Gut Microbiome Diversity and Community Structure Following Dietary Genistein Treatment in a Murine Model of Cystic Fibrosis

by

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ABSTRACT

Introduction: Cystic fibrosis (CF) is the most common life-shortening autosomal recessive genetic disease affecting Caucasians. The disease is characterized by a dysfunctional cystic fibrosis transmembrane regulator (CFTR) protein and aberrant mucus accumulation that subsequently alters the physicochemical environment in numerous organ systems. These mucosal perturbations have been associated with inflammation and microbial dysbiosis, most notably in the lungs and gastrointestinal (GI) tract. Genistein, a soy isoflavone and dietary polyphenol, has been shown to modulate CFTR function in cell cultures and murine models, as well exert sex-dependent improvement of survival rates in a CF mouse model. However, it is unknown whether dietary genistein affects gut microbiome diversity and community structure in cystic fibrosis. This study sought to examine associations between dietary genistein treatment and gut microbiome diversity and community structure in a murine model of CF. Methods: Twenty-four male and female mice homozygous for the DF508 CFTR gene mutation were maintained on one of three diet regimens for a 45-day period (n=11, standard chow; n=7, Colyte-treated water and standard chow; n=6, 600 mg dietary genistein per kg body weight). One fecal pellet was collected per mouse post-treatment, and microbial genomic DNA was extracted from the fecal samples, quantified, amplified, and sequenced on the Illumina MiSeq platform. QIIME 2 was used to conduct alpha- and beta-diversity analyses on all samples. Results: Measures of alpha-diversity were significantly decreased in the dietary genistein group as compared to either standard chow or Colyte groups. Measures of beta-diversity showed that community structure differed significantly between dietary treatment groups; these differences were further

illustrated by distinct clustering of taxa as shown by principal coordinates analysis plots. Conclusion: This 3-arm parallel experimental study showed that dietary genistein treatment was associated with decreased microbial diversity and differences in microbial community structure in DF508 mice.

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INTRODUCTION

Among Caucasians, cystic fibrosis remains the most common autosomal recessive genetic disease, and results in the inescapable shortening of the lifespan in affected individuals.[1] When cystic fibrosis (CF) was first identified in 1938, life expectancy was profoundly short at only six months of age.[2] In the 1960's and 1970's, children with CF fared better, yet still often passed away before reaching their twentieth birthday.[1] In most countries today, including the United States, the median age of death for individuals with CF is less than 30 years of age.[3,4] While treatment progress has increased lifespan, those with CF continue to experience a host of health complications that decrease quality of life and ultimately contribute to disease progression and death.[5–9] Many individuals with CF will not survive long enough to celebrate their fortieth birthday.[3,4]

Clinical manifestations of the disease result from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for ion channels found ubiquitously in epithelial tissues that transfer anions such as chloride and bicarbonate, and most notably impact the respiratory, digestive, and reproductive systems.[10,11] Symptom severity has been associated with specific gene variants, with the delta-F508 (DF508) mutation being the most common; individuals homozygous for the DF508 CFTR mutation have been shown to experience more severe symptoms than those with other variants of the gene.[12–14] In healthy individuals, the mucus layer lining the surface epithelia of the lungs and GI tract acts as a physiological barrier, innately allowing for nutrient exchange while defending against insults from environmental toxins and microbes. In CF, the loss of chloride and bicarbonate that result from CFTR dysfunction

affects the formation of gel that comprises the mucus layer, thereby inhibiting normal mucus barrier function.[15] Inflammation, which is commonly associated with CF, also increases production of mucus in these tissues.[16] The clinical manifestations resulting from these irregularities include thick, dense mucus accumulation, abnormal mucus adhesion to the epithelia, and increased epithelial permeability that leaves the host susceptible to further physical compromise and infection.[15–17]

As a recently emerging field in research, exploration of the human microbiome in relation to health and disease has provided new insight for clinicians working with CF populations.[18–20] Microbial dysbiosis of the lungs and GI tract resulting from mucosal perturbations have been identified in the literature and have been linked to increased mortality and morbidity in both children and adults.[21–23] Recent research efforts in the field have begun to characterize patterns in the CF microbiota community, noting that the microbiome tends to differ significantly from healthy controls.[20] However, the literature presents conflicting evidence on the microbial community structure in CF populations, often due to small sample size and differing study designs. Moreover, discrepancies in subject age and exposure to antibiotic therapies, as well as differing variants of the CFTR gene mutation play a role in microbiome composition.[24–26]

Groups that have conducted systematic reviews of CF microbiome profiles are in general agreement, noting that species found in the airway such as *Pseudomonas aeruginosa*, *Burkholderia cepasia*, and *Staphylococcus aureus* have been strongly associated with respiratory infection.[20] In the GI tract, suppression of *Bacteroidetes* and *Firmicutes* at the phylum level, enrichment of *Enterobacteriaceae* at the family level, and suppression of *Bifidobacterium* and *Clostridium* at the genus level have been reported.

Though respiratory infections and their subsequent complications have been identified as the leading cause of mortality in CF patients,[4,27,28] researchers are now starting to characterize complex relationships linking pathogenic colonization of the GI tract with colonization of the respiratory tract in these populations.[20,25,29–32] Ultimately, CFTR dysfunction is associated with a decline in microbial diversity of both the lungs and gut; a loss of beneficial organisms occurs as pathogenic microbes proliferate, leading to a state of dysbiosis.[33] These changes in the CF microbiota have downstream effects in tissues that further compromise the health of individuals with the disease.

While the respiratory tract has received substantial attention in CF patients, the gut is less well understood. It has been noted that CFTR dysfunction and the resulting aberrant mucosa are principal contributors to gut dysbiosis in CF. The mucosal barrier of the GI tract plays an integral role in maintaining the homeostasis of the gut, serving as the first line of defense against luminal microbes and their antigenic by-products.[34,35] The intestinal epithelium must be able to perform the functions of digestion and absorption while still preserving the integrity of the mucosal barrier, a feat achieved through dynamic communication between the intestinal epithelium and luminal microbes.[34] The physical barrier of the intestinal epithelium is made up of tight junctions, which are proteins that join intestinal epithelial cells at their apical side and seal off spaces between the cells. This physical barrier is crucial to gut homeostasis and serves as the principal determinant in regulating mucosal permeability of the intestines.[34,36] If tight junction integrity is compromised, microbes and their antigenic by-products are able to pass through the mucosal barrier into epithelial tissues via the paracellular route.[34] Several studies further highlight the role of intestinal inflammation in altering the composition of

the gut microbiota.[29,37,38] A possible bi-directional relationship between gut dysbiosis and intestinal inflammation has been suggested, indicating that microbe-derived products and other triggers contribute to inflammation, while intestinal inflammation conversely stimulates physiological changes that contribute to alterations in microbial communities.[38,39]

Importantly, physiological concentrations of inflammatory molecules have been found to increase permeability of epithelial tight junctions, allowing for paracellular transport of these molecules and other gut-derived bacterial antigens across the intestinal epithelia.[36,40] In addition, inflammatory molecules derived from gut microbes serve as a key contributor in inciting local inflammatory responses in the intestines as well as systemically.[40] In a healthy individual, concentrations of inflammatory molecules are either low or undetectable in circulating plasma. However, when the tight junction barrier is defective, inflammatory molecules are able to permeate intestinal tissues paracellularly, leading to increased translocation to the plasma.[40] Once into systemic circulation, exposure to inflammatory molecules induces the release of several inflammatory mediators, including pro-inflammatory cytokines and reactive oxygen species.[34,35] These inflammatory mediators further contribute to the degradation of tight junctions, thereby increasing intestinal permeability even more and resulting in chronic inflammation of the intestinal tissues.[34] In addition, excess mucus accumulation in the GI tract and impairments in intestinal motility and mucus clearance can also trigger intestinal inflammation, contributing greatly to the intestinal pathophysiology of CF patients.[33,41,42] Antibiotic therapy, used to treat bacterial infections frequently occurring in CF patients, contributes further to the development of dysbiosis seen in the respiratory and GI

tracts.[43–45] Regardless of how CF manifests, several studies illustrate that chronic intestinal inflammation is linked to gut dysbiosis and also hinders digestion and absorption of nutrients in the GI tract.[29,37,45,46]

Patients with CF often present with nutritional deficiencies, which arise from several factors related to chronic malabsorption. Those of primary interest include pancreatic insufficiency, aberrant intestinal mucosa, and decreased energy intake due to chronic intestinal inflammation and systemic infection.[47,48] Malnutrition, which has been indicated as a primary variable associated with death risk, is noted more often in CF patients than in healthy controls, and contributes greatly to disease severity and progression.[49,50] Poor nutritional status has also been attributed to GI dysbiosis as well as chronic intestinal inflammation in children with CF.[51]

Given the role of suboptimal nutrition in CF patients, strategies to improve nutritional status are paramount in improving health outcomes. At present, dietary therapies include pancreatic enzyme replacement as well as high-fat, high-calorie diets. Supplementation with fat-soluble vitamins are also recommended.[52] While dietary manipulation of the gut microbiota by including pre- and pro-biotics, as well as altering intake of indigestible carbohydrates has been proposed as a way to manage intestinal conditions associated with CF,[39] there is little research on dietary modification of the gut microbiome in these populations.[29,31,53] Considering the integral role of the gut microbiota in both the intestinal and overall health of CF patients, it is worth considering dietary strategies targeted at supporting a healthy gastrointestinal microbiome.

There has been rising interest in functional foods that may positively influence the gut microbiota. Bioactive polyphenols and other phenolics found in many edible plants

exert anti-inflammatory effects and also improve vascular health. Intestinal microbes metabolize polyphenols obtained through the diet and convert them to phenolic acids, which can modify the community structure of the gut microbiota.[54] Genistein, a non-steroidal dietary polyphenol that comprises 50% of the isoflavone composition of soy foods,[55,56] has demonstrated the ability to modulate activity of CFTR within cells, and has already shown promise in improving CFTR dysfunction in cell cultures and murine models of CF.[57–61] Studies suggest that activation of CFTR by genistein increases channel open probability, lengthens channel opening, stimulates Cl^- and bicarbonate (HCO_3^-) secretion, and alters localization of CFTR within cells.[59,60,62,63] These effects implicate genistein's role as a compound potentially capable of restoring the luminal environment of the GI tract by improving the ion channel function of CFTR. While dietary genistein may improve CFTR function in intestinal tissues, no studies to date have explored whether dietary genistein affects microbial community structure of the gut in CF populations.

Purpose of Study

The intent of this experimental study was to examine whether dietary genistein treatment altered gut microbe community structure in mice homozygous recessive for the delta-F508 gene variant.

Research Aims and Hypotheses

Aim 1: To explore associations between dietary genistein treatment and diversity of the gut microbe community structure in mice homozygous for the DF508 CFTR mutation following a 45-day experimental dietary study.

- H_1: We hypothesized that the gut microbiota of mice homozygous for the DF508 CFTR mutation receiving dietary genistein treatment would have similar or greater within-sample diversity (richness, evenness, Shannon's Diversity Index) compared to mice receiving standard chow plus Colyte treated water, and greater within-sample diversity compared to mice receiving standard chow.
- H_2: We hypothesized that overall microbial community structure (between-sample diversity via Jaccard, Bray-Curtis, and weighted and unweighted Unifrac metrics) would differ by dietary treatment group, as evidenced by differential clustering (PCoA plots).

Definition of Terms

- Cystic fibrosis: An autosomal recessive genetic disease that affects the rheological properties of mucus in various body systems.
- CFTR mutation: A genetic mutation that affects the cystic fibrosis transmembrane regulator gene, resulting in dysfunctional protein activity and localization.
- Genistein: A soy isoflavone that exerts a number of biological activities in the body.
- Colyte: A solution comprised of polyethylene glycol with electrolytes that is commonly used as a laxative.
- Microbiome: The collective genomes of all microorganisms present within the host ecosystem.
- Microbiota: The collective microorganisms that are present within the host ecosystem.
- Dysbiosis: Imbalance of the microbiota present within a given ecosystem.

- Prebiotic: “A substrate that is selectively utilized by host microorganisms conferring a health benefit,” as defined by ISAAP.[64]
- Probiotic: “Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host,” as defined by ISAAP.[65]
- Polyphenol: An organic compound that contains two or more phenolic groups.
- Alpha-diversity: Measures of microbial richness, evenness, or both that operate on a single sample from a given ecosystem.
- Beta-diversity: Measures of microbial composition and community structure that operate on a pair of samples from a given ecosystem.
- Relative abundance: The percent composition of a particular microorganism relative to the total number of microorganisms in a given sample.
- Differential abundance: Microorganisms that are present in different abundances across groups of samples.

CHAPTER 2

REVIEW OF THE LITERATURE

Cystic Fibrosis

History of cystic fibrosis. Cystic fibrosis was first identified in 1938, when Dr. Dorothy Andersen discovered cystic fibrosis of the pancreas during neonatal autopsy in patients suspected to have Celiac disease.[2] Over the next decade, researchers continued to characterize and distinguish the pathological differences between Celiac disease and CF, noting that the exocrine glands were largely affected by abnormal mucus accumulation and ductal plugging. The disease was further characterized by lung infection and chronic nutritional disturbances, including fat malabsorption, pancreatic insufficiency, steatorrhea, and malnutrition.[2,66] While the diagnosis of CF in earlier years was initially pathological in nature, it was later discovered that nearly all patients with CF exhibited elevated sweat chloride concentrations, allowing for a quicker and more effective means of diagnosis.[67] In 1989, several research teams made a pivotal breakthrough in the field after identifying the gene responsible for CF; this discovery provided an additional diagnostic tool to clinicians while simultaneously affording researchers new opportunities to explore solutions related to gene therapy.[68–70]

Current understanding and diagnosis. Today, CF is understood to be a life-shortening genetic autosomal recessive disease occurring most frequently among Caucasians.[4,71,72] CF manifests due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which codes for an ion channel found broadly across epithelial cell surfaces.[69,73] Epithelial cells throughout the body express CFTR and rely on it for normal cellular function.[74,75] When CFTR dysfunction occurs, the

rheological properties of mucus are negatively affected which results in a host of health complications for those with the disease, as shown by several reviews.[76–78] At present, the sweat chloride test remains the gold standard for the detection of CFTR abnormalities, and is generally performed according the Cystic Fibrosis Foundation guidelines.[4,79] Any value over 60 mmol/L signifies a positive CF diagnosis.[4,80]

Disease prevalence. Shortly after identifying CF as a distinct disease, the Cystic Fibrosis Foundation (CFF) was formed in the United States, with the introduction of the patient data registry in 1966.[81] While the US was one of the first countries to implement a national patient data registry, many countries have since established a national registry of their own in order to track health information, with most registries collecting data after 1990.[3] These global registries play an integral role in tracking CF data, as well as providing funding for research and drug development. The majority of registries publish a patient data report annually.[3] Several reviews have published epidemiological data regarding CF prevalence, with current global estimations placing the number of individuals with the disease at about 70,000; however, these numbers are likely underestimated due to underreporting in emerging registries as well as in geographical regions where CF is rare.[3,71] While the great majority of patients with CF are of European origin, a small percentage (<10%) of CF patients represented in global registries are of Asian, African, or Hispanic descent.[3,4,82,83]

Survival and mortality in cystic fibrosis. Although it was once considered a pediatric disease, reviews conducted in recent years show that the majority of patients diagnosed with CF now survive into adulthood.[3,50] In the United States, more than half of the patients registered with the CFF are over 18 years of age, with the predicted median

survival at approximately 48 years of age in 2016.[4] The increase in survival age is owed to significant progress in disease treatment over the past few decades, and can be attributed to factors including aggressive nutritional support, infection control, lung transplantation, pancreatic enzyme replacement therapy (PERT), CFTR modulator therapies, and decreased age at diagnosis, likely due to newborn screening implementation in healthcare facilities.[3,50,84] These trends have been identified across the United States, Canada, and Europe.[3,50,84]

While the upward trend in median age of survival is promising, a 2017 review found that the median age of death in CF patients globally remains less than 30 years in the majority of countries.[3] Disconcertingly, childhood deaths are not a rarity among children with CF. A large cohort study following over 5,000 pediatric patients over a decade showed several predictors of increased childhood mortality. These included early-age crackles (irregular lung sounds), clubbing (of digits), and *Pseudomonas aeruginosa* infection in the lungs, as well as being of the female sex. The authors also identified weight-for-age and height-for-age, at the age of four years old, as a major predictor for death before age 20 in CF populations.[22] Another two-year cohort study following CF patients 16 years of age and older found increased mortality rates for those with a low body mass index (BMI), as well as for those of shorter stature and those who had less lean body mass.[85] There also appears to be a gender gap in CF mortality risk, with females showing poorer survival rates.[50,86] It has been postulated that poorer outcomes in female CF patients are due to the rate of acquisition of pathogenic organisms. Compared to males, female CF patients acquire these organisms earlier and more readily.[86,87] Despite these trends, advancements in patient care have still greatly enhanced quality of

life and extended the lifespan in CF populations; however, it is important to note that with increasing age come new health concerns. Common comorbidities include CF-related diabetes, osteopenia, osteoporosis, arthritis, sinus disease, and mental health disorders such as anxiety and depression.[4,75] Cancer of the GI tract is also becoming increasingly more prevalent, with increased risk following immunosuppressive treatment for transplantation.[88,89]

Etiology of cystic fibrosis. Cystic fibrosis occurs due to mutations in the CFTR gene.[68–70] Found on the long arm of chromosome seven, the gene comprises 180,000 base pairs and codes for a 1480-residue membrane protein termed the cystic fibrosis transmembrane conductance regulator (CFTR).[74,90] CFTR belongs to the ATP-binding cassette (ABC) transporter family, which consist of membrane proteins responsible for the transport of various metabolites, as well as cell communication; however, CFTR is the only membrane protein in this family known to act as an ion channel.[73,90–92] CFTR is widely expressed throughout the body, forming ion channels at the apical membrane of epithelial cells.[93,94] These ion channels play a crucial role in the transport of anions, expressing a specificity for chloride (Cl^-) and bicarbonate (HCO_3^-).[11] Several review papers support the role of CFTR as a Cl^- and HCO_3^- transporter and have also shown that CFTR regulates chloride-bicarbonate exchange.[11,74,77] In addition to its role as an ion channel, CFTR performs an array of other cellular functions vital to health. These include the down-regulation of transepithelial sodium transport, regulation of calcium-activated chloride and potassium channels, and plasma membrane functions.[74]

Disease genotype and phenotype. All individuals with CF acquired two copies of mutated CFTR alleles and show clinical signs and symptoms associated with the disease,

whereas heterozygotes with a single mutated CFTR allele fail to express an abnormal phenotype.[69,95] CFTR mutations are categorized into classes I-V, with classes I-III exhibiting severe phenotypic disease expression. Class IV and V mutations are associated with more mild disease phenotypes.[96,97] After the CFTR gene was discovered in 1989,[68–70] researchers began exploring the relationship between genotype and disease phenotype. While there are more than 1900 known CFTR gene mutations, the majority of individuals (>80%) with CF possess at least one copy of the recessive Phe508del mutation (also referred to as DF508);[4] this mutation is characterized by the deletion of phenylalanine (F) at locus 508, resulting in destabilization and defective folding of the protein structure as well as preventing the mutated protein from reaching the cell surface.[4,74,96,98] The DF508 CFTR gene mutation has been categorized as a class II defect.[4,96]

In a retrospective cohort study examining US CFF mortality data in over 12,000 CF patients from 1991-1999, it was found that those homozygous for the DF508 mutation had much more severe manifestations of the disease than those who were heterozygous or had other gene variants. Pancreatic insufficiency and lung dysfunction appeared earlier in those homozygous for DF508; nutritional status in these patients also appears to be worse, as measured by patient height and weight.[14] The association between genotype and phenotype is also shown in other studies, supporting the view that individuals homozygous for the DF508 mutation demonstrate heightened symptom severity.[12,13]

Animal models of cystic fibrosis. As with most human pathologies, studies utilizing animal models serve as a specialized tool to identify and learn about illness, disease progression and health outcomes in humans. There are several animal models used to study CF in science today, including pig, ferret, and mouse models. While pig

and ferret models closely mimic the respiratory and pancreatic phenotype of CF in humans,[99–101] mouse models serve well to represent the intestinal environment of the human gut and also have the benefit of reproducing quickly and being much more cost-effective.[38] In the majority of CF mouse models, CFTR functions at less than 10% of optimal activity and most severely affects the gastrointestinal tract.[38] One study looking at three distinct CF mouse models found that each model exhibited bacterial overgrowth of the intestine.[102] Studies have shown that bacteria become trapped in the mucus layer of the intestinal epithelium, leading to decreased intestinal motility and bacterial overgrowth. Mice must be treated with laxatives and antibiotics, respectively, to alleviate these issues.[103] Researchers have also examined CF mice and found that they were significantly smaller than their wild-type counterparts. When given antibiotic treatment for intestinal infections, the CF mice were able to gain weight.[42] Key findings such as these have allowed scientists and clinicians to further research and develop targeted strategies to treat CF and improve health outcomes.

Pathophysiology of cystic fibrosis. The pathophysiology of CF is somewhat complex and multifactorial. However, decades of research have allowed for the dedicated exploration of the molecular and pathophysiological basis of the disease, and many in-depth reviews have recently been published summarizing the current understanding of CF pathophysiology. The disease is typified by accelerated mucus production and alterations in the physicochemical properties of mucus.[16] When individuals are healthy, the mucus layer that lines epithelial surfaces acts as a physiological barrier, defending against injury from environmental toxins, microbes, and microbially-derived products.[15,16] Mucins are the primary constituent of mucus, and are comprised of large glycoproteins. They are

manufactured by goblet and mucosal cells in epithelial tissues and are secreted into the lumen of organ systems with mucosal surfaces.[16] Transmembrane mucins are anchored on the apical side of the epithelial cell membrane, while gel-like mucins float freely above in order to collect microbial debris and particulate matter to shuttle out of the lumen.[104] Preserving health in organ systems with mucosal surfaces is therefore dependent on proper mucin release and mucus barrier function.

In CF, the loss of Cl^- and HCO_3^- that result from CFTR dysfunction are primarily responsible for the clinical manifestations of the disease.[15,104] While researchers initially looked at Cl^- transport to distinguish the effects of CFTR dysfunction, it was later discovered that HCO_3^- serves a crucial function in maintaining the normal rheological properties of mucus.[105,106] This is due to the action of CFTR as an ion channel for both Cl^- and HCO_3^-, as well as a driver for Cl^-/HCO_3^- exchange.[11,104] The resulting loss of CFTR function negatively influences the characteristics of extracellular mucus by locally influencing pH, thereby disrupting homeostasis of ionic and fluid content at epithelial surfaces. Recent reviews have shown that HCO_3^- secretion is essential for intestinal mucus release, proper mucus viscosity, and for normal mucin unfolding and expansion; these effects are vital in preserving the normal rheological properties of the mucus, and to discourage mucus adhesion to the mucosal surfaces of epithelia.[11,77,104]

Health outcomes associated with cystic fibrosis. Since CFTR is ubiquitous in epithelial tissues, CF affects a number of organs throughout the body, including the pancreas, airways, intestines, kidneys, salivary and sweat glands, and the reproductive organs.[77] A number of reviews show that aberrant mucus accumulates in the lumens of organ systems that produce gel-forming mucins at epithelial surfaces, and contributes to

the pathophysiologic triad seen in CF. This triad is signified by mucus-based obstruction, chronic infection, and inflammation.[16,37] Common manifestations of CF include persistent respiratory infection, intestinal obstruction, pancreatic insufficiency, pancreatitis, GI cancer, and CF-related liver disease.[37,75] Despite the widespread effects of CFTR dysfunction, the most common CF pathologies stem from abnormal mucus accumulation that interferes with normal cell function in the respiratory and digestive systems.[20,29,74,75] While respiratory pathologies and their subsequent complications have been identified as the leading cause of mortality in CF patients,[4,27,28] gastrointestinal manifestations generally present first and tend to be more severe depending on gene variation.[29,37]

Gastrointestinal health in cystic fibrosis. Though CFTR plays an important role throughout the body, functional CFTR is particularly important in maintaining GI health. Gastrointestinal pathologies are attributed to a cascade of events that alter the milieu of the GI environment, impairing proper organ function.[38,78] As discussed, dysfunctional CFTR results in defective Cl^- and HCO_3^- transport and exchange at epithelial cell surfaces along the digestive tract, and in its related organs.[15] Consequently, ion and fluid secretion is impaired, leading to shifts in local pH and poorly hydrated luminal fluid.[38,78,104] Lack of sufficient luminal HCO_3^- hampers normal mucin unfolding and expansion, which prevents proper mucus release and results in highly viscous mucus that accumulates in the GI lumen.[38,77] In addition to these deleterious effects on mucus properties, intestinal motility is disturbed due to smooth muscle inhibition, slowing intestinal transit and subsequently affecting mucus clearance throughout the GI tract.[38,41] These mucosal perturbations, paired with diminished mucus clearance and an altered

luminal environment, also promote microbial dysbiosis in the GI tract.[33,42,103] This cascade of events plays a significant role in the chronic intestinal inflammation seen in CF patients, and contributes to the aforementioned pathophysiologic triad that leads to the ensuing degradation of digestive organs, including the intestines, pancreas, liver, and gallbladder.[37,38,48]

Impact on nutrient digestion and absorption. Considering the many changes to the gastrointestinal environment in CF, it is unsurprising that digestion and absorption of nutrients is negatively impacted in this population. Nutritional deficiencies are common in CF and are owed to several factors, including pancreatic insufficiency, aberrant intestinal mucosa, suboptimal calorie intake, and increased energy expenditure.[47] Exocrine pancreatic insufficiency (PI) affects approximately 85% of patients at some point during life and is a key driver of the maldigestion and malabsorption seen in individuals with CF.[4,48,78,107] The pancreas is responsible for secreting digestive enzymes into the duodenum in order to break down and absorb nutrients from food, and also secretes HCO_3^- in order to neutralize gastric acids.[48] In patients with PI, bicarbonate transport is almost completely absent; pancreatic enzyme output is greatly diminished, and enzyme activity is impeded by insufficient HCO_3^-, resulting in intestinal luminal acidification from gastric contents.[48,78,104] While individuals with pancreatic sufficiency (PS) are able to secrete bicarbonate, its presence is still reduced,[104] hindering enzymatic ability to fully digest and absorb nutrients throughout the GI tract.

Nutritional status in cystic fibrosis. Nutritional status is paramount to achieving positive health outcomes in patients with CF, regardless of age. Because digestion and absorption of nutrients is considerably affected due to changes in the GI environment,

strategies to maintain adequate nutrition for those with CF remain an important component of patient care. The United States CFF nutrition guidelines designate height and weight above the 50th percentile as the baseline goal for infants, children, and teens up to 19 years of age. Adults 20 years of age and older are advised to maintain a BMI at or above 22 for females, and a BMI at or above 23 for males.[4] When patient height or weight fall below these values, advanced nutritional management is strongly encouraged.[4] It should be noted that there is an association between nutritional status and pulmonary function in CF patients. When measures of nutritional status or pulmonary function improve, there is a concurrent improvement in the other measure.[4] Since respiratory complications are chiefly responsible for death in this population,[4,27,28] combatting malnutrition and subsequently improving lung function may prove to be a key strategy in improving patient health and lengthening lifespan.

Several studies have examined data pertaining to patient body habitus and lung function. A prospective observational cohort study analyzed data from 3,142 patients and found that those who attained a higher weight-for-age percentile at 4 years of age experienced fewer pulmonary exacerbations and had greater survival at 18 years of age.[108] Similar findings were published in 2001, showing that patients who were >85% of their ideal body weight and had an FEV_1 (forced expiratory volume in 1 second) >30% had better outcomes at 5 years of age.[109] Studies have additionally confirmed that body wasting on its own presents a significant independent predictor of mortality in CF patients.[109,110] There is an increased risk of death in children with a BMI lower than the 10th percentile, and in adults with a BMI lower than 20 kg/m^2.[85,110] Researchers have also demonstrated that there is sufficient evidence to suggest a positive association between

measures of stature and increased survival in CF patients.[4,85,108,110] Further, these studies illustrate that decreased lung function, stunting, and wasting all significantly predict increased mortality risk. Individuals with CF undoubtedly face numerous health challenges throughout life. In order to promote optimal nutritional status and increase survival odds, there is a critical need to identify and implement nutritional strategies to improve the gastrointestinal environment and optimize digestion and absorption of nutrients in the CF population.

The Human Microbiome

Host-microbe interactions. Though they are microscopic, microbes comprise the vast majority of earth's life forms.[111] As such, humans and microorganisms have coexisted for millions of years and continue to foster a complex and dynamic relationship. The human body hosts a diverse community of microorganisms, including archaea, bacteria, viruses, protozoa, and fungi.[54,112] These collective microbial communities, referred to as the microbiota, are highly unique and consist of symbiotic, commensal, and pathogenic organisms alike.[54,113] Remarkably, the human microbiota play integral roles in numerous physiologic activities. Some of these roles include immune system function, xenobiotic metabolism, synthesis of vitamins and various metabolites, and nutrient digestion and absorption. The microbiota also play a role in mucosal barrier integrity and pathogen displacement, making it a key player in homeostasis preservation.[51,54,112,114]

The human microbiome in health and disease. Given the extensive participation of microbes in human metabolism, it should come as no surprise that the human

microbiome plays a vital role in health and disease. When in good health, the relationship between humans and resident microbes is mutualistic in nature, with each entity providing benefits to the other.[54,112] However, disturbances in the microbiome can negatively affect human health and have been identified as a contributory factor in the pathogenesis of a number of diseases.[112,115,116] These microbial disturbances are termed dysbiosis; when community composition shifts occur, dysbiosis of the microbiota can contribute to both pathologic and metabolic disturbances.[34,51,117] Reviews have shown associations between the microbiota and conditions such as cardiovascular disease, cancer, inflammatory bowel diseases, cystic fibrosis, obesity, and even behavioral disorders.[51,53,115–117] The colon, which is home to over 50 bacterial phyla, may hold the greatest potential in regard to the microbiome impacting human physiology.[29]

Taxonomic and functional diversity of the microbiome. There are several commonly used methods to obtain microbiome data.[118] Due to its fairly unbiased diversity characterizations of bacteria and archaea, as well as its relatively economical use, 16S rRNA gene sequencing is frequently used to measure and describe microbial community composition and structure. Microbial community composition is typically defined by measures such as diversity, richness, and evenness.[18,118] Other methods include obtaining metagenomic and metatranscriptomic data to describe functional potential and activity of gene expression, respectively.[118] The presence of diverse microbial communities is integral to human health, and human metabolism is the culmination of both human and microbial genomic interplay.[51] Diversity is particularly important in a community; all taxa present within a community affect phenotype expression to varying degrees, which can then interact with the host's environmental

state. This is referred to as functional diversity. Taxonomic diversity, which observes the composition and relative abundance of the species making up a community, plays a large role in functional diversity as different microbes encode unique information within their genomes.[119] These dynamic interactions between the host and the microbiome have the potential to strongly influence health and disease.

The gut microbiome. Of all body locales colonized with microbes, the gut serves as home for the great majority, with roughly 70% of the microorganisms within the human body taking up residence there.[114,120] Interestingly, the collective genome of the gut microbiota has a much greater number of genes when compared to the human genome.[54,121] It is therefore important to understand how these genes interact with the host environment, and how their metabolic products and functions may affect the host immune system. Microbes express their own gene function on the host, and the richer the microbiota, the higher the functional gene richness.[18] In a fascinating study published in 2014, researchers categorized people into either high or low microbial gene count groups; those with higher gene counts tended to be healthier individuals, with greater overall health owed to a more robust gut microbiome and increased microbial metabolic functions.[122] Overall, the more rich and diverse the gut microbial community, the greater the health benefits conferred to the host.[18]

The role of the gut microbiome in human health. Several studies have found that gut microbe metabolic activity is correlated with human health, as well as nutrition.[114,123–125] In addition to the roles already broadly described, microbial by-products are capable of modulating health through an array of mechanisms.[51] Some key roles of the gut microbiota include immune system development, regulation of intestinal endocrine

function, and biosynthesis of vitamins and neurotransmitters; the microbiota also serve to displace pathogenic organisms and assist with nutrient absorption in the gut.[112,113] In the intestines, the microbiota serve in the crucial function of water-soluble vitamin production. Several B-vitamins, including vitamin B_{12}, thiamine, riboflavin, pyridoxine, biotin, as well as vitamin K are all synthesized by microbes, and are then absorbed by enterocytes.[51,126] Additionally, the microbiota synthesize essential amino acids that humans cannot produce with their own enzymes.[54,126] The gut microbiome also has a number of genes coding for the metabolism of many simple sugars and starches.[54,127] These microbes colonizing the GI tract greatly enhance the metabolism of glycans and amino acids, and further play a role in metabolizing xenobiotics.[54,127] Other studies support these findings, reporting that core bacteria colonizing a healthy GI tract assist in the metabolism of carbohydrates and are also integral to synthesizing vitamins and other cofactors essential for metabolism.[18,128] One of the most appreciated roles of the gut microbiota is fermentation. Some intestinal microbes easily process indigestible polysaccharides from plant foods and subsequently produce SCFA, including acetate, propionate, and butyrate. These SCFA are understood to contribute to human metabolism as well as immune function.[51,54,111,126] Some of the neuroactive metabolites synthesized by microbes include gamma-aminobutyric acid (GABA), norepinephrine, dopamine, histamine, and acetylcholine.[129,130] Serotonin, an essential neurotransmitter, is chiefly produced in the GI tract through host-microbe interactions, with about 90% of serotonin coming from the gut microbiota.[131]

Taxonomic characterization of the gut microbiome. Several reviews have characterized the microbiota present within the GI tract. Among the various taxa present

in healthy adults, *Bacteroidetes* and *Firmicutes* represent the dominant phyla.[18,113,114,120] *Proteobacteria* and *Actinobacteria* are also present, though they are present in smaller numbers and generally account for less of the microbial community.[113,114] One review also found that the *Verrucomicrobia* phylum was present in small numbers in the healthy GI tract.[18] Several bacterial taxa within the gut have been correlated with adequate GI function and human health. From the *Firmicutes* phylum, identified taxa include *Roseburia, Eubacterium, Lactobacillus, Faecalibacterium,* and *Clostridium clusters XIVa* and *IVa*.[18,114] From the *Bacteroidetes* phylum, taxa from the *Bacteroides* genus have been identified, and from the *Actinobacteria* phylum, taxa from the *Bifidobacterium* genus have been associated with GI health.[18,114] While taxa from the *Proteobacteria* phylum represent a small portion of the gut microbiota in those with no intestinal pathologies, they represent a greater proportion of the bacterial community in those with GI disease.[18]

Factors influencing the gut microbiota. While studies suggest that the gut microbiome generally appears stable over time in healthy adult populations,[18,113,132] recent reviews have shown that a number of factors can produce alterations in gut microbiota composition. Both endogenous and exogenous factors, including age, diet, sex, genetics, epigenetics, and the use of antimicrobial agents, exert substantial effects on the microbiota and play a role in shaping the microbial community.[113,114] Additionally, the number and type of bacteria present differ depending on mucin composition, pH of the intestinal environment, and nutrient availability.[29] Reviews have found that the composition of the gut microbiota changes through the lifecycle.[120,133,134] Dissimilitude in gut microbial communities is greatest during infancy.[32] While scientists once believed that infants were sterile prior to birth, evidence demonstrates that the gut microbiome

may be present while in the prenatal environment.[18] Additionally, mode of delivery plays a significant role in the microbial colonization of infants. Those born vaginally are colonized with microbes found in the mother's vaginal tract, while infants born via Caesarean section acquire microbes from the mother's skin.[135,136] At birth, microbial communities are hardly developed and remain unstable through the first year of life before beginning to reach adult stability within one to three years of life.[32,113,132] Though rapid alterations in community composition still occur in early childhood, microbiome stability increases with age. By the time individuals enter adulthood, community composition tends to achieve and maintain stability through 70 years of age.[18,119,120] Nevertheless, deteriorations in composition may occur in old age.[113,120] Age-related effects observed regarding the microbiota include the decline in capacity for vitamin B_{12} biosynthesis via microbial metabolism, decreased activity among microbial reductases, and the potential for disturbances in the immune system, responses to stress, and higher likelihood of DNA damage.[18]

Individual dietary choices have a notable impact on the gut microbiota, and have been shown to quickly modulate gut microbiome composition and community structure.[18,113] In a 2013 study, healthy adults consuming meat-restricted or vegetable-restricted diets showed selective enrichment of bile-metabolizing or plant polysaccharide-fermenting microorganisms, respectively.[137] Dietary habits aside, alterations in gut microbiome composition can also be produced by acute or chronic illness, traveling overseas, or prescribed antibiotic use.[18,138] Certain practices, such as colonic lavage, have also been shown to reduce the microbiota associated with the mucosa of the GI tract, as well as the microbiota colonizing the GI lumen. One such bacteria known to colonize the

mucosal epithelium of the GI tract, *Mucispirillum*, has been repeatedly shown to disappear after the procedure.[139,140] Nearly all antibiotics, regardless of reason for use, have been linked to higher incidence of opportunistic infections.[54] During critical illness, the intestinal microbiome experiences a large degree of stress, especially following alterations in nutrient and oxygen availability, medication exposure, and antibiotic treatment, which can diminish the whole microbiome in patients. As more and more research is published on the effects of stress on the intestinal microbiome, health professionals are beginning to understand that preserving the integrity of the microbiome during periods of stress may itself enhance the immune system.[119]

Gut dysbiosis and physiological consequences. While many of the taxa present in the gut are commensal, beneficial microbes, there are primary and opportunistic pathogens present as well, though they account for less than 0.1% of the microbiota in the GI tract.[18,119] Reviews have shown that shifts in gut microbe communities have the potential to negatively impact mucosal health and disrupt epithelial barrier function, leading to dysregulation of the gut's innate immune system and chronic intestinal inflammation.[112,113] There are several other contributors to GI barrier function, including integrity of the epithelial mucus layer as well as the integrity of the tight junction complex.[113,141] Mucosal lesions may result from gut dysbiosis, further promoting an inflammatory state within the intestines.[112] When gut dysbiosis occurs, pathogenic organisms skew the taxa that normally predominate; this dysbiosis has been implicated in a myriad of diseases, ranging from neurologic disorders to GI, metabolic, cardiovascular, and cancer-promoting illnesses.[112,113] Interestingly, correlation studies conducted using both human and mouse models found associations between dysbiosis and common

chronic disorders that include autism spectrum disorder, atherosclerosis, asthma, and metabolic conditions, as shown by one review.[113]

The collective human microbiome in cystic fibrosis. As previously discussed, dysfunctional CFTR affects the physicochemical properties of the mucosal epithelium.[16,29,38] These mucosal perturbations promote shifts in microbial community structure and composition. While CFTR is ubiquitously expressed in epithelial tissues throughout the body, the most significantly affected sites for those with CF include the respiratory, GI, and reproductive tracts.[77] With recent improvements in sequencing technologies and bioinformatics software, many broad reviews have been published on the CF microbiome. CF patients are most known for the respiratory complications they experience, many of which are related to the chronic colonization of the lungs by pathogenic microbes.[27] Top contributors to morbidity and mortality in the CF population include bronchiectasis and chronic infection, both of which are widely acknowledged in the clinical etiology of CF.[20] The most common bacterial pathogen in the CF airway is *Pseudomonas aeruginosa*,[27,28] followed by *Staphylococcus aureus*.[27] Other respiratory pathogens include species from the *Burkholderia cepacia* group, of which there are around 17 phylogenetically related species.[28] Antibiotics used to treat recurrent respiratory infections in the CF population have also promoted the emergence of novel pathogens. For example, *methicillin-resistant Staphylococcus aureus* (MRSA) and *Mycobacterium abscessus* infection have become more prevalent, both of which are resistant to wide spectrum antibiotics and are challenging to treat.[4,27] Of note, the Cystic Fibrosis Foundation (CFF) Annual Data Report from 2016 identified that the prevalence

of *P. aeruginosa* has been declining over the last several years.[4] However, the CFF notes that multidrug-resistant *P. aeruginosa* prevalence has remained constant.[4]

To date, studies in humans have shown a decrease in microbial richness and diversity in CF populations as compared to healthy controls and siblings.[24,142] Microbial dysbiosis has been identified most notably in CF airways and GI tracts, which result from aberrant mucus accumulation, repeated antibiotic use, and irregular microbial colonization patterns in these systems.[32] Consequences of acute and chronic dysbiosis include systemic inflammation, altered immune function, and recurrent infection.[32] While respiratory pathologies are recognized as the leading cause of mortality in CF patients, GI complications also represent a considerable cause of disease morbidity and mortality.[20] Despite the strong focus on the respiratory microbiota in CF, researchers have begun to investigate the association between the GI microbiota and the microbiota of the airways in CF patients.[20] A 2016 review illustrated that recent studies conducted on the CF gut microbiota found associations between gut dysbiosis and lung pathologies seen in CF patients.[20] One study showed that the decline in specific GI bacteria, namely taxa from the *Parabacteroides* genus, preceded *P. aeruginosa* colonization in the CF airway.[30] More research is currently needed to understand the interactions of polymicrobial communities, including the relationship between the gut and lung microbiota in CF populations.

The gut microbiome in cystic fibrosis. Due to its critical role in immune and metabolic function, as well as its marked impact on nutrition, the GI microbiome is capable of affecting lifelong health.[32] Recall that CFTR is heavily expressed throughout the GI tract; the aberrant mucus that accumulates as a result of CFTR dysfunction is

considered a central pathological event in CF. The ramifications of these mucosal perturbations have been shown to contribute immensely to the progression of the disease.[78] At birth, individuals with CF display structural and functional alterations throughout the GI tract, many of which stem from CFTR dysfunction.[37,78] It has been postulated that atypical microbial patterns at birth, combined with recurring disruption of the gut microbiota community structure, may affect immune function in CF populations. Cumulatively, these events may be associated with disease progression of the lungs and GI tract.[32] Gut dysbiosis, especially in the context of CF where the intestinal environment is already significantly altered, is associated with a vast number of deleterious clinical concerns. Bacterial dysbiosis of the GI tract has been linked to impaired mucus barrier function, endotoxin accumulation, immune function disturbances, and translocation of microbially-derived lipopolysaccharides, which further aggravate the inflammatory response both intestinally as well as systemically.[16,34,54,121,143,144] Furthermore, alterations in intestinal permeability contribute to bacterial overgrowth in CF.[29] Even when GI symptoms are absent, there is evidence for chronic inflammation of the GI tract in CF populations; this is considered a primary driver of systemic inflammation in CF.[38] It is these significant alterations to the GI environment that affect the biodiversity of the gut microbiota.[78,145]

Taxonomic characterization of the cystic fibrosis gut microbiome. Thanks to advances in sequencing technologies, identifying the taxonomic and functional diversity of the gut microbiota in CF patients is much more attainable than could be achieved with culture-based approaches.[29] To date, a number of insightful papers have been published exploring the gut microbiota in CF patients. As previously discussed, some incongruities

exist among taxonomic characterization of the CF gut microbiome due to variances in sample size and study design. At the phyla level, several studies demonstrated decreased abundance of *Bacteroidetes*,[24,43,44] *Firmicutes*,[24,146] *Proteobacteria*,[43,44] and *Actinobacteria* in CF patients versus healthy controls.[43,146] However, two studies published in 2017 conversely found increased abundance of *Firmicutes* and *Actinobacteria* at the phyla level,[43,44] and a 2016 study showed increased abundance in the *Bacteroidetes* and *Proteobacteria* phyla.[146] One study also demonstrated decreased abundance of taxa from the *Verrucomicrobia* phylum,[44] while another study found *Verrucomicrobia* to be comparable in CF and non-CF controls.[43] Taxa from the *Escherichia*,[24] *Shigella*,[24] *Enterococcus*,[24,44] *Blautia*,[24] *Veillonella*,[24,31] and *Clostridium* genera were found in greater abundance in several studies.[24,43] Underrepresented genera in CF patients included *Roseburia*,[44] *Prevotella*,[44,145] *Faecalibacterium*,[44] and taxa from *Clostridium* cluster *XIVa*.[142] Taxa from the *Bifidobacterium* and *Bacteroides* were shown to be decreased in several studies.[43,44,142,145] However, two papers describe increased abundance of *Bifidobacterium* and *Bacteroides* genera.[31,44] As of yet, no discrepancies in species-level taxonomic characterization have been reported. *Ruminococcus bromii* was found in decreased abundance,[142] whereas *R. gnavus* and *R. torques* were found in increased abundance.[43,146] Several studies characterized a significant decrease in *Faecalibacterium prausnitzii* in CF patients compared to healthy controls.[43,142,146] Decreased abundance of *Clostridium coccoides* and *Bifidobacterium longum* was demonstrated in two studies in adults with CF, and one study demonstrated increased abundance of *Enterococcus faecalis*.[43]

Antibiotic use and the cystic fibrosis gut microbiome. As antibiotics are a regular treatment modality in CF patients, several studies examined the gut microbiota in the context of antibiotic use. An interesting study published in 2013 examined fingerprints from denaturing gradient gel electrophoresis (DGGE) in conjunction with qPCR to assess CF-related intestinal dysbiosis. All CF patients in the study were treated with antibiotics either before or during the sampling timeframe. The authors found that taxa from the *Bifidobacterium* genus and members of *Clostridium cluster XIVa* were significantly underrepresented in CF patients. Additionally, *Ruminococcus bromii* and *Faecalibacterium prausnitzii* (from *Clostridium cluster IV*) were underrepresented. Of note, the investigators highlight that lower abundance of *Clostridium cluster XIVa* taxa were seen in CF patients with poorer nutritional status as well as those with low weight-for-height, even when compared to healthy siblings that also presented with low weight-for-height.[142]

In a study published in BMC Microbiology, investigators collected and extracted metagenomic DNA from fecal samples and compared the gut microbiota of CF and non-CF individuals. Gut microbiota diversity and community composition was assessed using 16S rRNA gene sequencing, and study results showed that the CF group had significantly less diverse gut microbiota when compared with the non-CF group ($p<0.05$). Additionally, PCoA plots using weighted and unweighted UniFrac measures displayed separations in clusters between CF and non-CF groups. Taxonomic abundance differences were also significant ($p<0.05$) between CF and non-CF controls, notably in regard to decreases in *Bacteroidetes*, *Proteobacteria*, and *Verrucomicrobia* phyla, and significant increases in *Firmicutes* and *Actinobacteria* ($p<0.05$).[44] An important

characteristic of this particular study was the examination of antibiotic use. Those with CF were categorized into groups based on how many IV antibiotic treatments were received in the previous year. The study found that those in the low IV antibiotic group (less than or equal to one course, n=9) had significantly higher bacterial diversity ($p<0.05$) than those in the intermediate (two or four courses, n=8) or high (five or more courses, n=4) IV antibiotic groups. CF patients in the high IV antibiotic group displayed the greatest proportion of *Firmicutes* and the lowest proportion of *Bacteroidetes* at the phylum level compared to other CF patients. The paper shows a significant negative correlation between gut microbiota diversity and the frequency of IV antibiotics CF patients receive. This paper also examined FEV_1 (forced expiratory volume in 1 second) and found those with severe lung dysfunction had significantly diminished alpha diversity ($p<0.05$), whereas the highest alpha diversity measures were seen in those with mild lung disease.[44] The authors of a 2017 paper mention finding a greater number of gene families associated with antibiotic resistance in the CF group when compared to the control group, which is perhaps unsurprising when one considers the consistent exposure to antibiotics throughout the typical CF lifespan.[43]

Genotype and gut microbiome characterization in cystic fibrosis. A 2013 cohort study conducted by Schippa et al. examined CFTR gene variants and their relationship to gut microbiota composition and disease severity. A total of 36 patients (adolescents and adults) with CF underwent CFTR genotyping, and 26 different CFTR alleles were identified in the cohort. The majority of subjects had at least one DF508 mutation (66.7%), followed to a lesser extent by those with a N1303K mutation (16.7%). Subsequently, all patients were divided into classes which included those homozygous

for the DF508 mutation (n=5), those heterozygous for the DF508 mutation (n=19), and those absent for the DF508 gene mutation (n=5). Subjects who possessed one DF508 allele (n=24) underwent clinical evaluation for forced expiratory volume (FEV_1), exocrine pancreas function, and BMI. Patients were then given a score of either mild (n=10) or severe (n=14). The study results showed that patients homozygous for the DF508 mutation, as well as those who were given a score of severe, displayed a prominent reduction in beneficial bacterial species, including *Eubacterium limosum*, *Bifidobacterium spp*., and *F. prausnitzii*. These individuals also showed increased abundance of potentially detrimental species such as *Escherichia coli* and *Eubacterium biforme*. Even within the CF cohort, DF508 homozygotes and those classified as severe exhibited greater intestinal microbial dysbiosis.[25]

A proof-of-principle pilot study conducted by Fouhy and colleagues utilized shotgun metagenomic sequencing on freshly collected fecal samples from six adults with CF and six healthy controls. Of the CF patients, half were homozygous for the DF508 mutation and half were heterozygous for the mutation. In the CF group, taxa from the *Firmicutes* phylum were increased, whereas decreases were seen in the *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* phyla. The authors found *Verrucomicrobia* to be comparable in CF and non-CF controls. At the genus level in CF patients, significant decreases were seen in *Bifidobacterium*, whereas at the species level, *B. longum* was significantly decreased. Within the *Firmicutes* phylum, *Enterococcus faecalis* and *Clostridium* were elevated, as were *Ruminococcus gnavus* and *Ruminococcus torques*; however, significant decreases in *F. prausnitzii* were seen in the CF group.[43] Miragoli and colleagues also examined CF genotype in young adults with CF, though no

correlation was identified between gut microbiota composition and host genotype in the paper.[145]

Associations between lung and gut microbiomes in cystic fibrosis. A cohort study by Madan and colleagues observed the microbiota of the lungs and GI tract in CF infants. Seven infants were enrolled in the study within one month of birth, and followed for up to 21 months; samples were regularly collected at 3-month intervals. A total of 59 samples (26 respiratory samples and 33 intestinal samples) were collected and analyzed throughout the course of the study. Results from the study showed that *Veillonella, Bifidobacterium*, and *Bacteroides* genera dominated the GI tract, and made up 40% of the community. The most striking finding produced by the study was the concordance in taxa seen in both body sites. The great majority of taxa seen increasing the GI tract (14/16 genera) concurrently increased in the respiratory tract ($p<0.001$). Furthermore, gut colonization of seven of the observed genera preceded colonization of the airways.[31]

Hoen et al. also analyzed the respiratory and GI tract microbiota in a prospective longitudinal cohort study of infants and young children with CF. A total of 120 oropharyngeal and stool samples (54 respiratory samples and 66 intestinal samples) were collected from 13 subjects from birth up to 34 months of age, with sample collection occurring at 3-month intervals. Interestingly, this study demonstrated that changes in certain GI taxa preceded respiratory colonization. In the GI tract, significant decreases were seen in bacteria from the *Parabacteroides* genus prior to *P. aeruginosa* colonization in the airways ($p<0.001$). Taxa from the *Bifidobacterium* and *Bacteroides* genera were also shown to decrease in GI samples before initial respiratory colonization and CF exacerbation, though the results were not significant after correction for multiple

comparisons. Other significant findings of the study included an observed significant association between gut microbiome composition and CF exacerbation ($p=0.03$), as shown by PCA plots.[30]

Cystic fibrosis gut microbiome characterization using fecal proteomics. In 2016, Debyser and colleagues published a novel study examining taxonomic composition and protein expression using fecal proteomics. The study compared children with CF who had pancreatic insufficiency (n=15; ten DF508 homozygotes, five DF508 heterozygotes) with their non-CF siblings (n=15). At the phyla level, the study results yielded that samples from the CF children showed fewer *Firmicutes* and *Actinobacteria* and more *Bacteroidetes* and *Proteobacteria.* Interestingly, abundance of *F. prausnitzii* was reduced while *R. gnavus* abundance was increased. This is of interest as *R. gnavus* has been implicated as a potential mucin degrader,[147] which is why they may prefer the CF intestinal environment. Proteomes were also compared between healthy siblings and CF patients. The study demonstrated a significant difference ($q<0.05$) in the relative abundance of 79 protein clusters between the two groups. CF patients were shown to have lower abundance of proteins related to glycoprotein degradation and conversion pathways of carbohydrates, as well as higher abundance of acute phase protein clusters in CF patients. CF samples were also much higher in a number of inflammatory proteins. Other overrepresented proteins in the CF samples include those related to mucus, as well as goblet cells that secrete mucus. This is of interest considering the effects of CFTR dysfunction on the rheological properties of mucus in CF patients. In contrast to these findings, the healthy sibling group showed increased abundance of bacterial proteins generally seen in those without GI conditions, with higher numbers of proteins involved

in carbohydrate metabolism. Two proteins from *Faecalibacterium prausnitzii*, a species shown to be suppressed in CF patients in other studies, were also increased in the sibling group. Notably, non-CF samples were also found to contain pancreatic secretory granule membrane major glycoprotein (GP2), which may play a role in creating a physical barrier and inhibiting bacterial binding to receptors of host cells; this protein was not observed in the CF samples. In addition to decreased abundance of beneficial taxa, the increased abundance of acute-phase and inflammatory proteins in the CF group no doubt contribute to the altered intestinal environment observed in CF patients.[146]

Dietary Impact on the Gut Microbiome in Cystic Fibrosis.

Current dietary practices to address nutritional status in cystic fibrosis. The nutritional deficiencies and malnutrition that are commonly experienced by those with CF have been shown to contribute significantly to morbidity and mortality of the disease, as shown by several reviews.[47,49,50] Disturbances in nutritional status are owed primarily to maldigestion and malabsorption, both of which are consequences of a number of physiologic and metabolic derangements that occur in CF. These include increased energy expenditure, decreased calorie consumption, decreased GI transit time, pancreatic insufficiency (PI), abnormal mucus accumulation, intestinal inflammation, and gut microbial dysbiosis.[47,48] These derangements are at least partially responsible for the significant alterations seen in the CF intestinal environment. The central tenet to improving nutritional status in CF is the expectation that patients will be able to more readily cope with the acute and chronic clinical manifestations, as well as the upsurge in energy demands, that are commonly seen in the disease.[47] As pulmonary exacerbations

are the chief cause of mortality in CF and nutritional deficiencies are associated with declines in respiratory function, strategies to improve the nutritional intake of CF patients should be paramount given the correlation between nutritional status and enhanced clinical outcomes, most importantly increased survival.[4,47] At present, the standard nutrition recommendation given to CF patients is to liberally consume a high-calorie, high-fat diet to combat increased energy expenditure and fat malabsorption.[47,148]

Medical approaches to complement dietary strategies aimed at improving the nutritional status of CF patients include the use of nutritional supplements and pancreatic enzyme replacement therapy (PERT); these modes of nutritional support represent the foundation of medical nutrition therapy used to manage the disease.[4,47] Exocrine pancreatic insufficiency (PI) is one of the most common conditions associated with CF and is tied to the hindered digestion and absorption of nutrients in the GI tract.[48] The CFF reports that over 80% of patients registered with the organization required PERT in 2016, with that number climbing to 97% of individuals with genetic mutations in classes I-III (the most severely affected patients).[4] As the pancreas is a major organ tasked with macronutrient digestion via enzyme production, patients with PI generally need PERT in order to sufficiently digest and absorb nutrients, especially fat and fat-soluble vitamins.[48,149]

Functional food and nutrient approaches to modify the gut microbiome. Since microbial dysbiosis has also been linked to infection, morbidity, and mortality in CF patients, identifying dietary modifications that enrich diversity and normalize community composition of the gut microbiota may improve clinical outcomes.[39] Furthermore, attenuation of microbial dysbiosis may assist in optimizing intestinal interactions at the

mucosal epithelium, subsequently improving immune responses, nutrient digestion and metabolism.[39] Of all the factors that influence the composition of the gut microbiota, dietary modification stands out as a target of interest due to ease of manipulation.[39] Functional foods, which are considered foods or dietary products consumed for an intended health benefit,[150] have incited particular interest among researchers. Those that have elicited the most attention include prebiotics, probiotics, and polyphenols found within food and supplements. Prebiotics are substrates that the gut microbiota are able to selectively ferment, thus stimulating beneficial bacterial proliferation and activity in the GI tract.[151] Soy oligosaccharides, as an example, possess prebiotic properties as they arrive at the colon intact, and can then be broken down by microbes.[152] Probiotics have also been shown to modulate the intestinal microbiota.[126] Probiotics are live organisms that confer benefits to the host if given in sufficient amounts, and act in a number of ways to impact human health.[126] When introduced, they can act directly in the GI tract, or participate in the milieu of the intestinal environment and subsequently affect the microbiota, as well as barrier and immune functions.[126] Some of the actions of probiotics in the GI tract include luminal pH reduction, nutrient competition, prevention of bacterial epithelial adhesion, and secretion of anti-microbial compounds. In addition to altering gut microbiota composition, these mechanisms may also work to inhibit the proliferation of pathogenic microorganisms.[126] Microbes are also affected by the consumption of bioactive polyphenols and other phenolics found in many edible plants.

Polyphenols are plant-derived compounds with two or more phenolic groups. Among them are bioflavonoids such as anthocyanins, proanthocyanins, flavonols, flavones, and isoflavones, as well as lignans, stilbenoids, and coumestanes, among

others.[153] These compounds can exert anti-inflammatory effects and have also been shown to improve vascular health.[54] In the body, polyphenols are found to be most concentrated in the GI lumen.[153] However, absorption of dietary polyphenols is generally incomplete in the GI tract. Subsequently, the intestinal microbes metabolize polyphenols obtained through the diet and convert them to phenolic acids, which can modify the community structure of the gut microbiota.[54] In this way, polyphenols and their metabolic by-products may then accumulate in the GI tract and produce physiologic effects.[154] If the GI barrier is crossed by either polyphenols or their metabolites, they are able to enter into the systemic or lymphatic circulation and exert their effects there as well.[153] Studies reveal that plant-derived polyphenols impact the gut microbiota by either enhancing proliferation of beneficial microbes or by inhibiting pathogen proliferation.[155]

Microbial conversion of dietary polyphenols into phenolic acids modulates gut microbiota community structure by promoting *Bifidobacteria* proliferation; a decrease is also seen in the *Firmicutes* to *Bacteroidetes* ratio. Furthermore, microbial metabolism of polyphenols stimulates intestinal production of SCFA.[54,156] As previously discussed, SCFA are utilized by intestinal cells for energy, and when in the systemic circulation they can also be used by other tissues.[150] Other studies examining the effects of polyphenol ingestion on the gut microbiota support these findings. For example, studies examining tea polyphenols found that the intestinal microbial environment was modified by their intake, and suggested phenolics may inhibit the growth of pathogenic microbes.[154] Another study examining dietary intake of flavanols from cocoa found that the growth of certain gut microbes was affected, indicating potential prebiotic benefits exerted by consuming foods rich in dietary flavanols.[157]

Current studies examining functional food approaches in the treatment of cystic fibrosis. Prebiotics have not been extensively examined in the context of CF. Still, it has been shown that indigestible carbohydrates such as oligosaccharides and fructooligosaccharides (FOS) promote the proliferation of commensal microbes in the GI tract, which has been demonstrated as an effective method in reversing dysbiosis.[29] However, given the complex community structure of the gut microbiota in both healthy individuals and those with CF, it is problematic that prebiotic substrates may be acted upon by either commensal or pathogenic bacteria in the gut.[151] Conversely, the use of probiotics as a potential therapy in CF has been well studied. It has been noted that GI symptoms have been associated with the presence of distinctive taxa within the intestinal tract.[138] As such, it has been postulated that probiotic administration in CF may improve GI symptoms by altering the composition and community structure of the gut microbiota.[39] One review highlighted that probiotics may have the capacity to alleviate GI symptoms and attenuate the gut inflammation seen in CF, both of which are thought to disrupt dietary intake and inhibit nutrient digestion and absorption.[39] Another study reported that children consuming *Lactobacillus rhamnosus GG* for four weeks showed reduced gastrointestinal symptoms and biomarker concentrations related to intestinal inflammation.[158] In rat models, probiotics have been shown to be beneficial in preserving colonic barrier function; the result was decreased translocation of microbes and reduced concentrations of plasma endotoxin.[34]

Studies examining the use of probiotics in CF patients show they may modulate and restore commensal gut bacteria, thereby reducing pulmonary exacerbations, decreasing gut permeability, and increasing quality-of-life.[39,159] There have been a

handful of studies across the CF lifespan examining probiotic administration to modulate the gut microbiota, which were shown to benefit pulmonary function in CF.[160,161] Interestingly, these studies demonstrated that probiotic supplementation for six months significantly reduced the occurrence of respiratory exacerbations. The probiotic strains used in the supplements included either a combination of *Steptococcus* species, *Bifidobacterium*, and *Lactobacillus*, or *Lactobacillus rhamnosus GG* alone.[160,161] The authors of these studies suggested that the supplements worked by potentially restoring beneficial gut microbes, or by down-regulating the inflammatory response in the lungs.[160,161] In a systematic review published in 2017, investigators examining the effects of probiotic supplementation on intestinal inflammation and rate of pulmonary exacerbation in CF found limited evidence to provide general recommendations to those with the disease.[162] While the authors note that the five studies that met inclusion criteria showed positive effects of probiotic supplementation in patients overall, study quality was inadequate; high quality randomized controlled trials are needed to ascertain benefit to the CF population.[162]

It should also be noted that prebiotics and probiotics may work synergistically to provide benefits to the host, especially in cases where commensal microbes have been reduced.[29,39] This pairing, termed synbiotics, may help alleviate the chronic inflammation seen in CF, as shown by one study.[151] Other studies examining nutritional therapies in CF patients have been scant. One study that surveyed parents of children with CF reported increased use of CAM (complementary and alternative medicine) therapies and found nutritional supplements among the top CAM therapies used,[163] though no outcome measures were reported. Regarding studies examining the use of dietary polyphenols to

modulate the gut microbiota, there have been very few that contribute to the body of evidence. However, genistein, a soy isoflavone and dietary polyphenol, has been extensively studied in the context of CF.

Genistein, a soy isoflavone. Isoflavones, which exert numerous biological effects in the body, are found in large quantities in soybeans.[56] Soybean isoflavones are usually found as glycosides; the attached sugar is hydrolyzed to allow for absorption, creating aglycones. The common aglycones include glycitein, daidzein, and genistein. These aglycones and their pre-cursors account for the isoflavone content found in soybeans.[56,164] Genistein, a particular isoflavone of interest, constitutes about 50% of the total isoflavones found in soybeans, and is readily transported in the body via passive diffusion across cell membranes.[56,165] Unlike its glycoside genistin, the deconjugated genistein can also be absorbed in the stomach.[166,167] Genistein is recognized to have several biological functions, including antioxidant, phytoestrogen, and tyrosine kinase inhibitor activity.[168–172] Soybeans are classified as a prebiotic due to their high oligosaccharide content. These oligosaccharides, predominantly stachyose, are not digested well and travel to the colon where they are metabolized by bacteria. The resulting proliferation of certain bacterial species, such as *Bifidobacteria,* is beneficial to the individual's health.[56] In one ex-vivo study conducted in 2017, the soy isoflavone genistein was not shown to inhibit the aggregation of pathogens; however, probiotic biofilm formation was observed.[155] Other studies have demonstrated that genistein has shown antibacterial activity against pathogenic microbes, including *Staphylococcus aureus*, as well as *Bacillus anthracis* and *Vibrio fluvialis*. These studies also supported earlier findings of probiotic biofilm formation, showing increased aggregation of bacteria such as *Lactococcus lactis* and

Lactobacillus rhamnosus, as well as *Lactobacillus paracasei*.[155,173] Clearly, genistein is an interesting isoflavone exhibiting fascinating biological effects that warrant further attention.

To date, one of the most captivating characteristics of genistein is its role as a CFTR potentiator, as shown by numerous studies.[57–63,97] Recall that the most common defective CFTR allele inherited in CF is DF508. This mutation creates defects in the CFTR protein that inhibit proper folding and prevent the protein from reaching the cell membrane. In addition, the Cl^- ion channel function of the protein is exceedingly impaired. In short, such mutations prevent appropriate protein localization to the cell surface and impedes the ability to execute its intended biochemical role as an ion channel.[58] Furthermore, defective anion secretion causes aberrations in luminal pH and contributes to the disease pathogenesis in CF, especially in regard to the microbial dysbiosis that ensues following pH alterations.[38,78]

Thus far, genistein is the most well-studied CFTR potentiator.[57,58] Studies have shown that genistein, both in cell cultures and murine models, is a promising potentiator of CFTR and may be implicated in abating CFTR dysfunction.[57–59,61,97] Studies exploring the effects of genistein on CFTR activation have found genistein to stimulate Cl^- and HCO_3^- secretion,[58,59,62,63] both of which are essential to preserving the physicochemical properties of mucus in epithelial tissues. Moreover, genistein has been shown to increase the probability and lengthen the duration of channel opening.[60,63,97] Studies also support that genistein alters localization of CFTR to the apical membrane within epithelial cells, helping to overcome the defect in CFTR trafficking.[57,58,62,97] These effects implicate

genistein as a natural compound potentially capable of restoring the luminal environment of the GI tract by improving the ion channel function of CFTR.

Potential applications of genistein in cystic fibrosis treatment. Genistein is unquestionably an intriguing isoflavone. In addition to the various actions of genistein described above, genistein has also been shown to have low toxicity at physiologically relevant levels.[174] Genistein also boasts antimicrobial potential and may prove to be useful in combatting bacterial infections,[173,175–177] especially considering the increased prevalence of multi-drug resistant pathogenic infection in CF patients.[4,27] Since the gut microbiota may be altered by consumption of polyphenolic compounds, genistein may be a metabolite worthy of further exploration. Crucially, genistein has been shown to modulate CFTR function in cell cultures and murine models, which may confer clinical benefits to those with CF. In light of genistein's ability to affect CFTR location and biochemical function, and considering its low toxicity, genistein may present as one of the most promising candidates for developing therapeutic strategies to manage CF. While dietary genistein may improve CFTR function in intestinal tissues, no studies to date have explored whether dietary genistein affects microbial diversity or community structure of the gut in CF populations.

CHAPTER 3

METHODS

Research Subjects and Study Design.

In this randomized 3-arm parallel experimental study, twenty-four male and female mice homozygous for the DF508 CFTR gene mutation were maintained on one of three diet regimens for a 45-day period, following weaning at 20 days of age. Mice were stratified by weight and gender and randomly assigned to each diet group. Animal care and treatments were conducted in accordance with established guidelines and all protocols were approved by the Research and Animal Care and Use Committee of Case Western Reserve University. Note that this study was a secondary analysis of a sub-group of mice from a published survival study, with fecal pellets collected from 6-11 animals per group.[178]

Treatment Groups

The control group (n=11; 7 female and 4 male) was fed standard chow with regular untreated water, while a second treatment group was fed standard chow with Colyte (n=7; 3 female and 4 male), a laxative (considered standard of care), added to the water (polyethylene glycol 3350 with electrolytes; Kremers Urban, Princeton, NJ, USA). A third treatment group was fed chow containing 600 mg of dietary genistein per kg of chow with regular untreated water (n=6; 3 female and 3 male). All mice had ad-libitum access to food and water.

Protocol and Outcome Measures

DF508-CF mice were bred at Case Western Reserve University School of Medicine (Cleveland, OH, USA) and housed individually in cages in an animal care

facility with a 12-hour light-dark cycle. Body weight data were obtained before and after the 45-day treatment period, and mice were weighed every five days until euthanasia at 65 days of age using a calibrated balance scale. General health was monitored twice per week during the study, with laboratory technicians ensuring a clean environment and providing fresh water regularly. Collection of biological samples were conducted wearing the applicable personal protective equipment. Upon study completion, mice were placed in a separate box and euthanized via atmospheric asphyxiation using 100% CO_2, followed by thoracotomy to induce pneumothorax. Fecal pellets were collected just prior to euthanasia, flash-frozen in liquid nitrogen, and stored at -80˚C for later processing and analyses.

Laboratory Analyses

Frozen fecal samples were transferred to an Arizona-based institution to conduct gut microbiome community profiling. Samples were thawed on ice prior to processing. Gene amplification and sequencing were performed by Center for Fundamental and Applied Microbiomics (CFAM) staff using equipment in the Genomics Core Lab at Arizona State University. Fecal samples were processed to extract microbial genomic DNA using the DNeasy PowerSoil DNA Isolation Kit following standard manufacturer procedures (Catalog No. 12888, Qiagen, Germantown, MD, USA). Following extraction, DNA was quantified using the Quant-iT PicoGreen assay per manufacturer guidelines (Catalog No. P7589, Invitrogen, Carlsbad, CA, USA).

The 16s rRNA gene was amplified using 515F-806R primers as described by Caporaso et al.[179] The forward primer (515F) was ligated with a 5’ Illumina adapter, and the reverse primer (806R) was ligated with the reverse complement 3’ Illumina adapter; a

unique Golay barcode was attached to the reverse PCR primer sequence only to allow for sample identification after sequencing. The PCR products were validated on a 1.5% agarose gel, cleaned using the QIAquick PCR Purification Kit (Catalog No. 28106, Qiagen, Germantown, MD, USA), and quantified using qPCR using the KAPA Library Quantification Kit (Catalog No. KK4824, Kapa Biosystems, Wilmington, MA, USA). Sequencing was conducted on the Illumina MiSeq platform using 2x250 base read chemistry.

Demultiplexed sequences were then processed using the Quantitative Insights into Microbial Ecology 2 (QIIME 2) pipeline. Samples were quality checked and denoised to correct sequencing errors using DADA2; filtering, dereplication, and merging of paired-end reads were also performed using DADA2.[180] Taxonomy of DADA2 output sequences were determined using a naïve-Bayes classifier based on the GreenGenes 13.8 database. Sequence alignment was performed using MAFFT.[181] Fasttree (default) was used to produce a phylogenetic tree for diversity analyses.[182]

Statistical Analyses

Sample size for the parent study was determined based on published work examining this mouse model,[178] while this sub-analysis used a convenience sample comprised of animals having adequate fecal material for microbial sequencing analyses. QIIME 2 was used to conduct diversity analyses for all samples. The metadata file used for bioinformatic analyses was constructed in accordance with MIMARKS guidelines (BioSample package: specimen, host-associated; v.4.0).[183,184] An alpha-rarefaction curve using Observed OTUs was assessed to ensure sufficient sequencing depth. All diversity metrics and statistical analyses performed in QIIME 2 were reported using the

provenance function to allow for reproducibility of the workflow. Statistical significance was set at $p<0.05$, or $q<0.05$ for Benjamini-Hochberg corrected values following multiple comparisons. QIIME 2 (v. 2018.6) was used for quality control prior to statistical analyses of all samples.

Aim 1 Statistical Analyses

Hypothesis 1. Alpha-diversity (within-sample) metrics were assessed using Observed OTUs to assess richness, Pielou's Evenness to assess evenness, and Shannon's Diversity Index to assess richness and evenness.[180–182,185–189] Median alpha-diversity values were visually reported using distribution comparison plots (box-and-whisker plots), and group differences evaluated by Kruskal-Wallis tests.[190]

Hypothesis 2. Beta-diversity (between-sample) metrics to explore community composition were assessed qualitatively by 2D principle coordinates analysis (PCoA) plots visualized using EMPeror (wrapped in QIIME 2) and assessed statistically by running PERMANOVA and pairwise comparisons using four ß-diversity metrics: Jaccard, Bray-Curtis, Unweighted Unifrac, and Weighted Unifrac.[179–182,191–195] All metrics used in the analyses accounted for presence of taxa, though only the Bray-Curtis and Weighted Unifrac metrics tested for taxa abundance. Phylogenetic ß-diversity metrics included Unweighted Unifrac and Weighted Unifrac measures, and were used to evaluate evolutionary relationships between taxa, whereas Jaccard and Bray-Curtis measures assumed equal relationships between present taxa. Each β-diversity metric assessed group differences using pairwise PERMANOVA comparisons with the number of permutations set at 999.

Differential abundance. To further explore differences in gut microbial community structure across treatment groups, differential abundance analyses were conducted using ANCOM (QIIME 2)[179,180,186,189,196–198] and LEfSe (Galaxy Version 1.0). The DADA2 feature table from QIIME 2 upstream analysis was filtered for the minimum frequency that features appeared across all samples (minimum frequency = 2). The newly filtered feature table was then used to perform a taxa collapse at the phylum and genus level. These taxa-collapsed tables were uploaded directly for analysis into the LEfSe tool.[199] Following LEfSe analyses, one differential abundance test was conducted with all groups, and each analysis was additionally repeated with one treatment group removed to see intergroup differences. The algorithm utilized by LEfSe is fairly robust, and detects statistical significance using the non-parametric Kruskal-Wallis rank-sum test, with subsequent pairwise Wilcoxon rank-sum testing to detect differentially abundant OTUs. Finally, linear discriminant analysis was used to approximate the effect size of identified features.[200] Visual outputs provided by LEfSe analysis included LDA scores and a cladogram for differentially abundant taxa. For use with ANCOM, pseudocounts were added to the taxa-collapsed feature tables before running differential abundance tests in QIIME 2.[196] ANCOM utilizes log-ratio analysis to detect differentially abundant taxa and produces a W-value that denotes the number of times a sub-hypothesis is rejected; this indicates that the identified taxa are significantly different in abundance from W number of taxa. The higher the W-value, the more differentially abundant features were between samples.[196]

CHAPTER 4

RESULTS

Forward and reverse reads were trimmed by 10 base pairs; no truncations were performed on the forward reads (251 base pairs), while the reverse reads were truncated to 240 base pairs. A sampling depth of 32,800 sequences was identified as adequate for phylogenetic diversity metrics based on the minimum frequency per sample (**Figure 1**). Alpha rarefaction maximum depth was initially selected based on the median frequency per sample of 38,750 sequences. However, this depth was adjusted to 32,800 sequences to preserve the overall number of samples after seeing drop off in alpha-rarefaction curves.

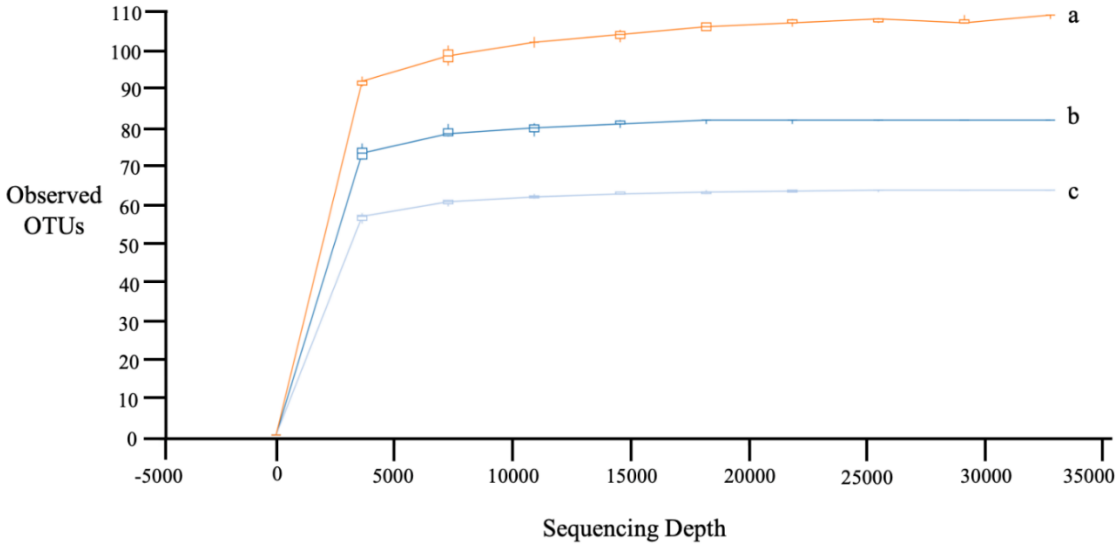

Figure 1. *Alpha rarefaction curve showing Observed OTUs by sequencing depth*. Treatment groups are indicated by letters (a, standard chow; b, Colyte; and c, Genistein).

Alpha Diversity Metrics

Kruskal-Wallis tests were used to assess group differences in alpha diversity metrics. After evaluating observed OTUs, the genistein diet group significantly differed from the

standard chow group ($p \leq 0.003$), as well as the group that received Colyte-treated water ($p=0.012$). The standard chow group and Colyte group also differed significantly ($p \leq 0.005$) (**Figure 2**). Similarly, Shannon's Diversity Index scores were significantly different between the genistein and Colyte dietary treatment groups ($p=0.006$), and between the genistein and standard chow treatment groups ($p \leq 0.003$). However, no significant difference was observed between the standard chow and Colyte groups (**Figure 3**). Lastly, no significant treatment group differences were observed for evenness of taxa within the microbial community, as measured by Pielou's Evenness (**Figure 4**). The results yielded following statistical analyses of the alpha-diversity metrics indicate significant differences in bacterial diversity of the gut within dietary treatment groups.

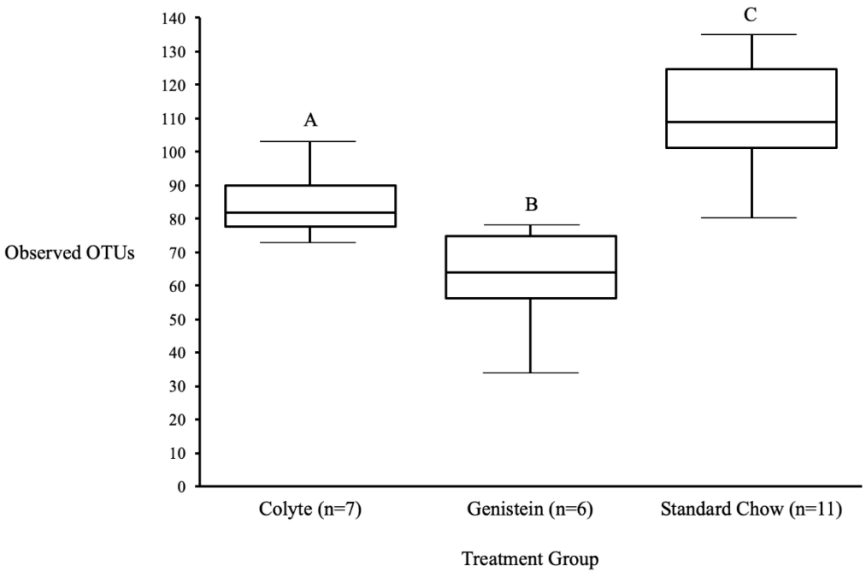

Figure 2. *Alpha diversity boxplot for Observed OTUs*. Unique superscript letters denote significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons.

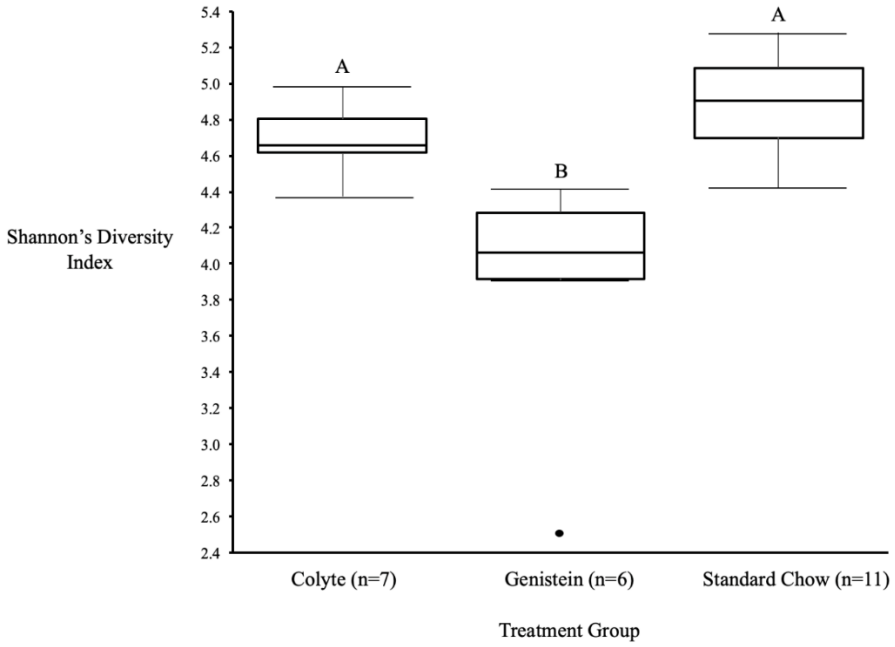

Figure 3. *Alpha diversity boxplot for Shannon's Diversity Index.* Unique superscript letters denote significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons.

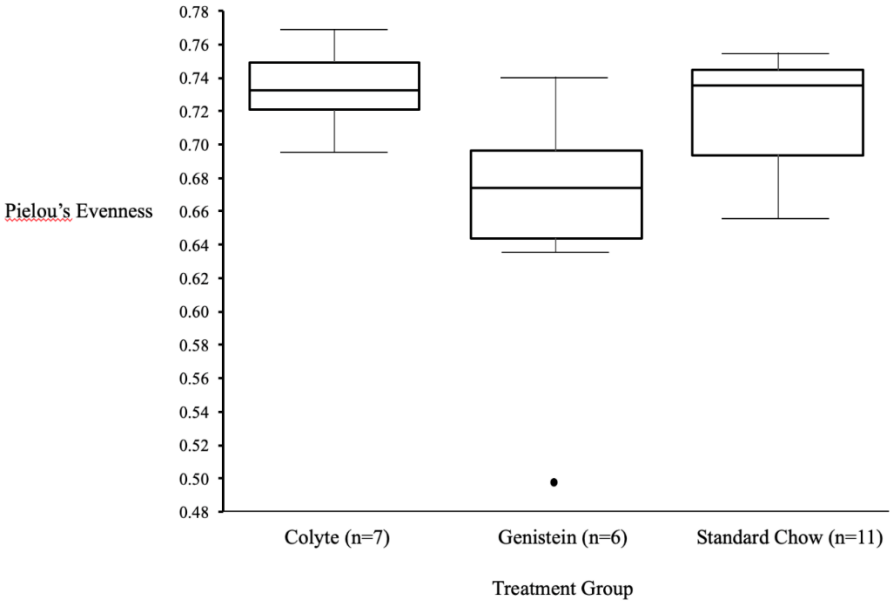

Figure 4. *Alpha diversity boxplot for Pielou's Evenness.* No significant differences were observed for evenness of taxa within the microbial community by dietary treatment groups.

Beta Diversity Metrics

PERMANOVA was used to assess pairwise differences in β-diversity metrics. Jaccard distance analyses (representative of taxa presence after assuming equal evolutionary relations between taxa) showed significant pairwise differences in the taxa present in feces from animals in all dietary treatment groups (genistein vs. standard chow, p=0.001; genistein vs. Colyte, p=0.001; standard chow vs. Colyte, p=0.001, **Figure 5**). Bray-Curtis distance measures (representative of taxa presence and abundance after assuming equal evolutionary relations between taxa) also significantly differed by dietary treatment group, with taxa from the genistein treatment group clustering significantly further from the standard chow (p=0.003) and Colyte (p=0.004) treatment groups. The Colyte and standard chow groups also differed significantly in distance, as shown in the PCoA plot (p=0.003) (**Figure 6**).

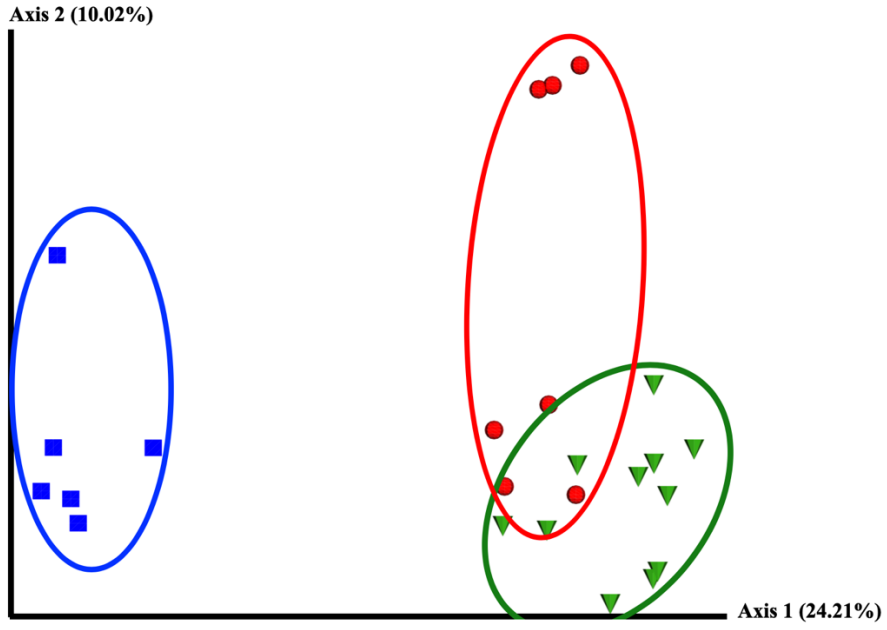

Figure 5. *Beta diversity PCoA plot of between group distances using Jaccard distance matrix values.* Treatment groups are as follows: genistein, blue squares; Colyte, red circles; standard chow, green triangles. Significant group differences (p<0.05) after

Benjamini-Hochberg correction for multiple comparisons were observed for all pairwise treatment comparisons.

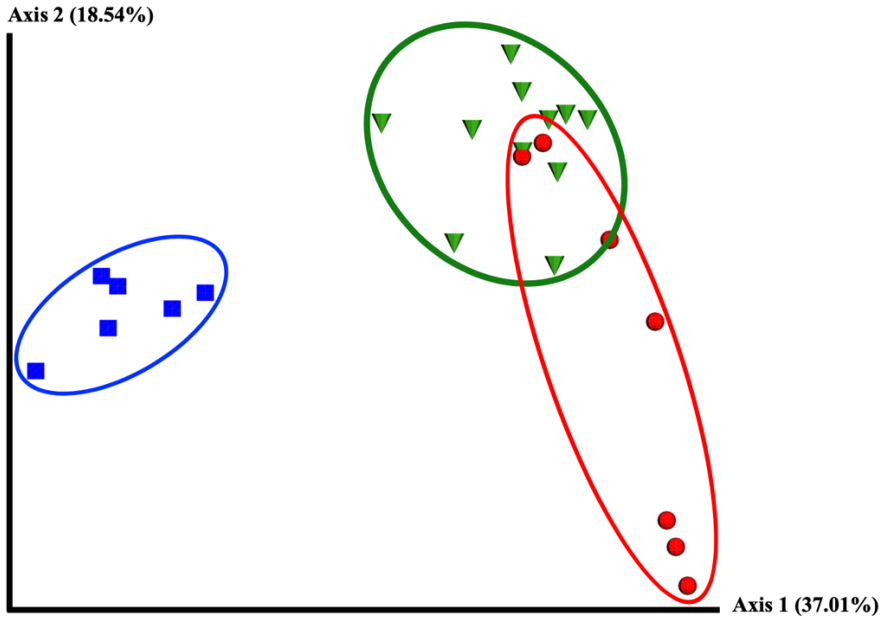

Figure 6. *Beta diversity PCoA plot of between group distances using Bray-Curtis distance matrix values.* Treatment groups are as follows: genistein, blue squares; Colyte, red circles; standard chow, green triangles. Significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons were observed for all pairwise treatment comparisons.

Pairwise PERMANOVA analysis was also conducted using the Unweighted Unifrac distance (representative of taxa presence alongside evaluation of evolutionary relationships between taxa) measure which suggested significant differences (genistein vs. standard chow, $p=0.001$; genistein vs. Colyte, $p=0.001$; standard chow vs. Colyte, $p=0.001$) in distances between all dietary treatment groups (**Figure 7**). Likewise, pairwise PERMANOVA results from the Weighted Unifrac distance (representative of taxa presence and abundance alongside evaluation of evolutionary relationships between taxa) measure showed significant differences between all dietary treatment groups (**Figure 8**).

In this final analysis, the genistein group differed significantly from the Colyte ($p<0.002$) and standard chow dietary treatment groups ($p<0.002$). The Colyte group also differed significantly from the standard chow treatment group ($p=0.005$). The results garnered from the aforementioned statistical analyses suggest significant differences in bacterial composition of the gut between dietary treatment groups.

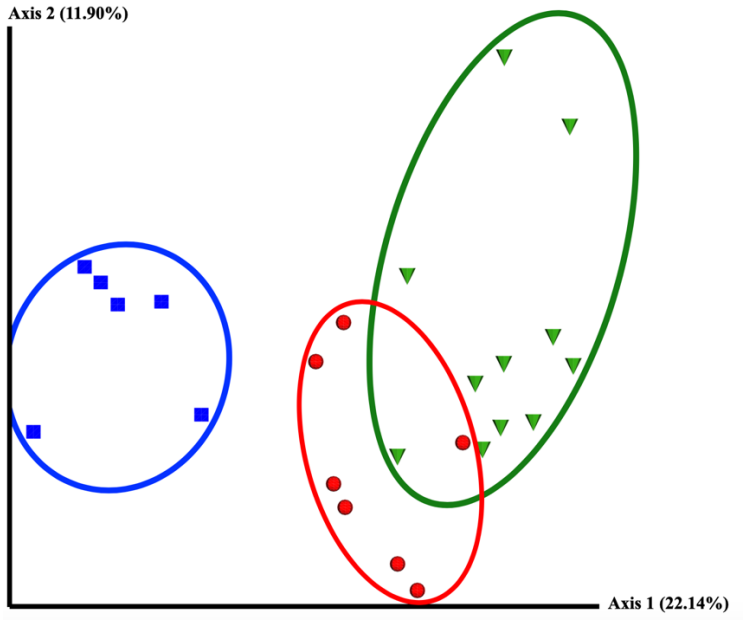

Figure 7. *Beta diversity PCoA plot of between group distances using Unweighted Unifrac distance matrix values.* Treatment groups are as follows: genistein, blue squares; Colyte, red circles; standard chow, green triangles. Significant group differences ($p<0.05$) after Benjamini-Hochberg multiple comparison correction were observed for all pairwise treatment comparisons.

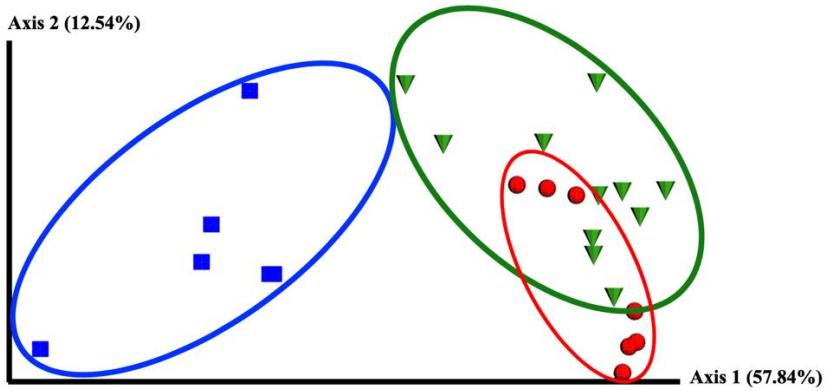

Figure 8. *Beta diversity PCoA of between group distances using Weighted Unifrac distance matrix values.* Treatment groups are as follows: genistein, blue squares; Colyte, red circles; standard chow, green triangles. Significant group differences ($p<0.05$) after Benjamini-Hochberg correction for multiple comparisons were observed for all pairwise treatment comparisons.

Relative Abundance

In order to portray predominant taxa across treatment groups, total counts of taxa were summed for all treatment groups; the most abundant taxa are shown at the phylum and genus level. At the phylum level (**Figure 9**), *Proteobacteria* (illustrated in orange) appeared to make up a larger proportion of the taxa present in the genistein group as compared to the Colyte and standard chow groups. *Firmicutes* (in yellow) members also made up a larger proportion of the community in the genistein group. The Colyte group appeared to be largely composed of *Bacteroidetes* members (in maroon), followed by *Firmicutes*. The predominant taxa in the standard chow group included members from the *Firmicutes* and *Bacteroidetes* phyla.

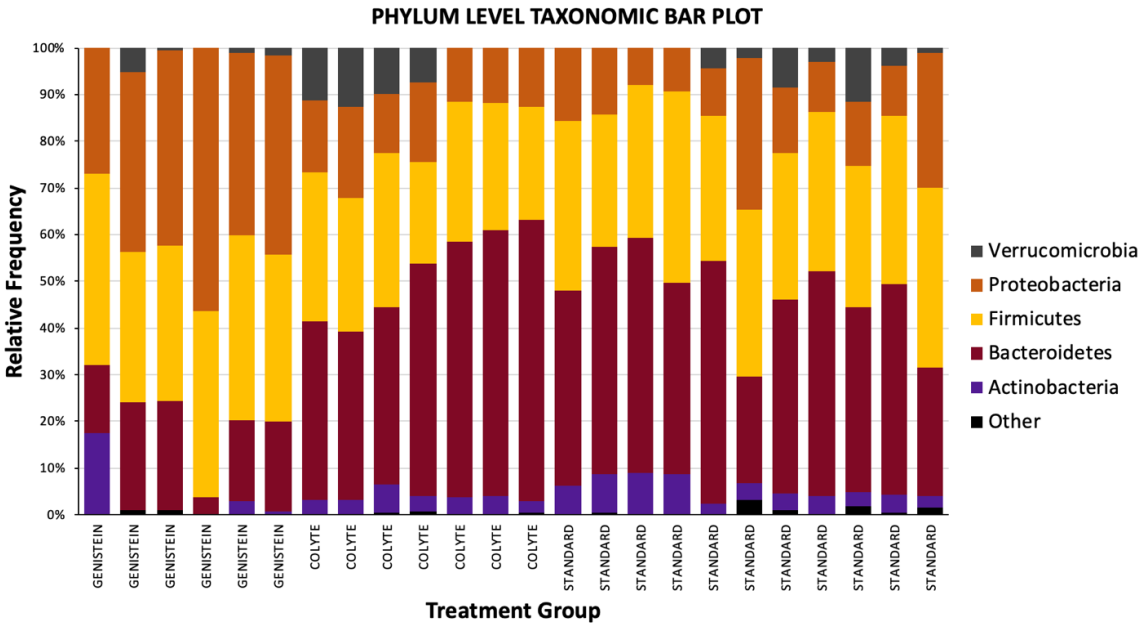

Figure 9. *Relative frequency of taxa by treatment group at the phylum level.* The top five phyla, summed across all treatment groups by highest count, are shown in the legend. Taxa present in lower proportions were summed and placed in the "Other" category.

Two taxonomic groups appeared to be overrepresented in the genistein group following qualitative observation of the relative frequency data (**Figure 10**). Taxa from the *Enterobacteriaceae* family (unspecified) and *Clostridium* genus were increased in the genistein group and were largely absent from both the Colyte and standard chow groups. Taxa from the *S24-7* family, while present in both Colyte and standard chow groups, appeared to be minimally present or absent altogether from the genistein group.

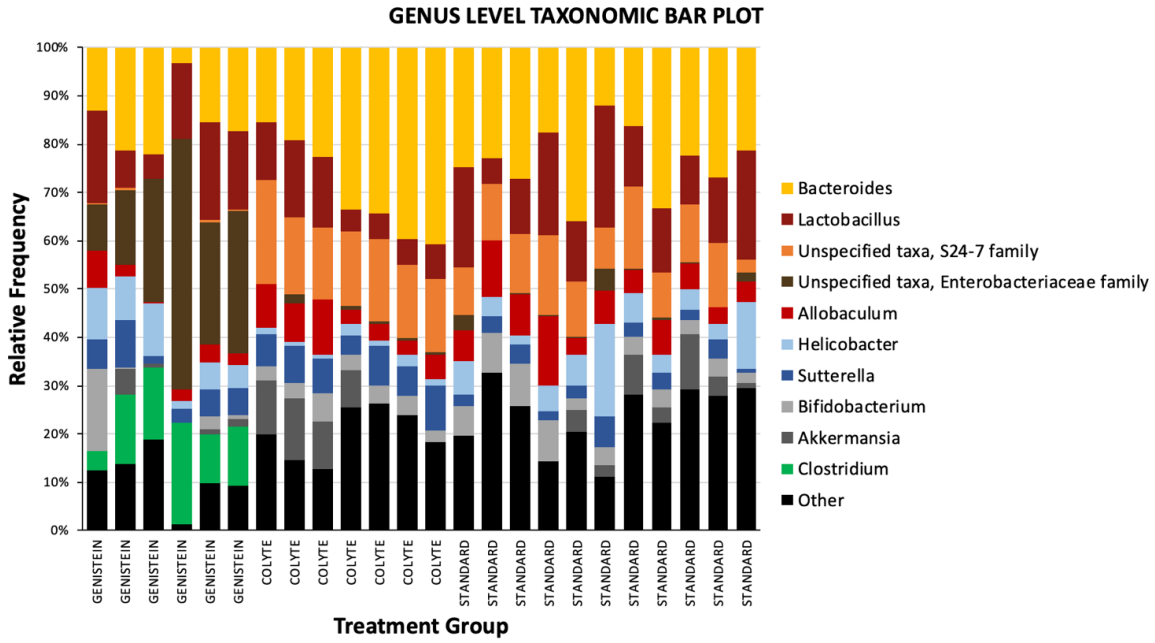

Figure 10. *Relative frequency of taxa by treatment group at the genus level.* Total frequencies of features were summed across treatment groups and truncated to the ten highest counts. Taxa present in lesser numbers were summed and placed in the "Other" category.

Differential Abundance

Differential abundance testing was added as an exploratory measure to assess differences in taxa abundance across treatment groups. ANCOM (QIIME 2) results allude that *Tenericutes* appeared to be higher in the Colyte group at the phyla level (W=6). At the genus level, ANCOM suggested that the standard chow group had more *Prevotella* members (W=108). Both Colyte and standard chow groups appeared to have more taxa from the *S24-7* family than the genistein group (W=101). The genistein group appeared to have a greater number of taxa from the *Clostridium* genus (W=111) and from the *Enterobacteriaceae* family (W=98) than either Colyte or standard chow groups.

Results from the LEfSe analyses at the phyla level showed that animals consuming genistein had significantly greater abundance of *Proteobacteria* than either

standard chow (LDA=5.3, p=0.002; **Figure 11**) or Colyte (LDA=5.1, p=0.003; **Figure 12**) diet groups. The Colyte group had significantly greater *Tenericutes* than both the genistein (LDA=5.2, p=0.015; **Figure 12**) and standard chow groups (LDA=4.4, p=0.006; **Figure 13**). Genistein also appeared to have a greater proportion of *Firmicutes* (LDA=4.7, p=0.007) and Colyte a greater proportion of *Bacteroidetes* (LDA=5.2, p=0.003) when compared against one another (**Figure 12**). *Bacteroidetes* did not differ significantly between Colyte and standard chow treatments but this phylum was differentially more abundant in the standard chow-fed animals when compared to the genistein group (LDA=5.3, p=0.002; **Figure 11**).

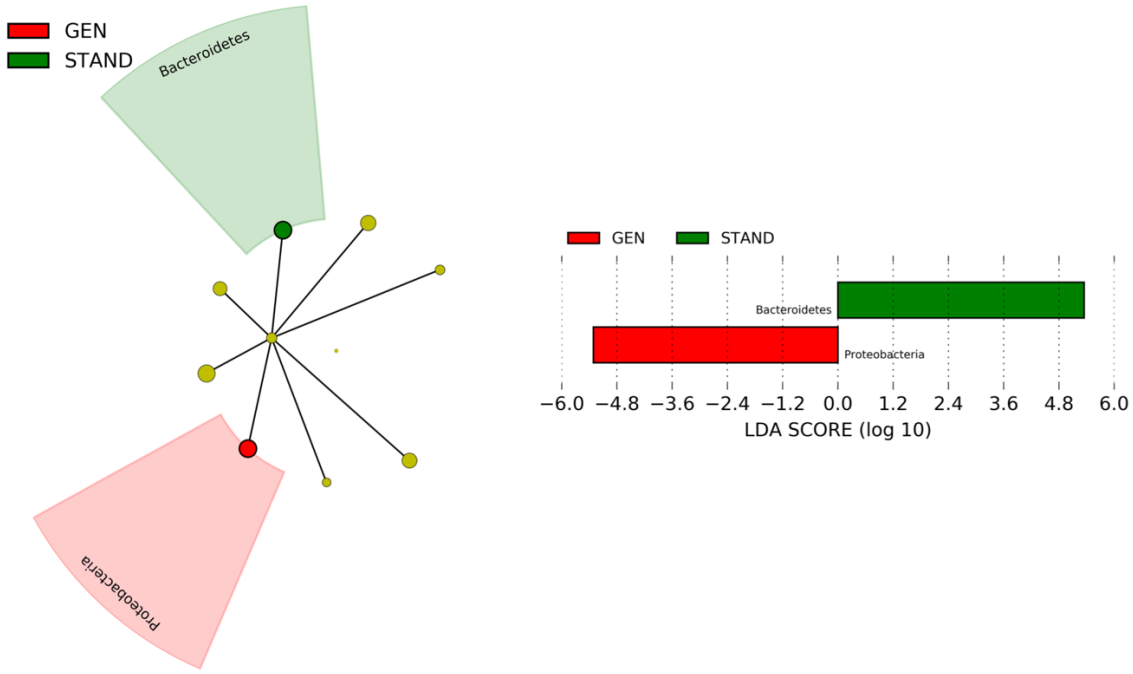

Figure 11. *Phylum level differential abundance analysis between genistein and standard chow treatment groups.* Number of discriminative features with an absolute LDA score greater than 2.0 = 2 features.

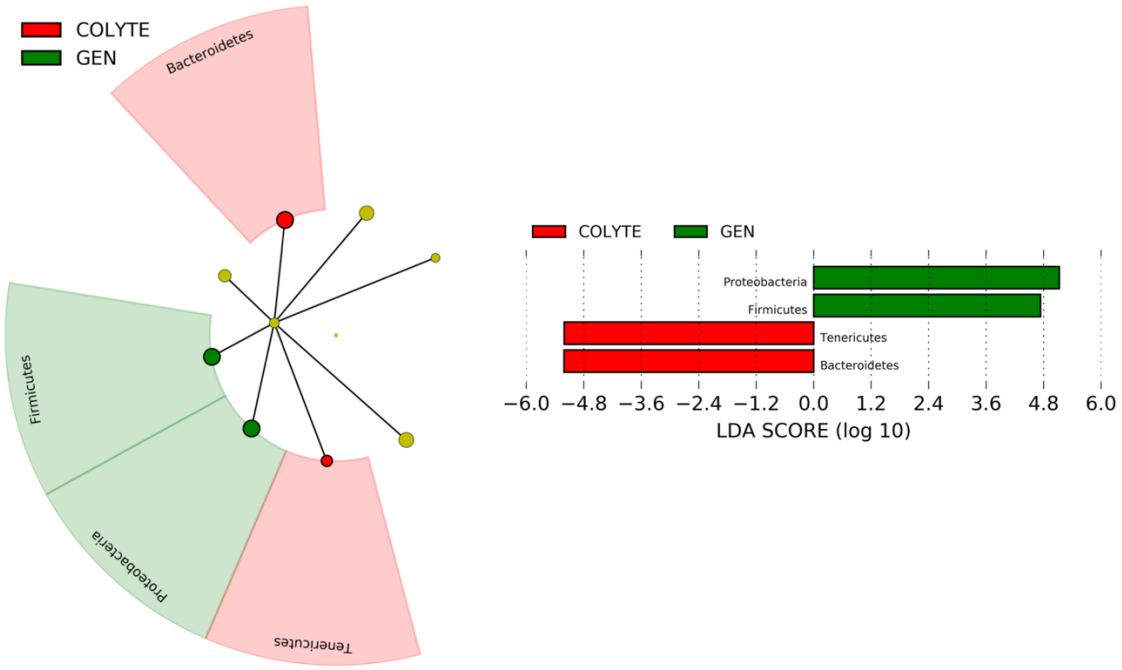

Figure 12. *Phylum level differential abundance analysis between genistein and Colyte treatment groups.* Number of discriminative features with an absolute LDA score greater than 2.0 = 4 features.

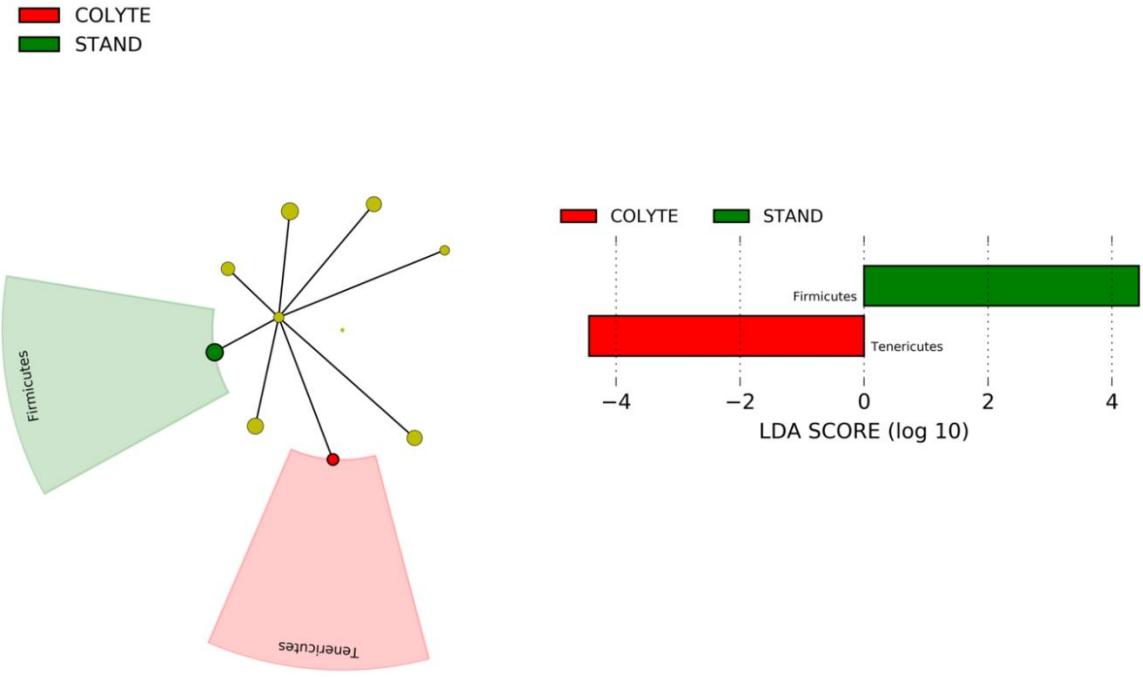

Figure 13. *Phylum level differential abundance analysis between Colyte and standard chow treatment groups.* Number of discriminative features with an absolute LDA score greater than 2.0 = 2 features.

At the genus level, no significant differences in differential abundance were detected when the standard treatment group was removed from the analysis, indicating that the Colyte and genistein groups were compositionally similar at this taxonomic level. However, when the standard group was compared to both Colyte and genistein, the standard diet group had significantly more taxa from the *Prevotella* genus (LDA=8.2, p=0.029, as well as members of the *Clostridiales* order (LDA=7.5, p=0.047; **Figure 14**). Other differentially abundant taxa for genera-level group comparisons can be seen in **Figure 15** and **Figure 16**.

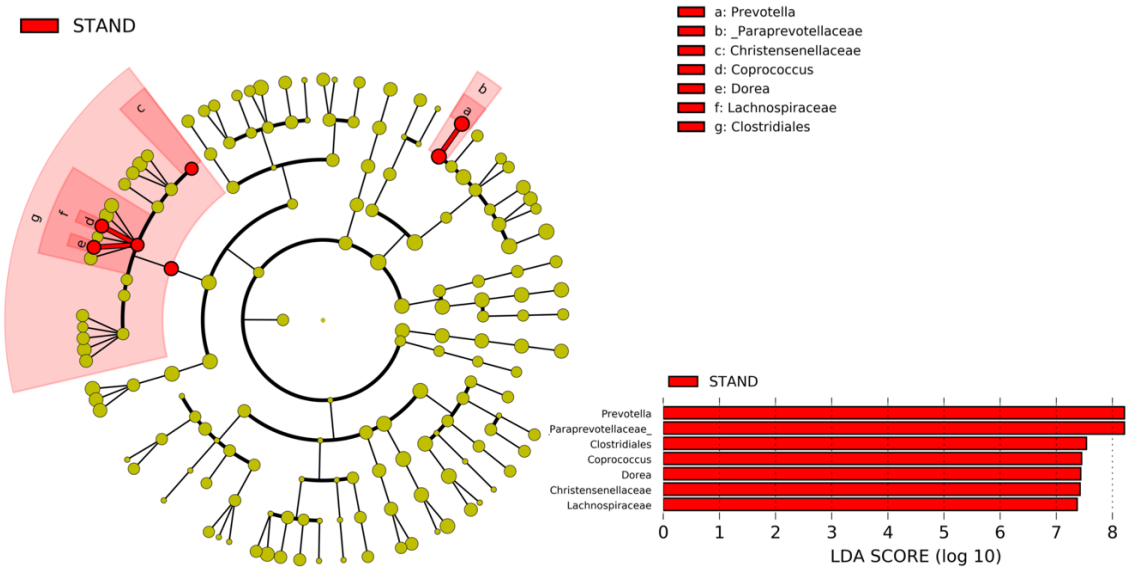

Figure 14. *Genus level differential abundance between all dietary treatment groups.* Number of discriminative features with an absolute LDA score greater than 2.0 = 7 features.

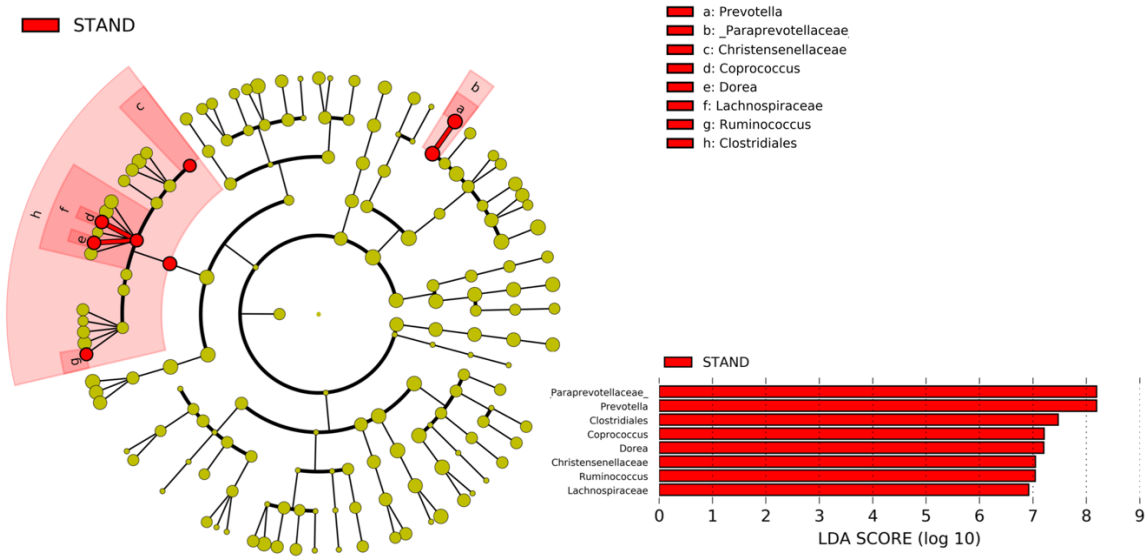

Figure 15. *Genus level differential abundance between genistein and standard chow treatment groups.* Number of discriminative features with an absolute LDA score greater than 2.0 = 8 features.

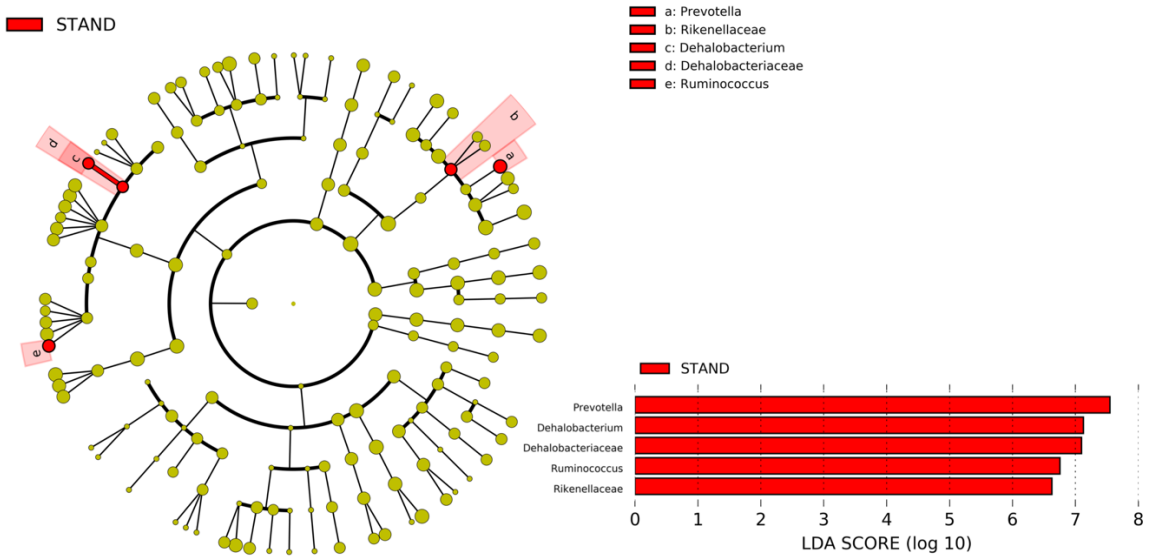

Figure 16. *Genus level differential abundance between standard chow and Colyte treatment groups.* Number of discriminative features with an absolute LDA score greater than 2.0 = 5 features.

CHAPTER 5

DISCUSSION

Overall findings. This study sought to examine associations between dietary genistein treatment and gut microbe diversity and community structure in DF508 mice, as compared to mice who received standard chow or standard chow plus Colyte-treated water. Results from diversity analyses suggest that dietary treatment is associated with within-sample diversity, between-sample diversity, and microbial abundance differences in the gut. Broadly, this study demonstrated that dietary genistein treatment was associated with depressed microbial diversity and significant differences in gut microbiome community structure as compared to Colyte and standard chow treatment groups.

Alpha diversity. Recall that alpha-diversity assesses measures of richness and evenness within a given sample. Richness is expressed as the number of unique taxa present in a sample, whereas evenness describes the distribution of the taxa present in a sample. These two indices are the two main components of alpha-diversity.[201] We hypothesized that alpha (within-sample) diversity of the genistein treatment group would be equal to or greater than that of the standard chow and Colyte groups. However, our data showed within-sample diversity to be significantly lower in the genistein diet group for two alpha-diversity metrics (observed OTUs and Shannon's Diversity Index). Each of these metrics account for species richness, suggesting that bacterial richness is depressed most significantly in the genistein diet group. While Shannon's Diversity Index also accounts for evenness, no significant treatment group differences were shown for evenness of taxa as measured by Pielou's Evenness. These results demonstrate that

dietary genistein treatment is associated with depressed microbial richness as compared to the Colyte and standard chow treatment groups.

Explanations for reduced bacterial diversity in the genistein diet group are challenging to elucidate. While studies in mice and humans with CF consistently demonstrate decreased gut microbial diversity,[24,42,44,142] studies exploring the impact of soy foods and genistein in otherwise healthy populations generally show beneficial effects. One study by Butteiger et al. demonstrated that soy-fed Golden Syrian hamsters had more diverse gut microbiota compared to hamsters fed a milk protein isolate diet, as measured by observed OTUs.[202] Similar findings were observed in an obese rat model, where alpha-diversity measures were increased in the soy protein group compared to the milk protein group.[203] In a study comparing mice fed a high-fat diet to mice fed the same high-fat diet with genistein, researchers found that measures of alpha-diversity (observed OTUs, Shannon's Diversity Index) were higher in the genistein group.[204] While soy foods and genistein appear to have positive effects on gut microbiota diversity in disease-free models, it is unclear how genistein influences the intestinal environment and interacts with the gut microbiota in CF models.

Given genistein's role as a polyphenol,[151,152] bacterial proliferation was an anticipated outcome with genistein acting as a potential substrate for bacterial metabolism. However, findings from this study showed decreased bacterial diversity in the genistein group. A potential explanation for the reduced bacterial diversity observed in the genistein group may be related to genistein's purported antimicrobial effects, as shown by several studies.[173,175–177] In order for isoflavones to exert antimicrobial effects, structural prerequisites must be met.[175,176] Isoflavones with prenyl groups, of which

genistein has none, exert potent antimicrobial activity.[175] Isoflavones containing phenolic hydroxyl groups, of which genistein has three, exert antimicrobial effects via protein affinity. This protein affinity is conducive for microbial enzyme and biosynthetic pathway inhibition.[175] Genistein has also been identified as a bacteriostatic compound, working as a DNA topoisomerase type I and II inhibitor and subsequently affecting DNA topology, ultimately impacting DNA replication.[173,177] Findings published by Mizushina et al. demonstrated that genistein caused cytotoxicity in cancer cells by inhibiting topoisomerase II, thereby disrupting the cell cycle and leading to cellular dysfunction.[205] A recent 2017 study by Vazquez and colleagues additionally revealed that genistein may exhibit stronger antimicrobial action due to its inhibition of DNA topoisomerase IV.[176] The exploration of genistein's antimicrobial activity is ongoing.

Interestingly, results from this study demonstrated that the Colyte treatment group had increased richness as compared to the genistein group, which was contrary to our initial hypothesis. In humans with CF, intestinal obstruction that occurs as a result of the altered intestinal environment can be treated with oral laxatives such as polyethylene glycol, which often precludes the need for surgical intervention.[206] Mouse models of CF exhibit a similar intestinal pathophysiology to humans and require treatment with laxatives in order to survive.[207,208] Furthermore, bacterial dysbiosis has been demonstrated in the CF mouse gut.[42,209] As such, studies that examined the effects of laxative use on the gut microbiota were evaluated. A 2007 study found that CF mice fed solid chow and treated with an osmotic laxative (polyethylene glycol 3350) had reduced mucus accumulation as well as decreased intestinal bacterial overgrowth by 92%, in addition to normalization of intestinal transit time. Furthermore, CF mice treated with

laxatives showed increased weight gain to levels comparable to laxative-treated wild-type mice.[103]

In addition to laxative use, many individuals with CF receive antibiotics to treat respiratory infections and small intestine bacterial overgrowth. One study showed that antibiotic treatment in CF mice attenuated bacterial overgrowth, subsequently decreasing mucus accumulation in the intestinal crypts.[208] As baseline fecal samples were not collected in the present study, changes in microbial richness were not observed. However, it is possible that microbial richness may have been elevated at baseline due to alterations in the CF intestinal environment, and Colyte treatment may have attenuated bacterial overgrowth, thereby decreasing overall microbial richness. Given the relationship between bacterial dysbiosis and irregular mucus secretion and accumulation, mitigating intestinal bacterial overgrowth may rescue intestinal function by reducing mucosal perturbations, thereby enhancing health in the CF gut.

Beta-diversity. Beta-diversity examines compositional differences in microbial communities.[210] Results from between-sample diversity analyses supported our hypothesis that overall microbial community structure would differ by dietary treatment group. Each metric used to assess beta-diversity produced a distance matrix that calculated dissimilarity between each pair of samples in the community and allowed for statistical and visual analysis of clustering patterns.[201,210] All four beta-diversity metrics used in the present study produced significant results, indicating significant differences in clustering of taxa between dietary treatment groups. These results were corroborated both visually (PCoA plots) and statistically (pairwise PERMANOVA).

It should be noted that different methods of analysis can skew interpretation of results. For this reason, multiple beta-diversity metrics were used to confirm that observed clusters were not contingent on a single set of parameters.[118] The different parameters used in this study accounted for presence, relative abundance, and phylogenetic relationships.[201] Beta-diversity measures of dietary genistein remained significantly different from both the Colyte and standard chow groups, even after accounting for presence or absence (Jaccard, Bray-Curtis, Unweighted Unifrac, Weighted Unifrac); abundance (Bray-Curtis, Weighted Unifrac); and phylogenetic relationships between taxa (Unweighted Unifrac, Weighted Unifrac). Significant differences in beta-diversity were also observed between the Colyte and standard chow groups using the same metrics.

Visual outputs were analyzed in this study using PCoA plots, an ordination technique that reduces dimensionality of the microbial data, allowing composition differences among communities to be visualized in 2D scatterplots.[118] Distinct clusters are indicative of compositional differences in microbial communities,[118] whereas samples with less distance between them (clustering closer together) signifies similarity between samples.[201] Visualizations of distance matrices were corroborated with PERMANOVA tests. These tests confirmed that microbial community structure differed significantly by dietary treatment group in DF508 mice. Overall, beta (between-sample) diversity metrics showed significant differences with all measures used, suggesting that dietary treatment was associated with dissimilarities in microbial community structure and composition after accounting for presence, abundance, and phylogenetic relationships between taxa.

It was noted earlier that alpha-diversity can remain unchanged in an ecosystem despite proportions of the microbiota shifting; overall taxonomic richness may remain the same even if the numbers of pathogenic and beneficial microbes are skewed disproportionally, which necessitates the analysis of microbial community structure. Studies examining shifts in microbial community structure in soy or genistein-fed non-CF models support changes in beta-diversity. Soy-fed Golden Syrian hamsters, for example, showed significantly different microbial community structure when compared to the milk protein group.[202] Adult non-obese diabetic mice who were fed genistein-containing or soy-free diets were shown in one study to have sex-dependent alterations in gut microbiota, with genistein-fed males exhibiting changes in beta-diversity.[211] Despite varied results in studies using human and animal models, one review examining the effects of soy food consumption on the gut microbiota provided evidence that soy foods can produce alterations in the *Firmicutes* to *Bacteroidetes* ratio and can increase proliferation of *Lactobacilli* and *Bifidobacteria*.[152] However, these findings have not been replicated in CF populations.

Different dietary polyphenols (including rutin, chlorogenic acid, caffeic acid, and quercetin), are capable of being metabolized into phenolic acids by intestinal bacteria, and have been shown to promote SCFA synthesis, decrease the ratio of *Firmicutes* to *Bacteroidetes*, and promote proliferation of *Bifidobacteria*.[156] While microbial metabolism of polyphenols has been shown to modulate community structure of the gut microbiota in these ways, proliferation of these taxa were not seen in the genistein group. Of consequence, it should be considered that the potential health benefits associated with

dietary polyphenol intake rely on microbial metabolism and the resulting microbial metabolites that are generated, and not necessarily on the initial compound introduced.[64]

The enzymes required for deglycosylation of genistin, β-glucosidases, are located within enterocytes and on the intestinal brush border. One paper published in 2003 demonstrated that genistein's biological activity may also be impacted by significant microbial degradation in the GI tract.[212] Notably, a number of microbial groups possess this enzyme activity; these groups include species from the *Bifidobacterium*, *Lactobacillus*, and *Bacteroides* genera.[212] The interaction of genistein with human vs. microbial cells may be influenced by the presence of other nutrients and functional food components. For example, researchers introducing prebiotic fiber with genistein found that FOS deterred microbial metabolism of genistein alongside increases in *Lactobacillus* and *Bifidobacterium*.[212] What is currently unknown, however, are the specific mechanisms by which the gut microbiota interact with genistein, and to what degree. Furthermore, it is unclear whether it is genistein itself or downstream metabolites from microbial degradation of genistein that contribute the positive biological effects associated with this isoflavone. More studies are certainly needed to substantiate these findings, especially in regard to the CF gut microbiota.

Despite genistein's role as a dietary polyphenol, proliferation of *Bifidobacteria* or *Lactobacillus* were not observed in the dietary genistein group, though microbial community structure was significantly different. Exploring the reasons for differences in microbial community structure associated with dietary treatment is challenging. While not significant, the genistein group demonstrated the lowest evenness of taxa among treatment groups. Given the differences in microbial community structure, it is possible

that some microbe or taxonomic group could be utilizing genistein. Taxa from the *Proteobacteria* phylum, which were increased in the genistein group, may be partly driving the differences in microbial community structure observed in the present study.

Laxative treatment has also been shown to affect microbial community structure. There are studies in both murine models and humans that have investigated the use of laxatives and their effect on the intestinal microbiota. In a human study observing the effects of colonoscopy bowel preparation on the intestinal microbiota, researchers found no significant alterations in gut microbial communities after taking a preparation that included 10 mL of Bisacodyl and two liters of polyethylene glycol. While a few participants experienced transient shifts in microbial communities, the majority of individuals exhibited no evidence of lasting intestinal microbiota changes.[213]

Rats who received polyethylene glycol-treated water (7%) were studied to determine the effects of laxative administration on intestinal sterol metabolism and gut microbiota composition, as compared to controls. Analysis of fecal microbiota showed that *Verrucomicrobia* increased and *Firmicutes* decreased at the phylum level. Changes detected at the genus level demonstrated that mucus-associated bacterial genera including *Ruminococcus*, *Bacteroides*, and *Akkermansia*, as well as *Proteobacteria* members, were significantly increased in relative abundance compared to controls, while *Clostridia* members were decreased in relative abundance. While the laxative-treated rats showed trends of lower microbial richness, only compositional changes in intestinal microbiota were significant, with a relative increase of bacteria associated with mucus seen in the rats given polyethylene glycol.[214]

A novel study published in 2018 utilized a humanized microbiota mouse model to examine alterations in gut microbiota following treatment with polyethylene glycol for six days compared to controls. Laxative treatment produced long-term changes in beta-diversity. The authors found that greater than 75% of the taxa present post-treatment in the laxative group were significantly altered, whereas only 5% of the control group showed variations in taxonomic abundance. While they initially comprised half of the total microbial abundance, members from the *S24-7* family became extinct within three days of beginning laxative treatment. Members from the *Bacteroidaceae* family were significantly increased in abundance, rising to 60% of the total microbial abundance. The authors attribute these changes in part to the thinned mucus layer at the epithelial surface following treatment with polyethylene glycol. However, the dysbiosis resulting from the laxative-induced mucosal perturbations were recovered within a few days without laxative administration when reintroduced into a seeded environment, though normalization was dependent on laxative dose.[215] In our study, while the Colyte group differed in microbial community structure, changes in specific taxa were not consistent with the literature described above.

Relative and differential abundance. Examining relative and differential abundance among treatment groups allowed for community comparisons. To date, a number of studies have been conducted to describe the role of genistein on microbial communities. Paul et al. examined the role of dietary genistein on microbial modulation in conjunction with chemotherapy status in a murine model of breast cancer. Germ-free mice were transplanted with patient fecal samples prior to and following chemotherapy and fed either a genistein or control diet. While not significant, the control group showed

a greater abundance of *Bacteroidetes*. *Verrucomicrobia* was significantly greater in the mice given genistein. Interestingly, no microbial community differences were noted in the pre- or post-chemotherapy samples, suggesting that variations in microbial community structure were attributed solely to the genistein diet.[216] Lopez et al. showed that mice who were fed genistein in addition to a high-fat diet had an increase in *Firmicutes/Bacteroidetes* ratio when compared to the high-fat diet group. At the genus level, *Prevotella* and *Akkermansia* were greater in relative abundance, whereas relative abundance of *Bacteroides* was decreased in the high-fat with genistein diet group.[204] Butteiger et al. demonstrated proliferation of *Bifidobacteriaceae* members and greater proportions of species from the *Clostridiales* order and *S24-7* family in soy-fed Golden Syrian hamsters.[202] These findings were not observed in the present study for the genistein group; however, greater abundance of *Clostridiales* order and *S24-7* family members were observed in the standard chow group.

LEfSe analyses of non-obese diabetic mice showed genistein-fed males to have significantly higher *Prevotella*, whereas genistein-fed females demonstrated increased *Enterobacteriaceae* (genus) and *Clostridia* (order) members.[211] Interestingly, results from our study also saw greater proportions in the relative abundance of *Enterobacteriaceae* members in the genistein diet group, as well as greater relative abundance of members from the *Clostridium* genus, though sex-dependent differences were not examined. Another study examining fecal microbiota changes following soy or milk protein consumption in obese rats fed a Western diet showed that relative abundance of *Proteobacteria* members were significantly increased in the soy protein group.[203] Notably, increased *Proteobacteria* was also observed in this study. It could be that

changes in the intestinal environment, whether mediated through obesity or CF, may contribute to these taxonomic shifts.

Differential abundance analysis revealed that the genistein diet group was associated with greater abundance of *Proteobacteria*, which are usually found in lesser proportions in the healthy human gut.[217] This finding is intriguing, especially given the beneficial role of genistein and soy food consumption as described above. Nevertheless, the CF intestinal environment is characterized by inflammation and bacterial dysbiosis.[32,38] Furthermore, increased relative abundance of *Proteobacteria* has also been correlated with antibiotic use, a common therapy used to treat bacterial dysbiosis in CF patients.[218] Several studies previously examined in this paper found similar increases in *Proteobacteria* abundance in CF populations,[24,25,146] though one study conversely found *Proteobacteria* to be decreased in the CF group.[43] In the context of CF, increased *Proteobacteria* holds significance due the taxa that comprise the phylum. For example, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, two of the most commonly identified respiratory pathogens in CF patients,[27,28] belong to the *Proteobacteria* phylum. *Enterobacteriaceae*, a family belonging to the γ-Proteobacteria subclass, is additionally comprised of a number of pathogens found throughout the GI tract, including *Escherichia coli* and *Shigella*, though some taxa from the group are innocuous symbionts.[219]

Ascertaining the reason for increased *Proteobacteria* in the genistein treatment group is perplexing. At this time, the effects of genistein on the gut microbiota and the nature of microbially-derived metabolites consequently produced are not clearly understood. As described previously, it is possible that genistein is being degraded by the microbiota before it is able to exert its positive biological effects.[212] Interestingly,

probiotic supplementation in CF populations may help mitigate the proliferation of taxa from the *Proteobacteria* phylum. One double-blind multicenter cross-over study conducted in Spain administered *Lactobacillus reuteri* or placebo once per day to CF patients and fecal samples were collected for metagenomic analysis. The basal samples of the CF patients were composed predominantly of members of the *Proteobacteria* phylum (68.2%). Following administration of *Lactobacillus reuteri* for six months, substantial decreases were observed in *Proteobacteria* members (30.7%), with a concomitant increase in both the *Bacteroidetes* (16.9%) and *Firmicutes* (38.2%) phyla.[220]

At the phylum level, both ANCOM and LEfSe analyses identified increased *Tenericutes* in the Colyte compared to other treatment groups. This finding was not shared among any of the CF literature previously described in this paper, or in other literature examining the use of laxatives on the gut microbiota in animal or human models. Both LEfSe and ANCOM analyses showed increased taxa from the *Prevotella* phylum and *Clostriadiales* order in the standard chow group. *Prevotella*, which is associated with vegetarianism in Western populations and in those with plant-based diets elsewhere, has been shown to be generally beneficial; however, it has also been implicated in chronic inflammatory conditions of the gut in some hosts.[221] Interestingly, *Prevotella* has also been identified as an opportunistic respiratory pathogen in CF patients.[27,222] It should be noted that the species comprising the *Prevotella* genus are highly variable in regard to genomic diversity; as such, it is hard to determine how members of *Prevotella* will interact in the GI environment, or with other bacterial residents in the host gut.[221]

Unspecified taxa from the *S24-7* family, which have been shown to be a dominant group in the murine gut,[223] were seen in higher frequencies in the Colyte and standard chow groups, and present in fewer numbers in the Genistein diet group. This microbial family belongs to the *Clostridiales* order, which LEfSe analysis showed to be significantly greater in the standard chow group. While largely uncharacterized and fairly novel, this family has been identified as a common bacterial resident within the GI tract.[223] While present in the human gut, members of the *S24-7* family represent a considerable proportion of the gut microbiota in murine models. In addition to their roles as primary fermenters, taxa from *S24-7* have demonstrated the ability to produce urease as well as degrade oxalate.[223] These microorganisms have also been shown to be IgA coated, though not substantially. In this regard, they are likely to be commensal bacteria. However, under the appropriate conditions these taxa may lead to opportunistic infection in the host.[223]

The overall taxonomic trends depicted in the relative and differential abundance data present an interesting microbial picture. As discussed previously, taxa from the *Bacteroidetes* and *Firmicutes* phyla typify a healthy gut microbiome.[18,113,114,120,221] Relative abundance data show that the standard chow group was predominantly composed of members of these two phyla. Intriguingly, the genistein diet group was shown to be higher in taxa from the *Proteobacteria* phylum, which was demonstrated in the differential abundance data (LEfSe). While *Proteobacteria* are typically present in the gut of both mice and humans, healthy individuals generally host these taxa in lesser numbers. However, *Proteobacteria* has been shown to be higher in proportion for those with GI disease.[18] A 2015 review examining elevated numbers of taxa from the

Proteobacteria phylum in various conditions suggested that increased proportions are indicative of dysbiosis and may be used as a potential marker for disease.[217] Chronic inflammation, which is commonly observed in CF patients, is further capable of disrupting gut homeostasis and may contribute to bacterial dysbiosis, which includes *Proteobacteria* proliferation within the GI tract.[217] Taxa from the *Enterobacteriaceae* family, while not shown to be differentially abundant, belong to the *Proteobacteria* phylum and likely contributed to the increased proportion seen in the genistein diet group.

Strengths and limitations. This novel study is among the first to explore associations between dietary genistein treatment and gut microbiota diversity and community structure in a murine model of CF. Nevertheless, the sample sizes for each treatment group were uneven, and the overall sample size was relatively small, which may have lessened statistical sensitivity of the diversity testing and reduced the likelihood of detecting significant differences in microbial community structure and composition. The most striking limitation of the present study is the lack of time-series data. Given that all mice are genetically similar due to the shared DF508 CFTR gene mutation, baseline characterization of the gut microbiota should have yielded similar results in terms of taxonomic diversity and composition. However, without obtaining these baseline fecal samples, examining alterations in the gut microbiota in the context of diet is not possible at this time. Given the contrasted diversity results of this study, obtaining baseline and post-treatment fecal samples would allow researchers to further explore how dietary genistein affects the gut microbiota in animal models of CF. Additionally, this study utilized common methods of analyzing diversity measures; more robust studies that

further explore functional diversity may help to distinguish mechanisms by which genistein affects metabolic and functional pathways of the CF gut microbiota.

CHAPTER 6

CONCLUSION

This study demonstrated that dietary genistein treatment is associated with decreased bacterial richness, but not evenness, in DF508-CF mice. Significant differences in gut microbial community structure were also detected between dietary treatment groups. PCoA plots of all beta-diversity metrics revealed distinct separation of clusters by treatment group. Of considerable importance is the increased differential abundance of *Proteobacteria* at the phylum level in the genistein diet group, given its role in GI disease and inflammation.

At this time, it is unclear why dietary genistein treatment was associated with lower gut bacterial diversity and changes in microbial community structure as compared to the Colyte and standard chow diet groups. Given genistein's ability to activate CFTR in cell cultures and murine models of CF,[57–59,61,97] it seems as though genistein offers physiological benefits beyond that of influencing the gut microbiota. Of key consideration, a captivating study published in 2018 demonstrated that dietary genistein treatment increased the survival rate of DF508 female mice, surpassing the survival rate of the Colyte-treated group. Moreover, the number of goblet cells were reduced in genistein-treated females, which may hold potential implications for rescuing the GI environment in regard to the chronic mucosal perturbations experienced in CF. While increased survival rates were not seen in the DF508 males, significant weight gain was observed compared to the Colyte-treated group.[178] Despite these novel findings, given the small sample size and lack of time-series data, it is difficult to determine precisely how

genistein influences the gut microbiota and whether any benefits remain to be seen from a clinical standpoint in alleviating the dysbiosis commonly observed in CF.

While this study was able to provide insight into differences of the gut microbiota within and between dietary treatment groups, explaining why these differences were observed presents a much more challenging task. Going forward, it will be necessary for researchers to identify the underlying mechanisms of how dietary genistein impacts the host microbiota, as well as interacts with other bacterial taxa present within the gut. Additionally, given the chronic inflammatory state of the CF gut, analyzing plasma biomarkers of inflammation in conjunction with microbiome diversity and composition analysis will help shape a clearer picture of the intestinal milieu in regard to bacterial dysbiosis. Understanding the etiology of this dysbiosis in the context of dietary genistein may prove to be consequential, especially as it pertains to diet and microbial competition, inhibition, and proliferation of the characteristic taxa in the CF gut microbiome.

Further studies are warranted to fully understand the taxonomic and functional diversity, as well as the role of microbial metabolites and their interactions with the host microbiota in both murine and human models of cystic fibrosis. Future studies exploring the effects of dietary genistein on gut microbiota composition and community structure should implement time-series data analysis, ensure a larger and more even sample size, and consider exploring functional diversity while assessing important covariates such as plasma biomarkers of intestinal inflammation, sex, age, and weight change. At this time, the role of genistein in modulating the CF gut microbiome for improved health outcomes requires further exploration.

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APPENDIX A

CASE WESTERN RESERVE UNIVERSITY SCHOOL OF MEDICINE IACUC

APPROVAL FORM

CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF MEDICINE

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date: **April 17, 2017**

Name: **Craig A Hodges**

Title: **Breeding mouse models of cystic fibrosis**

Protocol Number: **2014-0064**

Species: **Mice 36,000**

This Institution's Animal Care and Use Committee reviewed and approved the Animal Experimentation 2014-0064**.** The Committee has approved this protocol for a period of three years, beginning **4/17/2017** through and ending **4/17/2020.**

Before ordering animals, it is important to confirm that all animal handlers have completed their necessary training and have access to the animal facility. If animals are ordered prematurely, your staff will not have access to the animals or be able to schedule procedures associated with this protocol. For information on Training Requirements and Facility Access please visit the IACUC Training page http://casemed.case.edu/ora/iacuc/training.cfm

While the approved protocol covers 3 years, annual updates are due on the anniversary of the original approval. You will receive a continuing review email notification from eSirius3G sixty days prior to your annual due date and an Alert will remain in your eSirius3G Dashboard Mailbox until completed. You can access, complete, and submit your Continuing Review form directly from your eSirius3G Dashboard.

Please remember that any change to the scope of your animal protocol must be submitted to the IACUC office for Committee review and approval. No work may begin on a proposed addendum until the principal investigator has received either an email notification of approval through eSirius3G or a final written approval letter signed by the IACUC chair.

If this protocol supports a federally funded grant or sponsored project you must alert your office of grants and contracts if this protocol expires or is otherwise terminated. You must alert grants and contracts if a new protocol is approved to replace or add to the current protocol.
To aid in extramural grant submissions, the following description may be used as a guide for NIH and other grant submissions:

The animals described in this study will be housed in the AAALAC accredited facilities of the CWRU School of Medicine (SOM). Standard Operating Procedures and reference materials are available from the IACUC Office for animal use. The animal health program for all Case owned laboratory animals is directed by the Case Animal Resource Center Director, W. John Durfee, DVM, Diplomate ACLAM, and provided by two full-time clinical veterinarians. Animals in each room are observed daily for signs of illness by the animal technician responsible for providing husbandry. Medical records and documentation of experimental use are maintained individually for non-rodents and individually or by cage group for rodents. Veterinary technicians under the direction of the attending veterinarian provide routine veterinary medical care to all animals. Animal care and use is additionally monitored for training and compliance issues by the Training and Compliance Manager. The Case Assurance number is A-3145-01, valid until April 30, 2019**.**

Sincerely yours,

Agata A. Exner

Agata A Exner
IACUC Chair

APPENDIX B

QIIME 2 PROVENANCE (VERSION 2018.6)

Data imported into QIIME 2

§ qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]' --input-path rawdata --source-format CasavaOneEightSingleLanePerSampleDirFmt --output-path demux-paired-end.qza

Quality control with DADA2

§ qiime dada2 denoise-paired --i-demultiplexed-seqs demux-paired-end.qza --p-trim-left-f 10 --p-trim-left-r 10 --p-trunc-len-f 251 --p-trunc-len-r 240 --o-table dada2-table.qza --o-representative-sequences dada2-rep-seqs.qza --o-denoising-stats dada2-denoising-stats.qza

Sequence alignment with MAFFT

§ qiime alignment mafft --i-sequences dada2-rep-seqs.qza --o-alignment aligned-rep-seqs.qza

Masking and filtering alignment to remove highly variable positions

§ qiime alignment mask --i-alignment aligned-rep-seqs.qza --o-masked-alignment masked-aligned-rep-seqs.qza

Phylogenetic tree generation

§ qiime phylogeny fasttree --i-alignment masked-aligned-rep-seqs.qza --o-tree unrooted-tree.qza

Midpoint application to root phylogenetic tree

§ qiime phylogeny midpoint-root --i-tree unrooted-tree.qza --o-rooted-tree rooted-tree.qza

Core diversity metrics

§ qiime diversity core-metrics-phylogenetic --i-phylogeny rooted-tree.qza --i-table dada2-table.qza --p-sampling-depth 32800 --m-metadata-file cf-metadata.txt --output-dir core-metrics-results

Alpha-diversity, observed OTUs

§ qiime diversity alpha-group-significance --i-alpha-diversity core-metrics-results/observed_otus_vector.qza --m-metadata-file cf-metadata.txt --o-visualization core-metrics-results/observed-otus-group-significance.qzv

Alpha-diversity, Pielou’s evenness

§ qiime diversity alpha-group-significance --i-alpha-diversity core-metrics-results/evenness_vector.qza --m-metadata-file cf-metadata.txt --o-visualization core-metrics-results/evenness-group-significance.qzv

Alpha-diversity, Shannon’s Diversity Index

§ qiime diversity alpha-group-significance --i-alpha-diversity core-metrics-results/shannon_vector.qza --m-metadata-file cf-metadata.txt --o-visualization core-metrics-results/shannon-group-significance.qzv

Beta-diversity and treatment group, Jaccard

§ qiime diversity beta-group-significance --i-distance-matrix core-metrics-results/jaccard_distance_matrix.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization core-metrics-results/jaccard-txt-group-significance.qzv --p-pairwise

Beta-diversity and treatment group, Bray-Curtis

§ qiime diversity beta-group-significance --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization core-metrics-results/bray-curtis-txt-group-significance.qzv --p-pairwise

Beta-diversity and treatment group, unweighted UniFrac

§ qiime diversity beta-group-significance --i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization core-metrics-results/unweighted-unifrac-txt-group-significance.qzv --p-pairwise

Beta-diversity and treatment group, weighted UniFrac

§ qiime diversity beta-group-significance --i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization core-metrics-results/weighted-unifrac-txt-group-significance.qzv --p-pairwise

Taxonomic analysis

§ qiime feature-classifier classify-sklearn --i-classifier gg-13-8-99-515-806-nb-classifier.qza --i-reads dada2-rep-seqs.qza --o-classification taxonomy.qza

§ qiime metadata tabulate --m-input-file taxonomy.qza --o-visualization taxonomy.qzv

Feature-filtering for differential abundance analysis

§ qiime feature-table filter-features \
--i-table dada2-table.qza \
--p-min-frequency 2 \
--o-filtered-table feature-frequency-filtered-table.qza

Taxa collapse for differential abundance analysis, phylum and genus level

§ qiime taxa collapse --i-table feature-frequency-filtered-table.qza --i-taxonomy taxonomy.qza --p-level 2 --o-collapsed-table table-l2.qza

§ qiime tools export table-l2.qza --output-dir taxonomy-table

§ biom convert --to-tsv -i taxonomy-table/feature-table.biom -o taxonomy-table/feature-table.tsv

§ qiime taxa collapse --i-table feature-frequency-filtered-table.qza --i-taxonomy taxonomy.qza --p-level 6 --o-collapsed-table table-l6.qza
§ qiime tools export table-l6.qza --output-dir taxonomy-table
§ biom convert --to-tsv -i taxonomy-table/feature-table.biom -o taxonomy-table/feature-table.tsv

ANCOM with taxa collapse at the phylum and genus level

§ qiime composition add-pseudocount --i-table table-l2.qza --o-composition-table comp-table-l2.qza
§ qiime composition ancom --i-table comp-table-l2.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization l2-ancom-txt.qzv

§ qiime composition add-pseudocount --i-table table-l6.qza --o-composition-table comp-table-l6.qza
§ qiime composition ancom --i-table comp-table-l6.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization l6-ancom-txt.qzv

ANCOM without taxa collapse

§ qiime composition add-pseudocount --i-table dada2-table.qza --o-composition-table ANCOM-table.qza
§ qiime composition ancom --i-table ANCOM-table.qza --m-metadata-file cf-metadata.txt --m-metadata-column txt_group --o-visualization ancom-txt-all.qzv