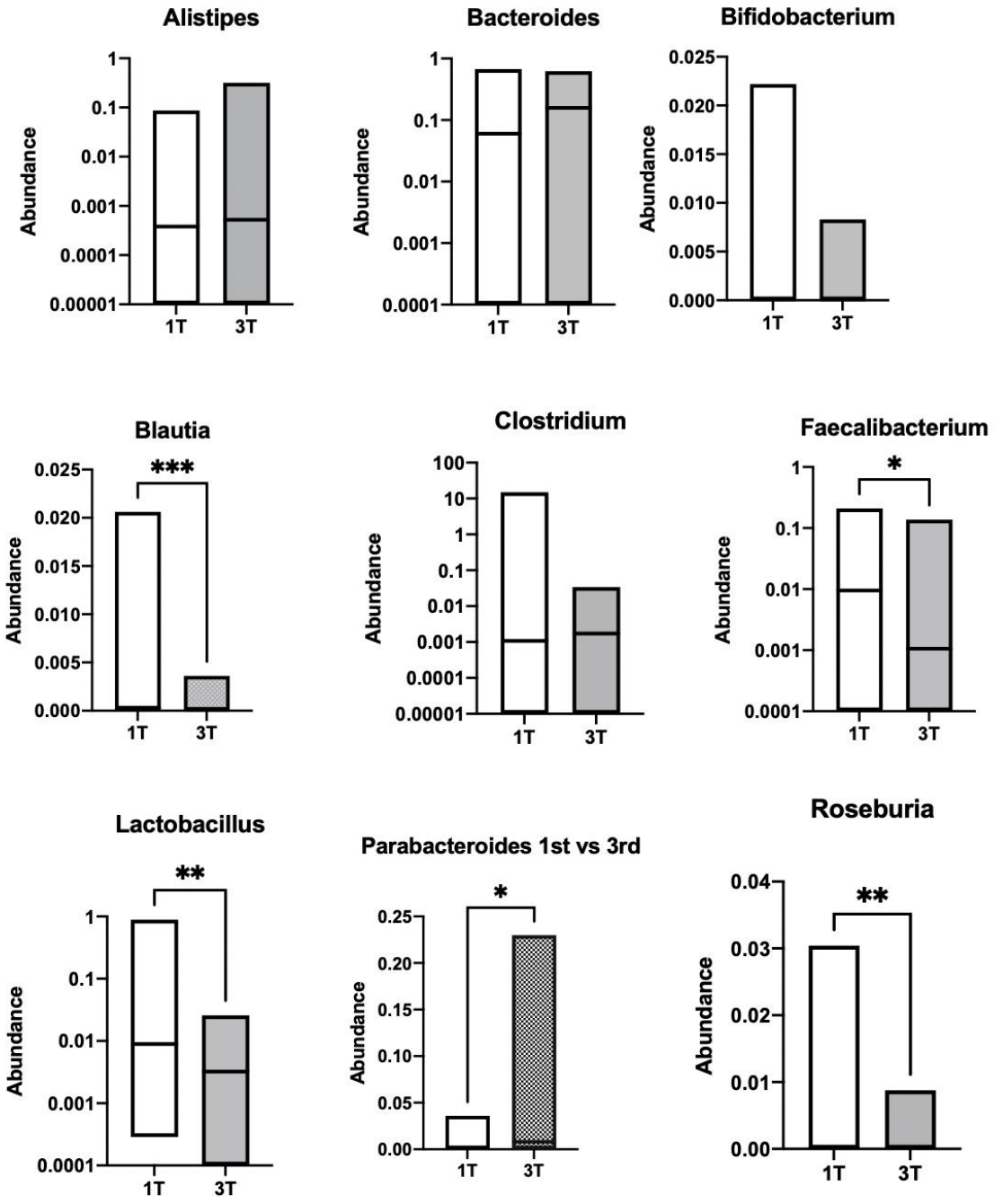


Figure 9. Box plot of log transformed relative abundance for microbiota implicated in SCFA production displays changes in abundance from first to third trimester.

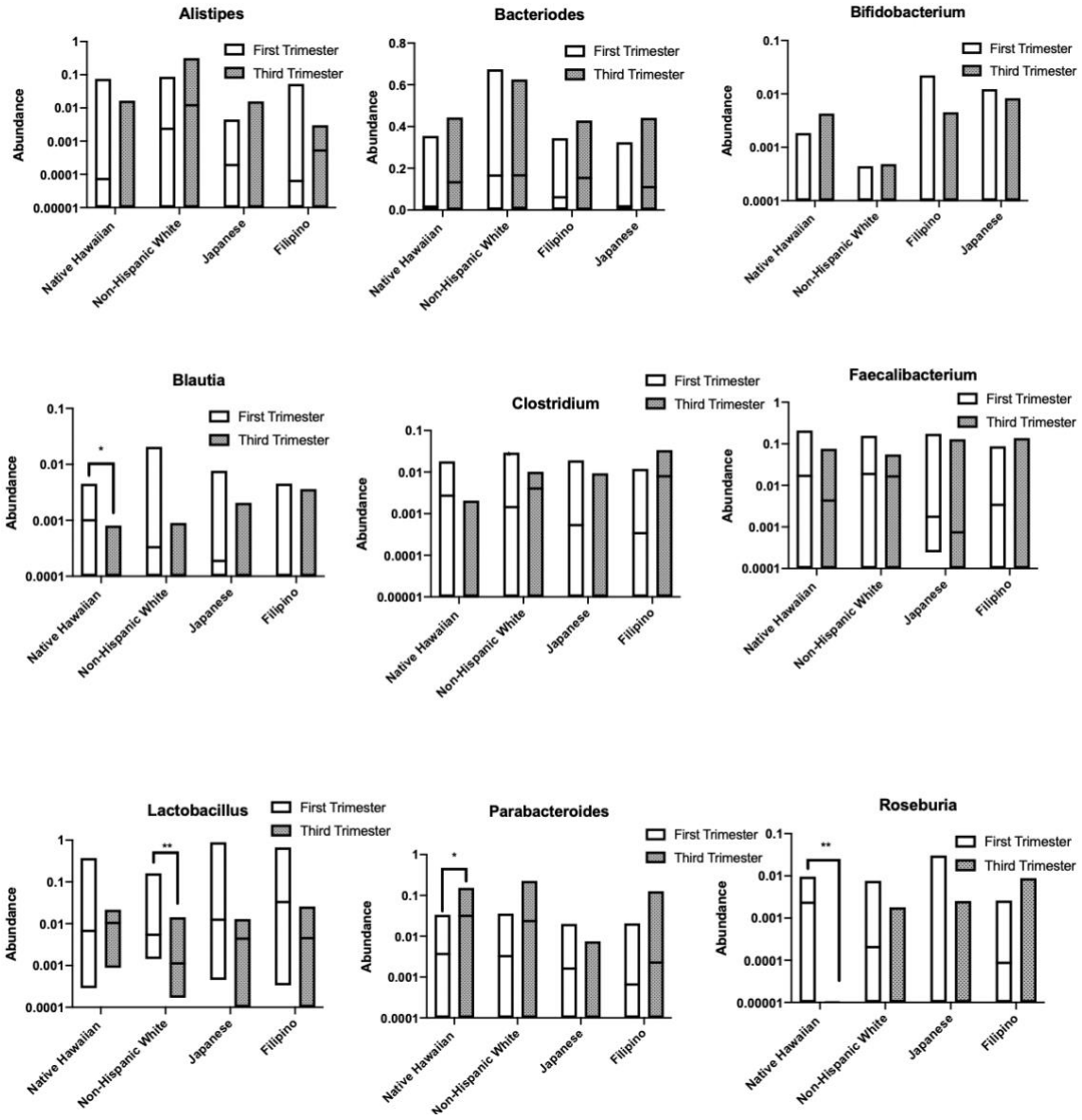


Figure 10. Box plot of log transformed relative abundance for microbiota implicated in SCFA production from first to third trimester stratified by ethnic group.

3.5 CYTOKINE DATA

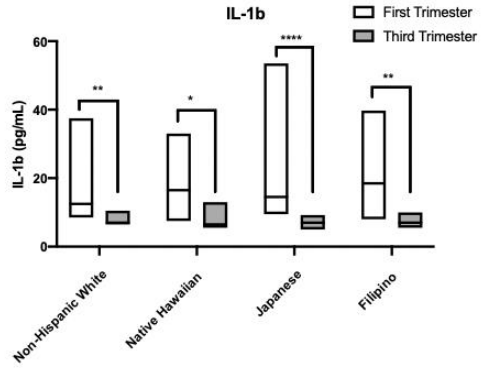
In a healthy and non-pregnant state, the microbiome plays a key role in the maintenance of systemic immune responses.[74] Thus, the occurrence of microbial modulations during pregnancy may be associated, because shifts in microbiome compositions can impact immune regulation. Therefore, characterizing the changes in inflammatory profiles offers insight into regulation of systemic immune responses in pregnancy. Table 8 and 9 report the median cytokine values of ethnic groups in the first trimester and postpartum. Within our cohort there were significant differences in cytokine profiles of ethnic groups from first trimester to postpartum as displayed in Figure 11. Among our cytokine panel, there were inflammatory markers that responded uniformly across ethnic groups, such as observed in IL-1b, IL-10, IFN-g, TNF-a and CRP. Significant reductions of IL-1b, IL-10, IFN-g, and TNF-a were reported in Native Hawaiians ($p<0.05$), ($p<0.0001$), ($p<0.0001$), ($p<0.005$); non-Hispanic Whites ($p<0.005$), ($p<0.0005$), ($p<0.0001$), ($p<0.0005$); Japanese ($p<0.0001$), ($p<0.0001$), ($p<0.0001$), ($p=0.0001$); and Filipino ($p<0.005$), ($p<0.0005$), ($p<0.005$), respectively. Cytokine IL-8 did not exhibit any significant changes over pregnancy for any ethnic groups but did have varying responses by ethnicity. Significant ethnic differences were observed for markers IL-6, MCP-1 and VEGF-A in which IL-6 was significantly decreased in non-Hispanic Whites ($p<0.05$), MCP-1 was significantly increased in non-Hispanic Whites ($p<0.05$) and Filipinos ($p<0.05$), and VEGF-A was significantly increased in non-Hispanic Whites ($p<0.05$) and Native Hawaiians ($p<0.05$).

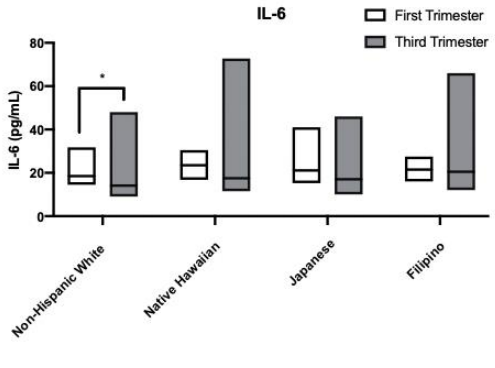
Table 8. Cytokine characteristics of the first trimester stratified by ethnicity. Data are presented as median [min, max] in concentrations of (pg/mL).

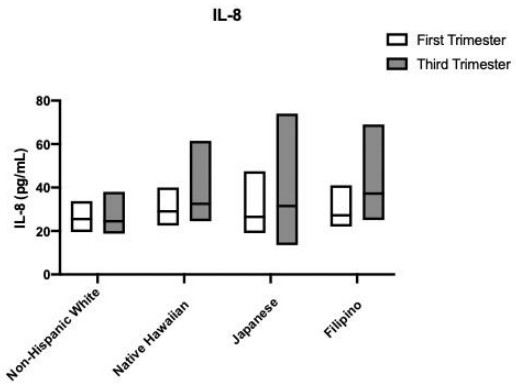
Ethnic Group	IL-1b	IL-6	IL-8	IL-10	IFN-g	TNF-a	MCP-1	VEGF-A	CRP
Non-Hispanic White	12.50 [8.50, 37.50] (n = 10)	18.50 [14.50, 31.75] (n = 9)	25.50 [19.50, 33.75] (n = 9)	18.00 [13.50, 49.00] (n = 9)	18.25 [11.00, 36.00] (n = 9)	14.00 [11.00, 33.25] (n = 9)	64.00 [25.00, 86.75] (n = 8)	41.74 [25.25, 57.25] (n = 9)	1843.00 [491.90, 2543.00] (n = 7)
Native Hawaiian	16.50 [7.50, 33.00] (n = 10)	23.50 [16.75, 30.50] (n = 10)	29.00 [22.50, 40.00] (n = 10)	22.75 [13.50, 37.50] (n = 10)	21.25 [15.00, 39.00] (n =10)	17.13 [10.75, 52.25] (n = 10)	65.75 [27.00, 150.00] (n = 10)	46.25 [27.25, 70.00] (n = 10)	849.80 [104.80, 3855.00] (n = 9)
Japanese	14.50 [9.50, 53.50] (n = 10)	21.13 [15.25, 41.00] (n = 10)	26.50 [19.00, 47.50] (n = 10)	23.25 [17.00, 59.50] (n = 10)	22.13 [17.50, 42.00] (n = 10)	17.25 [14.25, 44.25] (n = 10)	79.00 [32.00, 161.00] (n = 10)	41.50 [26.25, 96.00] (n = 10)	1482.00 [293.40, 3816.00] (n = 6)
Filipino	18.50 [8.00, 39.75] (n = 9)	21.50 [16.00, 27.50] (n = 8)	27.25 [22.00, 41.00] (n = 8)	24.75 [15.50, 40.00] (n = 9)	21.75 [15.50, 29.00] (n = 8)	17.75 [12.00, 27.50] (n = 8)	45.75 [34.00, 88.75] (n = 9)	47.50 [31.00, 68.25] (n = 9)	411.60 [56.60, 1906.60] (n = 8)

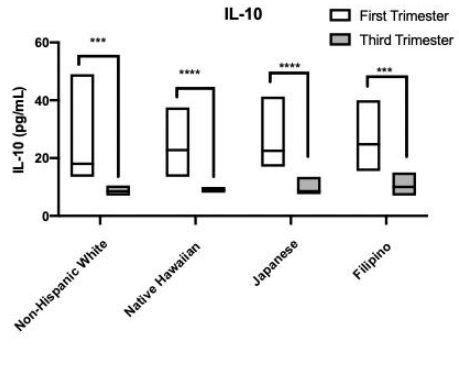
Table 9. Post-partum cytokine characteristics stratified by ethnicity. Data are presented as Median [min, max] in concentrations of (pg/mL).

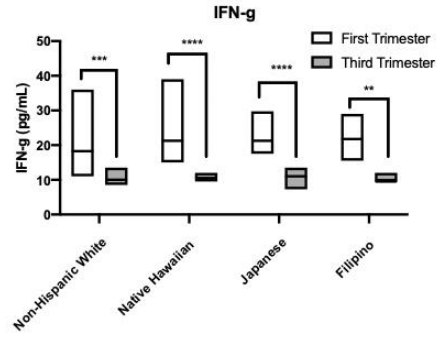
Ethnic Group	IL-1b	IL-6	IL-8	IL-10	IFN-g	TNF-a	MCP-1	VEGF-A	CRP
Non-Hispanic White	7.00 [6.50, 10.50] (n = 9)	13.50 [9.00, 17.50] (n = 7)	24.50 [18.75, 38.00] (n = 9)	9.00 [7.00, 20.00] (n = 8)	10.00 [8.50, 13.50] (n = 8)	9.13 [7.50, 12.50] (n = 8)	132.00 [23.50, 213.30] (n = 7)	108.30 [26.75, 172.8] (n = 8)	4640.00 [983.00, 4916.00] (n = 8)
Native Hawaiian	6.50 [5.50, 13.00] (n = 9)	12.75 [11.50, 32.25] (n = 7)	32.50 [24.50, 61.50] (n = 8)	9.00 [8.00, 10.00] (n = 8)	10.50 [9.50, 12.00] (n = 9)	10.00 [9.00, 13.50] (n = 9)	79.38 [59.50, 168.00] (n = 8)	70.63 [28.25, 212.50] (n = 8)	4590.00 [1608.00, 5460.00] (n = 7)
Japanese	7.00 [5.00, 9.25] (n = 9)	16.75 [10.00, 25.75] (n = 8)	31.50 [13.50, 74.00] (n = 9)	8.50 [7.50, 13.50] (n = 9)	11.00 [7.25, 13.50] (n = 9)	9.50 [7.50, 14.50] (n = 9)	164.00 [34.50, 295.30] (n = 7)	41.13 [12.00, 142.00 (n = 8)	4416.00 [1955.00, 4486.00] (n = 7)
Filipino	7.00 [5.50, 10.00] (n = 6)	20.50 [12.00, 66.00] (n = 5)	37.25 [25.00, 69.00] (n = 4)	10.00 [7.00, 15.00] (n = 6)	9.75 [9.50, 22.50] (n = 6)	11.25 [7.50, 22.00] (n = 6)	89.00 [59.74, 22.50] (n = 5)	75.50 [21.50, 192.30] (n = 6)	4338 [3662.00, 4629.00] (n = 6)

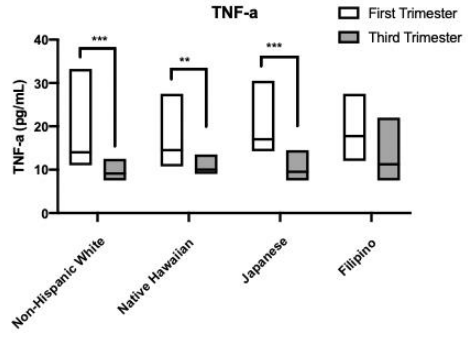

IL-1b
First Trimester
Third Trimester
IL-1b (pg/mL)
60
40
20
0
**
*
****
**
Non-Hispanic White
Native Hawaiian
Japanese
Filipino

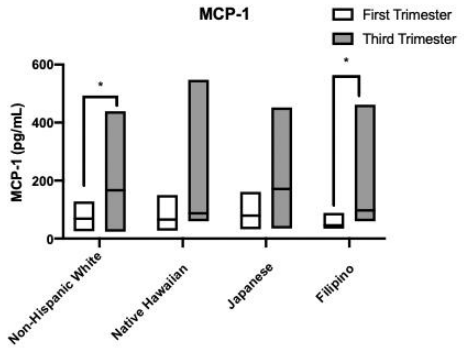

IL-6
First Trimester
Third Trimester
IL-6 (pg/mL)
80
60
40
20
0
*
Non-Hispanic White
Native Hawaiian
Japanese
Filipino

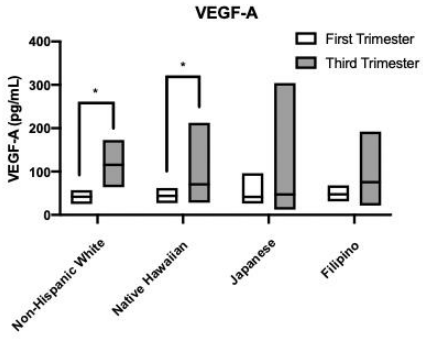

IL-8
First Trimester
Third Trimester
IL-8 (pg/mL)
80
60
40
20
0
Non-Hispanic White
Native Hawaiian
Japanese
Filipino


IL-10
First Trimester
Third Trimester
IL-10 (pg/mL)
60
40
20
0
***
****
****
***
Non-Hispanic White
Native Hawaiian
Japanese
Filipino


IFN-g
First Trimester
Third Trimester
IFN-g (pg/mL)
50
40
30
20
10
0
***
****
****
**
Non-Hispanic White
Native Hawaiian
Japanese
Filipino


TNF-a
First Trimester
Third Trimester
TNF-a (pg/mL)
40
30
20
10
0
***
**
***
Non-Hispanic White
Native Hawaiian
Japanese
Filipino


MCP-1
First Trimester
Third Trimester
MCP-1 (pg/mL)
600
400
200
0
*
*
Non-Hispanic White
Native Hawaiian
Japanese
Filipino


VEGF-A
First Trimester
Third Trimester
VEGF-A (pg/mL)
400
300
200
100
0
*
*
Non-Hispanic White
Native Hawaiian
Japanese
Filipino

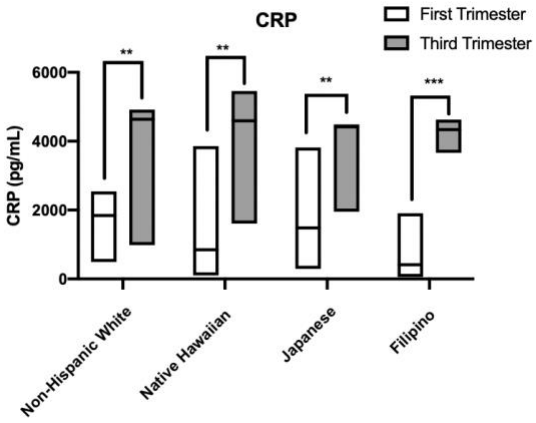


Figure 11. Boxplot of cytokine values stratified by ethnicity for first trimester and postpartum concentrations measured in (pg/mL).

3.6 ASSOCIATIONS BETWEEN MICROBIAL ABUNDANCE AND IMMUNE RESPONSE

The extent in which the microbiome modulates inflammation in pregnancy is unclear. At baseline levels, microbiome compositional shifts at the phylum level were generalizable across ethnicities. However, evaluation at the genus level suggested there are ethnic differences in compositional abundance for SCFA-producing genera. It is possible these baseline differences and variation in abundance can be implicated in observed ethnic differences of immune trajectories over gestation. One example of ethnic-specific differences was observed in the abundance of *Alistipes* that seemed to be associated with IL-8 concentrations seen in Figure 12.

Cumulatively, Figure 9 above showed the relative abundance of *Alistipes* in the first and third trimester was not greatly changed. However, Figure 12 indicated an ethnic-specific difference in modulation of abundance for *Alistipes* across timepoints. Breakdown of abundance by ethnic groups indicated non-Hispanic Whites had greater baseline representation of *Alistipes* than Native Hawaiians, Japanese and Filipinos. Upon continuation into the third trimester, these

differences became statistically significant. The change in median abundance values for non-Hispanic Whites and Filipinos were conserved across trimesters, although Filipino women had reduced variation and were typically less abundant. When third trimester relative abundance was compared across ethnic groups, non-Hispanic Whites had significantly greater abundance than Native Hawaiians ($p<0.05$), Japanese ($p<0.001$) and Filipinos ($p<0.05$).

Interestingly, a similar trend was observed in the immune response of inflammatory marker IL-8 that could indicate an association between *Alistipes* abundance and IL-8 production (Figure 12). At first trimester baseline levels, median IL-8 concentrations were consistent across ethnicities, but in the third trimester non-Hispanic Whites had lower levels of IL-8 than the other ethnic groups and were significantly lower than Native Hawaiians ($p<0.05$). Additionally, there was less variation observed in the non-Hispanic White group than Native Hawaiians, Japanese, and Filipinos. Possibly, the greater baseline abundance of *Alistipes* in non-Hispanic Whites can be protective of upregulation for IL-8 production in pregnancy.

Another indication of ethnic-specific difference was the observed relationship between *Parabacteroides* abundance and MCP-1 concentrations in Native Hawaiians (Figure 13). Native Hawaiians and non-Hispanic Whites had comparable relative abundance of *Parabacteroides* in the first trimester followed by Japanese and Filipinos which had descending abundances, although differences were not significant. The first trimester abundances were increased upon progression into the third trimester for Native Hawaiians ($p<0.05$), non-Hispanic Whites and Filipinos, however Japanese women had depleted *Parabacteroides* representation. Native Hawaiians were the only ethnic group to experience a significant increase. Although the median abundance was dramatically reduced in Japanese, there was large variation in the relative

abundance. Both Native Hawaiians ($p<0.05$) and non-Hispanic Whites ($p<0.05$) had significantly higher *Parabacteroides* abundance compared to Japanese women in the third trimester. These changes in abundance seemed to associate with MCP-1 concentrations.

Additionally, Figure 13 indicates the concentrations of baseline levels for MCP-1 and postpartum did not statistically differ between ethnic groups; however, MCP-1 postpartum in Native Hawaiians had the lowest median concentrations with least variance. Additionally, while non-Hispanic Whites, Filipinos and Japanese were seen to increase from first trimester to postpartum, the median concentration in Native Hawaiians remained steady. This in part may be due to the significant increase in *Parabacteroides* only experienced by Native Hawaiians, which could indicate that abundance of *Parabacteroides* is associated with MCP-1 production in the Native Hawaiian population, but not other ethnic groups.

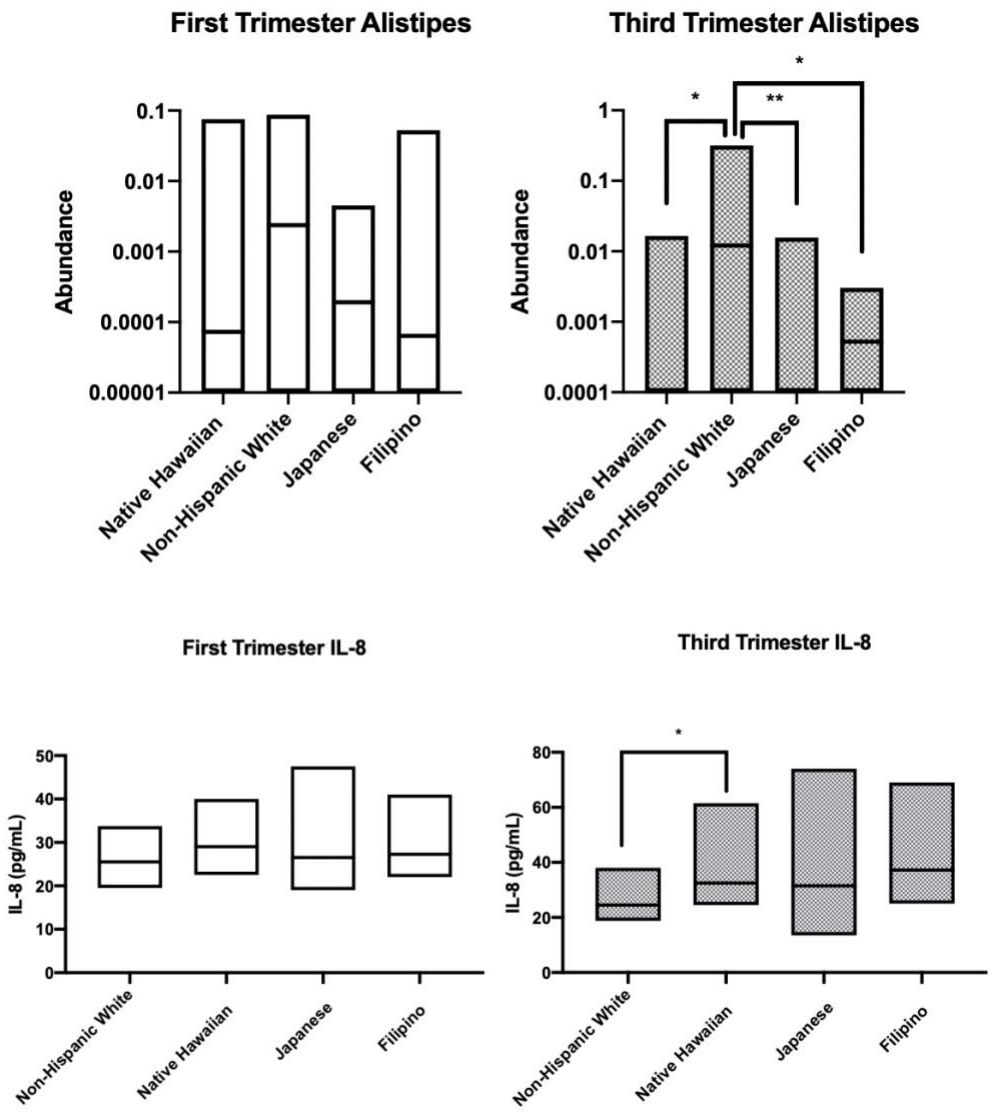


Figure 12. Boxplot of the relative abundance of *Alistipes* and IL-8 concentrations grouped by ethnicity for first trimester and postpartum. Data is represented as median [min, max] and cytokine concentrations are reported in (pg/mL).

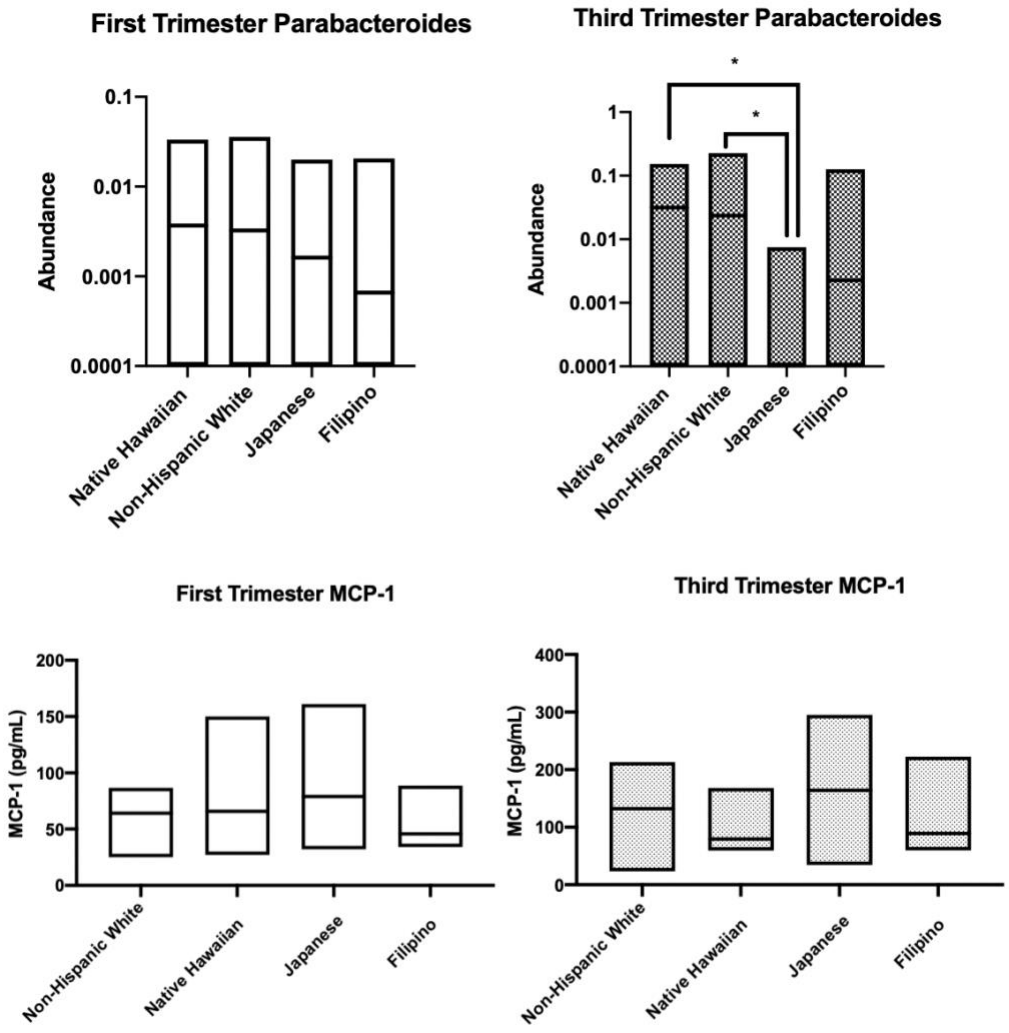


Figure 13. Boxplot of the relative abundance of *Parabacteroides* and MCP-1 concentrations grouped by ethnicity for first trimester and postpartum. Data is represented as median [min, max] and cytokine concentrations are reported in (pg/mL).

CHAPTER 4. DISCUSSION

The results of this study indicate unique relationships between gut microbiome composition and immune system regulation, which may occur in an ethnic-specific manner as a response to the physiological modulations experienced during pregnancy.

4.1 ETHNIC-SIGNATURES IN MICROBIOME COMPOSITION AND INFLAMMATION

Characterization of the gut microbiome offers insight into microbial stability, presence of dysbiosis and ability to effectively regulate immune and metabolic homeostasis. Within our cohort, all women irrespective of ethnic group experienced microbiome shifts at the phylum level throughout pregnancy that were consistent with current literature. The relative abundance of microbiome compositions in the first and second trimester of pregnancy was dominated by Firmicutes, Bacteroidetes and Proteobacteria resembling the state of healthy, non-pregnant women. As gestational age increased, alpha diversity trended downwards and the microbiome experienced increased enrichment of the Proteobacteria phyla, which has been corroborated in a similar study by Koren at al.[75] It is possible the increased abundance of Proteobacteria is a result of maternal weight gain due to the physiological adaptions in pregnancy that cause a shift to an obesogenic state in the third trimester.[71] While current literature has documented the overall composition of the gut microbiome in pregnancy, these studies were not well represented by the diverse ethnicities in Hawai‘i. It is possible ethnic-specific signatures of microbiome composition may have arisen to compensate for differences in genetics requiring varying regulation to maintain homeostasis.

Varying levels of production for SCFAs implicated in the modulation of metabolic and immune functions can be suggestive of distinct ethnic-specific requirements. Further investigation at the genus level of SCFA-producers was not consistent among ethnicities, elucidating the notion that despite a general uniformity of microbial shifts at the phylum level, there are ethnic-specific differences in genera abundances that lie within their respective phylum. There were discrepancies in the variation of abundance levels within ethnic groups and divergent distributions between ethnic groups across time points. Although these discrepancies in the changes of abundance are non-significant, it is possible the small sample size is concealing significant ethnic signatures at the genus level due to insufficient power of the study. Despite the lack of statistical differences between ethnic groups, it is evident that pregnancy modulates the microbiome in an ethnic-specific manner. This is suggested by inconsistencies across ethnic groups from baseline to third trimester abundances along with differences in the direction of change across trimesters. The observed differences in SCFA-producing genera between ethnic groups may be implicated in the trajectory of immune responses that were seen to differ by ethnicity.

Production of SCFAs such as butyrate promotes overall health by exerting anti-inflammatory activities and regulating energy metabolism, which is affected by the state of pregnancy.[71] Data evaluating butyrate production in human pregnancy is scarce, but animal studies have reported that butyrate enhances embryo survival in rats upon administration during early pregnancy.[59] The observed enrichment of butyrate kinase (*Buk*) gene within our cohort reinforces the protective role of butyrate in maintaining viable pregnancies. However, due to small sample size, *Buk* presence was unable to be evaluated across ethnicities, and it remains unclear whether this molecule is enriched in all ethnic groups, due to the observed variations of

abundances across timepoints for SCFA-producing genera. Therefore, the evaluation of immune responses across ethnicities can highlight the presence of associations between SCFA-producing genera and immune regulation.

There were demonstrated consistencies between ethnic groups for inflammatory markers IL-1b, IL-10, TNF-a, IFN-g, and CRP that may reflect commonalities in microbiome characteristics and composition. Yet, there were ethnic signatures observed among IL-6, IL-8, MCP-1 and VEGF-A that could associate with differences in microbial abundance at the genera level. Of the biomarkers that displayed ethnic differences, all first trimester median concentrations of these markers were not significantly different and in fact were very similar across groups. However, upon progression of gestation, discrepancies in immune markers arose in an ethnic-specific manner that was accompanied by increased variation in postpartum responses. This eludes that the abundance of genera in first and third trimesters may not be as important as the overall changes in abundance that occurs across time. Moreover, differences in IL-8 and MCP-1 production seemed to correspond with the relative abundance of *Alistipes* and *Parabacteroides*, respectively.

Associations between the conserved, high relative abundance of *Alistipes* in non-Hispanic Whites compared to other ethnic groups occurred in observance with conserved lower concentration levels of IL-8 in the first and third trimester. In this case, it would be the lack of robust change in abundance that may be implicated in maintaining IL-8 concentrations. This association is not considered to be a result of differences in BMI because we see varying responses in non-Hispanic Whites and Filipinos, which in our cohort were similar in BMI. A recent study investigating distribution characteristics of the microbiome during pregnancy in healthy women of Chinese origin living in China, reported a significant increase in *Alistipes*

abundance over pregnancy.[76] Among our cohort of Native Hawaiian and Pacific Islander (NHPI) women, *Alistipes* abundance was mostly depleted in the third trimester, except in Filipinos. This may suggest NHPI women have substantially deviated from cultural and traditional diets that has left their microbiome constrained due adoption of Westernized diets that typically reduce SCFA production. This is most likely due to depletion of necessary SCFA-producing microbiota and may be assumed to contribute to aberrant regulation of inflammatory states.

A similar association was observed in the relative abundance of *Parabacteroides* and MCP-1 production from first trimester to postpartum. While first trimester abundance of *Parabacteroides* and MCP-1 concentrations were not significantly different between ethnic groups, Native Hawaiians were the only group to experience significant enrichment of *Parabacteroides* in the third trimester. This significant increase associated with decreased variation in MCP-1 postpartum concentrations, while also keeping median concentrations lower than values observed in the other ethnic groups. This elucidates a possible ethnic-specific relationship that may regulate or avoid increased production of MCP-1 in Native Hawaiians. Moreover, the depletion of *Parabacteroides* abundance in Japanese women associated with the largest variation in MCP-1 responses and highest median MCP-1 levels. This contrasting response across ethnic groups for *Parabacteroides* abundance and MCP-1 concentrations further corroborates this potential association. Interestingly, non-Hispanic Whites had very similar abundance in both first and third trimesters along-side Native Hawaiians, but they did not experience the same response in MCP-1. This could indicate this relationship is only Native Hawaiian specific.

CHAPTER 5. CONCLUSION

5.1 LIMITATIONS

The most profound limitation of this pilot study was the small sample size due to limited funding. Despite the restricted sample size, the data postulated the presence of ethnic-specific signatures in both microbiome characteristics, composition, and inflammatory responses. Most likely increasing sample size would define additional or more significant differences between ethnic groups that would have allowed for clearer associations between microbiome and immune ethnic signatures. Additionally, the sample size was insufficient to accommodate for analysis of clinical characteristics and pregnancy outcomes. Further participant inclusion is necessary to elucidate relationships and regulatory potential of the microbiota-immune axis in pregnancy. Another considerable limitation was time collection of blood samples for inflammation analysis. Postpartum samples are not representative of third trimester inflammatory states; therefore, we were unable to evaluate if observed immune responses within our cohort were generalizable to other populations. Considering the highly labile nature of the microbiome, it was difficult to ascertain if microbiome modulations were due to metabolic adaptions which occur in pregnancy, or as a result maternal weight gain. Monitoring weight gain in mothers would have aided in controlling for this confounding factor.

5.2 FUTURE DIRECTIONS

The presence of ethic-difference in inflammatory responses within our diverse cohort requires further investigation. While baseline levels were generally similar, there were differences present among ethnicities that led to greater variation in postpartum cytokine responses. Assessing phenotypic differences of immune cell compositions across time points would be one way to confirm if ethnic-specific signatures of immune states were present. If

immune cell populations are consistent across trimesters within ethnic groups, we may assume there are general differences in immune responses based on ethnicity. However, if baseline immune cell populations are similar but become different in postpartum populations, it can be assumed the physiological adaptions consistent with pregnancy may differ based on ethnicity. A further way to corroborate this would be to evaluate the methylation states across time points using Epic Array technology to determine if differences in methylation states within the gene bodies are implicated in ethnic-specific expression of immune responses. This would further highlight the presence of ethnic signatures. Additionally, the observed differences in chemokine MCP-1 may suggest functional differences of monocytes within ethnic populations. Assays that utilize inflammatory stimulation of monocytes can elucidate whether monocytes are pre-dispositioned to initiate inflammatory or anti-inflammatory cascades that differ by ethnicity.

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