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The Impact of Exercise on Gut Microbiota in a Survivor to Germ-free Mouse Translational Model of Breast Cancer

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The Impact of Exercise on Gut Microbiota in a Survivor to Germ-free Mouse Translational Model of Breast Cancer

by

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Abstract

Breast cancer is the leading cause of global cancer incidence. Strategies to improve breast cancer treatment and health outcomes in survivors are needed to decrease mortality, mitigate side effects, and prevent recurrence. The gut microbiota is altered in individuals with breast cancer, can be affected by treatments such as chemotherapy, and plays a role in response to cancer treatments. It may be modified by environmental factors such as dietary components and exercise. In the present study, the Alberta Cancer Exercise (ACE) program was investigated as a strategy to favorably modify the gut microbiota of breast cancer survivors who had undergone chemotherapy. In a follow-up germ-free mouse study, the ability of exercise-responsive gut microbiota, alone or with prebiotic fiber supplementation, to alter tumor growth was interrogated using fecal microbiota transfer (FMT). In the cancer survivors, there was a significant enrichment in *Dialister*, *Oscillospiraceae*, and *Paraprevotella* following exercise ($p < 0.01$). In the germ-free mice, tumor volume trended consistently lower over time in the groups colonized with post-exercise gut microbiota compared to the group colonized with pre-exercise gut microbiota, with statistically significant differences found on day 16 and day 22. Tumor volume was further suppressed with prebiotic fiber supplementation. Gut microbial alpha and beta diversity differed across groups. Beta diversity and differential abundance analyses revealed that both the tumor cell injection and chemotherapy altered the gut microbial community in the mice. A potentially beneficial enhancement of *Parasutterella* and *Lachnospiraceae* and depletion of *Anaerostipes* and *Ruminococcus gnavus* was identified on day 22 in the group receiving prebiotic. Cytokine analysis of tumor tissue and serum indicated that the influence of the various FMTs resulted in distinct tumor microenvironments. The tumors of mice colonized with exercise-responsive microbiota exhibited lower levels of angiogenic VEGF among other markers, and greater levels of cytokines previously associated with positive Paclitaxel response. This was augmented with prebiotic supplementation. Exercise and prebiotic demonstrated potential to enhance anti-tumor immunity through advantageous gut microbiota modulation in breast cancer populations and should be further explored as adjuvants.

Preface

Portions of Chapters 1 & 2 were previously published in the International Journal of Molecular Science Volume 21, Issue 23. Material was modified as the author sees fit for this thesis.

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I truly stand on the shoulders of you all, and I thank you for your contribution to this work and my time at the University of Calgary.

Dedication

This thesis is dedicated with the deepest gratitude to my parents, Kimberley and Andy Sampsell. I have felt their unwavering belief in me every time I have shared a new pursuit, whether it would lead me away for a summer or across a continent for years. They have fostered my drive, curiosity, resilience, joy in learning, and propensity for dreaming and it has proven invaluable to me in life and in academics. They are a vision of hard work and kindness.

It is without question that this thesis belongs in part to the incredible people who raised me to climb metaphorical and physical mountains with a grateful heart.

For every trip to a science museum, book, and experience you provided me,
I dedicate this work to you,
with all my love.

Table of Contents

List of Tables

List of Figures

List of Symbols, Abbreviations, and Nomenclature

CTX	Cyclophosphamide
FOS	Fructo-oligosaccharides
5-FU	5-Fluorouracil
GOS	Galacto-oligosaccharides
ICI	Immune checkpoint inhibitor
IL-6	Interleukin-6
LPS	Lipopolysaccharide
ROS	Reactive oxygen species
SCFA	Short-chain fatty acids
SREBP	Sterol-regulatory element binding protein
IFN-γ	Interferon gamma
TNF	Tumor necrosis factor
GZMB	Granzyme b protein
PRF1	Perforin-1
COX-2	Cyclooxygenase-2
TNFα	Tumor necrosis factor alpha
PDL-1	Programmed Death Ligand-1
c-di-AMP	Cyclic dinucleotide adenosine monophosphate
Tregs	T regulatory cells
FMT	Fecal microbiota transfer
BMI	Body mass index
AMPK	AMP-activated protein kinase
mTOR	Mammalian target of rapamycin
S6K	S6 kinase
ZO-1	Zonular Occluden-1
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IP-10/CXCL10	Interferon gamma-induced protein 10
KC/CXCL1	Keratinocyte-derived chemokine/chemokine ligand-1
LIF	Leukemia inhibitory factor
LIX/CXCL5	Chemokine ligand 5
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
MIG/CXCL9	Monokine induced by interferon-gamma
MIP	Macrophage inflammatory protein
RANTES/CCL5	Regulated on activation, normal T cell expressed and secreted
VEGF	Vascular endothelial growth factor
OFS	Oligofructose

Epigraph

I then most always saw, with great wonder, that in the said matter there were many very little living animalcules, very prettily a-moving.

- Antonie van Leeuwenhoek, Letter to the Royal Society of London 1683

CHAPTER ONE: **INTRODUCTION**

1.1 Background

As of 2020, cancer is the second leading cause of death worldwide and its incidence and mortality rates continue to increase globally in alignment with population growth and aging.[1] Breast cancer has surpassed lung cancer as the leading cause of cancer incidence worldwide and is the most prevalent cancer among women with the highest mortality rate.[1] In 2020, breast cancer comprised 11.7% of the 19.3 million new total cancer diagnoses and it accounts for 1 in 4 female cancer cases and 1 in 6 female cancer deaths.[1] Breast cancer is the leading cause of female cancer incidence in 159 out of 185 countries, and the leading cause of cancer death in 110, emphasizing its significance as a global public health concern.[1] Several potentially modifiable lifestyle factors including dietary choices and physical activity levels have been associated with breast cancer risk. Although detection and treatment of breast cancer has improved over the last few decades, individuals face both acute and long-term health concerns related to the disease. Overweight and obesity are associated with greater breast cancer risk and recurrence risk.[2,3] This concern is compounded by the frequently reported weight gain that may result following chemotherapeutic treatment for breast cancer.[4] Adjuvant chemotherapy is often administered following surgery in this population and has been found to be associated with significant increases in weight, waist circumference, and metabolic markers such as low density lipoprotein (LDL) cholesterol, insulin, and glycated hemoglobin within two years of early stage breast cancer diagnosis.[5] Additional acute and long term side effects threatening patient health and quality of life include peripheral neuropathy, diarrhea, fatigue, anxiety, and depression.[6-9] The mechanisms underlying these effects are not yet fully described, but existing research indicating unique gut microbial profiles associated with diarrhea, psychological symptoms, and neuropathy, combined with recent research demonstrating a significantly different microbial profile in women who gain weight during adjuvant chemotherapy, suggest that the role of the intestinal microbiome in these relationships deserves further investigation.[10-13]

Within the past fifteen years, research into the gut microbiota has increased at an exponential rate, with insights into its relationship to several human diseases, including breast cancer, growing substantially.[14] The gut microbiota refers to the resident and transient bacteria, viruses, fungi, protozoa, and archaea present in the human gastrointestinal tract.[15] To date, the

vast majority of research on the gut microbiota has focused on bacteria because they comprise a significant component of the microbiota, their relationships to host health have been established, and investigative methodologies have developed more quickly for bacteria than those for other abundant members of the gut microbiota, such as viruses.[14,16] Although still ill-defined, a 'healthy' gut microbiota communicates with the host via various metabolic and signaling pathways, and in so doing, promotes host health.[14] However, disruptions to the gut microbiota also occur and this 'dysbiosis' is implicated in a growing list of disease states such as obesity, diabetes, and various cancers.[15] Alongside the host's environmental exposures and epigenetic and genetic susceptibilities, the gut microbiota is one aspect that can shape cancer risk.[17] The gut microbiota can potentially facilitate or impede carcinogenesis and influence how an individual will respond to certain cancer therapies.[17,18] The mechanisms through which the microbiota exerts its influences on carcinogenesis and cancer treatments require further investigation; however, some relationships are understood to exist via microbiota-derived metabolites, modulation of host metabolism, alteration of cytokine expression, intestinal barrier maintenance, and immune regulation.[19-22] The metabolic and immunological potential of an individual's gut microbiota may either serve as beneficial or detrimental in their disease progression and treatment. Additional research is needed to determine how the gut microbiota can be optimized in cancer patients and survivors and to identify safe and feasible methods to do so.

In addition to genetics, the gut microbiota is largely shaped by modifiable factors including an individual's environment, lifestyle, and diet.[23,24] These factors present as possible avenues to beneficially modify the gut microbiota of an individual with breast cancer before, during, and after treatment. Diet is an especially powerful intervention due to the metabolic capabilities of the gut microbiota.[25,26] Gut-resident microbes can break down various carbohydrate chains comprising dietary fiber for which humans lack the enzymes to digest. In doing so, additional energy is released, and short chain fatty acids are produced. This process influences energy balance and the local intestinal and systemic metabolic environment.[23,26] These metabolites can nourish microbes and influence host energy metabolism, inflammation, and gene regulation.[26,27] The diet can also be supplemented with prebiotics and probiotics which further support a health-associated microbiota.[28,29] The gut microbiota may also be influenced by an individual's physical activity level as evidenced by differential abundances in the gut microbiota of exercised vs. non-exercised mice and correlations between cardiorespiratory fitness and gut

microbial diversity.[30-32] A fruit- and vegetable-rich high fiber diet and a physically active lifestyle are widely recommended as cancer-protective and generally beneficial during treatment.[33-35] Therefore, employing these methods to shape the gut microbiota of individuals with breast cancer for optimized health, both surrounding their treatment and in the long-term, may prove effective and pose few implementation drawbacks.

Although breast cancer treatment has improved over time, individuals continue to face unique physical and psychological problems surrounding their diagnosis and treatment that can affect quality of life and clinical outcomes.[7,36] These include physical symptoms from the cancer itself, treatment side effects such as diarrhea, mucositis, or neuropathy, depression, loss of lean body mass, fatigue, and anxiety.[6-8,36-38] These health concerns may also compound on pre-existing conditions. In a cohort of Canadian women, 69-88% of individuals with breast cancer reported having one or more co-morbidity at the time of diagnosis which is comparable to the 73.8% who reported this in an American cohort.[39,40] Women who have had breast cancer also have a greater risk of developing an additional health condition such as obesity, cardiovascular disease, or mental illness following their diagnosis and treatment.[39,40] Treatment for breast cancer frequently involves a combination of modalities which may include chemotherapy, surgery, radiation, or endocrine therapy. These treatments can be accompanied by side effects that threaten overall health and decrease quality of life for affected individuals. In a cohort of 1945 women who received various chemotherapeutics to treat early-stage invasive breast cancers, 45% reported at least one toxicity which was severe or very severe.[41] Side effects and severe toxicities increase the likelihood of treatment discontinuation.[42] These factors must be addressed in order to improve patient outcomes and quality of life during and after breast cancer treatment.[6,43]Modulating the gut microbiota to improve host health and treatment tolerance may be a meaningful approach to this.[44,45]

1.2 Purpose of the Research

The overall purpose of this thesis is to explore the effect of exercise and prebiotic supplementation on gut microbiota in a translational model of breast cancer. In the clinical portion of the study, the goal was to determine whether the 12-week Alberta Cancer Exercise (ACE) program alters gut microbiota community composition in women with breast cancer who have undergone chemotherapy treatment. In the animal portion of the study, the goal was to

determine if transfer of human post-exercise gut microbiota into germ-free mice would reduce tumor growth compared to pre-exercise gut microbiota, and whether prebiotic fiber could enhance the effect of post-exercise microbiota.

1.3 Overview of Thesis Chapters

This thesis contains five chapters, beginning with this introduction to the thesis. The second chapter provides a review of the current literature on the relationship of the gut microbiota to breast cancer and explores existing evidence for diet, supplements, and physical activity as interventions in this population. Chapter three outlines the methodology used to conduct the research. Chapter four provides the results of this research study, and the fifth chapter discusses the findings, explores the strengths and limitations of the study, and identifies future directions that are guided by this research. The fifth chapter closes with an overall conclusion of the findings.

CHAPTER TWO: **LITERATURE REVIEW**

2.1 An Opportunity to Improve Breast Cancer-Related Health Outcomes

Considering the prevalence of cancer, and especially breast cancer, it has become increasingly important that scientists and clinicians understand the role that the microbiota plays in the development, progression, and treatment of the disease.[18] Harnessing this knowledge could lead to improved breast cancer prevention and help maximize treatment effectiveness. Cultivating a deeper understanding of the interactions between the gut microbiota and the host also has the potential to identify gut microbiota-targeted interventions.[18] The incidence of breast cancer and the number of survivors continues to grow, with many countries including the United States, Canada, England, Norway, Germany, Australia, and Japan reporting 85-90% five-year survival rates for women diagnosed between 2010-2014.[46,47] This large and growing population will benefit from novel interventions to support health during treatment, in survivorship, and to prevent recurrence of the disease.[39] As we move toward an era of increasingly personalized medicine, the status of an individuals' gut microbiota is emerging as a potentially useful tool for predicting likelihood of response to treatment, and with additional research, could serve as a possible intervention target to improve health outcomes in breast cancer.[14,18,38,48]

2.2 Gut Microbiota in the Context of Breast Cancer and Dysbiosis

Numerous studies have demonstrated a role for breast tissue microbiota and gut microbiota in the pathogenesis of breast cancer.[49] Microbial dysbiosis, which is characterized by an imbalance in the microbes of a given site or tissue, has been seen in women diagnosed with breast cancer compared to healthy controls. For example, Xuan et al (2014) showed that breast tumor tissue was enriched in *Methylobacterium radiotolerans* and that overall bacterial DNA load was reduced compared to paired healthy breast tissue.[50] Furthermore, Banerjee et al (2015) showed that breast cancer subtypes have unique microbiota signatures, with estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) positive subtypes showing greater similarity to triple-negative breast cancer tissues.[51] In terms of gut microbiota, Goedert et al showed that postmenopausal women with breast cancer had lower alpha diversity and higher relative abundance of *Clostridiaceae*, *Faecalibacterium*, and *Ruminococcaceae*, alongside reduced abundance of *Dorea* and *Lachnospiraceae* compared to paired healthy controls.[52] Of

functional significance, breast cancer in postmenopausal women has been associated with enrichment in gut microbial genes involved in lipopolysaccharide (endotoxin) biosynthesis, iron complex transport system, vitamin B12 transport system, phosphotransferase system, and secretion system, which have all been previously associated with inflammatory conditions including breast cancer.[53]

Although the composition of an ideal "healthy" microbiota remains elusive and in fact may not exist as a single entity, dysbiosis can increase risk for pathogenic infection, and is associated with inflammation and altered immune responses.[17,18,54,55] Dysbiosis commonly occurs through the loss of commensals, the proliferation of pathobionts (resident bacteria capable of causing disease under certain conditions), and/or a reduction in alpha diversity.[56,57] These shifts can occur following antibiotic treatment, chemotherapy, or radiation; all commonly utilized in cancer treatment.[10,18,58,59] Dysbiosis predisposes an individual to infection by opportunistic pathogenic bacteria capable of releasing toxins that can contribute to genomic instability and potentially, carcinogenesis.[18,60] In this dysbiotic state, the gut microbiota may lack sufficient diversity and resilience to prevent a bloom of bacteria such as certain strains of *Escherichia coli* which encode genes for toxins such as colibactin or cytotoxic necrotizing factor.[60] These group B2 and D *E. coli*-derived toxins are capable of altering the cell cycle, inducing DNA double strand breaks, and hijacking aspects of cell signaling which can contribute to genomic instabilities and abnormal cell activity in the intestinal tissues.[60] Dysbiosis is also associated with shifts in the metabolome (the metabolites in a given fluid such as serum or in a tissue) toward an inflammatory state which is favorable for carcinogenesis.[61]

The relationship between dysbiosis and inflammation may be bi-directional.[61] Inflammation-inducing events such as cytotoxic chemotherapy or radiation disturb the gut microbiota, and these disturbances are associated with inflammation through alterations in immune regulation, cytokine expression, and gut barrier function.[61-63] A healthy intestinal barrier consists of tightly packed epithelial cells with a thick mucus lining.[61,63] In a dysbiotic state, which is often caused by a chemical insult to the microbial community, the proteins (i.e. claudins, occludins, zona occludens) that maintain a tight junction between epithelial cells can be compromised and contribute to a 'leaky gut'.[61,63] Additionally, dysbiosis could include a reduction of bacterial populations that contribute to maintenance of a thick mucus lining to

supports gut barrier integrity or that produce protective metabolites such as the short chain fatty acid (SCFA) butyrate, which contributes to immune regulation. Degradation of the mucosal lining and tight junctions between intestinal epithelial cells also allows bacterial particles such as lipopolysaccharide (LPS) to translocate into the blood stream, inciting inflammatory responses from the immune system on a systemic level.[61] Elevated circulating LPS has been associated with both liver and colorectal cancers.[64,65] Increased circulating LPS presents as a risk factor for breast cancer metastasis through its ability to activate monocyte-mediated endothelial adhesion of circulating cancer cells.[66] Researchers have hypothesized that bacterial translocation from the gut to breast tissue is a possible mechanism through which distinct malignancy-associated breast tissue microbiomes may develop.[67] The increase in gut permeability is often referred to as "leaky gut" and has also been implicated in several chronic inflammatory disease states such as obesity and irritable bowel syndrome as well as cognitive conditions such as depression and chronic fatigue [68]. Therefore, maintenance and restoration of gut barrier integrity in women with breast cancer during and after treatment may improve clinical outcomes.

The interactions between the host and the gut microbiota are highly complex which likely explains some of the variability in research findings that show the presence of certain bacterial species to be beneficial and in other cases detrimental. For example, *Akkermansia muciniphila* abundance was higher in individuals with colorectal cancer in a study done by Sheflin, but it was key to the effectiveness of anti-PDL-1 immunotherapy in a study by Naito et al.[61,69] These contradictory findings regarding *A. muciniphilia's* relationship to cancer and treatment indicate the need for further research and underscore the likelihood that the desired abundance of key bacteria could be individual in nature based on the host, the tumor pathology, and the treatments.[17] It is also important to consider how previous treatments and co-morbid conditions may affect an individual's gut microbiota composition when considering what treatments will work best for them in the future since dysbiosis plays a potential role in the pathogenesis of cancer as well as in cancer therapy.[17,18,61,70]

2.3 The gut microbiota and breast cancer treatments

2.3.1. Chemotherapy

The gut microbiota can modulate host metabolism, inflammation, and immune responses; all crucial factors for tumorigenesis and dysregulated cell proliferation.[71] It is also through these physiological pathways that the gut microbiota has been shown to influence chemotherapy response and side effects.[71] The microbiota has the potential to affect treatment outcomes by metabolizing xenobiotic chemotherapy drugs, modulating immune response, or affecting local inflammation and gut barrier function directly or via its SCFA metabolites.[71,72] In *C. elegans*, researchers have shown that *E. coli* and *Comamonas* differentially influence efficacy of the chemotherapy drug 5-fluoro-2′-deoxyuridine through bacteria-dependent ribonucleotide metabolism.[73] This finding highlights the need for additional research on potential bacterial metabolism-dependent influences on drug efficacy for other cytotoxic chemotherapy agents.

The gut microbiota has also been linked to harsh side-effects of chemotherapy treatment related to its metabolic activity. β-glucuronidases are enzymes encoded by both humans and microbes. The human encoded β-glucuronidase functions within lysosomes to breakdown structural glycosaminoglycans, while microbe-derived β-glucuronidases can metabolize certain chemotherapy agents and are expressed by species such as *Clostridium perfringens, Streptococcus agalactiae,* and *Bacteroides fragilis*.[74,75] An example is Irinotecan, a chemotherapy agent commonly used to treat colon cancer and experimentally used to treat metastatic breast cancer, whose inactive metabolite can be re-activated by a β-glucuronidase present in the intestinal lumen, resulting in adverse drug effects such as severe diarrhea and intestinal damage.[74,76-78] β-glucuronidase-producing bacteria such as *Bacteroides* spp. and *Clostridium* spp. are associated with accumulation of diarrhea-inducing metabolites during chemotherapy treatments such as Irinotecan and 5-Fluorouracil (5-FU).[76] Diarrhea is a prevalent side-effect of several chemotherapeutic agents seen in 50-80% of patients and one that causes both discomfort and more severe complications such as dehydration.[79,80] *Bacteroides* spp. are common members of the gut microbiota, and an increased abundance of *Clostridium* spp. is typical of dysbiosis following chemotherapy.[71] For example, the chemotherapy agent 5-FU was associated with post-treatment dysbiosis characterized by an increase of *Staphylococcus* and *Clostridium* spp. and a decrease in *Enterobacteriaceae*, *Lactobacillus* and *Bacteroides* in the

colon of rats treated with 5-FU compared to untreated controls.[81] Diarrhea in response to treatment with 5-FU in mice was found to be associated with increased expression of mRNAs encoding for inflammatory cytokines TNF-α, IL-1β, IL-6, Il-17A and IL-22 as well as altered expression of intestinal and colonic aquaporins which are responsible for modulating fluid transfer through the gut barrier.[82] Inhibition of TNF-α did not affect the 5-FU-induced diarrhea, suggesting that the mechanism of the diarrhea may be independent from the associated upregulation of inflammatory cytokine expression.[82]

Chemotherapy is also known to induce mucositis in many patients.[83] Gastrointestinal mucositis is an inflammatory condition that, like diarrhea, appears to develop in association with bacterial species shifts in the gut microbiota due to chemotherapy treatment.[83,84] For example, in mice treated with 5-FU, a decrease in the relative abundance of *Actinobacter* and an increase in *Verrucomicrobia* has been observed.[83] In a murine model of 5-FU-induced mucositis, levels of several chemokines and cytokines, including chemokine-1,-2,and -9, as well as Interleukin-4 (IL-4) were elevated.[85] IL-4 can be pro-inflammatory and contributes to increased gut barrier permeability following 5-FU treatment, while chemokine-9 has been associated with gut epithelial damage due to its ability to phosphorylate S6K1 (S6 kinase beta 1) which inhibits intestinal cell proliferation.[21,85,86] In mice, 5-FU-induced mucositis was associated with upregulation of several inflammatory cytokines at both the mRNA and protein level in serum and colon tissue. This upregulation occurred alongside a decreased *Firmicutes/Bacteroidetes* ratio and additional phylum level shifts consisting of increased relative abundances of *Verrucomicrobia,* and decreased *Proteobacteria*, and *Cyanobacteria*.[85] Although our understanding is growing, more work is needed to fully describe the mechanism(s) by which gut microbiota shifts influence expression of key signaling molecules that affect gut epithelial injury from chemotherapeutics.[85]

It is also important to note that response to chemotherapy treatment can be affected by microbe-modulated immune responses and microbial translocation to lymphoid organs.[71] Platins, Cyclophosphamide, and 5-FU are among chemotherapeutics used in the treatment of breast cancer. Platinum-based chemotherapeutic drugs such as oxaliplatin and cisplatin have been shown to be ineffective in mice whose gut microbiota were depleted with antibiotic treatment as well as in germ-free mice.[71,87,88] Disrupting the gut microbiota with antibiotics reduced the

production of reactive oxygen species (ROS) by tumor-associated inflammatory cells, the production of which is required for oxaliplatin genotoxicity.[87] Lam. et al. (2021) recently reported a mechanism underlying the gut microbiota's role in oxaliplatin efficacy and potentially other chemotherapeutics which involves gut microbial signaling molecules that enhance immune activity in the tumor microenvironment.[88] Ongoing mechanistic research is likely to provide deeper insights into microbiota-treatment relationships, including exciting work in mice showing that supplementation with *Lactobacillus acidophilus* alongside cisplatin offered a synergistic effect with the greatest survival and smaller tumor size compared to the groups treated with antibiotics and cisplatin or cisplatin alone.[89]

Similar to platinum-based drugs, cyclophosphamide (CTX) effectiveness is similarly negatively affected by antibiotic administration.[90,91] Gut-resident *Enterococcus hirae* and *Barnesiella intestinihominis* have been reported as necessary for successful CTX response.[92] Both species support Th1 immune response.[92] *E. hirae* translocates from the gut to lymph nodes, increasing the CD8/Tregs ratio to support anti-tumor immunity while *Barnesiella intestinihominis* was found to promote infiltration of anti-tumor immune cells at the cancer site.[91,92] Although the mechanism is not known, treatment with 5-FU has been found to be less effective in murine models of antibiotic-induced dysbiosis compared to controls.[93] Researchers note that 5-FU efficacy may be dependent on the pre-existing community of gut microbes and the mechanism may be related to the immune activation or xenobiotic metabolism capabilities of these microbes.[93] The antibiotic-treated animals demonstrated an enrichment of *Firmicutes* and the *Proteobacteria* species *Escherichia shigella* and *Enterobacter*, while relative abundances of protective butyrate-producing bacteria including *Roseburia* and *Bacteroidetes* decreased, which may be related to the subsequent poor 5-FU response.[93] Although this effect was seen in the treatment of colorectal tumors with 5-FU, future studies should investigate the impact of dysbiosis on 5-FU treatment for other commonly treated tumor types, including breast tumors. Response to these chemotherapy agents is inhibited when the gut microbiota lacks species capable of supporting the necessary immune responses, and the treatments themselves are dysbiosis-inducing. At this time there are no published studies on the persistence (over a period of months) of dysbiosis following chemotherapy nor documentation of whether exercise or dietary interventions could resolve the dysbiosis and restore species that would benefit treatment effectiveness and tolerability. Additional clinical research is needed on the associations between

gut microbiota and chemotherapy, but the composition of gut microbiota is being promoted as a potential predictive biomarker for individual treatment response and target for improving outcomes.[48,71,94]

2.3.2 Radiotherapy

Research addressing the impact of the gut microbiota on radiotherapy remains sparse; however, variability in gut microbiota has been reported as a potential contributor to the heterogeneity seen in tumor responses.[71] Radiotherapy is commonly used to treat breast cancer, so understanding potential influences on patient responses is clinically relevant. In one mouse study investigating the relationship between the circadian rhythm and radiotherapy, researchers found that mice with normal 12h light/12h dark schedules had improved survival and this was correlated with greater species richness in comparison to cohorts with altered light/dark schedules.[95] Radiotherapy may also induce dysbiosis which has been postulated to potentially mediate radiation toxicity.[96] In a mouse model of severe radiotoxicity, fecal microbiota transfer (FMT) from control mice to irradiated animals significantly improved survival and mitigated toxicity.[96] Clinically, Ferreira et al.[97] reported that in individuals receiving pelvic radiotherapy, an association existed between gut microbiota composition and enteropathy, with an enrichment of *Clostridium*, *Roseburia*, and *Phascolarctobacterium* in those experiencing toxicities. In individuals with cervical cancer who underwent a combination of chemoradiation therapy, high gut microbial diversity at baseline was associated with greater median overall survival compared to those with low diversity.[98] It is important to consider that the radiation was localized closer to the gut microbiota for individuals in the studies described here than it would be in an individual undergoing radiotherapy for breast cancer. These studies indicate the potential role for gut microbiota optimization during radiotherapy. More research is needed to elucidate the potential mechanisms and species-specific relationships present between gut microbes and radiotherapy response in various cancer types, including breast cancer.

2.3.3 Immunotherapy

The use of immune checkpoint blockade agents represents an important advance in the treatment of cancer. Immune checkpoint inhibitors (ICIs) suppress the interaction of immune-response inhibiting receptors on T lymphocytes with their respective ligands which are found on

the surface of cancer cells.[99] This prevention results in a greater T lymphocyte-mounted immune response to cancerous cells characterized by elevated CD4+ and CD8+ T cells in circulation and in the tumor microenvironment.[99] Recent pre-clinical studies and clinical trials support the use of anti-programmed cell death protein 1/programmed death ligand 1 pathway (anti-PD-1/PDL-1) therapy in some individuals with metastatic or triple negative breast cancer, which are difficult to treat.[100,101] Not all breast cancers are PD-1+, but this therapy has been reported to be well tolerated for those individuals and it is being investigated as a potential adjuvant to conventional breast cancer therapies.[100] Multiple studies have demonstrated the necessity of the gut microbiota for response to the anti-PD-1/PDL-1 blockade.[99,102] The mechanism occurs at least partly through immune modulation.[99] T regulatory cells (Tregs) play a role in immune regulation and tolerance of self-antigens which are both key to anti-cancer immunity. Intra-tumoral Treg activity is measured via biomarkers such as the CD4/FOXP3 cell ratio, which was found to be elevated in mice who received an FMT from responder or non-responder cancer patients and were subsequently co-treated with PD-1 and *Akkermansia muciniphila.*[99]

In murine models, antibiotic treatment impedes response to the ICIs, indicating the necessity of the gut microbiota for treatment response.[18] Primary resistance to this treatment is associated with dysbiosis and clinical outcomes are correlated with the relative abundance of *Akkermansia muciniphila*, whose presence was proven necessary for response to the therapy in a murine model.[99] *Bifidobacterium* spp., a well-known health-associated bacteria, has been linked to successful response to anti-PD-1/PDL-1 therapy.[103] In depleted mice, supplementation with *Bifidobacterium* spp. rescued a favorable anti-PD-1/PDL-1 response.[103] Of interest regarding the work in this thesis is that *Bifidobacterium* spp. can be increased with consumption of prebiotic fibers such as oligofructose.[29] The mechanism governing the relationship between gut microbiota and ICIs is not well understood but appears to occur partly through the recruitment of key immune cells to the tumor site.[18,99,103] A recent study identified the gut microbe-derived metabolite inosine to play a key mechanistic role in immune cell activation at the tumor site during ICI treatment in murine models of colon cancer, bladder cancer, and melanoma.[19] Inosine is produced by *Bifidobacterium pseudolongum* and *A. muciniphila* in the upper gastrointestinal tract.[19] Monocolonization with *Bifidobacterium pseudolongum* in murine models of colon cancer prior to ICI treatment with anti-PDl-1 or anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) significantly increased anti-tumor immunity when compared to the response in germ-

free mice.[19] Elevated intra-tumoral IFN-γ+ CD4+ and IFN-γ+ CD8+ T cell infiltration was characteristic of increased immune checkpoint blockade efficacy with inosine administration.[19] *Lactobacillus johnsonii* and *Olsenella* species monocolonization also yielded significantly enhanced efficacy of ICIs, albeit to a lesser extent than *B. pseudolongum* monocolonization which aligns with the species' ability to produce inosine.[19] Pre-treatment optimization of the gut microbiota may be a strategy to improve response to ICIs; however, additional research is warranted on how to achieve that optimization as well as on the mechanism of microbial influence over ICI response. The influence of the gut microbiota should be considered as ICIs continue to be investigated for use in breast cancer treatment.

2.3.4 Hormone therapies

Hormone receptor positive breast cancers are often treated with hormone therapies aimed at either lowering the amount of estrogen in the body or impairing the action of estrogen on breast cancer cells. Presently, no studies have investigated a link between response to hormone therapies and the gut microbiota, however this may be of interest considering the role of the gut microbiota in estrogen metabolism. The group of gut microbiome genes active in estrogen-related metabolism is termed the "estrobolome" and may serve as a useful biomarker.[104,105] Estrogen metabolized in the liver enters enterohepatic circulation which allows bacterial β-glucuronidase enzymes to deconjugate it to free estrogen that will enter systemic circulation.[106] β-glucuronidase producing bacteria belong to the genera *Clostridia* and *Ruminococcaceae*.[106] In addition to direct metabolism of estrogen, the gut microbiota can metabolize compounds known as phytoestrogens that are similar in structure and are capable of binding estrogen receptors.[105] This is true of microbiota derived metabolites of soy isoflavonoids and other plant lignans that act as phytoestrogens.[105] Higher circulating estrogen levels are linked to increased breast cancer risk in post-menopausal women.[104] Elevated deconjugation activity by the estrobolome can lead to excess re-entrance of deconjugated estrogen into circulation when it would have otherwise been excreted, potentially increasing breast cancer risk.[104] However, phytoestrogenic compounds processed by the estrobolome have been known to regulate transcription factors to promote metabolism and clearance of carcinogens.[105] Although a recent meta-analysis showed that higher dietary intake of isoflavone (a phytoestrogen) was inversely associated with overall mortality and cancer recurrence in patients with breast cancer [107], the risk/benefit profile of phytoestrogens

remains a topic of ongoing debate and has been reviewed in Senthilkumar et al. (2018).[108] The relationship between the gut microbiota, estrogen, and breast cancer is complex and further investigation is needed, particularly with regard to the impact of the gut microbiota and estrobolome on hormone therapies for hormone receptor positive breast cancers.

2.4 The Interplay of Obesity, Breast Cancer, and the Gut Microbiota

Obesity prevalence has risen dramatically in the past thirty years in North America and globally.[109,110] Obesity is characterized by a BMI $\geq$ 30 and is associated with excess fat mass, chronic low-grade inflammation, insulin resistance, and impaired signaling of several hormones key to maintaining metabolic health.[3,111,112] The presence of obesity increases the risk of developing breast cancer, breast cancer recurrence, and all-cause mortality for individuals with breast cancer.[2,3] Additionally, treatment efficacy is decreased in women with obesity undergoing systemic chemotherapy, and endocrine therapies prove to be significantly less effective compared to treatment in women without obesity.[2,3] Individuals with obesity are also at higher risk for complications during surgery and radiation.[2] The mechanisms for these increased risks are complex.

Adipose tissue is biologically active and capable of contributing to estrogen levels and producing inflammatory cytokines.[2,3,113] An elevated production of estrogen and inflammatory cytokines such as IL-1, IL-6, and TNF due to excess adipose tissue in individuals with obesity may contribute to biological processes that support carcinogenesis.[113,114] For example, TNF secretion from adipose tissue and circulating plasma IL-6 were highly associated with obesity-driven insulin resistance in a human cohort.[114] Insulin resistance is recognized as a risk factor for breast cancer.[115] Obesity causes increased circulating levels of insulin and insulin-like growth factors 1 and 2 which bind insulin receptors and upregulate pathways that ultimately promote protein and lipid biosynthesis, supporting dysregulated cell proliferation.[113] Elevated estrogen and inflammation levels are also recognized risk factors for recurrence of breast cancer.[3] In murine models of HER-2 positive breast cancer, obese animals show markedly faster tumor recurrence in comparison to non-obese controls, further indicating a strong link between the biological environment that is characteristic of obesity and increased breast cancer risk.[3] The notably detrimental impacts of obesity for breast cancer indicate a need to address or prevent the

condition in individuals with active disease and in survivors. A potential avenue for this lies in the relationship between the gut microbiota and obesity and potential mediation by exercise.

An obesity-associated gut microbiota differs significantly from a lean-associated gut microbiota.[116-118] In addition to lower bacterial diversity in those with obesity, elevated levels of *Lactobacillus* species and a relatively low abundance of *Bacteroides vulgatus, Bifidobacterium* and *Akkermansia,* as well as a higher *Firmicutes/Bacteroidetes* ratio have been reported.[116,117,119] Fecal transplants from obese mice into healthy germ-free mice resulted in the development of an obese phenotype in the previously healthy mice, demonstrating the existence of an obesity-associated gut microbiota.[23] An obesity-associated gut microbiota also modulates host gene expression to promote greater deposition of fats into adipose tissue.[23] More recent studies have demonstrated the ability of FMT from a calorie-restricted mouse as well as FMT from a mouse fed a normal-fat diet to mitigate weight gain in models of obesity.[120,121] In humans, fecal microbiota transplants from healthy individuals into those with obesity and metabolic syndrome increased insulin sensitivity over a 6-week follow-up period.[122,123] These studies showcase the influential role that the gut microbiota play in energy harvest, inflammation, and hormone signaling and highlight its potential as a target to improve the metabolic environment of individuals with breast cancer.[124] Diet and exercise are two first-line interventions aimed at improving the health of individuals with obesity. In an observational study of women who had been treated for breast cancer, an associated 50% reduction in mortality risk was found for both non-obese and obese individuals who consumed at least five servings of fruits and vegetables per day and performed physical activity equivalent to six 30-minute walks per week.[125] This study indicates that the potential impact of incorporating healthy lifestyle choices may be beneficial even if BMI remains in the obese range.[125] Additionally, diet and exercise are modifiable lifestyle factors that are investigated for their potential as interventions to address or prevent gut microbiota dysbiosis associated with obesity or cancer treatments.

2.5 Diet and Prebiotics in Relationship to Breast Cancer

2.5.1 The Metabolic Capabilities of Gut Microbiota in Response to Dietary Components

Overall diet quality, prebiotic intake, and probiotic intake are important factors that can shape an individual's gut microbiota [126]. Prebiotics are defined as "substrates that are selectively

utilized by host microorganisms and confer a health benefit", while Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".[29,127] The known associations between gut microbiota composition and certain diseases[24,126,128-131], point to the potential therapeutic avenues for dietary modifications and prebiotic or probiotic supplementation to support cancer prevention and/or treatment.[126] Of the dietary factors, fiber intake plays a notable role in shaping the gut microbiota.[25] The American Institute of Cancer Research endorses a diet high in fruits, vegetables, whole grains, and legumes and low in processed and red meats as cancer preventative.[35] In contrast, other diet components can be cancer promoting through their modulation of the microbiota.[132] Certain microbes are known to cause direct inflammation of tissues or contribute to a carcinogenic metabolic environment.[132] For example, species of bacteria capable of fermenting protein, such as *Fusobacterium* genera, are known to be enriched in individuals with colorectal cancer compared to healthy cohorts.[133] Hydrogen sulfide resultant of microbial amino acid fermentation disrupts colonocyte barrier function.[134] Butyrate oxidation is necessary for ion absorption, mucin synthesis, membrane lipid synthesis, and detoxification processes in colonocytes and is dependent on an enzyme that is inhibited by hydrogen sulfide.[134] These processes support gut barrier function and normal intestinal epithelial cell proliferation and function.[134] As previously described, the gut barrier is integral to preventing endotoxemia and translocation of bacteria and its products from the intestinal lumen to distant locations in the body.[63] Loss of gut barrier integrity facilitates development of an inflammatory phenotype that is favorable for carcinogenesis.[63,66,131] This is not to say that individuals should not consume an adequate quantity of protein, but emphasis on other protective dietary factors is critical. Researchers have noted that inflammatory metabolic profiles associated with high proteolytic activity are attenuated in individuals who consumed diets that were also high in fiber.[132,135]

Recent research demonstrated that supplementation with the prebiotic inulin was associated with increased relative abundance of bifidobacteria and decreased levels of proteolytic metabolic products including ammonia and branched chain fatty acids in human fecal samples.[136] Consumption of fermented foods may also contribute to a protective metabolic environment due to their probiotic contents.[137-140] In murine models of breast cancer, consumption of milk fermented with *Lactobacillus casei* resulted in suppression of tumor angiogenesis and metastasis which was associated with decreased levels of pro-angiogenic factor IL-6, decreased infiltration

of macrophages, and increased CD8+ and CD4+ lymphocyte response.[137-139] Adjuvant consumption of milk fermented with *L. casei* alongside administration of the chemotherapeutic Capecitabine was reported to decrease metastasis of breast cancer in mice, increase survival, decrease IL-6 levels, and mitigate common side effects such as weight loss, diarrhea, mucositis, and low red and white blood cell counts when compared to mice who consumed non-fermented milk alongside Capecitabine.[140] Overall, it is important for individuals at risk for breast cancer or in treatment for breast cancer to consume a diet that will help maintain a robust community of gut microbes that can inhibit an excess of inflammation-inducing microbes and molecules.[61,126] A primary goal should be to avoid or attenuate dysbiosis and metabolic conditions such as obesity which are associated with more favorable conditions for carcinogenesis and less favorable conditions for successful treatment response.[3,61,91,93,99,113]

The gut microbiota profile can potentially play a protective role against cancer partly through its production of protective metabolites such as SCFAs.[141] Butyrate is the primary protective SCFA and its intestinal concentration is dependent on both diet and the intestinal microbiota.[141,142] Short chain fatty acids produced by the gut microbiota include butyrate, propionate, and acetate which are metabolic products of microbial fermentation of dietary fiber, including prebiotic fibers.[25] Aside from serving as an energy source for colonocytes, butyrate can prevent histone de-acetylases from making epigenetic modifications that can lead to tumorigenesis.[141,143] Butyrate is also known to repress angiogenesis; therefore, slowing or inhibiting tumorigenesis.[144] Prominent butyrate producers include *Faecalibacterium prausnitzii, Roseburia intestinalis, Eubacterium rectale,* and *Roseburia* spp..[142,145] Butyrate shows promise as an anti-cancer metabolite due to its anti-inflammatory properties, ability to induce cell differentiation and cancer cell apoptosis, and its protective histone hyper-acetylation activity.[141,143,144,146] Therefore, facilitating butyrate production may be of interest in individuals with breast cancer or at risk for breast cancer. Important for this thesis, is the demonstration that butyrate is increased in exercised versus sedentary rats[147] and in humans, high levels of butyrate have been associated with a lean phenotype and increased cardiorespiratory fitness.[147,148] While the mechanisms by which bacterial metabolites exert their effects are largely unknown and will require further investigation [149,150], lifestyle interventions such as exercise and prebiotic/fiber supplementation that favorably modulate these metabolites warrant investigation.

2.5.2 Prebiotics

Prebiotics are substrates that are nondigestible by the host but promote the growth of beneficial microbiota.[29] Common prebiotics include oligofructose, inulin, and galacto-oligosaccharides (GOS).[29] Prebiotics are found naturally in foods such as asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, banana, and barley, but oligofructose, GOS, and inulin can be consumed in higher concentrations in supplemental form.[151] These prebiotics increase the abundance of *Lactobacillus* and *Bifidobacterium*.[29,152] In a murine model of obesity, oligofructose supplementation decreased circulating serum LPS levels by 40% over 12-weeks, thereby demonstrating its ability to attenuate systemic inflammation.[153,154] Serum LPS has also been implicated in breast cancer metastasis, further highlighting the relevance of decreasing LPS levels in circulation.[66] To date, no studies have focused on prebiotic supplementation alongside breast cancer therapy; however, prebiotics have been investigated in relationship to other tumor types. In a murine model, supplementation with inulin or mucin inhibited melanoma growth through distinct shifts in gut microbial taxa that increased anti-tumor immune activity.[155] In addition, inulin was also found to limit growth of colon cancer tumors in a murine model.[155] *Akkermansia muciniphila* was most significantly enriched in the inulin-fed mice, who presented with colon-cancer growth inhibition.[99,155] The mice in this study consumed a diet of 15% wt/wt inulin which exceeds human consumption levels; however, the associated gut microbiota shift and anti-tumor immune activation provide valuable mechanistic insight into the relationship between prebiotics, the gut microbiota, and tumor growth.[155] These findings indicate that further research on the benefits of prebiotics for other cancer types may be warranted. The ability of prebiotics to increase the abundance of taxa that benefit host health and decrease inflammation levels could be helpful to individuals with breast cancer before and during treatment to promote health, and after treatment to attenuate dysbiosis; however, more research is needed on this topic.

2.6 Exercise and the Gut Microbiota

2.6.1 A Developing Understanding

While the impact of diet on gut microbiota is supported by a robust body of research, the impact of exercise on the microbiota is lesser known. Support for exercise as a beneficial modifier of the gut microbiota originated from observational studies that demonstrate that a

greater ratio of *Firmicutes* to *Bacteroidetes* is correlated with higher maximum oxygen consumption (VO2 max) and that women who performed at least three hours of exercise per week had greater abundance of several butyrate-producing bacteria, including *Akkermansia muciniphila*.[156] Although these studies show positive correlations between beneficial microbiota and exercise, they were done in healthy individuals and failed to control for other factors such as diet which are known to affect the gut microbiota, indicating that the results may not be attributable to exercise alone.[156,157] Additionally, because these studies were cross-sectional, it is difficult to determine whether the microbiota profile or the higher VO2 max was present first.[156,157] More recent studies have been performed longitudinally in a controlled setting and demonstrate that 30-60 minutes of aerobic exercise performed three times per week is enough to induce significant changes in gut microbiota, although the changes differ in lean individuals compared to individuals with obesity.[157] For example, *Faecalibacterium* species increased in lean subjects but decreased in subjects with obesity and *Bacteroides* species decreased in the lean subjects and increased in the subjects with obesity.[157] It is also important to note that six weeks of sedentary behavior following the exercise intervention reversed any changes that were seen in the gut microbiota during the intervention, indicating that the effects of exercise are transient and easily reversed.[157] In pre-menopausal women, those meeting the World Health Organization's recommendation for 150 minutes of moderate aerobic activity each week presented greater abundance of *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Roseburia hominis* compared to those who were sedentary.[158] These species have demonstrated health-promoting effects such as maintenance of the intestinal barrier.[158] When investigating the effect of exercise on the gut microbiota, it is important that additional microbiota-modifying factors such as diet be taken into account. Additional research is needed on the effects of different exercise types and intensities, exercise in combination with prebiotics and/or probiotics, and exercise in a variety of populations.[159,160]

2.6.2 Exercise for Women with Breast Cancer

It is important to note the additional benefits of exercise for women with breast cancer alongside the potential favorable modification of the gut microbiota. A sedentary lifestyle has been associated with cancer and many other chronic diseases.[158] There is strong evidence that being physically active decreases the risk of breast cancer in pre- and post-menopausal women.[34]

Exercise has been proven as a safe intervention for cancer patients to improve their fatigue, physical function, and quality of life.[161] The current American College of Sports Medicine guidelines for exercise in cancer populations states that exercise should play a key role in the prevention and control of cancer.[33] In murine models of breast cancer, exercise increases sensitivity to chemotherapeutics through decreased hypoxia and regulation of vascularity, leading to improved outcomes via direct tumor suppression.[162] Additionally, exercise slows the growth of varying tumor types through its ability to induce vascular normalization and increase immune activity at the tumor site in murine models.[161] In addition to mechanistic evidence, observational data demonstrates a link between self-reported exercise and reduced rates of development and recurrence of multiple cancers.[161] An observational study on women with breast cancer and high recurrence risk found that meeting the minimum guidelines for physical activity both before and after diagnosis is associated with a 55% reduction in risk of recurrence and a 68% reduction in risk of mortality.[163] Interestingly, those who performed comparatively lower levels of activity pre-diagnosis, during treatment, and post-diagnosis experienced similar benefits to those who performed higher levels of activity at each time point.[163] This indicates that even limited amounts of regular physical activity yield significant benefits for this population.[163] It is also important to note that those who did not meet guidelines pre-diagnosis but met them post-treatment completion still experienced a 46% decreased risk of recurrence and 43% decreased risk of mortality, emphasizing the benefit of incorporating exercise regardless of previous activity levels.[163]

These beneficial associations can be explained by exercise's ability to improve body composition, decrease sex hormone bioavailability, improve insulin sensitivity, decrease levels of inflammatory biomarkers, and promote DNA repair.[164] Key growth and energy metabolism pathways including mTOR and AMPK are differentially regulated during exercise which could impact tumor growth, though researchers note that the impact is not yet understood from a mechanistic standpoint.[165] High levels of intra-tumoral lactate inhibits the infiltration of natural killer cells and T-lymphocytes, so exercise's ability to lower intra-tumoral lactate levels could potentially enhance anti-tumor immunity.[165] In a murine model, exercised animals demonstrated enrichment of *Faecalibacterium prausnitzii* and decreased expression of the inflammatory enzyme cyclooxygenase-2 (COX-2) in the proximal and distal gut epithelium which indicates improved gut barrier integrity and is hypothesized to be resultant of increased butyrate

production.[166] In addition to the above benefits, resistance exercise increases lean body mass.[167] According to a meta-analysis, 27.7% of individuals with cancer have low muscle area based on computed tomography tests, and this is associated with poor survival rates.[167] Therefore, it is important to promote and maintain lean body mass in individuals with breast cancer, and exercise may address this. It is possible that these benefits are also related to changes in the gut microbiota, but research is needed to explore these areas.

2.6.3 Exercise May Beneficially Modulate the Gut-Brain Axis

Exercise has been associated with improved psychosocial outcomes and health-related quality of life in individuals with cancer.[168] The mechanism has not been elucidated, but one area of investigation is possible modulation of the gut-brain axis.[169] The gut-brain axis is a paradigm that seeks to characterize the way that the gut microbiota and brain communicate bidirectionally.[170] The presence of mood-related hormones in the gut is a key component of the gut-brain axis.[170] Serotonin is a primary mood and cognition regulating hormone that plays a role in both gut and brain function and 90-95% of an individuals' serotonin is found in the gut.[171] Levels of blood serotonin have been noted to change in response to exercise and are associated with decreased depressive symptoms compared to non-exercised controls.[172] Many gut microbes have been reported to produce neurotransmitters.[171] Currently, no data exists as to whether exercise is associated with shifts in abundance of these species. However, these gut-microbiota derived neurotransmitters are postulated to exert effects through the gut-brain axis.[173] Increases in *Firmicutes*, SCFA production, and diversity in response to exercise have been negatively correlated with anxiety and depression.[174] Additional research is needed to investigate the potential relationship between those factors. In a pilot study investigating the effect of exercise in breast cancer survivors, correlations between gut microbiota beta diversity and fatigue and depression were seen as well as between gut microbiota composition and cardio-respiratory fitness.[169] These findings call for further research investigating the influence of exercise in cancer populations and the correlations between gut microbiota and psychosocial outcomes [169], including those captured in self-report questionnaires such as the FACT-G (Functional Assessment of Cancer Therapy – General).

2.7 Mechanistic Indications for Targeting the Gut Microbiota to Improve Breast Cancer Outcomes

Recent studies have explored ways in which the gut microbiota is able to modulate physiological influences on chemotherapy treatment response.[71] In several cases, these mechanisms have been demonstrated to manifest through immune regulation and alteration of gut barrier function.[18,71,88,91] It is largely through these influences that the gut microbiota's association with treatment effectiveness and prevalence of side effects is understood to exist.[71] An individual's ability to tolerate a treatment plays a critical role alongside their physiological anti-cancer response in determining treatment efficacy. Gastrointestinal mucositis and diarrhea are two common side effects of chemotherapeutic agents that are associated with dysbiosis or detrimental shifts in the gut microbiota during treatment, and they can negatively affect tolerability.[8,61,82] This dysbiosis may also contribute to a pro-carcinogenic environment as it is associated with greater levels of inflammatory cytokines.[82] For these reasons, it is critical that we strive to better understand exactly how the gut microbiota may be beneficially modified before, during, and after chemotherapy treatment to optimize an individuals' response to treatment and health status.

The gut microbiota of individuals with breast cancer varies from that of healthy individuals[175,176], providing a potential target for improving health outcomes. Although the directionality of this association is likely complex, the potential implications of gut microbial alteration on inflammation, immunity, and treatment response are significant. FMT is a valuable tool that can aid in understanding these influences. FMT into germ-free mice has enabled exploration of the functional outcomes associated with the gut microbiota of individuals with specific health conditions or microbial profiles.[177] FMTs allow the gut microbiota of an individual or experimental animal to be seeded into an otherwise germ-free model with the intention of investigating whether the transplanted microbiota are causally inducing the phenotype of the host animal.[177] For example, germ free mice colonized with the gut microbiota of patients with colorectal cancer for eight weeks displayed significantly greater tumor incidence and proliferation than control groups.[178] The pro-carcinogenic gut microbiota was associated with greater mRNA expression of pro-inflammatory cytokines TNF-α and IL-1β in the germ-free mice.[178]

Using similar FMT methodology, it is possible to evaluate tumor growth and response to a chemotherapy agent in germ-free mice colonized with pre-exercise and post-exercise gut

microbiota. Whether or not the prebiotic fiber oligofructose could enhance the potential beneficial effects of post-exercise microbiota on tumor growth and response to treatment is also of great interest. A beneficial synergistic effect of oligofructose supplementation and cytotoxic chemotherapy has previously been described.[179] A 44% increase in lifespan was observed for liver-tumor bearing mice that were treated with cyclophosphamide and consumed a 15% oligofructose diet when compared to un-supplemented controls.[179] The researchers also found that in mice with EMT-6 mammary adenocarcinomas, the group fed 15% oligofructose displayed significantly smaller tumor size than controls over the course of 46 days following tumor injection.[180] Given the beneficial effects described for oligofructose, it is possible that a synergistic effect between oligofructose and an exercise-modified gut microbiota could exist that would reduce tumor growth and enhance protection over either factor alone.

2.8 Research Justification

The gut microbiota's relationship with host physiology allows it to influence the development, progression, and treatment of breast cancer. The gut microbiota presents as a novel target to improve treatment efficacy and long-term health outcomes by beneficially shaping host metabolism, molecular signaling, and immune responses. Further research is needed to elucidate the mechanisms through which associations exist between the gut microbiota and response to treatments for breast cancer. Additionally, further characterization of the gut microbiota associated with response to these treatments is necessary and could potentially translate into strategies to optimize the gut microbiota prior to treatment or development of predictive models. Modulation of the gut microbiota could potentially decrease several common side effects of breast cancer treatment and make effective treatments more tolerable. Diet, prebiotic supplementation, and exercise show promising signs as strategies to optimize the gut microbiota pre-treatment and attenuate treatment or disease-associated dysbiosis to promote health and reduce recurrence risk. These interventional strategies may also improve overall metabolic health and reduce the negative impact of common comorbidities such as obesity. Additional animal and clinical studies are needed to identify safe and effective ways to incorporate these strategies into treatment pathways. Strengthening the current understanding of the interactions between the gut microbiota, breast cancer risk, and breast cancer treatments could lead to safe and effective gut microbiota-based interventions that will improve health outcomes in this population.

2.9 Objectives and Hypotheses

The overall goal of this thesis is to understand the impact of exercise on breast cancer in a translational model.

Objective 1: To examine the effect of a 12-week exercise program on gut microbiota composition in women with breast cancer who had undergone chemotherapy. Potential relationships between microbiota and patient-reported quality of life outcomes were also assessed. We hypothesized that gut-microbial alpha diversity would improve from baseline to 12-weeks (completion of the exercise program).

Objective 2: Using FMT and germ-free mice, we examine the influence of human donor pre-exercise and post-exercise microbiota (with or without prebiotic supplementation) on tumor growth following injection of mammary tumor cells and subsequent treatment with the chemotherapy agent paclitaxel. Gut microbial diversity, tumor and serum cytokine levels, and intestinal tight-junction protein expression were examined as potential mechanisms governing the effects of FMT and/or prebiotic. We hypothesized that mice colonized with post-exercise gut microbiota would have reduced tumor volume compared to those receiving pre-exercise microbiota. It was hypothesized that prebiotic oligofructose supplementation would further enhance the beneficial effects of post-exercise FMT.

CHAPTER 3: **METHODS**

3.1 ACE gut microbiota and breast cancer study

3.1.1 Alberta Cancer Exercise (ACE) program

Study participants were recruited from individuals who had enrolled in the Alberta Cancer Exercise program (ACE). The study population for ACE is southern Alberta. Participants adhered to the ACE study protocol which involves attending 12-weeks of bi-weekly 60-minute exercise classes. Historically, this program is delivered in person but was shifted to a virtual delivery via Zoom due to the restrictions imposed by the COVID-19 pandemic in March 2020. The exercise classes include aerobic, strength, and flexibility components and were taught by trained individuals.[181] The intensity of the classes ranges from mild to moderate. Participants at any stage of cancer can directly contact ACE personnel to enroll or are referred by a healthcare provider, and may be actively in treatment or in a survivorship stage up to 3-years post-treatment completion.[181] Several psychosocial and fitness measures are included in the ACE study protocol. For the purposes of this study, we accessed data on demographics, the Functional Assessment of Cancer Therapy-General (FACT-G) questionnaire, and Godin's Leisure Time Exercise questionnaire which are described in greater detail below. The results of the questionnaires were available to us through the REDcap online database.

3.1.2 Recruitment

Participants who met the inclusion criteria **(Table 1)** were sent an email by ACE personnel on our behalf to inform them of the opportunity to participate. Interested participants contacted us and an eligibility questionnaire and a detailed explanation of the study were distributed to them. Follow-up calls were made to individuals who did not respond to the initial email to bolster recruitment. Following confirmation of eligibility, participants signed the informed consent document to indicate their participation in the study. Twenty-four individuals replied to the initial contact by ACE personnel, seventeen individuals expressed interest and were provided additional information and the eligibility questionnaire, four individuals were deemed ineligible, and three were no longer interested, leaving a cohort of ten participants.

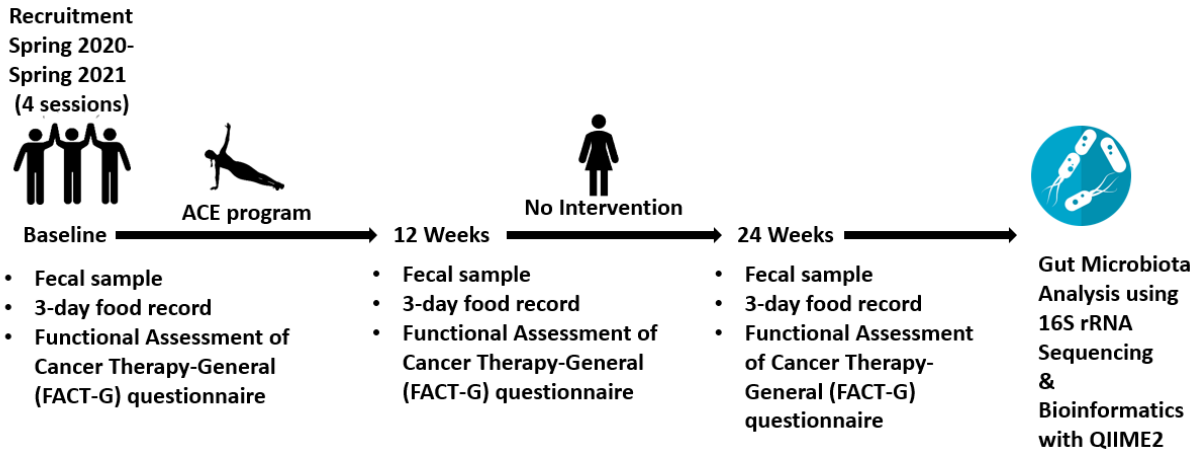
Table 1. **Inclusion and exclusion criteria for participant recruitment**

Inclusion Criteria	Exclusion Criteria
• Biologically female • Clinically diagnosed with breast cancer • Have undergone chemotherapy as a part of their treatment	• Intestinal diseases such as ulcerative colitis/Crohn's • BMI >35 kg/m^2 • Type 1 or 2 diabetes, cardiovascular disease, liver disease, pancreatic disease, or any disease of the gastrointestinal disease. • Antibiotic use within the last 3 months (if a patient is required to take antibiotics during the study they will be withdrawn) • Probiotic or prebiotic supplementation (this does not include consumption of foods which contain probiotic or prebiotic) • History of major gastrointestinal surgery • Regular consumption of > 1 alcoholic beverage/day • Pregnant or lactating • Currently undergoing chemotherapy or immunotherapy

3.1.3 Sample Size and Power

A target sample size of n=26 was determined for this study. This target sample size was calculated based on previous studies on the gut microbiota of individuals with cancer or cancer survivors.[11,32,182] Additional considerations included a low expected drop-out rate based on historical compliance with the ACE program as well as the estimated size of the available target population. The given sample size would allow us to detect significant group differences (estimated with a power of 0.80, $\alpha = 0.05$). Statistical calculations were done utilizing an online statistical calculator provided by the University of British Columbia. Due to limitations resultant of the COVID-19 pandemic, our recruitment resulted in n=10 participants who were recruited from four ACE sessions between Spring 2020 and Spring 2021. The outline of the study is provided below **(Figure 3-1).**

Figure 3-1. **A timeline of the ACE microbiota sub-study**



3.1.4 Demographic Information

Demographic information was collected as part of the ACE program via a baseline questionnaire. We accessed information including participant age, ethnicity, education, income, and employment status. The ACE program also collected information on participants' past treatment history including whether they had received surgery, radiation, or hormone therapy in addition to chemotherapy.

3.1.5 Godin's Leisure Time Exercise Questionnaire

At baseline, 12-weeks, and 24-weeks, participants completed a Godin's leisure time questionnaire as part of ACE. The questionnaire consists of four questions which query how frequently the individual performs mild, moderate, or strenuous physical activity.[183] Respondents indicate how many times per week they perform each category of physical activity for a time period of 15 minutes or more. Time spent in mild, moderate, and strenuous exercise sessions are multiplied by 3, 5, and 9 respectively which are then totaled to yield a final score in metabolic equivalents (METs). This questionnaire has been found to correlate closely to measures of physical fitness such as VO_2 max and is widely utilized in oncology research.[184]

3.1.6 Patient-reported Psychosocial Outcomes

Through ACE's REDcap database, we were able to access the results from the Functional Assessment of Cancer Therapy (FACT-G) health-related quality of life questionnaire. This 27-item questionnaire provides a cumulative score based on measures of physical, social, emotional,

and functional well-being in cancer patients. The general questionnaire is designed and validated for use in any clinical cancer population.[185] Each item presents a statement to which the respondent is asked to choose a numeric rating from 0-4 to represent how the statement applies to them over the past 7 days. A rating of 0 indicates "not at all", 1 indicates "a little bit", 2 indicates "somewhat", 3 indicates "quite a bit", and 4 indicates "very much". For example, item GE4 under the emotional well-being heading states *"I feel nervous"* and the participant rates how this statement applied to them in the past 7 days on the scale from 0-4. The total score is calculated by summing the totals multiplied by the number of items for each of the four categories after reverse scoring the indicated items. This results in a final score of 0-108 with a higher numeric score indicating greater QOL.

3.1.7 Dietary Intake

Study participants were required to document their food intake in a 3-day dietary record (two weekdays and one weekend day) at 0, 12, and 24 weeks. Instructions were provided on what to include in the report and how to quantify foods. Participants were given a document for recording their intake which was accompanied by detailed instructions and examples. Dietary records were analyzed using Food Works 18.0 software and the Canadian Nutrient File (The Nutrition Company, Long Valley, NJ).[186]

3.1.8 ACE Participant Fecal Samples and 16S rRNA analysis

Participants collected their own stool samples using at-home collection kits and stored them in their home freezer until pick-up. Samples were picked-up and transported to the University of Calgary for storage at -80C within three days of collection. The gut microbial content was analyzed according to protocols established and standardized in the Reimer lab.[33] Bacterial DNA was extracted from ~250mg of fecal sample using FastDNA Spin Kits (MP Biomedicals, Lachine, QC, Canada) and quantified using a PicoGreen DNA quantification kit (Invitrogen, Carlbad, CA, USA). The purified samples were diluted to 20ng/ul and sent to the Centre for Health Genomics and Informatics to undergo Illumina's 16S rRNA sequencing protocol. The V3 – V4 regions of the 16S rRNA gene were amplified and sequenced on 2×300 bp MiSeq Illumina platform as previously described (Centre for Health Genomics and Informatics, University of Calgary).[187] The pooled and indexed library set was denatured, diluted, and sequenced on an Illumina MiSeq (Illumina Inc., San Diego, USA). Demultiplexed

16S rRNA gene sequences were analyzed in QIIME2 platform using DADA2 for denoising and amplicon sequence variants (ASVs) extraction. ASV sequences were aligned to Silva 138 reference database and Genome Taxonomy Database for current taxonomy assignment. The resultant reads were analyzed using Shannon and Simpson indices, Weighted UniFrac, and DESeq2 analysis in the QIIME2 bioinformatics pipeline. Alpha and beta diversity analyses were calculated after rarefying the number of reads to 8683 using QIIME2 pipeline (version 2021.4).[188] Differential abundance analysis was carried out with the unrarefied ASV counts table using the DESeq2 package in R (version 4.0.0) with the model designed to control the random effect of participant variation.

3.1.9 Statistical Analysis

All data are presented as means ±SEM. Data normality was tested using Shapiro-Wilk's normality test. Godin's Leisure Time Exercise Questionnaire, FACT-G, and Dietary Intake were analyzed using a paired samples t-test to compare time points. Significance values were resultant of the two-tailed test. Significance is denoted as $p < 0.05$. Data analyses for non-microbial metrics were performed using SPSS statistics 27 (IBM). 16S rRNA statistical analyses and Spearman's correlations were completed in R (version 4.0.0). Alpha diversity was analyzed using a Kruskal-Wallis pairwise test in QIIME2 (version 2021.4). Beta diversity underwent analysis of similarity (ANOSIM) with 999 permutations. Adonis analysis using Weighted UniFrac values with 999 permutations was performed to investigate the %variance in beta diversity explained by exploratory factors.

3.2 Murine FMT study

Given the negative impact of COVID-19 on recruitment into the clinical study, an animal study was designed that would allow us to glean greater insight into the potential for an exercise-modified gut microbiota to affect breast cancer outcomes. The following methods describe the mechanistic study designed to specifically investigate the relationship of an exercise-responsive gut microbiota to breast cancer tumor growth and chemotherapy treatment. FMT allowed us to colonize germ-free mice with the pre- and post- exercise gut microbiota from one select participant in our clinical cohort and assess tumor- and microbiota-related outcomes in the recipient mice. In addition to the exercise-responsive microbiota, oligofructose supplementation was also assessed in this murine breast cancer model to investigate a potential synergistic

protective effect of exercise and prebiotic supplementation in breast cancer treatment. Based on the cost associated with running a study of this magnitude in the germ-free facility, one participant who showed a positive microbial effect to exercise, was selected as the fecal donor for the FMT. An overview of the study design is provided in **Figure 3-2**.

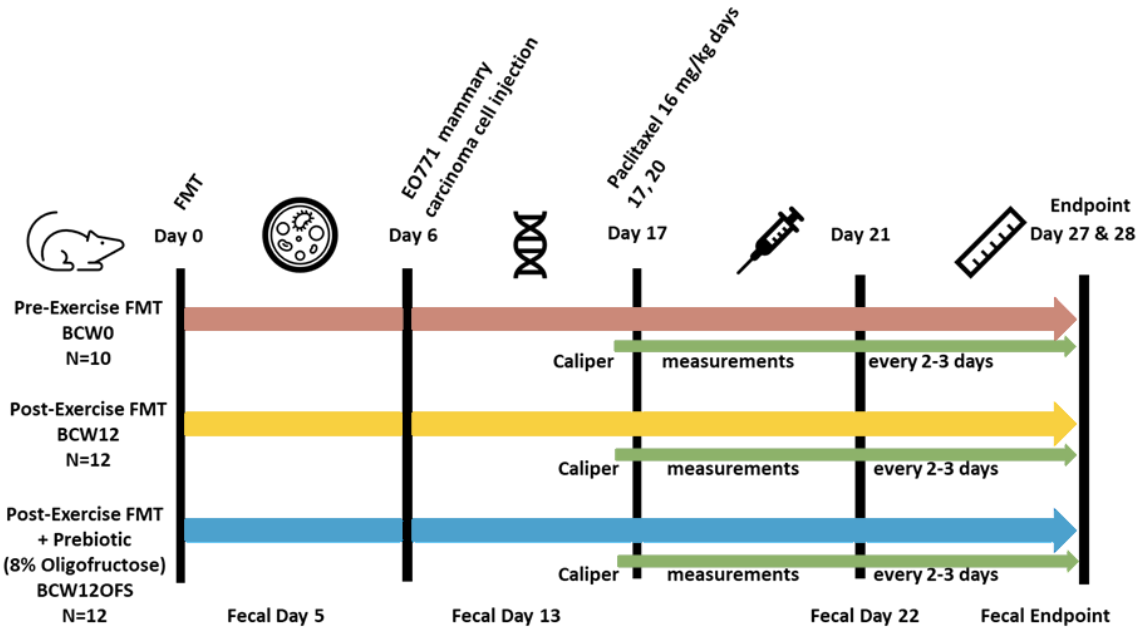


***Figure 3-2*. Murine study schematic.**

3.2.1 Animals

Forty-eight female 18–20-week-old C57BL/6 germ-free mice were bred and housed by the International Microbiome Center (IMC) at the University of Calgary, Canada. Germ-free status was confirmed via culture-dependent and independent methods prior to the study. All animals were kept on a 12-hour light-dark cycle on standard 4% fat chow. Animals were housed with litter mates in HEPA filtered iso-cages during the study.

3.2.2 Groups

Animals were randomly allocated to four weight and age-matched groups. The four groups were comprised of BCGF (germ-free control), BCW0 (received FMT of the participant's baseline, pre-exercise fecal sample), BCW12 (received FMT of the participant's 12-week, post-exercise fecal sample), and BCW12-OFS (received the same FMT as BCW12 and consumed

oligofructose-supplemented water). Each group consisted of n=12 mice at the start of the study. Two animals were euthanized in the BCW0 group following FMT, resulting in n=10 mice in the BCW0 group.

3.2.3 Cell Culture

The EO771 murine breast carcinoma cell line, originally isolated from a spontaneous tumor in a C57BL/6 mouse, was used in the animal experiment.[189] The cell line was generously provided by the S. Liao lab at the University of Calgary. Cells were cultured in 15 or 30ml of Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) in T-75 and T-150 flasks respectively. Cultures were maintained in an incubator at 37°C and 5% CO2. Prior to injection preparation, the cells were screened to ensure absence of mycoplasma (PCR Mycoplasma detection kit, Thermo Scientific).

3.2.4 EO771 Cell Injections

To prepare injection aliquots, cells were detached from the flasks with a 3-minute incubation in 1x Trypsin. Once the cells were visibly detached, DMEM was added to inactivate the Trypsin. Cells were then pooled in a 50ml Falcon tube, and an aliquot was removed to perform a cell count. The Falcon tube of cells was centrifuged for 5 minutes at 4°C. Following centrifugation, the supernatant was removed, and the cells were resuspended in DPBS (Sigma) to the proper concentration. The cell solution was aliquoted into designated microcentrifuge tubes for each group and diluted 1:1 with Corning® Matrigel Matrix (Millipore Sigma, Oakville, Canada) on ice to reach the final injection concentration. On day 6, each mouse received a 50ul subcutaneous injection into the right flank which delivered $1x10^6$ EO771 cells.

3.2.5 Paclitaxel Injections

On days 14 and 20, all mice were administered paclitaxel (Invitrogen, Themo-Fisher, Waltham, MA, USA) dissolved in DMSO and PBS via 100ul intraperitoneal injection. Paclitaxel is a common first-line chemotherapeutic for breast cancer which acts as a microtubule stabilizer, preventing mitotic cell division and inducing cell-death.[190] The mice received a cumulative 16mg/kg dose over the two days in accordance with previously published work indicating dose tolerability.[191] The total dose was calculated based on the common low-dose used in breast cancer [192] with conversion from human to rodent dosing as per Reagan-Shaw et al. [193] The solution was filtered with 0.2 micron filters to ensure sterility prior to injection.

3.2.6 Oligofructose supplementation

On day 0, the water bottles of the prebiotic intervention group BCW12-OFS (n=12) were replaced with oligofructose (Orafti P95, Beneo, Germany) solution which they consumed ad libitum to accrue an 8% dose of oligofructose for the remainder of the study. The oligofructose powder was weighed and mixed into water. The resultant solution was sterilized by filtration with a 0.2 micron filter to ensure sterility prior to consumption.[194] The dose calculation was based on an average 6ml/mouse daily water intake and water bottles were weighed every third day to ensure adequate intake.[195]

3.2.7 Mouse Fecal Samples and 16S rRNA Analysis

Fecal samples were collected by handling the mice until they provided a sample directly into an autoclaved Eppendorf tube. A fecal sample was collected on day 5 to assess if the FMT had colonized the mice, on day 13 which was one week after the tumor cell injections, day 22 which was two days after the second paclitaxel injection, and on day 27 or 28 (endpoint). Cecal contents were collected during endpoint tissue collection and snap frozen in liquid nitrogen. DNA extraction was performed as described in section 3.1.7. Samples were stored at -80C until extraction. The V3 – V4 regions of the 16S rRNA gene were amplified and sequenced on 2×300 bp MiSeq Illumina platform as described above (Centre for Health Genomics and Informatics, University of Calgary).[187] Analysis of the resultant reads was performed for Shannon and Simpson indices, Weighted UniFrac, and DESeq2 analysis as described above. Control germ-free mice (n=12) that did not receive FMT but underwent all study procedures were included as a control for contamination. During analysis, their samples were removed from the set after confirming germ-free status as evidenced by low number of reads compared to the colonized groups. Alpha and beta diversity analyses for the experimental groups were performed after rarefying the number of reads to 10,011 using QIIME2 pipeline (version 2021.4).[188] Differential analysis was carried out with the unrarefied ASV counts table using the DESeq2 package in R (version 4.0.0) and controlled for cage effect in the model design.

3.2.8 Tumor Measurements

Tumor measurements were taken every third day beginning on day 13 which is when tumors were consistently palpable (measurement days were 13, 16, 19, 22, 24, and 27 or 28 as

endpoint). Tumor length and width were measured with metal calipers and the modified ellipsoid formula (V = 1/2(AB2)) was used to calculate subcutaneous tumor volume.[196]

3.2.9 Tissue Collection

Mice were euthanized on days 27 and 28. Half of the mice from each group were euthanized on each day due to the substantially increased time it takes to perform tasks in the germ-free facility. Mice were anesthetized with isoflurane and blood was collected via retro-orbital bleed. Blood was allowed to clot for 30 minutes, and serum was collected following a 10-minute centrifugation at 4°C and 2500 rpm. Cervical dislocation was performed. The tumor was resected, and the distal ileum, proximal colon, and cecum were dissected. All tissues, fecal samples, and cecal contents were immediately snap-frozen in liquid nitrogen. Tissues and serum were stored at -80C until analysis.

3.2.10 Real-Time PCR Analysis

Ileum and colon samples were processed using real-time PCR as previously described.[197] Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription to cDNA was performed using 2 µg of total RNA and cDNA synthesis kit (Invitrogen). Primers for ileal and colonic genes (ZO-1, occludin, claudin-3) are listed in **Table 2**. The mRNA levels were calculated from PCR results using the $2^{-\Delta CT}$ method.[198]

Table 2. ***Primer Sequences for intestinal tight junction proteins***

Gene	Forward Sequence	Reverse Sequence
ZO-1	AGGGGCAGTGGTGGTTTTCTGGTTCTTTC	GCAGAGGTCAAAGTTCAAGGCTAAGAGG
Occludin	TCAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTTCAC
Claudin-3	CACCGCACCATCACCACTAC	CTTCCAGCCTAGCAAGCAGAC

Abbreviations: Zonular Occluden-1 (ZO-1)

3.2.11 Serum and Tumor Cytokine Analysis

Serum samples were sent to Eve Technologies (Calgary, Alberta, Canada) for measurement of the cytokines and chemokines listed below. Tumors were snap frozen in liquid nitrogen during tissue collection and stored at -80°C. Protein was extracted from tumor tissue by homogenizing in NP40 lysis buffer (Invitrogen) with protease and phosphatase inhibitor (Sigma).

The homogenate was centrifuged to allow collection of the supernatant. Samples were diluted to a concentration of 400µg/mL after protein quantitation with BCA Protein Assay Kit (Pierce, Themo-Fisher, Waltham, MA, USA). A panel of 31 cytokines including Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, VEGF-A were measured using BioPlex 200 Mouse Cytokine Array/ Chemokine Array 31-Plex Milliplex Immunoassay (Millipore). The array of cytokines and chemokines in the panel is designed to analyze markers of immune activity, inflammation, and cancer.

3.2.12 Statistical Analysis

All data are presented as means ±SEM. Data normality was tested using Shapiro-Wilk's normality test. Tumor volume was analyzed using a repeated measures ANOVA (RMANOVA) with timepoint as the within-subject factor and group as the between-subject factor. Post-hoc Tukey tests were used to detect between group significance for all ANOVA analyses. One-way ANOVA was used to analyze single-timepoint data (e.g. tight junction proteins, tumor tissue cytokines, serum cytokines). A paired samples t-test was used to analyze average fluid intake between BCW12OFS and the non-OFS groups. Significance of t-test results were from the two-tailed test. Statistical analyses of non-microbial measures were done with SPSS statistics 27 (IBM). Statistical analysis of 16S gut microbiota metrics were done in R (Version 4.00). Alpha diversity was analyzed using a Kruskal-Wallis pairwise test. Beta diversity underwent analysis of similarity (ANOSIM) with 999 permutations.

CHAPTER FOUR: **RESULTS**

4.1 Clinical Study Results: ACE's Impact on Gut Microbiota in Breast Cancer Survivors

4.1.1 Demographics

The demographics of the participants in the human ACE gut microbiota and breast cancer sub-study are presented in **Table 3**. Participants were primarily middle-aged (57.9 ±2.8 years old) and highly educated with 100% having completed at least some university, 60% completed university, and 10% completed graduate school. Most reported belonging to a mid to upper annual income bracket with 40% making >$99,000 per year. Participants in the study were largely of European descent. Occasional drinking was reported most frequently (50%) for alcohol consumption. An additional 20% reported never drinking, and the remainder of participants drank previously (10%), socially (10%), or regularly (10%). A substantial portion (40%) smoked previously, and no participants reported smoking currently. The demographics information indicates a homogeneous population sample that is highly educated, financially well-off, and low in ethnic diversity.

Table 3. ***Demographic information on participants recruited from the ACE program***

Demographic		Mean	Frequency	Percent
Age	Average Age (years)	57.9 ±2.8		
	Under 65		8	80
	Over 65		2	20
Biological Sex	Female		10	100
Marital Status	Never Married		1	10
	Married		8	80
	Divorced		1	10
Education Level	Some University		3	20
	Completed University		6	60
	Some Graduate School		1	10
Annual Income	Between $20 000-$39 999		2	20
	Between $40 000-$59 999		1	10
	Between $60 000-$79 999		1	10
	Between $80 000-$99 999		2	20
	Over $99 999		4	40
Employment Status	Disability		2	20
	Retired		2	20
	Part Time		3	30
	Full Time		2	20
	Temporarily Unemployed		1	10
Ethnic Background(s)	Britain		4	40
	Western Europe		2	20
	Eastern Europe		4	40
	Northern Europe		3	30
	Southern Europe		2	20
	Asia		2	20
Smoking Status	Never		6	60
	Previously		4	40
Drinking Status	Never		2	20
	Previously		1	10
	Occasionally		5	50
	Socially		1	10
	Regularly		1	10

4.1.2 Participant Clinical Characteristics

Table 4 provides the clinical characteristics of the participants. All participants in the study completed chemotherapy prior to the study start and had also undergone surgery for their breast cancer. An additional 80% of participants were previously treated with radiation therapy and one participant had completed hormone therapy treatment. Treatments that coincided with the study period included hormone therapy for 50% of participants and zoledronic acid infusions for one participant. BMI was variable among participants and a combined 70% of participants met criteria for placement in the overweight (40%) or obese (30%) BMI categories. Of the remaining participant BMIs, one fell into the underweight BMI category and two fell into the healthy BMI category.

Table 4. ***Clinical characteristics of the participants recruited from ACE***

Clinical Characteristic		Mean	Frequency	Percent
Completed Treatments				
	Chemotherapy		10	100
	Surgery		10	100
	Radiation Therapy		8	80
	Hormone Therapy		1	10
Current Treatments				
	Hormone Therapy		5	50
	Zoledronic Acid Infusions		1	10
Anthropometrics				
	Average Height (cm)	163.6 ±1.1		
	Average Weight (kg)	71.5 ±4.2		
	Underweight BMI (< 18.5)		1	10
	Healthy BMI (18.5-24.9)		2	20
	Overweight BMI (25-29.9)		4	40
	Obese BMI (≥ 30)		3	30

Abbreviations: Body Mass Index (BMI)

4.1.3 Godin's Leisure Time Exercise Questionnaire

The results of the Godin's Leisure Time Exercise Questionnaire are reported in **Table 5**. A significant difference in mean reported MET hours spent per week exercising was found between weeks 0 and 12 ($p=0.002$) and weeks 0 and 24 ($p=0.030$), but the change between week 12 and 24 was not significant ($p=0.535$). The increase from week 0 to week 12 coincides with the duration of the ACE program. The mean MET hours/week increased from 18.44 ±4.16 to 33.64 ±5.21. Reported strenuous, moderate, and mild exercise increased from 0 to 12 weeks; however, only the increase in strenuous exercise was statistically significant, changing from a mean 4.75 ±2.61 MET hours per week to 14.25 ±3.82 over this time ($p=0.016$). Reported flexibility and resistance exercise increased from week 0 to week 12, but the pattern was not significant ($p=0.276$ and $p=0.133$, respectively). No other statistical significance was found between timepoints or across exercise intensities and types.

Table 5. ***Godin's Leisure Time Exercise Questionnaire***

Week & Exercise Category	MET hours / week	P value 0 to 12 weeks	P value 12 to 24 weeks	P value 0 to 24 weeks
0 total	18.4 ±4.2	0.002		
12 total	33.6 ±5.2		0.535	
24 total	38.1 ±7.7			0.030
0 strenuous	4.7 ±2.6	0.016		
12 strenuous	14.2 ±3.8		0.280	
24 strenuous	20.5 ±7.0			0.075
0 moderate	8.8 ±2.2	0.301		
12 moderate	12.2 ±3.2		0.452	
24 moderate	10.2 ±1.9			0.626
0 mild	4.8 ±0.8	0.072		
12 mild	7.1 ±1.5		0.891	
24 mild	7.3 ±1.5			0.150
	Minutes / week			
0 resistance	34.4 ±20.4	0.113		
12 resistance	69.4 ±22.0		0.767	
24 resistance	63.3 ±17.1			0.224
0 flexibility	45.5 ±11.5	0.276		
12 flexibility	71.1 ±21.3		.816	
24 flexibility	74.4 ±16.1			0.078

Abbreviations: Metabolic Equivalent (MET)

4.1.4 Three-day Food Record Dietary Analysis

Results of the 3-day food record analysis for key nutrient intakes are provided in **Table 6**. There were no significant differences between 0 and 12 weeks for any macronutrients examined. Total caloric intake decreased significantly from 12 to 24 weeks, dropping from 2260.2 ±115.0 kcal/day to 1785.3 ±196.9 kcal/day (p=0.017). All macronutrient intakes displayed a trend to increase from 0 to 12 weeks followed by a decrease between 12 and 24 weeks. Omega-6 polyunsaturated fat intake increased from 2.5 ±0.4 grams/day at week 0 to 3.9 ±0.4 grams/day at week 12 (p=0.007). During the post-ACE exercise (washout) period, total fat intake decreased significantly from 107.4 ±9.0 grams/day to 74.2 ±12.9 grams/day (p=0.012) between weeks 12 and 24 and included significant decreases in polyunsaturated and monounsaturated fat. The decrease in saturated fat intake between week 12 and week 24 also approached significance (p=0.057). Vitamin E intake decreased significantly between week 12 and week 24 from 10.86 ±1.25 µg/day to 6.61 ±1.19 µg/day (p=0.029). The only other micronutrient that changed significantly between weeks 12 and 24 was selenium (p=0.021). No differences were not found for the remainder of nutrients analyzed which included cholesterol, calcium, copper, phosphorus, zinc, iron, magnesium, potassium, sodium, vitamin A, beta-carotene, B vitamins, vitamin C, vitamin D, Vitamin K, isoleucine, leucine, valine, butyric, alcohol, caffeine, and phytosterols.

Table 6. ***Dietary intake at baseline, 12 and 24 weeks***

Nutritional Measure	Time Point	Daily Average	Consecutive Time Point p value
Calories (kcal)	Baseline	2069.3 ±188.8	
	12 weeks	2260.2 ±114.9	0.404
	24 weeks	1785.2 ±196.9	0.017
Protein (g)	Baseline	79.5 ±7.9	
	12 weeks	87.3 ±5.0	0.352
	24 weeks	73.7 ±6.9	0.125
Carbohydrate (g)	Baseline	232.3 ±20.9	
	12 weeks	234.3 ±10.8	0.936
	24 weeks	210.4 ±21.2	0.359
Total Fat (g)	Baseline	92.6 ±11.2	

	12 weeks	107.4 ±9.0	0.338
	24 weeks	74.1 ±12.9	0.012
Fiber (g)	Baseline	23.9 ±4.4	
	12 weeks	29.7 ±4.9	0.352
	24 weeks	27.8 ±6.2	0.478
Omega-3 Polyunsaturated			
Fatty Acid (g)	Baseline	3.02 ±1.0	
	12 weeks	4.7 ±2.1	0.539
	24 weeks	2.9 ±1.2	0.123
Omega-6 Polyunsaturated			
Fatty Acid (g)	Baseline	2.5 ±0.4	
	12 weeks	3.9 ±0.3	0.007
	24 weeks	3.3 ±0.8	0.453
Polyunsaturated Fat (g)	Baseline	17.7 ±1.9	
	12 weeks	24.1 ±3.2	0.244
	24 weeks	13.1 ±2.0	.004
Monounsaturated Fat (g)	Baseline	30.5 ±3.5	
	12 weeks	38.1 ±3.2	0.153
	24 weeks	24.6 ±3.9	< 0.001
Saturated Fat	Baseline	29.9 ±3.9	
	12 weeks	36.0 ±5.3	0.387
	24 weeks	22.6 ±3.9	0.057
Vitamin E (mg)	Baseline	8.7 ±1.5	
	12 weeks	10.9 ±1.3	0.365
	24 weeks	6.6 ±1.2	0.029
Selenium (mcg)	Baseline	109.9 ±8.9	
	12 weeks	121.5 ±8.9	0.466
	24 weeks	100.9 ±8.0	0.021

4.1.5 Health-Related Quality of Life Results: FACT-G

The results of the Functional Assessment of Cancer Therapy – General questionnaire for each time point is summarized in **Table 7**. There were no significant changes in total score between baseline, week 12, and week 24. The average score out of 108 possible points was 84.6 ± 5.2 at baseline, 84.9 ± 4.8 at week 12, and decreased to 81.4 ± 5.3 at week 24, but this was not statistically significant. Scores in the four categories of well-being assessed in the questionnaire did not differ significantly between timepoints. Average emotional well-being score decreased from 16.2 ±1.1 to 14.9 ±1.6 between week 12 and week 24. The total possible score for emotional well-being in the questionnaire was 20 points.

Table 7. ***FACT-G questionnaire results***

Time Point	FACT-G Score (108 max)	Consecutive timepoint P value	Median Score	Sample Size	Completion %
Baseline	84.6 ± 5.2		87.5	N = 10	100
12 Weeks (post-ACE)	84.9 ± 4.8	0.869	92	N = 9	90
24 weeks (Follow-up)	81.4 ± 5.3	0.302	82	N = 9	90

Well-being Category	Timepoint	Categorical Score	Consecutive timepoint p value	Median Score
Physical	Baseline	23.9 ±1.3		25.0
	12 weeks	24.7 ±0.9	0.560	24.0
	24 weeks	24.9 ±0.7	0.816	25.0
Social	Baseline	23.7 ±1.2		23.5
	12 weeks	23.2 ±1.4	0.688	22.0
	24 weeks	22.4 ±1.4	0.065	21.0
Emotional	Baseline	16.4 ±0.9		17.0
	12 weeks	16.2 ±1.1	0.327	21.0
	24 weeks	14.9 ±1.6	0.291	14.0
Functional	Baseline	21.2 ±2.0		20.0
	12 weeks	18.3 ±1.5	0.091	18.0
	24 weeks	20.4 ±1.7	0.082	21.0

4.1.6 Gut Microbial Composition

4.1.6.1 Alpha Diversity

Alpha diversity, a measure of microbial diversity within a sample, is shown in Figure 4-1. Alpha diversity indices weight two components, richness (count of the number of different taxa in the sample) and Evenness (equitability of taxa frequencies in a sample). Gut microbial Evenness, shown as pooled participant data (**Figure 4-1 A1**; $p = 0.87$) or assessed as individual data **(Figure 4-1, A2),** did not differ between baseline (pre-exercise) and 12 weeks (post-exercise), nor between 12 weeks and the end of the washout period at 24 weeks ($p = 0.15$). Similarly, observed species, which measures richness, did not differ between pre- and post-samples **(Figure 4-1 B1, B2)**, nor between 12 and 24 weeks. The Shannon index, which equally weights evenness and richness, did not change over time (**Figure 4-1, C1, C2)**.

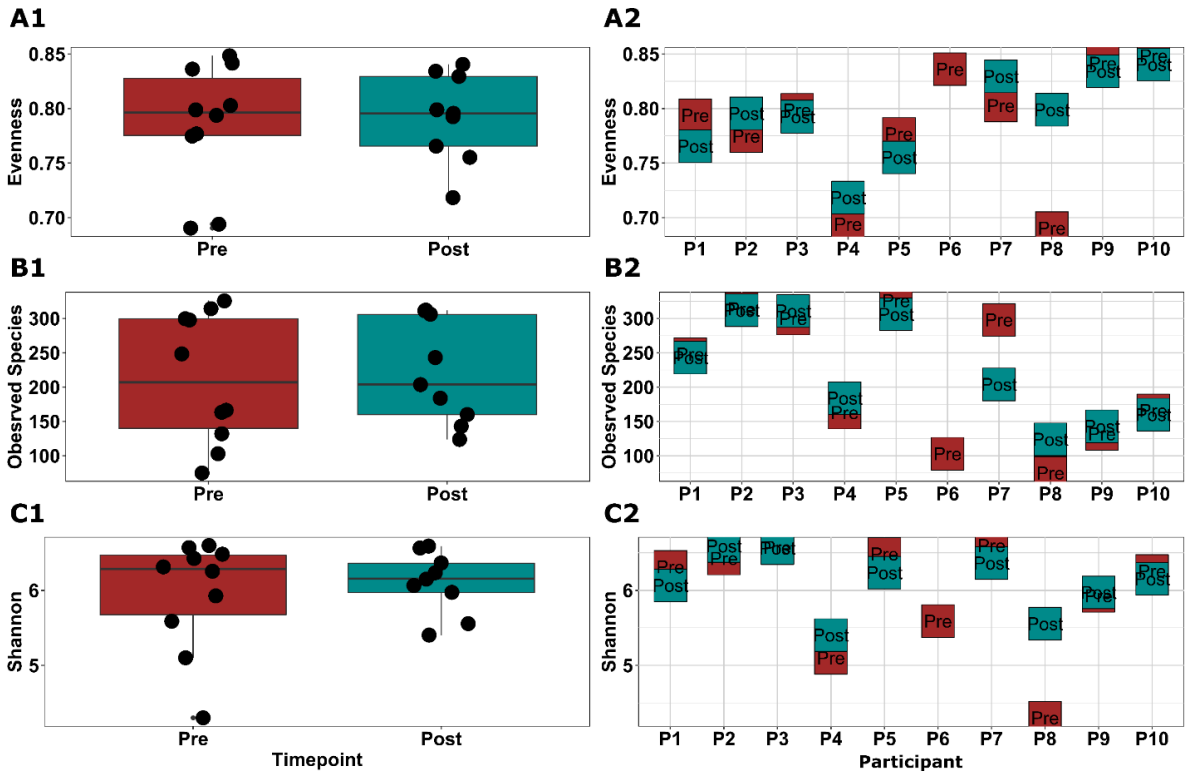


Figure 4-1. **Gut microbiota alpha diversity indices in ACE participants.**

Measures of alpha diversity pre (baseline) and post (12 weeks) ACE program. A1-C1 show pooled samples while A2-C2 visualize pre and post samples for each participant individually. The alpha diversity metrics include Pielou's Evenness (A1, A2), Observed Species (B1, B2), and Shannon (C1, C2). No statistical significance was found using Kruskal-Wallis pairwise tests.

4.1.6.2 Beta Diversity

Analysis of beta diversity, a measure of the similarity or dissimilarity between two samples, indicated that gut microbial communities did not differ significantly between baseline (pre-exercise) and 12 weeks (post-exercise) as shown in **Figure 4-2 A** ($p = 0.99$). An R value far below 1 ($R = 0.122$) indicated low dissimilarity between the communities. Had exercise significantly altered the microbial community composition, we would have expected to see distinct clustering of samples according to pre- and post-exercise time points. This was not the case and samples largely cluster together. Additional analysis was conducted to take age (over or under 65 years old) and BMI (underweight, healthy, overweight) into account as potential beta diversity influencers. There was no difference in community composition based on age category (**Figure 4-2 A**; $p = 0.205$) but there was a statistically significant difference in community composition found when participant BMI was taken into account **(Figure 4-2 B)**. There was a significant ($p=0.008$) dissimilarity in gut microbial composition ($R=0.27$) according to BMI **(Figure 4-2 B)** which was likely driven by the underweight participant. Of note, overweight and obese BMI category groups were combined due to the similar effects of these characteristics on gut microbial composition.

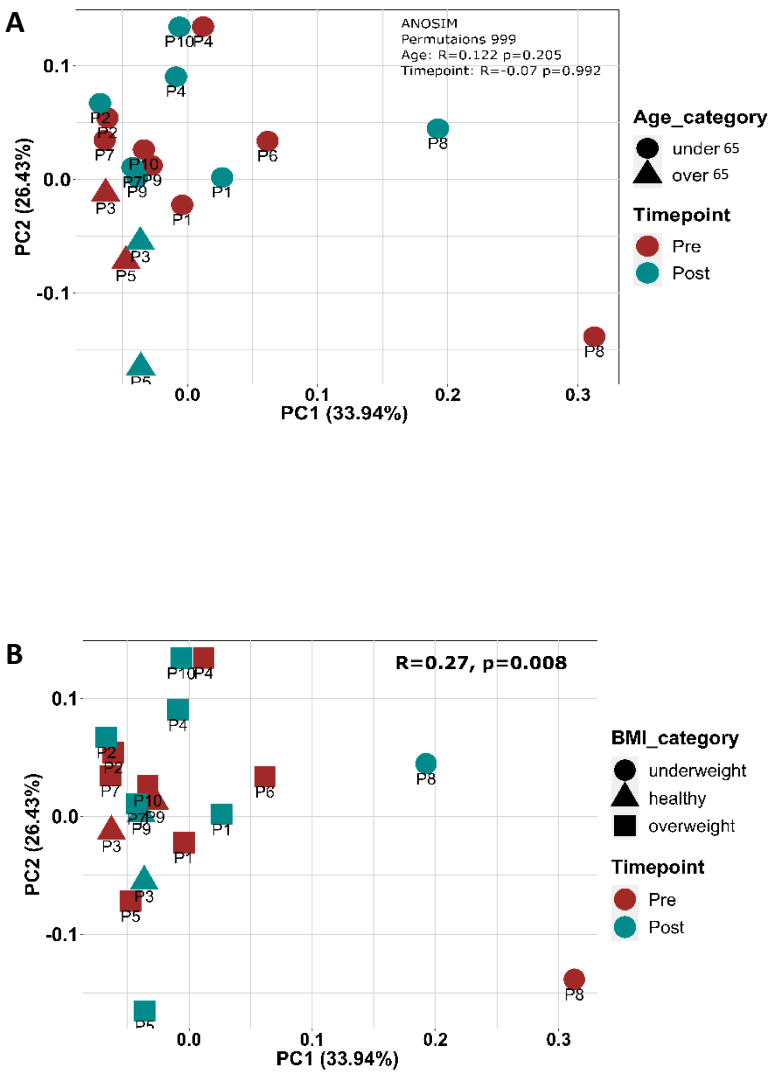


Figure 4-2. **Gut microbiota beta diversity analyses on ACE participants.**

Beta diversity was measured by Weighted UniFrac Distance Matrix-based PCoA for pre- (baseline) and post- (12 weeks) exercise in the ACE program and analyzed with ANOSIM. Age (over or under 65) and time point did not significantly influence community diversity (A). BMI category significantly influenced community diversity (p =0.008) (B).

4.1.6.3 Taxonomic Community Characteristics

The relative abundance of three health-associated genera (*Bifidobacterium, Faecalibacterium, Roseburia)* and three inflammation-associated genera (*Enterobacteriaceae, Klebsiella, Escherichia-Shigella*) at pre-exercise and post-exercise time points are presented in **Figure 4-3 A**. No statistically significant differences were found in the relative abundance of these bacteria between time points **(Figure 4-3 A)**. **Figure 4-3 B** presents the results of DESeq2 analysis to investigate whether any microbiota differed significantly between pre- and post-exercise time points with participant accounted for as a covariate in the analysis. The relative abundance of *Dialister*, *Oscillospiraceae*, and *Paraprevotella* was significantly higher in post-exercise samples compared to pre-exercise samples ($p < 0.01$) **(Figure 4-3 B)**. Pre-exercise samples exhibited enhanced relative abundance of *Pseudomonas*, *Gastranaerophilales*, *Barnesiella*, *Phascolarctobacterium*, and *Butyribrivio* ($p < 0.01$) **(Figure 4-3 B)**. The absolute log2FoldChange value represents the magnitude of the difference in relative abundance.

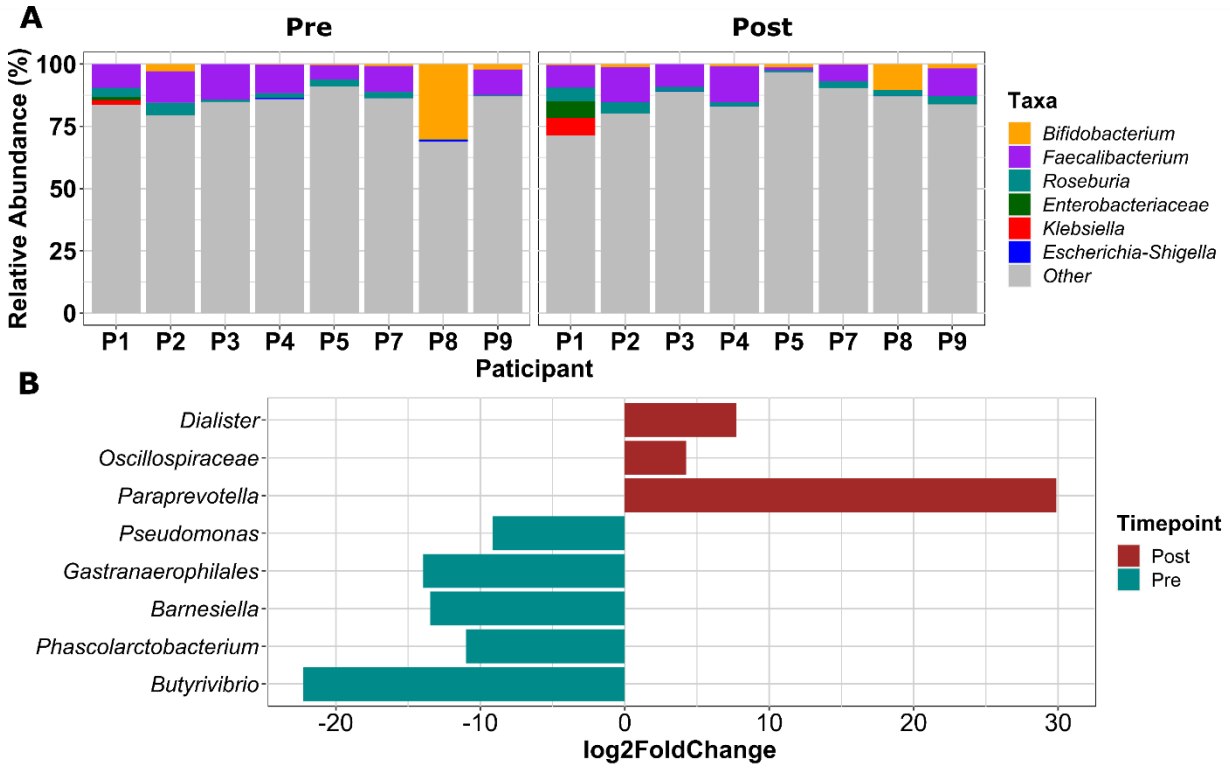


Figure 4-3. **Gut microbiota differential abundance analyses from 16S rRNA sequencing.** Relative abundance of three health-associated and three inflammation-associated microbiota were analyzed using DESeq2, resulting in no significant differences (A). Eight microbiota were significantly differentially abundant between pre- (baseline) and post- ACE (12 weeks) samples when analyzed with DESeq2 ($p < 0.01$) (B).

4.1.7 Correlations Between Nutrient Intake or Emotional Well-being and Diversity Metrics

Spearman's correlational analysis was completed to investigate whether our secondary outcomes of interest were correlated with alpha diversity metrics. Key macronutrients and nutrient subsets were chosen based on their foundational role in dietary intake, known ability to shape the microbiota, or because they demonstrated significant differences in average intake between time points. No significant correlations were found between these factors and either Observed Species, Evenness, or Shannon diversity (**Table 8**). At baseline, total fat intake demonstrated a trend for positive correlation with Pielou's Evenness index ($p=0.07$). The same trend toward a positive correlation with Evenness was seen with both omega-3 fatty acid intake ($p=0.08$) and omega-6 fatty acid intake ($p=0.09$) at baseline. At week 12, protein intake exhibited a trend toward negative correlation with observed species ($p=0.08$). No other nutrients were correlated with alpha diversity metrics at any of the three time points. Emotional well-being was not correlated with alpha diversity metrics at any time point (**Table 8**). To investigate the potential influence of nutrient intake factors on beta diversity, Adonis analysis was completed. The analysis was based on Weighted UniFrac distance matrix data across all three time points for all participants. When samples were categorized by allocating participants into 3 equal groups (low, medium, high intake) according to numerically ordered total fat values, fat intake group demonstrated a significant 13.7% contribution to variance seen in beta diversity between samples ($p = 0.034$). The low total fat intake group averaged 55.6 ±6.0 grams/day, the medium group averaged 94.6 ±2.2 grams/day, and the high intake group averaged 138.3 ±9.0 grams/day. No other nutrients listed in **Table 8** or combinations of factors contributed significantly to variance in gut microbial communities as measured by Adonis analysis on the Weighted UniFrac matrices.

Table 8. ***Spearman's correlational analysis between exploratory variables and alpha diversity***

Spearman's correlation coefficient

Alpha diversity	Time point	Calories	Protein	Fiber	Total fat	Omega-3	Omega-6	Saturated fat	Emotional well-being
Evenness	Overall	0.11	0.07	0.19	0.19	0.3	0.27	0.17	0.1
	Baseline	0.4	0.16	0.4	0.59	0.58	0.56	0.56	0.29
	Week12	0.39	0.33	0.36	0.27	0.42	0.28	0.24	0.37
	Week24	-0.17	0.01	-0.37	-0.24	-0.04	-0.11	-0.3	0.06
Observed species	Overall	-0.12	-0.25	-0.07	-0.11	-0.2	-0.13	-0.09	-0.1
	Baseline	-0.1	-0.33	-0.12	-0.05	-0.32	0.05	-0.18	-0.11
	Week12	-0.34	-0.61	-0.38	-0.16	-0.3	0.02	-0.18	-0.2
	Week24	-0.18	-0.06	0.17	-0.36	-0.11	-0.39	-0.06	-0.11
Shannon diversity	Overall	0.04	-0.12	0.1	0.08	0.04	0.12	0.07	-0.05
	Baseline	0.2	-0.08	0.19	0.33	0.11	0.4	0.21	0.06
	Week12	0.01	-0.3	-0.11	0.11	0	0.22	0.04	0.01
	Week24	-0.19	-0.06	0.11	-0.4	0.01	-0.34	-0.14	-0.13

p value of Spearman's correlation coefficient

Alpha diversity	Time point	Calories	Protein	Fiber	Total fat	Omega-3	Omega-6	Saturated fat	Emotional well-being
Evenness	Overall	0.57	0.74	0.34	0.34	0.13	0.17	0.39	0.62
	Baseline	0.26	0.65	0.26	0.07	0.08	0.09	0.10	0.42
	Week12	0.31	0.39	0.34	0.48	0.26	0.46	0.54	0.33
	Week24	0.69	0.97	0.37	0.57	0.93	0.80	0.47	0.89
Observed species	Overall	0.57	0.21	0.73	0.57	0.31	0.53	0.65	0.63
	Baseline	0.78	0.36	0.75	0.89	0.36	0.89	0.62	0.77
	Week12	0.38	0.08	0.32	0.69	0.43	0.95	0.64	0.60
	Week24	0.67	0.88	0.69	0.38	0.79	0.34	0.89	0.79
Shannon diversity	Overall	0.85	0.56	0.61	0.70	0.83	0.55	0.72	0.82
	Baseline	0.57	0.83	0.61	0.35	0.76	0.25	0.55	0.86
	Week12	0.98	0.43	0.78	0.78	0.99	0.57	0.91	0.98
	Week24	0.65	0.89	0.79	0.33	0.99	0.42	0.75	0.75

4.1.8 Choosing an FMT Donor for the Germ-free Murine Study

A single participants' samples from baseline and 12 weeks (post-exercise intervention) were selected to use in the germ-free study as FMT donor material. The selection decision was made based on identification of a response to the exercise intervention as evidenced by the gut microbiota analyses. Although there were no profound shifts in microbiota due to exercise, likely in part due to our low sample size, we examined individual responses to exercise in order to select a donor participant. A beneficial response was defined by us as an increase in alpha diversity accompanied by an increase in health-associated gut microbiota and/or decrease in inflammation-associated gut microbiota. Participant 4 was selected as the FMT donor because their pre- and post- samples met the response to exercise criteria we specified. **Figure 4-4 A** demonstrates that relative abundance of *Faecalibacterium* and *Roseburia* increased from baseline to week 12 in participant 4's samples. Although not statistically significant, **Figure 4-4 B** and **Figure 4-4 C** show the increase in alpha diversity between baseline and week 12 in participant 4's samples as measured by Shannon index and Pielou's Evenness index. Taken together, participant 4's pre- and post-exercise samples indicated a promising gut microbial response to exercise and were chosen for investigation of the potential physiological effects of this response in a germ-free mouse model. In addition to gut microbial profile, a description of the selected participants' characteristics and questionnaire outcomes is provided in **Table 9**. The participant was 59 years of age with a BMI of 25.1. The participants' fiber intake increased slightly by 3.3g/day between baseline and 12 weeks but remained under the daily recommended intake (DRI) for their sex and age category (21g/day). Total fat intake increased by 39.1g/day between baseline and week 12 and was characterized by an increase in both saturated and polyunsaturated fat intakes. The participant's emotional well-being score increased by 1 point between baseline and week 12 and decreased a clinically significant amount between 12 weeks and 24 weeks. Their total physical activity per week increased from 9 MET hours at baseline to 13.5 MET hours at week 12. The individual's class attendance was 75% compared to the 87.6% average and their time spent doing resistance exercise increased from 0 minutes to 60 minutes per week between baseline and week 12 (**Table 9**).

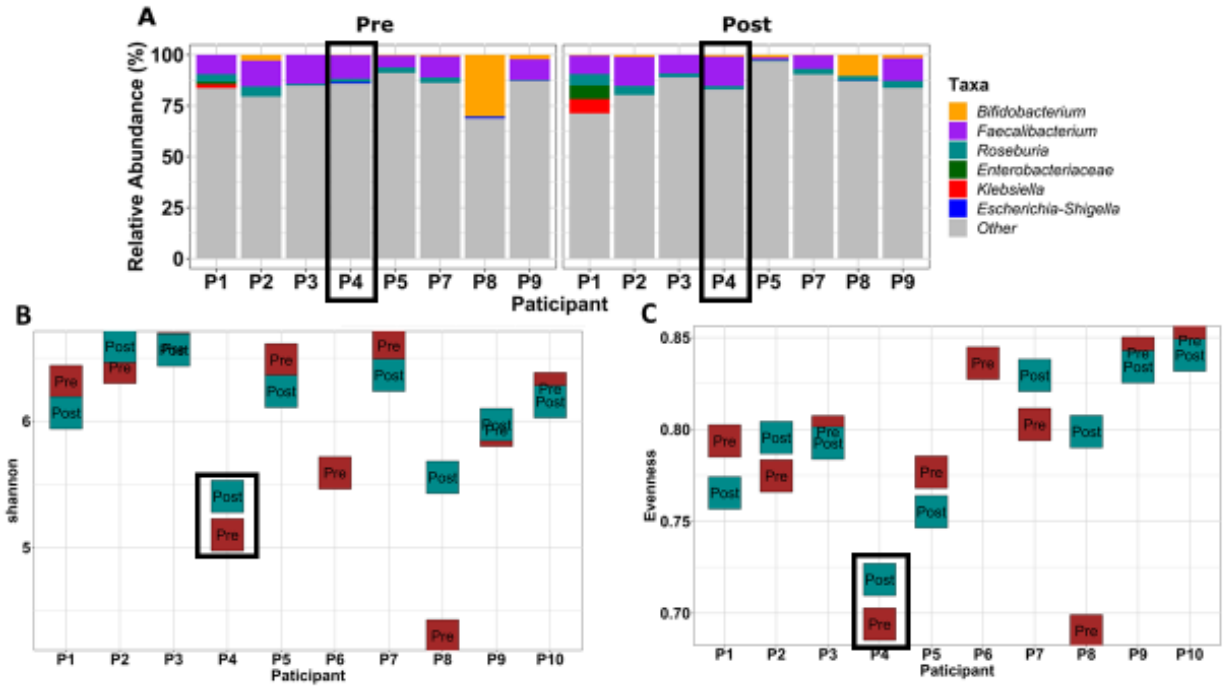


Figure 4-4. **FMT donor selection informed by relative abundance and alpha diversity.**

Relative abundance of three health-associated and three inflammation-associated genera were analyzed. Participant four exhibited an increase in *Faecalibacterium* from baseline to 12 weeks (ns) (A). Alpha diversity as measured by Shannon (B) and Evenness (C) indices increased from baseline to 12 weeks in participant four (ns).

Table 9. ***Profile of the participant chosen as the FMT donor***

Metric	Baseline	Week 12	Week 24
Age (years)	59		
BMI (kg/m^2)	25.06		
Calories (kcal/day)	2393	2484	2598
Fiber (g/day)	16.2	19.5	24.6
Total Fat (g/day)	88.6	127.7	140.4
Total Protein (g/day)	114.2	89.1	85.7
Total Carbohydrates (g/day)	257.9	200.5	215.8
Saturated Fat (g/day)	30.5	40.1	37.3
Omega-3 FA (g/day)	1.68	2.06	1.14
Omega-6 FA (g/day)	2.67	3.20	3.40
FACT-G Total	69	68	64
Physical	22	23	24
Functional	15	10	13
Social	19	18	16
Emotional	13	14	11
GLTAQ Total (MET hours per week)	9	13.5	13.5
Mild (MET hours/week)	9	13.5	4.5
Moderate (MET hours/week)	0	0	0
Strenuous (MET hours/week)	0	0	9
Resistance (minutes)	0	60	0
Flexibility (minutes)	0	0	0
Completed Treatments: Surgery, Chemotherapy, Radiation **Smoking Status:** Previously **Drinking Status:** Regularly **Class Attendance:** 75%			

Abbreviations: Godin's Leisure Time Exercise Questionnaire (GLTEQ), Metabolic Equivalent (MET), Body Mass Index (BMI), Fatty Acid (FA)

4.2 Results of the Germ-free Mouse Study Investigating the Impact of Exercise-Responsive Gut Microbiota in a Murine Model of Breast Cancer Treatment

4.2.1 Fluid Intake

Since the oligofructose supplement was delivered dissolved in water, fluid intake was recorded over the course of the study for the OFS and non-OFS groups. The results of analysis of daily average fluid intake per mouse are presented in **Table 10**. Fluid intake did not differ between mice receiving the oligofructose solution and those who had water (p = 0.192).

Table 10. ***Fluid intake in treatment groups***

Oligofructose Status	**Average Fluid Intake (ml/mouse/day)**	**Number of Intake Periods Recorded**	**Paired Samples T-test p value**
Non-OFS	5.84 ±0.38	4	
OFS	6.53 ±0.42	7	0.192

4.2.2 Tumor Volume

The results of caliper measurements to assess tumor volume over the course of the study are presented in **Figure 4-5**. Measurements were taken on days 13, 16, 19, 22, 24, and at endpoint which fell on either day 27 or 28. The overall pattern of average tumor volume was consistently higher in the BCW0 mice compared to BCW12 and BCW12OFS mice. There was a statistically significant difference in tumor volume between BCW0 and BCW12 (p=0.034) as well as between BCW0 and BCW12OFS (p=0.006) on day 16 of the study. On day 22, tumor volume was significantly smaller in the BW12OFS group compared to BCW0 (p=0.043). No difference between groups was found on days 13, 19, 24, or at endpoint; however, the lower tumor volume in BCW12OFS mice approached significance compared to BCW0 at endpoint (p = 0.055).

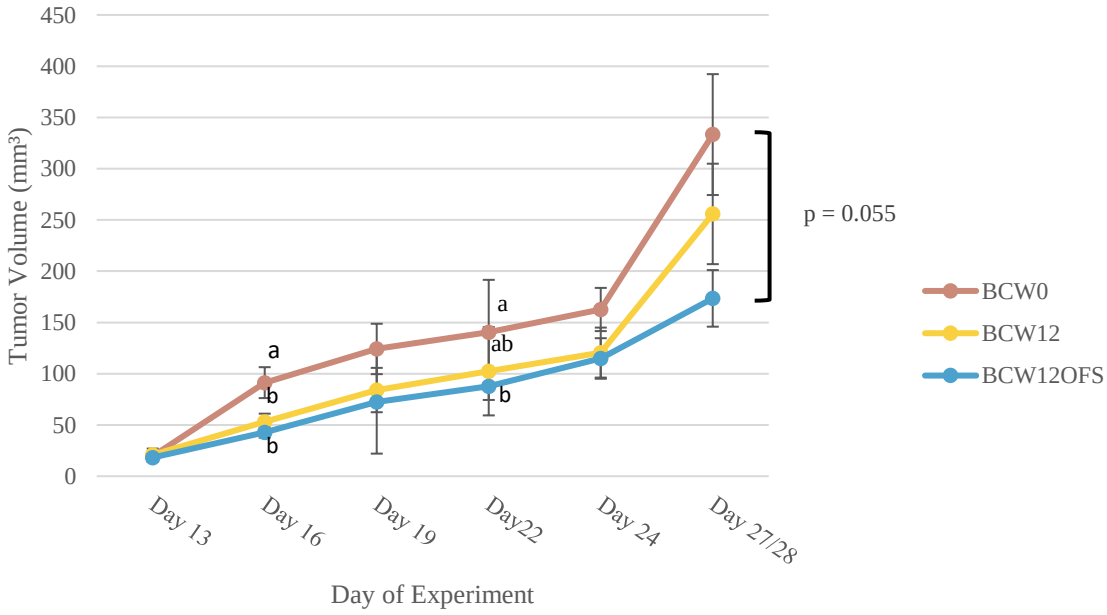


Figure 4-5. **Tumor volume over time.**

Average tumor volume at each measurement time point is plotted for each group. Average volume differed significantly between groups on day 16 and day 22. Values without a common superscript are significantly different (p<0.05; i.e. 'a' is different from 'b' but 'ab' is not different from 'a' or 'b'). Data are presented as mean ± SEM.

4.2.3 Mouse Gut Microbial Composition

4.2.3.1 Mouse Gut Microbial Alpha Diversity

Alpha diversity as measured by Observes Species, Shannon index, and Pielou's Evenness index are presented in **Figure 4-6**. No significant differences in observed species were found between groups at any of the four time points **(Figure 4-6 A)**. On day 5, Pielou's Evenness was significantly lower in BCW12OFS compared to BCW12 (0.02) **(Figure 4-6 B)** and lower in BCW0 compared to BCW12 (p=0.014) **(Figure 4-6 B)**. On day 13, which fell after tumor cell injection, BCW12OFS maintained significantly lower Evenness than BCW12 ($p < 0.001$) and also BCW0 (p=0.006) while the difference between BCW0 and BCW12 was no longer significant **(Figure 4-6 B)**. Post-chemotherapy treatment on day 22, Evenness was once again significantly lower in BCW12OFS compared to both BCW12 ($p < 0.001$) and BCW0 (p=0.021) **(Figure 4-6 B)** and BCW0 was lower than BCW12 (p=0.011) **(Figure 4-6 B)**. At endpoint, Evenness became more similar across groups; however, BCW12OFS mice remained lower than BCW12 mice (p=0.015) **(Figure 4-6 B)**. Shannon diversity did not differ between groups on day 5 **(Figure 4-6 C)**. Post tumor cell injection the BCW12OFS group exhibited decreased Shannon diversity compared to both BCW12 (p=0.005) and BCW0 (p=0.002) **(Figure 4-6 C)** which was no longer seen on day 22 and at endpoint **(Figure 4-6 C)**.

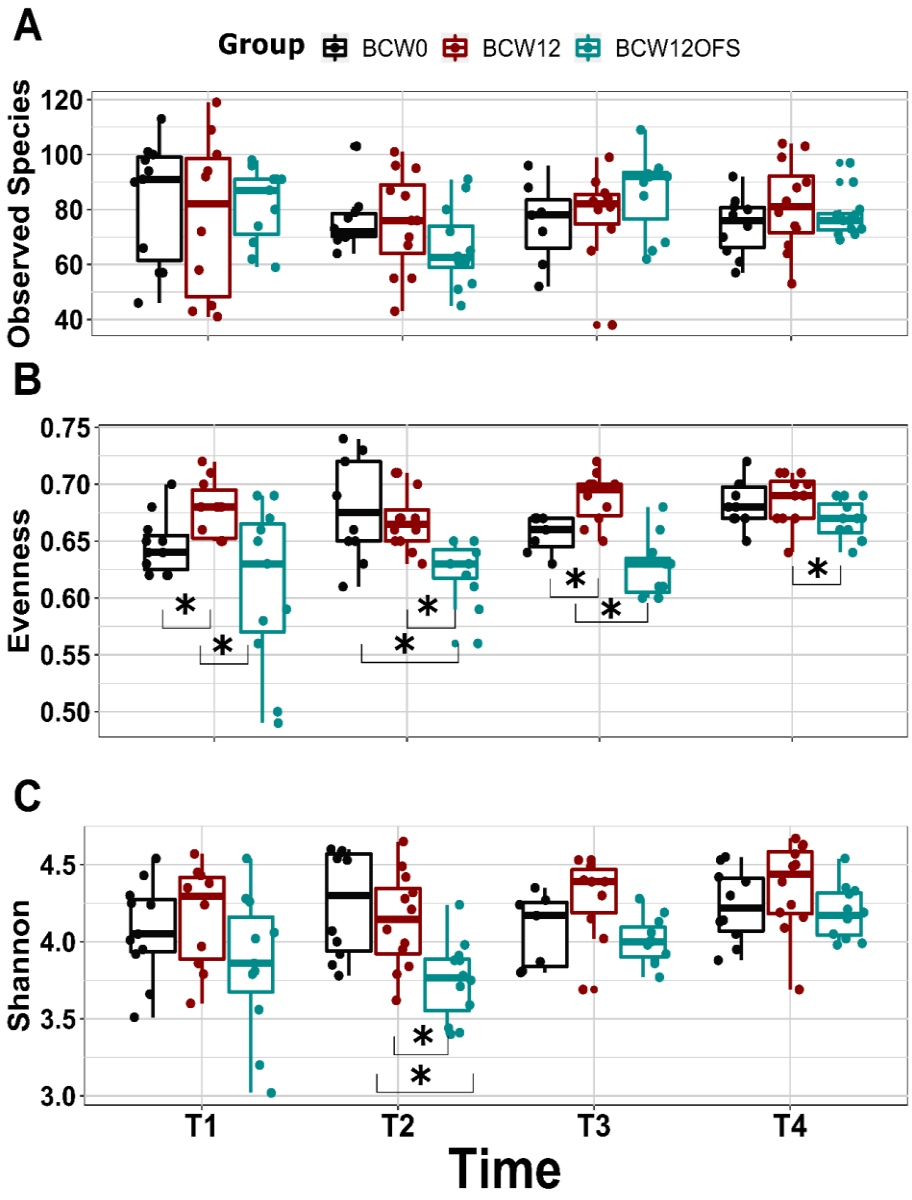


Figure 4-6. **Mouse gut microbial alpha diversity across time points.**

Alpha diversity metrics measured at T1 (day 5), T2 (day 13), T3 (day 22), and T4 (day 27/28) and compared between groups using Kruskal-Wallis pairwise tests (A-C). Observed Species did not differ significantly between groups (A). Evenness differed significantly between groups at each timepoint (B). Shannon diversity differed significantly between groups at T2 (day 13) (C). * indicate a significant difference ($p < .05$). Data are presented as ±SEM and each point represents an individual mouse's results.

4.2.3.2 *Mouse Gut Microbial Beta Diversity*

Beta diversity is presented in **Figure 4-7** . **Figure 4-7 A** presents a comparison of beta diversity between groups at each time point while **Figure 4-7 B** shows the within-group comparisons over all of the time points. In **Figure 4-7 A** it is apparent that the gut microbial community in BCW12OFS mice differed significantly when compared to BCW0 and BCW12 at each of the four time points ($p < 0.05$). The gut microbial community in BCW12 mice differed significantly from BCW0 on day 5, day 22, and at endpoint ($p < 0.05$) but did not differ significantly from BCW0 on day 13 which was after tumor cell injection **(Figure 4-7 A)**. A significant difference in beta diversity between all four times is evident within each group as displayed in **Figure 4-7 B** ($p <0.05$) indicating that the microbial community was altered from baseline to when tumor cells were injected to when chemotherapy was administered to the end of the study.

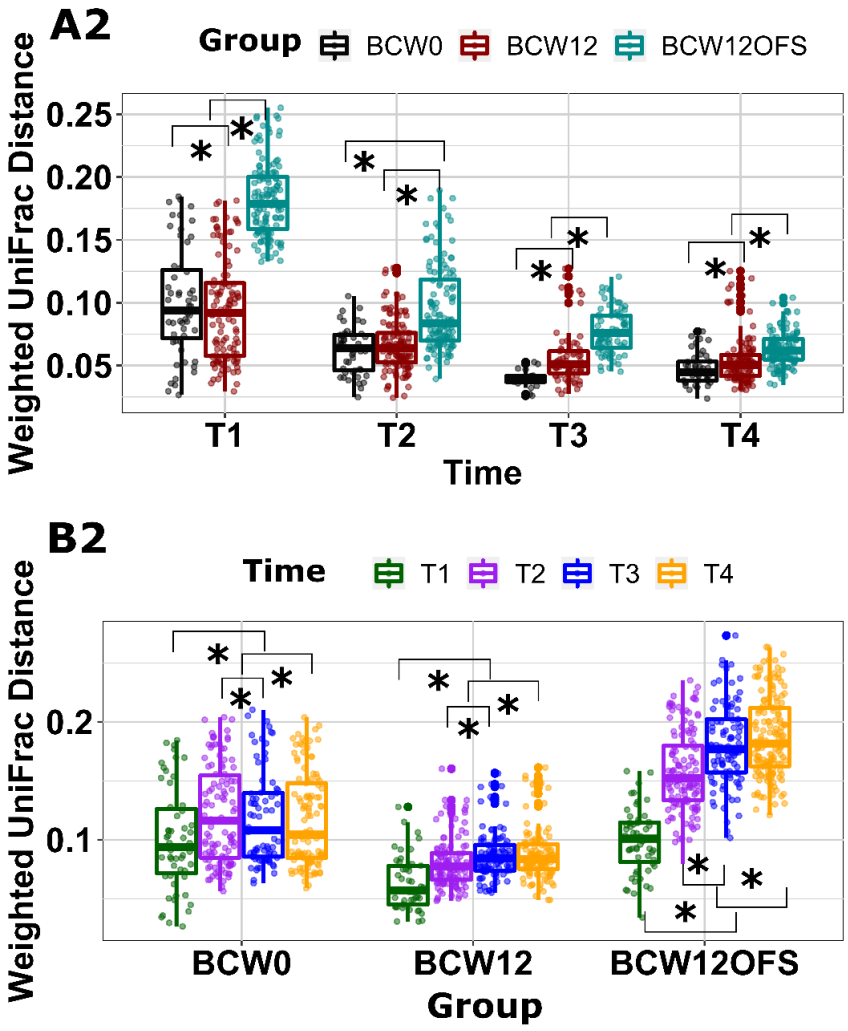


Figure 4-7. **Mouse gut microbial beta diversity across time points.**

Beta diversity as measured by Weighted UniFrac Distance at T1 (day 5), T2 (day 13), T3 (day 22), and T4 (day 27/28) and analyzed using ANOSIM to detect significant between-group community differences at each time are presented (A). Weighted UniFrac Distance analyzed with ANOSIM to detect community differences over time within each group are also presented (B). * indicate a significant difference ($p < .05$). Data are presented as ±SEM.

4.2.3.3 *Mouse Gut Microbial Differential Abundance Analysis*

Differential abundance analysis with DESeq2 was completed to determine differences in taxonomic composition at day 13, which was after tumor cell injection **(Figure 4-8 A),** and at day 22, which was after chemotherapy conclusion **(Figure 4-8 B)**. Differentially abundant genera with $p < 0.001$ are displayed. On day 13, BCW12OFS had significantly greater abundance of *Tyzerella*, *Ruminococcus gauvreauii*, and *Eubacterium hallii* compared to BCW0 **(Figure 4-8 A)**. At the same time point, BCW12 also showed enrichment in *Tyzzerella* and *Ruminococcus gauvreauii* compared to BCW0 **(Figure 4-8 A)**. BCW12 had significantly greater abundance of *Enterococcus* and decreased abundance of *Bifidobacterium* compared to BCW12OFS at day 13 **(Figure 4-8 A)**. On day 22, following chemotherapy, the greatest number of bacteria were found to be significantly differentially abundant between groups **(Figure 4-8 B)**. BCW12OFS mice exhibited greater *Enterococcus*, *Blautia*, *Parasutterella*, *Eubacterium iraeum*, *Colidextribacter*, *Tyzzerella*, *Ruminococcus gauvreauii*, and *Lachnospiraceae* alongside lesser abundance of *Hungatella*, *GCA-900066575*, *Anaerostipes*, *Coprococcus*, *Phocea*, and *Ruminococcus gnauvus* compared to BCW0 **(Figure 4-8 B)**. On day 22, BCW12 similarly presented with increased abundance of *Eubacterium iraeum*, *Colidextribacter*, *Tyzzerella*, *Ruminococcus gauvreauii*, and *Lachnospiraceae* compared to BCW0 **(Figure 4-8 B)**. Compared to BCW12OFS, the BCW12 group exhibited greater abundance of Incertae_Sedis, Ruminococcus torques, Hungatella, *GCA-900066575*, and *Anaerostipes*, coinciding with significantly lower abundance of *Enterococcus*, *Blautia*, and *Parasutterella* **(Figure 4-8 B)**.

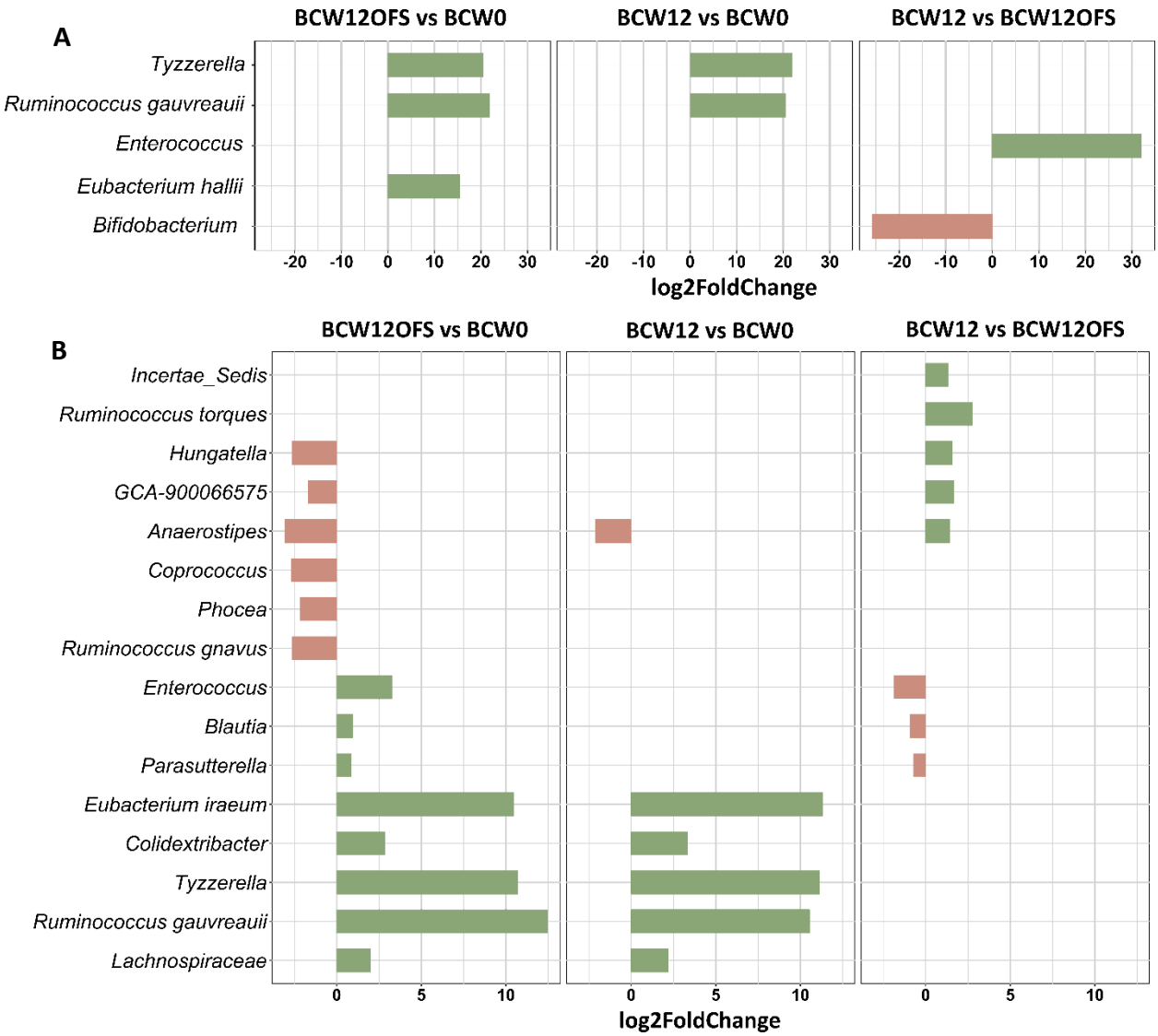


Figure 4-8. **Mouse gut microbiota differential abundance analyses.**

Taxa found to be differentially abundant between groups are represented by log2FoldChange from DESeq2 analysis. The group serving as the base for comparison comes after "vs" (i.e. BCW12OFS vs BCW0 is showing values for the bacteria in BCW12OFS compared to BCW0). Positive log2FoldChange indicates greater relative abundance, while a negative value indicates lesser relative abundance. Significantly differential abundance for each comparison at day 13 are presented (A). Significantly differential abundance for each comparison at day 22 are also presented (B). Only differences with $p < 0.001$ from DESeq2 analysis are represented.

4.2.4 Ileal and Colonic Tight Junction Proteins

Results of RT-PCR analysis for tight junction proteins involved in maintaining intestinal barrier function from both ileal and colonic tissues are presented in **Table 11**. mRNA expression of Occludin, ZO-1, and Claudin-3 were analyzed. No significant differences were found in the ileal or colonic expression of the three genes.

Table 11. ***Gene expression of ileal and colonic tight junction proteins***

Tissue Type	Tight Junction Protein	BCW0 (group1)	BCW12 (group2)	BCW12OFS (group3)	P value 1&2	P value 2&3	P value 1&3
Ileal							
	ZO-1	102.71 ±13.56	97.13 ±11.07	91.47 ±12.13	1.00	1.00	1.00
	Occludin	3.77 ±0.58	3.14 ±0.47	2.56 ±0.52	1.00	1.00	0.386
	Claudin-3	5.66 ±0.81	5.77 ±0.66	3.95 ±0.73	1.00	0.225	0.386
Colonic							
	ZO-1	126.96 ±16.29	140.05 ±14.74	164.16 ±14.11	1.00	0.741	0.285
	Occludin	2.71 ±0.33	2.76 ±0.30	2.72 ±0.29	1.00	1.00	1.00
	Claudin-3	7.11 ±0.84	7.43 ±0.76	6.99 ±0.73	1.00	1.00	1.00

4.2.5 *Tumor and Serum Cytokine Levels*

Given that inflammation is a critical component of tumor progression[199] and the microbiota can affect inflammation in the host[200], we examined tumor and serum cytokine levels (**Tables 12 & 13)**. Tumor cytokines which exhibited statistically significant differences in levels between groups are visualized in **Figure 4-9**. There were no significant differences in levels of Eotaxin, G-CSF, GM-CSF, IFNy, KC, MIP-1α, MIP-2, IL-1α, IL-1β, IL-6, IL-5, IL-4, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, LIF, M-CSF, or MIG. In **Table 12** it is evident that several targets exhibit a trend toward differential levels between groups. For example, although the difference is not statistically significant, tumor KC (CXCL1) levels in BCW0 mice were increased compared to BCW12 mice ($p = 0.055$) and BCW12OFS mice ($p = 0.08$) **(Table 12)**. Tumor IL-9 levels were significantly higher in BCW0 mice compared to both BCW12 and BCW12OFS mice ($p < 0.001$) whereas levels between BCW12 and BCW12OFS did not differ significantly as seen in **(Figure 4-9 B)**. Similarly, MCP-1 levels were increased in BCW0 tumors compared to that of BCW12 ($p=0.007$) and BCW12OFS ($p=0.002$), which did not differ from each other **(Figure 4-9 A)**. Tumor TNFα levels were significantly higher in BCW12OFS mice compared to BCW0 mice ($p=0.009$) and approached significance compared to BCW12 ($p = 0.071$) **(Figure 4-9 C)**. Tumor levels of VEGF were significantly higher in BCW0 mice compared to both BCW12 ($p=0.001$) and BCW12OFS ($p < 0.001$)**(Figure 4-9 D)**. **Figure 4-9 E** shows that IP-10 levels were significantly increased in BCW0 compared to BCW12 tumors ($p=0.036$) and approached significance in comparison to BCW12OFS tumor levels ($p = 0.068$). Levels of tumor RANTES were significantly higher in BCW12OFS mice compared to both BCW0 mice ($p=0.005$) and BCW12 mice ($p=0.037$) as displayed in **Figure 4-9 F**.

Serum cytokines were far more refractory to change compared to tumor cytokine levels. The results of serum cytokine analysis are presented in **Table 13**. There were no significant differences in serum levels of any of the cytokines apart from serum levels of LIX (CXCL5) which were significantly higher in BCW12 mice compared to BCW0 ($p < .05$).

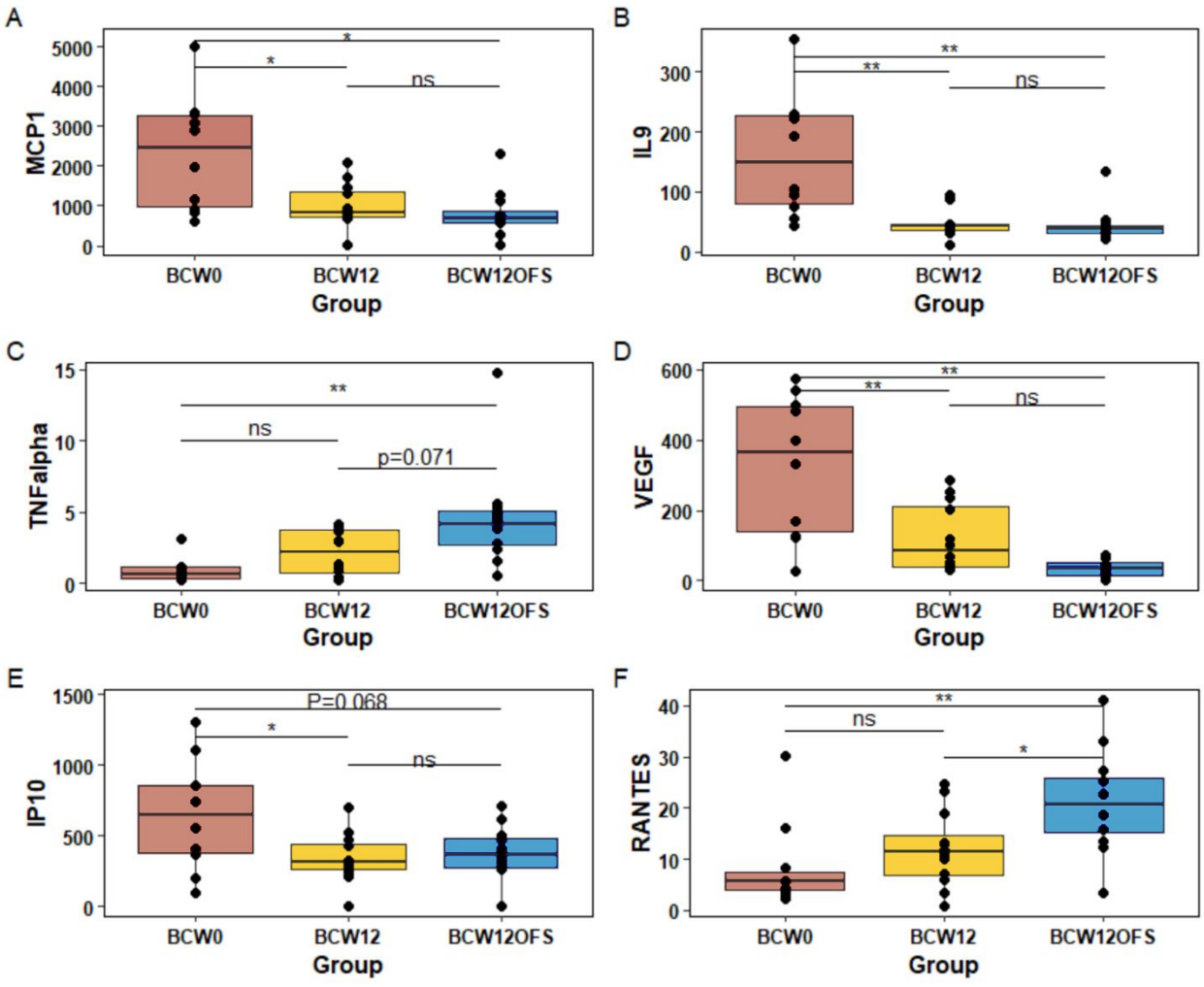


Figure 4-9. **Levels of key tumor cytokines.**

Tumor cytokine levels as measured by multiplex assay which differed significantly between groups are presented (A-F). These include levels of MCP-1 (A), IL-9 (B), TNFα (C), VEGF (D), IP-10 (E), and RANTES (F). * Indicate a significant difference of $p < .05$ and ** indicate a significant difference of $p < .01$. Data are presented as ±SEM and each point represents an individual mouse's results.

Table 12. ***Tumoral cytokine levels***

Tumor Cytokine (pg/mL)	BCW0 (group1)	BCW12 (group2)	BCW12OFS (group3)	P value 1&2	P value 2&3	P value 1&3
Eotaxin	113.5 ±24.4	136.3 ±22.3	154.2 ±22.3	1.00	1.00	0.679
G-CSF	579.6 ±220	26.8 ±200.8	148.0 ±200.8	0.219	1.00	0.472
GM-CSF	147.8 ±62	7.4±62	15.2 ±65.8	0.369	1.00	0.467
IFNy	6.4 ±6.2	11.9 ±4.4	17.3 ±3.6	1.00	1.00	0.418
IL-9	160.5 ±18.5	46.7 ±16.9	45.0 ±16.9	< .001	1.00	< .001
MCP-1	2317.3 ±288.0	1016.3 ±262.9	804.3 ±262.9	0.007	1.00	0.002
KC	454.7 ±116.4	61.8 ±106.3	87.9 ±106.3	0.055	1.00	0.08
MIP-1α	236.8 ±46.8	96.2 ±42.7	141.3 ±42.7	0.102	1.00	0.426
MIP-2	3595.9 ±1235.8	119.7 ±1235.8	282.2 ±1128.1	0.169	1.00	0.172
TNFα	0.93 ±0.9	2.2 ±0.7	4.5 ±0.7	0.844	0.071	0.009
VEGF	327.8 ±38.92	122.3 ±35.5	34.4 ±35.5	0.001	0.27	< .001
IL-1α	98.4 ±40.1	23.3 ±36.6	51.4 ±36.6	0.531	1.00	1.00
IL-1β	6.6 ±1.2	4.2 ±1.1	7.6 ±1.0	0.468	0.099	1.00
IL-6	3.0 ±5.6	9.8 ±5.3	19.2 ±5.1	1.00	0.65	0.125
IL-5	2.4 ±0.8	3.5 ±1.0	1.5 ±1.0	1.00	0.502	1.00
IL-4	1.6 ±0.6	1.1 ±0.6	1.8 ±0.6	1.00	1.00	1.00
IL-10	5.4 ±1.8	3.2 ±1.8	1.2 ±7.1	1.00	1.00	0.322
IL-12p40	0.66 ±0.3	0.3 ±0.1	0.5 ±0.2	0.891	1.00	1.00
IL-12p70	30.5 ±2.11	27.8 ±1.9	26.1 ±1.9	1.00	1.00	0.391
IL-13	19.35 ±4.29	12.5 ±5.1	7.7 ±5.5	0.946	1.00	0.331
IL-15	4.24 ±0.70	3.2 ±0.8	2.5 ±0.7	0.937	1.00	0.263
IP-10	651.3 ±82.6	353.5 ±75.4	382.9 ±75.4	0.036	1.00	0.068
LIF	66.5 ±25.5	7.3 ±24.3	9.5 ±24.3	0.309	1.00	0.349
M-CSF	137.8 ±52.7	17.4 ±48.1	21.7 ±50.3	0.306	1.00	0.365
MIG	331.3 ±69.8	245.5 ±63.7	354 ±63.7	1.00	1.00	0.714
RANTES	8.5 ±2.8	11.9 ±2.5	21.4 ±2.5	1.00	0.037	0.005

Table 13. ***Serum cytokine levels***

Serum Cytokine (pg/mL)	BCW0 (group1)	BCW12 (group2)	BCW12OFS (group3)	P value 1&2	P value 2&3	P value 1&3
Eotaxin	183.1 ±18.1	220.0 ±16.4	216.1 ±15.7	0.436	1.00	0.549
G-CSF	3128.6 ±1037.7	265.6 ±938.6	716.1 ±898.7	0.150	1.00	0.268
IFNy	2.8 ±0.7	2.4 ±0.7	3.1 ±0.7	1.00	1.00	1.00
IL-9	6.4 ±10.1	5.8 ±8.9	19.5 ±9.4	1.00	0.904	1.00
MCP-1	56.4 ±8.8	52.1 ±7.9	56.6 ±7.9	1.00	1.00	1.00
KC	154.8 ±26.6	181.6 ±24.1	175.2 ±23.1	1.00	1.00	1.00
MIP-1α	40.9 ±5.6	26.5 ±5.1	27.9 ±5.3	0.205	1.00	0.315
MIP-1β	48.1 ±7.9	34.4 ±6.9	30.0 ±10.4	0.628	1.00	0.552
MIP-2	91.7 ±34.5	76.8 ±29.4	50.1 ±29.4	1.00	1.00	1.00
TNFα	7.0 ±1.0	5.4 ±0.9	7.0 ±0.9	0.755	0.680	0.755
VEGF	0.5 ±0.1	0.4 ±0.1	0.4 ±0.1	0.873	1.00	0.876
IL-1α	218.0 ±132.6	378.2 ±119.9	372.9 ±114.8	1.00	1.00	1.00
IL-1β	1.1 ±0.3	0.9 ±0.3	1.2 ±0.3	1.00	1.00	1.00
IL-2	3.2 ±0.8	3.1 ±0.5	2.6 ±0.8	1.00	1.00	1.00
IL-6	15.4 ±5.6	4.1 ±5.1	10.1 ±4.9	0.439	1.00	1.00
IL-5	15.2 ±2.2	13.3 ±2.0	13.2 ±1.9	1.00	1.00	1.00
IL-10	3.2 ±0.7	2.0 ±0.6	2.5 ±0.6	0.651	1.00	1.00
IL-12p40	17.4 ±7.8	15.7 ±7.1	5.5 ±6.8	1.00	0.925	0.786
IL-12p70	28.1 ±11.9	9.6 ±9.7	10.4 ±8.8	0.723	1.00	0.729
IL-13	100.6 ±20.4	48.0 ±18.5	60.2 ±17.7	0.198	1.00	0.435
IL-15	25.1 ±19.1	67.4 ±23.4	39.6 ±19.1	0.558	1.00	1.00
IL-17	3.9 ±0.7	3.2 ±0.6	3.2 ±0.6	1.00	1.00	1.00
IP-10	68.9 ±5.9	59.7 ±5.4	66.6 ±5.1	0.772	1.00	1.00
M-CSF	16.2 ±7.7	3.6 ±7.7	2.6 ±6.2	0.788	1.00	0.548
MIG	614 ±78.2	512.8 ±70.8	584.0 ±67.8	1.00	1.00	1.00
RANTES	17.4 ±3.2	13.7 ±2.9	11.2 ±2.8	1.00	1.00	0.472
LIX/CXCL5	27634.0 ±484.0	4523.7 ±437.8	3442.8 ±419.2	0.035	0.255	0.894

CHAPTER 5: **DISCUSSION**

Making up more than 1 in 10 new cancer diagnoses each year, breast cancer impacts human health significantly on a global scale.[1] Of these individuals, up to 17% of those with stage I & II disease, 62% with stage III, and 66% with stage IV will undergo chemotherapy during their treatment.[201] Chemotherapy can cause microbial dysbiosis which has the potential to compromise the gut barrier, enabling negative side effects of treatment such as neuropathy and diarrhea.[10,37,71] These intestinal changes have the potential to influence cognitive symptoms via the gut-brain axis which could decrease health-related quality of life.[202] Additionally, individuals with breast cancer are at risk for disease recurrence and related mortality which is positively associated with overweight and obesity[2,3] and negatively associated with regular exercise and adherence to nutritional recommendations.[125,163] The gut microbiota is a powerful influencer of metabolism, energy harvest, the immune system, and inflammation.[20,23,124,203] For these reasons, this study was designed to investigate the effects of an exercise intervention on the gut microbiota of women who underwent chemotherapy for breast cancer, including exploration into the mechanistic impacts that exercise-responsive gut microbiota may have on tumor growth and chemotherapy treatment via FMT in a germ-free murine model of the disease. The primary outcome from the clinical portion of the study was gut microbial diversity and composition, while health-related quality of life and dietary components were explored for potential relationships to the gut microbiota. The main objectives of the mouse study were to investigate the impact of exercise-responsive gut microbiota on tumor volume and the gut microbial impacts surrounding chemotherapy. Adjuvant oligofructose supplementation's effect on these outcomes was also explored and tight-junction protein gene expression and tumor and serum cytokine levels were analyzed as possible mechanistic links between the gut microbiota and tumor outcomes.

5.1 Clinical Study Outcomes

5.1.1 Gut Microbiota Diversity and Composition in ACE Participants

5.1.1.1 Alpha Diversity Did Not Differ Significantly Between Time Points

Alpha diversity frequently serves as a metric used to gauge the state of the gut microbiota due to its associations with host health and inverse relationship to several disease states.[204] Dysbiosis is often associated with a drop in alpha diversity[56], and this decrease is frequently reported to be associated with chemotherapy treatment in humans and mice.[10,205,206] Alpha diversity has been observed to be lower at diagnosis in individuals with breast cancer compared to healthy controls as reported in multiple case-control studies.[52,175] Since dysbiosis may negatively affect cancer treatment outcomes and overall health, we were interested in the potential for the ACE program to increase alpha diversity between baseline and 12 weeks in individuals with breast cancer who had undergone chemotherapy. In healthy adults, higher reported physical activity levels and greater cardiorespiratory fitness have been associated with increased alpha diversity.[207] Although a previous study demonstrated promising positive correlations between cardiorespiratory fitness and alpha diversity metrics in breast cancer survivors[32], alpha diversity as measured by Pielou's Evenness index, Shannon index, and Observed Species did not differ significantly between pre- and post- exercise time points in our participants. This finding is consistent with the participants pooled at each timepoint and with individual pre/post comparisons. However, some participants demonstrated a trend toward increased alpha diversity between 0 and 12 weeks based on the three indices. Namely, participants 2, 4, 8, and 9. Studies investigating the impact of exercise on the gut microbiota in diverse populations remain limited, but "individualized response" and responses which varied between lean and obese BMI individuals have previously been reported in regard to the effects of an exercise intervention on gut microbial composition.[208,209] We included a 24-week time point to investigate whether a "washout" period would reverse gut microbial changes after exercise as it has been reported previously that gut microbial changes induced by exercise are reversible with exercise cessation.[208] We found no differences between 12 and 24 weeks which is likely due to the lack of robust change due to the exercise in the previous 12 weeks. It has been hypothesized that some individuals could have a more exercise-responsive gut microbiota than others. This responsiveness may be influenced by a multitude of environmental, genetic, or

epigenetic factors known to shape the gut microbiota including diet, BMI status, and stress.[210] The variability of participant BMI likely influenced individual alpha diversity results as individuals belonging to underweight and overweight BMI categories have been reported to have decreased alpha diversity compared to those with a healthy weight category.[211] A larger sample size in this population might allow for better investigation of variation in response. Additionally, a program with strenuous rather than mild to moderately intense exercise may be necessary to yield the higher cardiorespiratory fitness levels that were associated with increased alpha diversity reported by Carter et al..[32]

5.1.1.2 Beta Diversity was Influenced by BMI but not Time Point or Age in ACE Participants

Beta diversity metrics allow for a comparison between gut microbial communities. Beta diversity was analyzed between time points with the participants pooled to investigate whether the exercise program and washout period was associated with differences in the composition of the gut microbial community between time points. In a previous pilot study with 12 breast cancer survivors by Paulsen et al., a significant difference in beta diversity based on magnitude of cardiorespiratory fitness change was found.[169] A similar finding was reported by Carter et al. whereby cardiorespiratory fitness had a significant effect on beta diversity in 37 breast cancer survivors, whereby the microbiota from those in the higher quartiles of fitness clustered separately from those with lower fitness.[32] In individuals with overweight and obesity, moderate, vigorous, and bike-commute exercise resulted in significant changes in beta diversity compared to the non-exercising control group.[212] Thus, we were interested in investigating whether the moderate intensity ACE program would illicit significant community changes between baseline and 12 weeks in individuals with breast cancer. Gut microbial diversity decreases with aging[213] and has been previously controlled for when investigating the gut microbiota of women with breast cancer[52], but we found no significant effect of age on beta diversity in our small sample. Using Unweighted UniFrac matrix, no significant differences in beta diversity were found between baseline and 12 weeks or between 12 weeks and 24 weeks. Although Kern et al. demonstrated that moderate intensity exercise produced shifts in beta diversity in healthy overweight individuals, they found that vigorous exercise had a more powerful effect[212], and it is possible that this is not the case for all demographics since current research is limited outside of healthy populations. Additionally, in mice, 8-weeks of forced or voluntary moderate exercise

was not capable of inducing significant changes in beta diversity.[160] Similar to the possible explanation for the findings in participant alpha diversity, it is possible that the ACE exercise intervention was not capable of generating the magnitude of cardiorespiratory fitness increase necessary to produce the shifts in beta diversity reported by Paulsen et al. and Carter et al.[32,169] A more vigorous or cardiorespiratory-focused exercise intervention may be the path to achieving this result. The relationship between cardiorespiratory fitness and beta diversity may also lack strict directionality. Since there were no significant changes induced by exercise in this cohort, a "washout" was not observed at 24 weeks. Although no significant effect of time point or age were found, BMI category was found to apply a significant effect on the beta diversity of the 10 participants. Several studies have reported the powerful influence of BMI on gut microbial diversity in humans and mice[116,214] and specifically, on beta diversity.[208,215,216] In an exercise study done using a diet-induced obesity model in mice, groups clustered according to obesity status and voluntary exercise status after 12 weeks.[217] BMI is a factor that could influence an individual's gut microbial response to an exercise intervention that should be considered in further studies in this population and others.

5.1.1.3 Exercise is Associated with Limited Taxonomic Changes in the Gut Microbiota

Exercise and cardiorespiratory fitness have been linked to changes in taxonomic profiles characterized by increases in species that may offer health benefits to the host in both humans and mice.[32,158,166,218] When analyzing participant taxonomic data, three health-associated bacteria were chosen, and three inflammation-associated bacteria were chosen to serve as markers of overall gut microbial health. *Bifidobacterium*, *Faecalibacterium*, and *Roseburia* are SCFA-producing bacteria known for competitive exclusion of pathogens, participation in nutrient metabolism, and support of colonocyte function.[219-221] *Enterobacteriaceae, Klebsiella*, and *Escherichia-Shigella* are bacteria belonging to the phylum Proteobacteria that make up a very small percentage of the commensal gut microbiota, are known as opportunistic pathogens, and have been associated with inflammatory conditions.[222-224] The relative abundance of these genera was not found to differ significantly between time points. Bressa et al. found that active women exhibited greater abundance of *Bifidobacterium* compared to sedentary women[225], which is what we hypothesized we would see in exercise response. Carter et al. observed that breast cancer survivors with higher cardiorespiratory fitness had a higher relative abundance of

Faecalibacterium than those with lower cardiorespiratory fitness[32] which aligns with gut microbial findings in participants 2, 4, and 9 who appeared to be more exercise-responsive in the present study, albeit with increases that were not statistically significant. The trend is also in alignment with increased relative abundance of *Faecalibacterium prausnitzii* and *Roseburia hominis* observed in women exercising 150-minutes or more per week compared to sedentary women.[158] *Faecalibacterium rodentium,* a specific species within the genera *Faecalibacterium,* was found to rescue the accelerated breast tumor growth caused by antibiotic treatment in a mouse model, highlighting the potential benefit of increasing abundance of this genera.[226] It is possible that some individuals are more exercise-responsive, and individuals may need different exercise types, intensities, or durations to produce a gut microbial response.

Eight genera were found to be differentially abundant between baseline and 12 weeks which may indicate that despite the lack of statistical significance in the overall community metrics of alpha and beta diversity, exercise still influenced specific bacteria within the gut microbiota of the participants. Pre-ACE samples exhibited higher relative abundance of *Pseudomonas, Gastranaerophilales, Barnesiella, Phascolarctobacterium,* and *Butyrivibrio* compared to post-exercise samples. Members of *Pseudomonas* are opportunistic pathogens that are not abundant in healthy individuals and known to cause infection in individuals with cancer[227], so a decrease may be beneficial. In particular, *Pseudomonas aeruginosa* is known to cause intestinal infection in individuals with cancer and targeted elimination of it from the gut microbiota could decrease morbidity and mortality in the population.[227] In a mouse model of aging, *Gastranaerophilales* spp. appeared in the gut microbiota at later time points, indicating a possible association with aging.[228] *Barnesiella* is a bacteria that is often found in the gut microbiota of healthy individuals[229], so a decrease in its presence may not necessarily be beneficial. The same is true of SCFA-producing *Phascolarctobacterium* which is also found in the gut microbiota of healthy individuals and in greatest abundance between the ages 30-60.[230] *Phascolarctobacterium* may flourish when exposed to a high-fat diet as demonstrated in a high-fat-diet model in rats.[231] The DRI for fat intake in adults is 20-35% of total caloric intake which would be equivalent to about 46-80 grams/day for the average 2,069 kcal/day intake at baseline. This indicates that the reported average total fat intake of 96.2 grams per day at baseline exceeds the DRI. This high average fat intake could contribute to proliferation of *Phascolarctobacterium;* however, total fat intake was increased at week 12 compared to baseline, potentially indicating

that exercise may mitigate the proliferative effect of high fat intake on *Phascolarctobacterium. Butyrivibrio* is a butyrate-producing bacteria known for its anti-inflammatory activity. Our finding that exercise decreased its abundance does not align with findings indicating that 6 weeks of forced treadmill running increased its abundance in mice.[232] The discordance between the present results and the proliferation noted by Allen at al. may be related to the primarily aerobic nature of the treadmill running exercise compared to ACE or potentially to a stress response to "forced exercise" in mice that is propagated by the gut-brain axis and did not occur in response to ACE.

The relative abundance of *Dialister, Oscillospiraceae,* and *Paraprevotella* increased between baseline and 12 weeks with the ACE program. *Dialister* has been found to be enriched in individuals who presented with cardiovascular diseases and type-2 diabetes mellitus. However, increased *Dialister* may be of benefit to host health in an anti-inflammatory and neuro-protective manner as it has been linked with greater decreases in IL-6 levels with whole grain incorporation and the genus is depleted in individuals with depression.[233,234] The role of *Dialister* in host health may therefore be host-state-dependent. Evidence for the roles of *Oscillospiraceae* are in their infancy, however some members such as *Oscillospira* are associated with leanness, produce butyrate, and inversely correlate with inflammatory conditions.[235] Conversely, greater *Oscillospiraceae* abundance has previously been associated with depression[236], indicating the need for further research on its activity and specifically, in response to exercise. The observed increased abundance of *Paraprevotella* with exercise is in alignment with findings from Bressa et al. which noted that active women had greater abundance of *Paraprevotella* compared to sedentary women.[225] These bacteria may be particularly exercise responsive. Overall, though significant findings indicating changes in the gut microbiota associated with the exercise intervention are limited, they may influence the metabolic potential of the community and the intestinal environment in ways that could benefit host health.

5.1.2 Alberta Cancer Exercise Program Supports Health-Related Quality of Life

Although no statistical significance was found between total scores, results from the FACT-G questionnaire indicated that total scores remained similar between baseline and 12 weeks then receded between 12 and 24 weeks when the ACE program ended. The drop between week 12 and week 24 meets the 3–7-point criteria for being minimally clinically significant.[237]

This indicates a clinically important association between the end of the exercise program and a decline in health-related quality of life. It was originally hypothesized that FACT-G scores would increase between baseline and 12 weeks in conjunction with the ACE program; however, it is possible that the idea and process of beginning the program may benefit perceived quality of life at baseline. Additionally, it is important to note that this research was conducted during the COVID-19 pandemic which may have contributed unusual levels of stress capable of affecting resultant scores. From 12 to 24 weeks there was a decline in emotional well-being from levels seen at to baseline and 12 weeks which may indicate a positive influence of the intervention on emotional well-being as well. The ability of exercising with others, but not alone, to increase emotional well-being in women with cancer has previously been reported.[238] It is interesting to note that greater well-being was associated with group exercise classes that were held online rather than in-person. Taken together, these results indicate that ACE was clinically beneficial to quality of life in breast cancer survivors.

5.1.3 Total Fat Intake Influences Beta Diversity but Alpha Diversity Shifts Independently

No significant correlations were found between nutrient intakes and alpha diversity metrics, indicating that the limited shifts seen in the gut microbiota of participants between baseline and week 12 were likely due to the exercise program rather than dietary perturbations. Some trends toward a positive correlation of Evenness with total fat intake, omega-3 fatty acid intake, and omega-6 fatty acid intake were noted at baseline. An increase in gut microbial Evenness in response to dietary fat intake is not widely reported[239], and the influence of high fat diets (27.1%-65% kcal) in 25 mouse models did not demonstrate consistent gut microbial responses as measured by alpha diversity.[240] Polyunsaturated fatty acid intake does not seem to have a consistent impact on alpha diversity metrics.[239] Since these trends are only present at baseline, it is possible that exercise attenuated the impact of dietary fat intake on gut microbial Evenness. After allocating participants to low, medium, and high fat intake groups, total fat intake level was found to contribute 13.7% of the variance seen in weight UniFrac beta diversity, indicating the influence that total fat intake may have on gut microbial communities. Total fat intake has been demonstrated to influence beta diversity clustering in murine models as well.[240] Our analysis included all three time points, so although total fat intake levels appear to play some role in shaping the gut microbial community response to exercise, the effect is present at each

time point. Total fat intake level may be a factor of interest in future investigations of the impact of exercise on the gut microbiota.

5.2 Murine Study Outcomes

5.2.1 Exercise-responsive Microbiota Slows Tumor Growth and Prebiotic Enhances the Effect

Groups with post-exercise gut microbiota (BCW12 and BCW12OFS) demonstrated a pattern of attenuated increases in tumor volume over the course of the study. Previous studies in mice have linked exercise performed pre-tumor cell injection and post-tumor injection with suppressed breast tumor growth.[241,242] These studies posit that the breast tumor suppressive effects of the exercise were mediated by the observed alterations in relevant circulating immune cells that are widely known to occur in response to acute exercise.[243] Here it is demonstrated that exercise may also beneficially alter gut microbiota in a way that promotes tumor suppression independent from the direct acute effect of the exercise, although the suppression may still be mediated through immune activity alteration. The group that received prebiotic oligofructose in addition to exercise-responsive gut microbiota exhibited the smallest tumor volume, and greatest number of statistically significant time points compared to the pre-exercise group. This indicates an enhancement of the tumor-suppressive effect of the exercise-responsive gut microbiota with adjuvant oligofructose administration. The anti-tumor and anti-mammary tumor effect of oligofructose has previously been documented in rats and mice whereby tumor incidence was decreased and oligofructose acted synergistically in combination with various chemotherapeutics[179,180], which aligns with the present finding that the group who received prebiotic supplementation maintained the smallest tumor volume on average compared to other groups. The mechanisms underlying these effects have not been thoroughly explored in the literature, so here, the gut microbiota, tight junction proteins, and cytokine levels were investigated to provide context to findings indicating a protective effect of exercise and oligofructose against breast tumor progression.

5.2.2 Gut Microbiota Differs Between Groups and is Altered by Tumor and Chemotherapy

5.2.2.1 Alpha Diversity Metrics Vary Significantly Between Groups

High alpha diversity is often associated with gut microbial health; however, here the BCW12OFS mice exhibited a trend towards or significantly decreased alpha diversity compared

to the other groups at most time points. This is likely due to oligofructose's ability to support proliferation of select beneficial bacteria[244,245] which may in turn dominate the community structure and competitively exclude other bacteria, thus reducing alpha diversity. A decrease in alpha diversity with prebiotic consumption has been noted in a human intervention trial as well in which lower alpha diversity was in fact associated with improved obesity-related health outcomes.[246]

Paclitaxel treatment has previously been reported to decrease gut microbial alpha diversity in a murine model[205]. It is possible that the present results indicate a potential Observed Species-protective effect of oligofructose supplementation surrounding Paclitaxel treatment. BCW12OFS was found to have decreased Evenness compared to BCW12 at each time point and compared to BCW0 on day 5, 13, 22, and at endpoint. Evenness of BCW12 gut microbiota was significantly greater than BCW0 on day 5 and on day 13, after tumor cell injection. Shannon diversity was significantly lower in BCW12OFS compared to BCW12 and BCW0 at the post-tumor cell injection and demonstrated this trend at each time point. Based on Evenness and Shannon diversity results, it seems that the tumor cell-injection may influence gut microbial alpha diversity. Although directionality of the relationship between tumor and altered gut microbiota in humans cannot be confirmed, this aligns with data indicating that women with breast tumors have decreased alpha diversity at diagnosis compared to healthy individuals.[175] Considering that BCW12 alpha diversity trended higher and BCW12OFS trended lower overall when compared to BCW0, and both exercise-responsive groups demonstrated a trend for lower tumor volume compared to BCW0, the specific members and structure of the gut microbial community may be more influential than alpha diversity when considering the tumor-promoting or tumor-protective properties of the gut microbiota.

5.2.2.2 Beta Diversity Varies Significantly Between and Within Groups Over Time

Beta diversity as measured by Weighted UniFrac distance was analyzed to detect differences between the gut microbial communities across groups. The results indicate that communities differed significantly between all groups at each time point. In the ACE participants, beta diversity between time points for individual participants could not be analyzed statistically, but in the germ-free mice it is evident that communities differed between the baseline gut microbiota (FMT for BCW0 mice) and the post-exercise gut microbiota (FMT for

BCW12 and BCW12OFS mice) from participant 4. Although gut microbial beta diversity continued to differ significantly throughout the study, they tended to cluster more closely over time. Injection of the breast tumor cells may have induced changes to the gut microbiota which would support findings in humans which demonstrated that disease-specific community differences strongly associated with either individuals with breast cancer or healthy individuals.[175] Additionally, significant gut microbial community differences between mice receiving no treatment and those treated with Paclitaxel in murine models of breast cancer have previously been reported[247], so the communities may become more similar at day 22 and at the endpoint due to the effects of the cytotoxic exposure alongside tumor progression. Beta diversity also differed significantly between each time point within each group. In a sample of women with breast cancer, researchers found that beta diversity differed significantly between groups based on tumor size (T1 vs T2&T3) which indicates that progression could influence the gut microbial community over time.[248] These findings provide further evidence that breast tumor initiation, progression, and chemotherapeutic treatment with Paclitaxel are influential environment-modifying events associated with alterations within the gut microbial community.

5.2.2.3 Differential Abundance of Specific Taxa May Contribute to Host Tumor Response

Results were presented for differential taxonomic abundances at key time points which included day 13, following tumor cell injection, and day 22, following completion of Paclitaxel treatment. These time points were chosen for in-depth analysis due to evidence in the present diversity analyses, previous studies indicating that tumor presence and Paclitaxel treatment both alter the gut microbiota[37,175,247], and the greatest number of bacteria appeared to be differentially abundant between groups at these time points. On day 13, *Tyzzerella*, *Ruminococcus gauvreauii*, and *Eubacterium hallii* were significantly more abundant in BCW12OFS mice compared to BCW0. Increased relative abundance of *Tyzzerella* has been associated with high cardiovascular disease risk[249], and may be a mediator between diet and ectopic fat deposition[250] which suggests that the increased presence is not beneficial, although a relationship with breast cancer has not been established. Enhanced *Ruminococcus gauvreauii* abundance may be beneficial to the host as is has been associated with decreased depressive symptoms and an increase in inflammation-regulatory metabolic pathways as analyzed by KEGG following probiotic and vitamin B7 supplementation.[251] Additionally, *Ruminococcus gauvreauii* has been negatively associated with

coronary artery disease incidence, strengthening its potentially protective role.[252] Increased relative abundance of *Eubacterium hallii* in BCW12OFS compared to BCW0 may also be positive considering its ability to metabolize glucose, fermentative products, and metabolic products into butyrate or propionate which could benefit the intestinal microenvironment, supporting intestinal barrier health and host immunity.[253] *Eubacterium hallii* are also capable of supporting host health by producing essential vitamin B12.[253]

Compared to BCW0, gut microbiota of BCW12 mice were also enriched in *Tyzzerella* and *Ruminococcus gauvreauii* after tumor cell injection, but not *Eubacterium halli*. This suggests that oligofructose supplementation was responsible for the uniquely increased *Eubacterium halli* in BCW12OFS mice while the other bacteria were resultant of the exercise-responsive gut microbiota colonization. On day 13, BCW12OFS exhibited a significantly greater abundance of *Bifidobacterium* and lesser abundance of *Enterococcus* compared to BCW12. Oligofructose is known to support proliferation of beneficial Bifidobacterium[245], and inulin, a slightly longer chain fructo-oligosaccharide, has been reported to diminish enterococci in conjunction with the bifidogenic effect.[254] *Bifidobacterium* are beneficial in their immunomodulatory and anti-inflammatory activity.[219,255] *Enterococcus* is common in the gut microbiota and includes some opportunistically pathogenic and antibiotic-resistant bacterial strains known the cause infections in humans, making it a potential reduction target.[256] However, the species *Enterococcus hirae* is key to anti-tumor immune response with cyclophosphamide (CTX) treatment[91] and its relevance for treatment with other chemotherapeutics has not been investigated. Its species have also been found to produce cadaverine[150] which has been shown to mitigate breast cancer aggressiveness.[149] BCW12 and BCW12OFS gut microbiota were enriched for potentially health-supporting strains compared to BCW0 after tumor cell injection, with BCW12OFS exhibiting additional benefit from oligofructose compared to BCW12.

On day 22, following chemotherapy treatment, more genera were differentially abundant between groups compared to day 13, which suggests that the cytotoxic chemotherapy Paclitaxel influences the gut microbiota more significantly than breast tumor presence alone. Following Paclitaxel treatment conclusion, 14 bacterial groups differed in BCW12OFS mice compared to BCW0 mice. One genus that was increased was *Blautia*, which remains controversial for women with breast cancer. In a cohort of 31 women, its abundance was reported to be negatively

correlated with overweight (BMI $\geq$ 25 kg/m^2)[257], which is generally breast cancer-protective, and it has been demonstrated to be decreased in individuals with diabetes and negatively correlated to HbA_{1c}, indicating a potential influence in supporting glucose homeostasis.[258] However, the study by Luu et al. (2017) also found that *Blautia* was more abundant in individuals with clinical stage II or III disease compared to 0 or I and posited that it may be due to a member of the genera's (*B. producta*) ability to metabolize plant lignans into phytoestrogenic compounds capable of binding estrogen receptor's relevant for breast cancer progression.[257] Since the 16S rRNA sequencing analysis performed here does not reliably reach the species level, we cannot determine whether the presence of this *Blautia* poses such a concern, but the evidence highlights a potentially dualistic role for the genera in breast cancer.

Parasutterella presence seems to play a role in anti-tumor immunity as it has previously been reported to increase in abundance with inulin supplementation which resulted in greater abundance of tumor infiltrating lymphocytes at the tumor site in a murine model of melanoma.[259] Another species that was enhanced in BCW12OFS at day 22, *Lachnospiraceae,* may also play an important role in anti-breast tumor immunity specifically, as its abundance was reported to be decreased in non-responders to Trastuzumab treatment for HER-2 positive breast cancer[260] and in pre-menopausal individuals with breast cancer in general.[52]

In a cohort of 267 breast cancer patients and healthy controls, *Anaerostipes* were increased in individuals with pre-menopausal breast cancer compared to their controls, and negatively correlated with abundance of *Bifidobacterium* in the controls.[176] This indicates a potentially protective effect of oligofructose supplementation (which reduced *Anaerostipes* abundance) against breast cancer in that it appeared to significantly increase abundance of *Bifidobacterium* when compared to BCW12 at day 13, which may be of particular relevance for women with pre-menopausal breast cancer.

In a heterogenous cohort of 26 cancer patients receiving cytotoxic chemotherapy, targeted chemotherapy, and/or immunotherapy, *Ruminococcus gnavus* abundance was significantly lower in responders to treatment compared to non-responders.[48] Further evidence that the significantly decreased abundance of *Ruminococcus gnavus* seen in our BCW12OFS mice is likely beneficial in a breast cancer context can be found in the complex polysaccharide glucorhamnan secreted from *R. gnavus* which elicits an inflammatory immune response

characterized by TNFα release from affected systemic dendritic cells.[261] Not only is the genus negatively associated with response to chemotherapies, but it also may incite local and systemic inflammation via its polysaccharide metabolite. Taken together, the differentially abundant species in BCW12OFS compared to BCW0 following chemotherapy demonstrate potentially protective attributes, especially in the increased abundance of *Parasutterella* and *Lachnospiraceae* and decreased abundance of *Anaerostipes* and *Ruminococcus gnavus*.

BCW12 also exhibited significantly greater relative abundance of *Eubacterium iraeum, Colidextribacter*, *Tyzzerella*, *Ruminococcus gauvreauii*, and *Lachnospiraceae* and decreased abundance of *Anaerostipes* compared to BCW0, strengthening the possibility that these characteristics may benefit anti-tumor response. Compared to BCW12OFS, BCW12 differed in that there was a significantly enhanced abundance of *Hungatella*, GCA-900066575, and *Anaerostipes*, further indicating that enhancement of these genera as seen in BCW0 compared to BCW12OFS may be negative for tumor progression since BCW12OFS exhibited the lowest average tumor volumes. It is evident that both BCW12 and BCW12OFS exhibited differential relative abundances compared to BCW0 that may indicate protection from key gut microbial disruptions caused by Paclitaxel treatment. The relative enhancement or depletion of these species may beneficially modulate the host immune response to cancer, contributing to the decreased tumor volume in BCW12 and BCW12OFS mice.

5.2.3 Trends in Ileal and Colonic Tight Junction Protein Expression

The intestinal tight junctions are a key component of the gut barrier separating the environment of the intestinal lumen and that of the rest of the body.[262] Tight junction structural proteins, such as ZO-1, and proteins that regulate the passage of substrates through the tight junction, such as occludin and claudins, play an integral role in the tight junction and maintaining the perma-selectivity of the intestinal barrier.[262] Treatment with chemotherapeutics and associated dysbiosis have been shown to compromise the integrity of the intestinal barrier[61], justifying investigation of the expression of the tight junction proteins, ZO-1, occludin, and claudin-3 in the ileum and colon of the mice. Fermentation of dietary fiber into SCFAs by gut microbiota appears to benefit the intestinal tight junctions.[262] For example, butyrate has been demonstrated to stimulate expression of claudin-3 using an in vitro model of human intestinal epithelium.[263] Therefore, it was hypothesized that oligofructose supplementation might be

associated with greater expression of the tight junction proteins, maintaining a more functional intestinal barrier despite chemotherapy treatment. Conversely, the results indicate that although not statistically significant, ileal expression of all three junction proteins trended lower in BCW12OFS mice compared to BCW12 and BCW0. Greater expression of intestinal tight junction proteins is generally regarded as beneficial because the tight junctions prevent bacterial translocation and movement of bacterial particles such as lipopolysaccharide (LPS) into systemic circulation which can increase risk of sepsis and systemic inflammation.[264] However, disruption of the intestinal tight junction that allows bacterial translocation may play a positive role in specific chemotherapy effectiveness since bacterial presence can increase activation of the adaptive immune system, enhancing the anti-tumor immune response.[91] This has been shown in the case of Cyclophosphamide treatment. Translocation of *Lactobacillus* spp. and *Enterococcus hirae* to mesenteric lymph nodes and the spleen resulted in an increased Th17 cell-differentiation mediated anti-tumor immune response with CTX treatment in a murine model of sarcoma.[91] It is possible that greater acute ileal permeability could play a role in anti-tumor immune response in specific clinical conditions, but further investigation into intestinal permeability's relationship with anti-breast tumor immunity and Paclitaxel treatment are needed.

5.2.4 Cytokine Levels Reveal a Gut Microbial Influence on the Tumor Microenvironment

Tumor and serum cytokines were analyzed to investigate a potential mechanism influencing the differences in tumor volume between groups. Cytokines are cellular signaling molecules pertinent to inflammation and immune response that can provide insight into the tumor microenvironment. Circulating cytokine levels can also serve as useful biomarkers for tumor prognosis in breast cancer.[265] BCW0 mice exhibited significantly higher intra-tumoral levels of MCP-1(CCL2), IL-9, and vascular endothelial growth factor (VEGF) compared to BCW12 and BCW12OFS mice whose levels did not differ significantly from each other, indicating that the pre-exercise gut microbiota was a driver for the increased levels. IP-10 (CXCL10) was more prevalent in BCW0 tumor tissue compared to BCW12 and demonstrated that trend compared to BCW12OFS.

MCP-1(CCL2) is a chemoattractant cytokine active in recruiting monocytes from the blood to the tumor site where they then differentiate into macrophages.[266] Depending on environmental factors, monocytes will differentiate into immune-activating (M1) or immune-

suppressing (M2) tumor associated macrophages at the tumor site.[266] Macrophages may also exist on a spectrum between M1 and M2 in breast cancer[267]. MCP-1(CCL2) could therefore play a role in immune-suppression, allowing for greater tumor proliferation in the BCW0 mice compared to the other groups. Interestingly MCP-1 levels were shown to be increased in ovarian cancer models of Paclitaxel resistance[268,269] which could be related to unfavorable tumor-associated macrophage activity. Additional factors can affect monocyte polarization. Lam et al. (2021) recently demonstrated that favorable gut microbiota mediate polarization of monocytes in the tumor microenvironment through microbiota derived molecules such as the cyclic dinucleotide c-di-AMP. The molecules can activate stimulator of interferon genes (STING) to produce IFN Is (IFNα and IFNβ), beneficially modulating the tumor immune environment by regulating monocyte to macrophage polarization and influencing activity of natural killer and dendritic cells which contribute to anti-tumor immunity.[88] The effects of this pathway are lost with gut microbiota disruption with antibiotics or in a germ-free mouse, and the pathway is enhanced by the microbiota of mice fed a high fiber[88], which may present a possible mechanism to explain decreased tumor volume with oligofructose supplementation. This microbiota-mediated pathway may have beneficially shaped the tumor microenvironment in ways that led to volume differences between groups.

Findings in the literature regarding intra-tumoral IL-9 levels are mixed, with one study reporting that IL-9 blockade re-activated natural killer and CD8+ cells in individuals with high IL-9+ cell bladder cancer[270], while it has also been reported that tumor infiltrating leukocytes, which play an important role in anti-tumor immunity, secrete IL-9.[271] Here we report greater intra-tumoral IL-9 associated with increased tumor volume in the BCW0 mice, but the mechanisms underlying high IL-9 levels are unclear.

VEGF stimulates the growth of new blood vessels and breast cancer cells secrete it in response to hypoxia to incite the angiogenesis necessary to supply adequate blood flow for cell proliferation and tumor growth.[272] VEGF also contributes to breast cancer's metastatic potential and resistance to apoptosis.[273,274] The elevated levels in the BCW0 tumor microenvironment reflect increased growth and metastatic potential which are negative for overall prognosis. Increased vascularity would support the greater tumor volume. The significantly decreased intra-tumoral VEGF levels observed in post-exercise microbiota mice may be indicative of improved

Paclitaxel treatment response or lower overall vascularity potential due to a possible gut microbial influence on the tumor microenvironment.

Tumoral IP-10 (CXCL10) has been correlated with tumor stage and lymphoid metastasis in women with breast cancer, indicating a poorer prognosis with higher expression.[275] IP-10 (CXCL10) has also been demonstrated to induce cell proliferation, migration, and epithelial to mesenchymal transition in MCF-7 and MDA-MB-231 breast cancer cell lines[276], which would contribute to a more aggressive breast tumor profile supportive of increased tumor volume in BCW0 mice.

Compared to BCW0, TNFα was significantly increased in the BCW12OFS tumor microenvironment and presented a strong trend toward elevation compared to BCW12, while BCW0 and BCW12 levels did not differ significantly. High serum TNFα is associated with systemic inflammation characteristic of metabolic dysfunction[114] and its role in breast tumor initiation, progression, and metastasis has also been investigated, with indication that TNFα may be a treatment target.[277] However, Paclitaxel treatment has been demonstrated to increase TNFα production by mouse macrophages[278] and in A2780 ovarian tumor cells and MDA-MB-231 mammary adenocarcinoma cells, it has been found to ultimately contribute to the cytotoxicity of the taxane.[279,280] This response to Paclitaxel treatment may have been permitted/accentuated in BCW12OFS tumors compared to the other groups. Additionally, M1 subtype macrophages are known to secrete TNFα to incite immune activation in an anti-tumorigenic manner.[281].

RANTES (CCL5) levels were significantly enhanced in BCW12OFS tumors compared to the other groups whose levels did not differ significantly from each other. In an investigation of ovarian cancer resistance to Paclitaxel, an increase in tumoral RANTES (CCL5) was characteristic of positive response to the treatment.[269] Although RANTES (CCL5) has been implicated in metastasis of breast cancer[282], the effect occurs largely through its attraction of macrophages to the tumor site.[283] The type of macrophage induced at the tumor site plays an important part in its immunomodulatory influence[88], so the ability of RANTES to attract macrophages to the tumor site may support an active immune environment in the context of our results. Additionally, secretion of CCL5 from natural killer cells to recruit dendritic cells to the tumor site is noted to be increased via microbiota-STING-IFN I pathway activation[88].

Unfortunately, the cytokine panel that was analyzed did not contain IFN Is, which would have provided more insight on the possible activity of this mechanism.

Tumor KC (CXCL1) levels demonstrated a strong increased trend in BCW0 tumors compared to BCW12 and BCW12OFS, just missing statistical significance. This chemokine powerfully enhances breast tumor growth and metastasis through its interaction with tumor-associated macrophages.[284] Most serum cytokines were not altered significantly between groups. Serum CXCL5(LIX) levels were significantly higher and trended to be higher in BCW12 and BCW12OFS mice respectively compared to BCW0 mice, but the mechanism for this is unknown. In humans, serum CXCL5 is a marker associated with adverse breast cancer prognosis[285]; however, additional investigation is needed in the context of this study since that does not align with the rest of our data. The differences in several tumor-relevant cytokine levels between groups indicate that the gut microbiota exerted influence on anti-tumor immunity. Several of the findings discussed here contribute evidence toward a more angiogenic, immuno-suppressed tumor microenvironment being observed in the BCW0 mice and a more active anti-tumor immune environment in the BCW12, and especially BCW12OFS mice. Evidence of immune activity stimulation within the tumor microenvironment provides a mechanistic insight into the gut microbiota-potentiated benefit of exercise and prebiotic supplementation during breast cancer treatment.

5.3 Strengths and Limitations

The primary strength of this project is the translational design of the study whereby the mechanistic impacts of exercise-responsive gut microbiota from a breast cancer survivor could be investigated in germ-free mice. This allowed for exploration into how the microbiota would respond to further perturbation and how it may be immunomodulatory to host response to breast cancer and chemotherapy treatment. The findings contribute clinical relevance to the gut microbiota shifts that may be induced in exercise-responsive individuals. A limitation of the design lies in the pilot design of the human trial. A fully powered randomized clinical trial would provide greater insight into the effect of exercise on the gut microbiota with an active exercising arm and control arm in the study (perhaps a waitlist control protocol). This study is also influenced by a self-selection bias which may mean that those interested in the study have a higher baseline interest in modifiable lifestyle factors to change their microbiota or improve

overall health. The small sample size was not representative of the larger breast cancer population's socioeconomic and ethnic diversity as indicated by the results of the demographic questionnaires, and therefore, the results may not be applicable to the broader breast cancer population.

Additional limitations stemmed from the ongoing COVID-19 pandemic. The ACE program was moved to an online format so that it could continue to run within the limits of gathering restrictions. Recruitment to the human portion of the study was lower than expected with this change, and it may have affected participant efforts during class. This change resulted in a smaller sample size for analysis of the outcomes. A larger sample size would provide more robust data on the effects of exercise on the gut microbiota, which tend to be variable. Some indication that response is variable between individuals has been shown here, which emphasizes the benefit of a larger sample size. Additionally, a larger sample size would have provided more participants to choose from when selecting the samples to use as FMT donor material for colonization of germ-free mice. It is possible that additional subjects could have shown a more significant response to exercise or exhibited other beneficial characteristics such as less dietary variability.

Strength can be found in the analyses performed in this study. 16S rRNA sequencing is the most widely used next-generation technique currently used to characterize the gut microbiota. It provides robust data on microbial community diversity and composition. Although subject to self-reporting bias, Godin's Leisure time Exercise Questionnaire, 3-day dietary record, and Functional Assessment of Cancer Therapy-General are valid and reliable measurement tools on their respective metrics. Repetitive collection of this information provided time point-specific information. By collecting information on potential influences of the gut microbiota outside the exercise intervention, the information could be used to understand factors that could influence response to exercise. Additionally, assessing participants' health related quality of life provides support for the feasibility of exercise interventions for individuals with breast cancer and the provision of benefits beyond an influence on the gut microbiota.

Strengths of the murine study included controlling for fluid intake to ensure that the oligofructose was delivered as intended. Caliper measurements are regularly used in murine cancer research to assess tumor volume. Metal calipers were used for tumor measurement, and

although measurements were taken by a single individual with consistent technique, they may be subject to individual bias. Due to the necessity of autoclaving materials when working in a germ-free facility, digital calipers, which would reduce measurement bias, could not be used. Additional strength lies in the frequency of fecal sample collection so that gut microbiota could be assessed at multiple time points, providing insight into the effects of specific events such as tumor cell injection and Paclitaxel treatment, which are clinically relevant. The chemotherapeutic Paclitaxel and dosing were both chosen to be clinically relevant for breast cancer treatment in humans, promoting the translational potential of the findings. An additional strength is the investigation of a prebiotic supplement alongside the exercise-responsive microbiota which has not been previously explored. A limitation relevant to both studies is the use of 16S rRNA sequencing rather than shotgun sequencing to analyze the gut microbiota. Shotgun or whole genome sequencing allows for the function of the microbiota to be assessed but is at present still expensive and requires highly specialized bioinformatics expertise. Intestinal permeability was inferred from relevant gene expression of tight junction proteins; however, a functional test of intestinal permeability (i.e. FITC dextran gavage) would be a better measure that was not an option due to the time and procedural restrictions of the germ-free facility. Finally, cytokine levels are used here to explore immune activity, but tumor rRNA sequencing or characterization and quantification of immune cells within the tumor microenvironment and spleen would have provided a deeper assessment of immune activity.

Finally, a strength for the application of this research is evident in the existing support for exercise and a fiber-rich diet in cancer prevention, during treatment, and in survivorship. The present findings corroborate this evidence and provide mechanistic background in support of this messaging. Additionally, prebiotic supplementation is a way to provide the microbiota with fermentable substrate without drastically changing dietary intake, which can be a barrier to increasing fiber intake. This strengthens its feasibility as an adjuvant intervention for individuals with cancer and cancer survivors. Oligofructose is soluble and can be added to any liquid, smoothie, or nutritional shake, making it accessible for those who cannot tolerate solid food.

5.4 Future Directions

This is the first study to investigate the effects of an exercise intervention on the gut microbiota of women with breast cancer and the downstream effects of the exercise-responsive

microbiota on tumor and treatment response, both alone and in conjunction with prebiotic oligofructose supplementation. As such, further investigations are necessary to explore this relationship and characterize mechanisms of action. Future studies should be conducted with a control group and include a larger sample size of women with diverse socioeconomic and ethnic backgrounds to increase the application of the findings. Multiple exercise types, intensities, and/or durations should be explored to assess which conditions are most favorable for gut microbial exercise response in breast cancer survivors. The significant differences in gut microbiota based on cardiorespiratory fitness levels reported by Carter et al. (2019) are promising, and unexplored exercise conditions may be needed to elicit fitness increases that would yield comparable differences in gut microbiota. It would be informative to explore microbiota from multiple fecal donors as there are likely insights to be gained from those who do not respond to exercise and the potentially different microbial profiles that may arise in individuals in response to the same exercise intervention. It is possible that an 'ideal' microbiota profile exists in response to exercise that would maximize favorable tumor growth outcomes in a FMT mouse study.

When considering study design and analyses for mechanistic studies, future work should consider interrogation of additional treatment groups. For example, it would be beneficial to investigate the effect of prebiotic supplementation in a group colonized with pre-exercise intervention gut microbiota in addition to those explored here. Since chemotherapeutics have varying mechanisms of action, exploring response of the tumor microenvironment to multiple agents in mice that are colonized with the same exercise-responsive microbiota or receiving the same prebiotic may provide insights for precision oncology. Along the same lines, exploring response between groups injected with tumor cells with varying characteristics will also be of importance when seeking to understand the role that microbiota profiling, exercise, and prebiotic might play in precision oncology.

Deeper molecular and immunological experiments and analyses should be done in future work to elucidate mechanisms linking the gut microbiota and the tumor microenvironment. Recent work by Lam et al. (2021) demonstrates the influence that the microbiota has on the tumor microenvironment via microbe-derived molecules. Further research should focus on identifying genera and species that may be enriched in exercise-responsive and prebiotic-

responsive gut microbiota that secrete immunologically active molecules capable of stimulating this pathway. Additionally future research should investigate whether enriched species are translocating to elicit enhanced immune response as seen in the case of cyclophosphamide.[91] This would include microbial characterization of lymphoid and tumor tissue. Finally, identification and quantification of immune cells such as macrophages and tumor infiltrating leukocytes in the tumor microenvironment and spleen would provide additional understanding of the immune responses associated with different gut microbial colonizations and deeper insights on the potential mechanisms underlying tumor immunity modulation by the gut microbiota in future studies.

5.5 Conclusions

This project sought to investigate the potential for the ACE program to beneficially modify the gut microbiota of women with breast cancer and to explore the mechanistic influence exerted by exercise-responsive gut microbiota in a germ-free mouse model of breast cancer. The human portion of the study indicated a limited effect of the exercise intervention on participant gut microbiota which was primarily distinguished by differential relative abundance of several genera between baseline and the conclusion of the ACE program. Select participants also exhibited an increase in alpha diversity with exercise which we hypothesize is indicative that some individuals will experience a greater gut microbial response to exercise compared to others. Exercise may serve as a possible intervention to attenuate treatment and breast cancer-associated gut microbial dysbiosis, but additional research is needed to clarify effective exercise types, intensities, and durations. Health related quality of life scores exhibited a clinically meaningful drop following the conclusion of the exercise program which may indicate the positive influence of a group exercise intervention on quality of life. This does not indicate a causal role for the gut microbiota in shaping cognition around exercise, but the gut-brain-axis may be an area for further research in this context. The greater emotional well-being scores surrounding the program, albeit not statistically significant, also suggest a positive effect of the exercise intervention on health-related quality of life. These results strengthen support for group exercise recommendations in this population. Although shifts in gut microbial communities in response to exercise seemed minimal, FMT of a participant's baseline and exercise responsive microbiota in a germ-free model of breast cancer resulted in significant differences in tumor volume, gut

microbiota, and immunologically active tumor and serum cytokines over time. Some of these effects, such as decreased tumor volume, decreased angiogenesis markers, and increased markers of Paclitaxel response in the tumor microenvironment, were enhanced by prebiotic oligofructose supplementation in mice colonized with exercise-responsive gut microbiota. These findings support the hypothesis that exercise and prebiotic supplementation beneficially modulate anti-tumor immunity in part through favorable modification of the gut microbiota. Further research will be necessary to characterize the interaction between gut microbiota and the tumor microenvironment more completely, but taken together, the results of this study demonstrate a benefit of exercise and prebiotic supplementation as adjuvant interventions, or for recurrence prevention in individuals with breast cancer and survivors.

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