MICROBIOME-MEDIATED METABOLIC ACTIVITY AND WEIGHT DYSREGULATION IN ANOREXIA NERVOSA

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ABSTRACT

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Microbiome-Mediated Metabolic Activity and Weight Dysregulation in Anorexia Nervosa
(Under the direction of Cynthia M. Bulik)

Anorexia nervosa (AN) is a serious and often life-threatening psychiatric disorder that continues to perplex clinicians and researchers. Treatment outcome is poor, and despite significant morbidity and mortality, the evidence base for treatment is weak. The biology of AN is poorly understood, which has hindered development of novel interventions. Compelling evidence that the intestinal microbiota regulates key features of AN, including weight, energy metabolism, anxiety, and depression, provides a strong rationale for exploring the role of this complex microbial community in relation to the disorder. Changes in gut microbial communities associated with extreme weight loss may perpetuate and contribute to AN via direct effects on weight and mood.

To better understand the role of the intestinal microbiota in physiologic changes associated with AN, we (i) characterized the composition and diversity of the intestinal microbiota in acutely ill patients with AN (n=16) before and after hospital-based renourishment and compared with healthy controls (n=12); (ii) examined associations between microbial composition and diversity and measures of psychopathology in patients with AN (n=15) and healthy adult females (n=91); (iii) investigated changes in fecal energy content during hospital-based renourishment and associations with the intestinal microbiota in patients with AN (n=15); and (iv) examined daily changes in the intestinal microbiota during hospital-based renourishment in patients with AN (n=3).

We found evidence of an intestinal microbial dysbiosis in patients with acute AN, marked by lower microbial diversity and taxonomic differences from healthy controls. Moreover, we found compositional changes to the intestinal microbiotas of patients with AN during hospital-based renourishment, as well as relative changes in fecal energy content. Although we saw associations between microbial markers and psychopathology in patients with AN, there were no significant associations between microbial composition and diversity and psychiatric measures in healthy adult females.

This work introduced a novel approach to studying the pathophysiology of AN by profiling the intestinal microbiota in individuals with AN using high-throughput 16S rRNA sequencing. Ultimately, we may identify bacterial taxa whose promotion or elimination would improve the efficacy and efficiency of therapeutic weight restoration, as well as the psychological and physical treatment experience of patients with AN.

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AN	Anorexia nervosa
BAI	Beck Anxiety Inventory
BDI	Beck Depression Inventory-II
BMI	Body mass index
CEED	Center of Excellence for Eating Disorders
EDE-Q	Eating Disorder Examination-Questionnaire
FMT	Fecal microbial transplantation
FDR	False discovery rate
GABA	Gamma-aminobutyric acid
GF	Germ-free
GI	Gastrointestinal
HCG	Healthy comparison group
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
Mini IPIP	Mini International Personality Item Pool
PC	Principal coordinate
PCR	Polymerase chain reaction
PSS	Perceived Stress Scale
QIIME	Quantitative Insights Into Microbial Ecology
UNC	University of North Carolina at Chapel Hill

CHAPTER 1

GUT FEELINGS: A ROLE FOR THE INTESTINAL MICROBIOTA IN ANOREXIA NERVOSA?[1]

1.1 The pathophysiology of anorexia nervosa remains unclear

Anorexia nervosa (AN) affects 0.9% of women and 0.3% of men in the United States(1) and continues to perplex clinicians and researchers. Treatment outcome is poor, especially in adults, and AN has the highest mortality rate of any psychiatric disorder.(2, 3) Despite significant morbidity and mortality,(1-4) the evidence base for the treatment of AN is weak.(5, 6) Although a fundamental first step in treatment, few clinical trials exist that explore how best to renourish individuals with AN, and treatment approaches are typically based on clinical opinion or guidelines.(7) Moreover, gastrointestinal effects of refeeding are uncomfortable, distressing, and painful, and body fat redistribution after refeeding is unequal, with disproportionate central adipose tissue deposition.(8) Low treatment acceptance and high treatment dropout are common. To date, the biology of AN and the physical adaptations that occur during weight restoration are poorly understood. New lenses through which to view the disorder are essential to advance understanding and enhance treatment.

Genetic epidemiological investigations indicate that AN has a strong genetic etiology.(9, 10) Indeed, a significant familial association has been reported for AN,(11, 12) and twin studies estimate heritability at 48-74%.(9, 10, 13, 14) Although a strong genetic component exists, twin

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studies clarify that environmental factors or gene environment interactions are also implicated and are worthy of investigation.

Humans coexist with numerous diverse microbial communities, or microbiotas, living in and on the human body. The intestinal microbiota refers to living organisms, including prokaryotes, eukaryotes, archaea, and viruses, while the microbiome refers to the cumulative genomes of these organisms. Although each person has a unique microbiota, a core set of microorganisms is common across individuals.(15, 16) The environment, including long-term dietary patterns, exerts profound influence on the intestinal microbiota.(17, 18) Short-term dietary changes can also induce measurable microbial shifts.(19)

Compelling evidence that the intestinal microbiota regulates key features of AN, including weight regulation, energy metabolism,(20) anxiety, and depression,(21) provides a strong rationale for exploring the role of this complex microbial community in the disorder. However, the functional role of enteric microbes in AN has never been comprehensively explored.(22, 23) Based on the existing literature, it is logical to posit that changes to gut microbial communities associated with extreme weight loss may perpetuate and contribute to AN via direct effects on weight and mood. *Defining alterations and functional effects of AN intestinal microbiotas on adiposity and behavior could provide new mechanistic insights into this perplexing illness and guide new treatment paradigms.*

1.2 A dysbiosis or microbial imbalance in the intestinal microbiota exists in patients with anorexia nervosa

A small culture-based study of a stool sample from a single patient with AN at hospital admission identified 11 completely new bacterial species in the Firmicutes (n=7), Bacteroidetes (n=2), and Actinobacteria (n=2) phyla, which may suggest distinct characteristics of the

intestinal microbiota in AN.(23) However, further research is needed to investigate whether these new species are uniquely associated with AN. Moreover, a molecular-based study analyzing the intestinal microbiota of nine patients with AN found increased levels of the archaeon *Methanobrevibacter smithii*.(22) Although intriguing, this study was cross-sectional and used a narrow approach [quantitative polymerase chain reaction (PCR) of specific bacterial taxa] to determine the abundances of a limited number of microbial groups (n=4). Given that the intestinal microbiota encompasses 500–1,000 different microbial species,(24) a more comprehensive characterization of the intestinal microbiota in AN, how it changes following treatment, and how it differs from healthy controls is warranted. *Exploration of longitudinal changes in the intestinal microbiota in patients with AN over the course of medically supervised weight restoration would provide new insights into how current AN treatment impacts enteric microbes and how microbial shifts may contribute to adipose distribution and behavior.*

1.3 The intestinal microbiota influences adiposity in humans and animal models

Consistent evidence implicates the intestinal microbiota in excessive accumulation and storage of fat in humans.(25, 26) Additionally, in mice, obese intestinal microbiotas are more effective at extracting calories from food and stimulating host accumulation of fat than microbiotas of lean mice.(27) Transfer of microbiotas from mouse models of diet- or genetically-induced obesity to germ-free (GF) mice is sufficient to stimulate increased adiposity and metabolic dysfunction.(27) Similarly, fecal samples transplanted to mice from obese adult humans transmit obesity-associated phenotypes via the intestinal microbiota.(20) *Given that AN is marked by extreme weight dysregulation and is treated initially by weight restoration,(28) exploring the functional impact of a dysbiotic intestinal microbiota on adiposity in AN is a logical and critical step toward better understanding of AN pathophysiology and treatment*

response.

1.4 The intestinal microbiota influences behavior in humans and animal models

A majority of individuals with AN report a lifetime history of comorbid anxiety disorders (75%)(29-31) or major depressive disorder (80%).(32) Animal models provide evidence that enteric microbes significantly influence both anxiety and depression, and many studies have documented behavioral changes following pathogenic infection or manipulation of the intestinal microbiota. For example, infection with a pathogenic microbe increases anxiety-like behavior,(33-35) and GF mice exhibit reduced anxiety-like behavior that is reversed upon reconstitution with a gut microbiota.(36-39) Furthermore, GF BALB/c mice colonized with an NIH Swiss intestinal microbiota show reduced anxiety-like behavior compared to GF NIH Swiss mice colonized with a BALB/c microbiota, indicating that gut microbe-associated changes in anxiety are transmissible and are affected by the composition of the intestinal microbiota.(40) Probiotics have been shown in animal models to reduce depressive and anxiety-like behavior at effect sizes similar to antidepressant treatment,(41, 42) and prebiotics may reduce stress-induced anxiety-like behavior and stimulate changes in microbial diversity.(43)

These findings suggest that changes within the intestinal microbiota may be of central importance to the development or maintenance of depression and anxiety, but few studies have examined associations of the enteric microbe-gut-brain axis in human samples—and significant results generally lack replication. Two studies comparing the intestinal microbiotas of individuals with mild-to-severe depression to healthy controls generated mixed findings, with one failing to find significant between-group differences,(44) while the other found increased diversity in individuals with major depressive disorder and significant taxonomic differences.(45) Prebiotic and probiotic supplementation have also emerged in human clinical

studies as potential means for altering mood, with improvement in measures of depression, anxiety, cognitive reactivity, and stress levels in healthy volunteers after placebo-controlled supplementation trials of prebiotic or probiotic formulas.(46-48) However, prebiotic or probiotic supplementation has not always been associated with observable compositional changes to the intestinal microbiota.

There are many possible physiologic mechanisms involved in the enteric microbe-gut-brain axis. Bravo *et al.* (2011) suggest that a naturally occurring enteric microorganism used as a probiotic impacts behavior via the vagus nerve (the main gut-brain communication pathway) and gamma-aminobutyric acid (GABA) expression in the brain.(41) However, the intestinal microbiota also (i) stimulates release of cytokines and chemokines that can elicit immune response; (ii) synthesizes neurotransmitters and short-chain fatty acids that have neuroactive properties and can influence a range of other physiologic functions; and (iii) regulates permeability of the blood-brain barrier.(49)

The investigation of the specific enteric microbes that influence anxiety and depression in AN has never been attempted. *Yet the centrality of anxiety and depression in AN, and the demonstrated role of the intestinal microbiota on these traits, support research to identify whether microbial shifts in patients with AN correlate with anxiety and depression measures and whether they can confer anxiety and depression to GF mice.*

1.5 The intestinal microbiota is a valid intervention target

Studies involving transplantation of intact uncultured microbiotas from healthy humans to individuals with *Clostridium difficile*-induced colitis or patients with metabolic syndrome have provided proof-of-principle that the intestinal microbiota represents a valid therapeutic tool for treating or preventing disease.(50-52) These findings provide a basis for the of use of fecal

microbial transplants beyond the treatment of *C. difficile* and metabolic syndrome, but this research is in its infancy and the mechanism by which these transplants (via enema or capsule) induce a beneficial outcome is unclear. Supporting this concept, a probiotic originally isolated from the intestinal microbiota of a healthy individual (*Lactobacillus rhamnosus* JB-1) reduced anxiety- and stress-related behavior in mice via modulation of the expression of GABA in the brain.(53) These biological and behavioral effects were not seen in vagotomized mice, illustrating the critical role of microbe-gut-brain communication. Thus, an enteric microbe, originating from the intestinal microbiota, is known to regulate behaviors that are prominent in AN. *Identifying microbes within the intestinal microbiota of patients with AN associated with specific AN traits (weight regulation, anxiety, and depression) that are transmissible to GF mice would provide a rationale to develop new, microbiota-based treatments for this disorder.*

This research would pioneer the combination of large scale 16S rRNA gene sequencing-based studies of intestinal microbiotas in AN with exploration of their functional influence on weight regulation and behavioral traits associated with AN. Correlating the configuration of an individual's intestinal microbiota with health status is a fundamental first step in testing for a causative role of enteric microbes in AN. A unique challenge for this research is the inability to compare individuals with AN to similarly malnourished individuals who do not have AN or other medical conditions that result in malnutrition. This does not preclude other informative designs that could, for example, explore whether the presence of specific enteric microbes are associated with successful maintenance of therapeutically restored weight after renourishment.

A major challenge for current microbiome-related research is how to move from observational to functional and mechanistic studies that dissect how these microbes impact a host's biology. One option is transplanting an intact uncultured human intestinal microbiota

(from feces) from patients with AN into GF mice and measuring anthropometric, metabolic, and behavioral changes that occur, along with the composition of the intestinal microbiota. This would test the ability of donor microbiotas to functionally impact AN-related phenotypes (i.e., weight regulation, anxiety, and depression) in the recipient animals. Identifying enteric microbes that have a potential detrimental or beneficial impact on weight regulation and behavior in patients with AN would generate target microbes that can then be studied further on a platform that enables the investigation of host–microbe interactions, namely gnotobiotics.

Novel interventions for AN are essential. Microbiota-modulating strategies could comprise a significant therapeutic advance in the treatment of AN. Our incomplete understanding of the pathophysiology of AN has hindered the development of novel, safe, acceptable, and effective interventions. Concerted attention to this area could identify bacterial taxa whose promotion or elimination would improve the efficiency of therapeutic weight restoration, as well as the psychological and physical treatment experience of patients. We need new information about the biology of AN at a microbial level to inform innovative therapies targeting enteric microorganisms, which may fundamentally alter the way we understand and treat AN.

As a critical first step, the overarching goal of this dissertation is to gain a greater understanding the role of the intestinal microbiota in the physiologic changes associated with AN. *We hypothesize that a unique intestinal microbial dysbiosis arises from prolonged starvation in patients with AN.* To address our knowledge gap related to the underlying biologic mechanisms of this disorder, my research sought to:

(i) Characterize the composition and diversity of the intestinal microbiota in acutely ill patients with AN before and after hospital-based renourishment and compared to healthy controls (Chapter 2);

Hypothesis: the intestinal microbiota in patients with AN will differ in measures of composition and diversity from that of healthy controls and change over the course of hospital-based renourishment.

(ii) Examine associations between microbial composition and diversity and measures of depression, anxiety, and eating disorder psychopathology in patients with AN (Chapter 2) and healthy females (Chapter 4);

Hypothesis: the composition and diversity of the intestinal microbiota is significantly associated with measures of psychopathology in patients with AN and healthy females.

(iii) Investigate changes in fecal energy content during hospital-based renourishment and associations with the intestinal microbiota (Chapter 3);

Hypothesis: the intestinal microbiota in AN, which is altered during renourishment, adaptively responds to a low-energy environment (i.e., prolonged caloric restriction).

and

(iv) Characterize daily changes in composition and diversity of the intestinal microbiota in three acutely ill patients with AN over the entire course of hospital-based renourishment and identify enteric bacterial groups associated with metabolic changes during treatment (Chapter 5).

Hypothesis: changes in composition and diversity of the intestinal microbiota in patients with AN during hospital-based renourishment will be associated with metabolic changes in these patients.

CHAPTER 2

THE INTESTINAL MICROBIOTA IN ACUTE ANOREXIA NERVOSA AND DURING RENOURISHMENT: RELATIONSHIP TO DEPRESSION, ANXIETY, AND EATING DISORDER PSYCHOPATHOLOGY[2]

2.1 Introduction

The robust and documented role of the intestinal microbiota in metabolic function and weight regulation provides a strong rationale for exploring the role of this complex microbial community in the emergence, maintenance, and recovery from anorexia nervosa (AN).(26) AN is a severe, life-threatening mental illness(54) associated with dangerously low body weight and biochemical, metabolic, immunologic, and sensory abnormalities,(55-60) as well as mortality rates among the highest for any psychiatric disorder.(3) Despite the significant morbidity and mortality associated with AN(1-4) and decades of research, the evidence base for its treatment is weak—especially during the initial renourishment phase.(5, 61) Current models are unable to account for how individuals with AN can achieve and defend such low body weights.

The composition of the human microbiota, which includes the diverse microbial communities living in and on the human body, as well as the genetic material of these microorganisms (microbiome) and their interactions with the surrounding environment, has become a burgeoning area of study. The composition of these microbial communities can vary with age, sex, environment, geography, diet, and disease, but we understand little about the nature of these variations or their impact on human development, physiology, immunity, and

[2]Kleiman SC, Watson HJ, Bulik-Sullivan EC, Huh EY, Tarantino LM, Bulik CM, Carroll IM. The Intestinal Microbiota in Acute Anorexia Nervosa and During Renourishment: Relationship to Depression, Anxiety, and Eating Disorder Psychopathology. Psychosom Med. 2015 Nov-Dec;77(9):969-81. PMID: 26428446.

nutrition.(62) Seminal work by the Human Microbiome Project has characterized the microbiome in a cohort of healthy individuals,(63) whereas other investigators have focused on how deviations from the norm could contribute to diseases such as inflammatory bowel diseases (IBD),(64) asthma,(65-70) and obesity.(22, 25, 27, 71-74)

A growing body of evidence from both animal models and human studies shows communication between the intestinal microbiota and the brain (i.e., the so-called gut-brain axis).(75) This phenomenon has not been studied in individuals with AN, and the specific mechanism(s) through which enteric microbes affect brain function remains unclear. However, individuals with AN often present with comorbid anxiety and depression—up to 80% will experience major depression at some point in their lifetime,(32) whereas up to 75% will have some form of anxiety disorder, including social phobia, specific phobia, and generalized anxiety disorder.(29-31)

The intestinal microbiota plays a demonstrable role in weight gain/loss(22, 25, 27, 71-74) and energy extraction from the diet(27, 74, 76) in human and animal models. Given that AN is marked primarily by extreme weight dysregulation,(28) exploring the role of the intestinal microbiota in AN is a logical and inevitable next step. Consistent evidence implicates this enteric microbial community in obesity and metabolic outcomes, although the degree of that contribution is controversial.(20, 25, 27, 52, 73) Findings suggest that the composition of the intestinal microbiota differs between obese and lean individuals,(25, 73) and that obese individuals may extract more energy from a given diet than their lean counterparts,(27) but very little is known about the gut microbiota in individuals with AN.

Intriguing published and preliminary findings suggest a role for the intestinal microbiota in AN. A culture-based study of a stool sample from an AN patient at hospital admission

identified 11 completely new bacterial species in the Firmicutes (n=7), Bacteroidetes (n=2), and Actinobacteria (n=2) phyla, suggesting distinct characteristics of the gut microbiome in AN.(23) Further research is needed to investigate whether these new species are uniquely associated with AN. In addition, a molecular-based study(22) analyzing the intestinal microbiota of nine patients with AN found increased levels of the archaeon *Methanobrevibacter smithii*. Because *M. smithii* and other methanogens play an important role in removing excess hydrogen gas from the gut and improving efficiency of microbial fermentation (and associated energy yield), this could demonstrate an adaptive response toward optimizing energy extraction from a very low-calorie diet. Although novel findings were reported, this study analyzed a limited number of microbial groups (two phyla: Bacteroidetes and Firmicutes; one genus: *Lactobacillus*; and one archaeon: *M. smithii*). Animal models also suggest that the intestinal microbiota influences satiety mechanisms through interaction with peptide signaling(77) and protective adaptation in a starvation state.(78) A more comprehensive characterization of the intestinal microbiota of individuals with acute AN is required, along with exploration of changes in enteric microbes over the course of medically supervised weight restoration.

This study sought to i) gain insight into the composition and diversity of the intestinal microbiota in a cohort of patients with acute AN; ii) measure changes in the intestinal microbiota of patients with AN after hospital-based weight restoration; iii) compare the intestinal microbiota in acutely ill patients with AN to that of a healthy comparison group (HCG); and iv) examine associations between these microbial measures and depression, anxiety, and eating disorder psychopathology.

2.2 Methods and Materials

The study was approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill (UNC). All participants provided written consent before study participation.

Females (n=16) admitted for inpatient treatment at the UNC Center of Excellence for Eating Disorders (CEED) participated in the study. Participants were recruited from consecutive inpatient admissions from December 2012 to May 2013, and inclusion criteria were as follows: a) age 15 to 64 years; b) meet the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision criteria for AN; and c) present at less than 75% of ideal body weight. Exclusion criteria were based on factors known to influence the composition of the intestinal microbiota: history of gastrointestinal tract surgery (other than appendectomy or cholecystectomy); history of IBD, irritable bowel syndrome, celiac disease, or any other diagnosis that could explain chronic or recurring bowel symptoms; treatment in the last two months with antibiotics, nonsteroidal anti-inflammatory drugs, or steroids; or intentional use of probiotics during the last two months.

Data from HCG (n=12) with no recurring gastrointestinal symptoms were obtained from a previous study.(79) This study recruited controls via advertisement from the general population in the same geographical region (central North Carolina) and from UNC outpatient clinics. HCG participants were subject to the same exclusion criteria as patients with AN and were selected for this analysis based on sex (female), age (15 to 64 years), and body mass index (BMI; 18.5–24.9 kg/m^2). They were not screened for psychopathology during recruitment.

Weight and height were assessed using a calibrated digital scale and stadiometer. HCG participants were measured once. AN patients were weighed daily as part of standard treatment.

Height was measured at admission for all AN patients and again at discharge for those younger than 21 years. Eating disorders diagnosis and psychopathology were established via the Eating Disorder Examination(80) and the Structured Clinical Interview for DSM-IV-TR Axis I Disorders(81) conducted by credentialed members of the CEED Assessment Core. AN patients also completed electronic versions of the Beck Anxiety Inventory (BAI),(82) Beck Depression Inventory-II (BDI),(83) and Eating Disorder Examination–Questionnaire (EDE-Q)(84) within 24 hours of admission.

The first stool sample produced after admission (T1) was collected for all AN patients (n=16), and for a subset of these patients (n=10), an additional sample was collected before discharge (T2). Input and output are measured as part of routine treatment, minimizing risk of missing samples, and all samples were collected by nurses trained in collection protocols. Fresh stool samples were collected from HCG in the same manner as AN patients, as previously reported.(79) All samples were transferred to the laboratory, where they were mechanically homogenized with a sterile spatula, aliquoted into sterile 2 ml cryovials, and stored in a -80 °C freezer for future DNA isolation and nucleotide sequence analysis.

Bacterial DNA was isolated from collected samples using a phenol/chloroform extraction method combined with physical disruption of bacterial cells and a DNA clean-up kit (Qiagen DNeasy Blood and Tissue extraction kit [Qiagen, Valencia, CA]), as previously described.(79, 85)

Bacterial community composition in isolated DNA samples was characterized by amplification of the V1-3 (forward, 8f: 5'-AGAGTTTGATCMTGGCTCAG-3'; reverse, 518r: 5'-ATTACCGCGGCTGCTGG-3') variable region of the 16S rRNA gene by polymerase chain reaction (PCR), as previously described.(79) 16S rRNA PCR products were quantified, pooled,

and purified for the sequencing reaction. Sequencing was performed on a 454 Life Sciences Genome Sequencer FLX machine (Roche, Florence, SC) by the Microbiome Core Facility in the UNC School of Medicine.

16S rRNA sequence data generated by the 454 sequencer were processed by the Quantitative Insights Into Microbial Ecology (QIIME) pipeline.(86) Sequences that were less than 200 base pairs or greater than 1000 base pairs in length, contained incorrect primer sequences, or contained more than one ambiguous base were discarded.(87) Sequences were clustered into Operational Taxonomic Units (similar to species level) based on their sequence similarity at a 97% threshold using BLAST and assigned taxonomy using the Greengenes database.(88) Principal coordinates were generated using unweighted and weighted UniFrac distances.(89-91) The richness of the intestinal microbiota was characterized by the number of observed bacterial species in each sample and the Chao-1 estimator of diversity.(92, 93)

Differences in alpha diversity (expressed as number of observed species and Chao-1 estimator), beta diversity (UniFrac distances), and taxa abundance of bacterial groups (at the phylum, class, order, family, and genus levels) were examined in AN patients (n=10) at T1 versus T2 using 16S rRNA sequence data. Bacterial groups present in at least 25% of all samples at T1 or T2 were included in analyses. Because response variables were not normally distributed, nonparametric testing was used. Depending on the symmetry of the distribution of the paired differences, differences were tested at the univariate level using Wilcoxon matched-pairs rank test (-2 <= skewness <= 2) or the sign test (skewness <= -2 or >= 2). Power analysis was conducted in G*Power 3 to determine the effect size that could be detected with n=10, a two-tailed test, an [alpha] of 0.05, and power of 80%; under these conditions, the Wilcoxon matched-pairs rank test can detect a large effect (dz=1.1), as can the sign test (g=0.41). The false

discovery rate (FDR) procedure addressed multiple testing(94) and was applied to the number of comparisons per outcome and per taxonomic rank. A global, multivariate hypothesis test developed for high-dimensional small-sample data (i.e., the type of data acquired through high-throughput technology in metabolomics, genomics, and proteomics) was also used to test for differences in alpha diversity, beta diversity, and taxa abundance across all bacterial groups.(95) A global test offers additional conceptual advantages to a univariate test because microbiota can work together or in a pathway and may have greater explanatory power when considered collectively.

Differences in alpha diversity, beta diversity, and taxa abundance of bacterial groups (at the phylum, class, order, family, and genus levels) were compared in AN patients at T1 (n=16) versus HCG (n=12) and AN patients at T2 (n=10) versus HCG with two-tailed Wilcoxon-Mann-Whitney tests. The Chi *et al.*(95) global multivariate test was used for beta diversity and per taxonomic level, and FDR correction was applied as described earlier.

Associations between T1 psychopathology scores measured as continuous variables (BDI [depression], BAI [anxiety], and EDE-Q [total + subscales for Dietary Restraint, Eating Concern, Shape Concern, and Weight Concern]) and alpha diversity, beta diversity, and taxa abundance of bacterial groups (at the phylum, class, order, family, and genus levels) were examined in AN patients (n=15; one patient did not complete the surveys) with the tau-b correlation coefficient. Bacterial groups present in at least 25% of T1 samples were considered. Univariate analyses used the Wilcoxon-Mann-Whitney test, and the FDR procedure was used to adjust for multiple testing, implemented per outcome and per taxonomic rank. The global multivariate test was implemented for beta diversity and per taxonomic level.

The [alpha] level used was 0.05, but for FDR correction, a more lenient criterion of 0.1

was used given the exploratory nature and small sample size. All analyses were conducted in SAS 9.3 (Cary, NC).

2.3 Results

Fecal samples were collected at T1 from female patients with AN (n=16). Average age was 28.0 (11.7; mean [standard deviation]) years, and mean BMI at T1 was 16.2 (1.5) kg/m^2. A subset of patients (n=10) provided an additional sample at T2, when they had reached a mean BMI of 17.4 (0.9) kg/m^2. Female HCG (n=12) who provided samples had a mean age of 29.8 (11.6) years and mean BMI of 21.5 (1.9) kg/m^2. Participants were predominately white (n=14 patients with AN; n=7 HCG), with a small representation of African American participants (n=2 patients with AN; n=1 HCG). Four HCG participants did not provide information on race.

At T1, patients with AN (n=15) had mean BDI and BAI scores of 26.6 (13.4) and 17.7 (11.9), respectively, reflecting moderate depression and anxiety.(96, 97) Most patients endorsed at least mild levels of depression (80.0%) and anxiety (66.7%). Mean EDE-Q total scores of 3.6 (1.8) and scores on subscales for Dietary Restraint (3.7 [1.9]), Eating Concern (3.4 [1.9]), Shape Concern (3.8 [1.9]), and Weight Concern (3.4 [2.1]) are consistent with other clinical samples of patients with AN.(98, 99)

From the 26 fecal samples analyzed from patients with AN, a total of 197,956 16S rRNA sequences with acceptable quality were obtained with an average of 7,613 reads per sample (range, 4,101–9,511). From the 12 fecal samples analyzed from HCG, a total of 122,461 16S rRNA sequences with acceptable quality were obtained with an average of 10,205 reads per sample (range, 5,265–15,596). Using a 97% similarity threshold, we found a total of 1,666 and 2,020 Operational Taxonomic Units in the samples analyzed from patients with AN and HCG, respectively.

Table 1 presents changes in bacterial composition and diversity over the course of inpatient weight restoration. Global tests indicated significant differences between T1 and T2 in beta (between-sample) diversity ($p<0.001$) and at the phylum ($p=0.042$) and genus ($p=0.041$) taxonomic levels (**Figure 1**). Based on unweighted UniFrac distances, three principal coordinates (5, 6, 10) were significantly different at hospital admission and discharge and remained significant at an FDR level of 0.1. The average unweighted UniFrac distances were significantly different between groups ($p<0.0001$), with T2 samples showing greater similarity to each other than T1 samples (**Figure 2**). The strongest taxonomic changes were seen in the family Ruminococcaceae, with significant changes in specified (*Ruminococcus*; $p=0.002$) and unspecified ($p=0.004$) subgenera.

We compared the intestinal microbiota in patients with AN at T1 and T2 to that of age- and sex-matched HCG. At both time points, the alpha (within-sample) diversity remained significantly lower in patients with AN versus HCG, measured as either the number of observed species or Chao-1 estimator (**Figure 3**; **Tables 2 and 3**). However, the bacterial composition of samples from patients with AN at T1 showed greater differences with HCG than samples collected at T2. At T1, patients with AN had greater levels of class Bacilli ($p=0.007$) and the unspecified genus in family Coriobacteriales ($p<0.001$) and reduced levels of class Clostridia ($p=0.007$), order Clostridiales ($p=0.006$), and genera *Anaerostipes* ($p=0.003$) and *Faecalibacterium* ($p=0.002$) versus HCG (with all differences remaining significant at an FDR level of 0.1; **Table 2**). At T2, the only one of these differences that remained significant was in the unspecified genus in family Coriobacteriales ($p<0.001$), although there were additional differences between patients with AN at T2 and HCG among the family Ruminococcaceae ($p=0.002$) and the genus *Parabacteroides* ($p=0.006$; **Table 3**).

Alpha (within-sample) diversity, measured as bacterial richness or the Chao-1 estimator, was significantly associated with scores on the BDI and EDE-Q. Greater levels of depression were negatively associated with the number of observed bacterial species (p=0.026) and Chao-1 estimator (p=0.026; **Figure 4** and **Table 4**). Lower number of observed species was also associated with greater levels of eating disorder psychopathology, measured as EDE-Q total score (p=0.026) or scores on subscales for Shape Concern (p=0.008) and Weight Concern (p=0.025; **Table 5**). All associations remained significant at an FDR level of 0.1.

Significant associations were also seen between specific bacterial taxa and BDI, BAI, and EDE-Q scores, but none remained significant at an FDR level of 0.1. The strongest (negative) associations were seen with the family Ruminococcaceae (**Tables 4 and 5**).

2.4 Discussion

In examining the composition and diversity of the intestinal microbiota in patients undergoing inpatient treatment of AN, we report i) changes over the course of hospital-based weight restoration; ii) significant differences between patients with AN and HCG; and iii) associations between microbial measures and depression, anxiety, and eating disorder psychopathology. These results extend findings from earlier, smaller studies of patients with AN and provide strong support for future work, including mechanistic studies of gut-brain interaction, to better understand the biological mechanisms at work in the risk and maintenance of AN.

Significant changes in the composition of the intestinal microbiota were seen in patients with AN during renourishment, particularly among genera falling under the family Ruminococcaceae. This family of bacteria has been associated with intestinal disorders marked by inflammation, including irritable bowel syndrome (IBS) and IBD.(100, 101)

In comparing the intestinal microbiota of patients with AN to that of HCG, we found that alpha diversity was significantly lower in patients with AN both before and after inpatient weight restoration. Alpha diversity was also significantly associated with depression and eating disorder psychopathology in our patient group, with a lower number of observed bacterial species associated with greater depression and greater weight concern, shape concern, and overall eating disorder psychopathology. These results show intriguing associations and underscore findings across various other disease states, including IBD(64) and arthritis,(102) which have shown that a healthier gut is a more diverse one. Moreover, as we found greater differences in bacterial composition between AN and HCG before versus after hospital-based renourishment, our results may suggest that the intestinal microbiota is trending toward a healthier state during treatment.

Although there has been limited research to date into the role of the intestinal microbiota in AN, some parallels can be drawn to microbial changes associated with malnutrition. Studies have demonstrated that acute malnutrition in children is marked by an intestinal dysbiosis and that the malnutrition phenotype (marked by severe weight loss) can be transmitted via the intestinal microbiota in a gnotobiotic mouse model.(103, 104) This microbial dysbiosis may also interact with a nutrient-deficient diet to affect energy metabolism and cause malnourishment to persist.(103, 105)

Mounting evidence from animal studies in which the intestinal microbiota have been manipulated through probiotics, antibiotics, or microbial transfer to gnotobiotic mice has shown that behavior is associated with changes in bacterial composition and diversity.(40-42, 106-111) This includes models of depression and anxiety, which are common among patients with AN.(30, 31) However, we have little evidence supporting associations between the intestinal microbiota and depression or anxiety disorders in humans.(21, 44, 112) Our results, particularly

those showing that lower bacterial diversity is associated with greater depression and anxiety, are at the forefront of providing evidence for the gut-brain axis in a human population.

Several limitations should be taken into account when considering these results. First, we did not control for diet of either patients with AN or HCG. The composition of the intestinal microbiota is strongly influenced by long-term dietary patterns,(17, 18) and short-term dietary changes can also induce dramatic microbial shifts.(19) Because patients resided on an inpatient hospital unit, dietary intake was controlled, and all participants consumed a standard diet, with far less variation across individuals than what would be expected from those in a free-living environment. In addition, our sample size limited power to detect differences between patients and controls over the course of renourishment. However, we did see some significant compositional changes during inpatient treatment, as well as significant global changes in composition and diversity using a statistical method that provides greater explanatory power by considering the intestinal microbiota collectively. Third, all of our study participants were female, limiting generalizability of the results to males, who comprise approximately 10% of individuals with AN.(113) Given that we would be unlikely to recruit a sufficient number of male participants to allow testing for sex differences, we focused recruitment on females to maximize sample size. Lastly, we are unable to distinguish between changes to the intestinal microbiota that reflect weight gain versus recovery from AN, which will be important in future work, as BMI alone is associated with abundance of specific bacteria.(114)

Although genetic and neurobiological research underscores that AN is most accurately considered a biologically based mental illness, the neurobiology of AN remains an enigma, which has hindered the development of novel, safe, and effective treatments. These findings are an important first step in uncovering the role of the intestinal microbiota in AN. Future

mechanistic studies examining the impact of specific taxa on behavior and adiposity, including transplantation of the intestinal microbiota of patients with AN into gnotobiotic mice, will allow us to distinguish between microbial markers of renourishment and recovery from psychopathology and move us even closer to designing innovative therapies for AN targeting enteric microorganisms. Such studies could identify specific bacterial taxa whose promotion or elimination would improve the efficiency of therapeutic weight restoration, as well as the psychological and physical treatment experience of patients, and lead to pathophysiological-directed therapeutic approaches for the management of AN via probiotic, prebiotic, symbiotic, or antibiotic means.

Table 1. Hospital admission (T1) vs. hospital discharge (T2): differences in microbial taxa and diversity measures in females with AN (n=10)

Taxonomic/diversity level	Classification	Test statistic	*p*	FDR corrected *p*
Global Tests				
Phylum		2.66	0.042	
Class		2.14	0.067	
Order		1.77	0.064	
Family		1.70	0.064	
Genus		1.74	0.041	
Beta diversity	Weighted	1.50	0.22	
	Unweighted	6.07	0.0003	
Univariate Tests				
Family	*Eubacteriaceae*	-3.5	0.039	0.63
Genus	*Ruminococcaceae_genus*	-26.5	0.004	0.10
	Oscillospira	-22.5	0.020	0.34
	Ruminococcus	27.5	0.002	0.10
Beta diversity	Unweighted (PC 5)	-23.5	0.014	0.070
	Unweighted (PC 6)	-21.5	0.027	0.090
	Unweighted (PC 10)	26.5	0.004	0.040

[a] FDR = false discovery rate; PC = principal coordinate

[b] Alpha = 0.05; FDR level = 0.1

[c] A global multivariate test (95) was used to test for differences in alpha diversity, beta diversity, and abundance per taxonomic level. Depending on the symmetry of the distribution of the paired differences, differences were tested at the univariate level using Wilcoxon matched pairs rank test ($-2 \leq$ skewness ≤ 2) or the sign test (skewness ≤ -2 or ≥ 2).

Table 2. Hospital admission (T1): differences in microbial taxa and diversity measures in females with AN (n=16) vs. healthy comparison group (n=12)

Taxonomic/diversity level	Classification	Test statistic	*p*	FDR corrected *p*
Class	*Bacilli*	-2.72	0.007	0.026
	Clostridia	2.72	0.007	0.026
Order	*Clostridiales*	2.76	0.006	0.068
	Lactobacillales	-2.25	0.024	0.15
Family	*Actinomycetaceae*	-2.03	0.042	0.23
	Lachnospiraceae	2.02	0.043	0.23
	Porphyromonadaceae	-2.60	0.009	0.14
	Ruminococcaceae	2.53	0.011	0.14
	Streptococcaceae	-1.97	0.049	0.23
Genus	*Anaerostipes*	2.99	0.003	0.042
	Blautia	2.06	0.031	0.17
	Coribacteriales_genus	-4.62	<0.0001	0.005
	Faecalibacterium	3.18	0.002	0.034
	Lachnospira	2.16	0.030	0.17
	Parabacteroides	-2.60	0.009	0.10
	Ruminococcaceae_genus	2.30	0.022	0.16
	Ruminococcus	2.39	0.017	0.15
Alpha diversity	# of observed species	4.02	<0.0001	
	Chao-1 estimator	3.83	0.0001	

[a] FDR = false discovery rate
[b] Alpha = 0.05; FDR level = 0.1
[c] Differences in alpha diversity, beta diversity, and taxa abundance of bacterial groups were compared with two-tailed Wilcoxon-Mann-Whitney tests. A global multivariate test (95) was used for alpha diversity, beta diversity, and abundance per taxonomic level.

Table 3. Hospital discharge (T2): differences in microbial taxa and diversity measures in females with AN (n=10) vs. healthy comparison group (n=12)

Taxonomic/diversity level	Classification	Test statistic	*p*	FDR corrected *p*
Phylum	*Bacteroidetes*	2.08	0.038	0.11
	Firmicutes	-2.08	0.038	0.11
Class	*Bacteroidia*	2.08	0.038	0.35
Order	*Bacteroidales*	2.08	0.038	0.42
Family	*Porphyromonadaceae*	2.74	0.006	0.15
Genus	*Coribacteriales_genus*	4.29	<0.0001	0.004
	Parabacteroides	2.74	0.006	0.065
	Ruminococcaceae_genus	-3.07	0.002	0.047
Alpha diversity	# of observed species	-2.80	0.005	
	Chao-1 estimator	-2.41	0.016	

[a] FDR = false discovery rate
[b] Alpha = 0.05; FDR level = 0.1
[c] Differences in alpha diversity, beta diversity, and taxa abundance of bacterial groups were compared with two-tailed Wilcoxon-Mann-Whitney tests. A global multivariate test (95) was used for alpha diversity, beta diversity, and abundance per taxonomic level.

Table 4. Hospital admission (T1): microbial taxa and diversity measures associated with depression and/or anxiety in females with AN (n=15)

Taxonomic/diversity level	Classification	Behavioral measure	Test statistic	*p*	FDR corrected *p*
Class	*Clostridia*	BDI	-0.394	0.042	0.50
Order	*Actinomycetales*	BDI	0.406	0.040	0.40
	Clostridiales	BDI	-0.394	0.042	0.40
	Coriobacteriales	BAI	0.410	0.036	0.40
Family	*Actinomycetaceae*	BDI	0.406	0.040	0.46
	Rikenellaceae	BDI	-0.488	0.016	0.34
	Ruminococcaceae	BDI	-0.587	0.002	0.16
		BAI	-0.566	0.004	0.16
Genus	*Blautia*	BDI	-0.433	0.026	0.47
	Faecalibacterium	BDI	-0.386	0.047	0.47
	Lachnospira	BDI	-0.526	0.008	0.47
		BAI	-0.421	0.037	0.47
	Rikenellaceae_genus	BDI	-0.488	0.016	0.47
	Roseburia	BDI	-0.406	0.040	0.47
		BAI	-0.503	0.012	0.47
	Ruminococcus	BDI	-0.490	0.011	0.47
		BAI	-0.527	0.007	0.47
	Veillonella	BDI	0.460	0.034	0.47
Alpha diversity	# of observed species	BDI	-0.433	0.026	0.045
	Chao-1 estimator	BDI	-0.433	0.026	0.090
Beta diversity	Weighted (PC 2)	BDI	-0.510	0.009	0.45
		BAI	-0.488	0.013	0.45

[a] FDR = false discovery rate; PC = principal coordinate; BDI = Beck Depression Inventory-II; BAI = Beck Anxiety Inventory

[b] Alpha = 0.05; FDR level = 0.1

[c] Associations were examined with the tau-b correlation coefficient.

Table 5. Hospital admission (T1): microbial taxa and diversity measures associated with eating disorder psychopathology in females with AN (n=15)

Taxonomic/diversity level	Classification	Behavioral measure	Test statistic	*p*	FDR corrected *p*
Order	*Actinomycetales*	ShapeC	0.450	0.024	0.40
		WeightC	0.434	0.030	0.40
	Clostridiales	EatingC	-0.452	0.020	0.40
		EDEQ	-0.448	0.020	0.40
	Turicibacterales	EatingC	-0.484	0.018	0.40
Family	*Actinomycetaceae*	ShapeC	0.450	0.024	0.34
		WeightC	0.434	0.030	0.38
	Clostridiaceae	Restraint	-0.437	0.025	0.34
	Clostridiales_family	WeightC	-0.453	0.022	0.34
	Odoribacteraceae	Restraint	0.450	0.024	0.34
	Ruminococcaceae	Restraint	-0.554	0.005	0.16
		EatingC	-0.529	0.006	0.16
		ShapeC	-0.534	0.006	0.16
		WeightC	-0.579	0.003	0.16
		EDEQ	-0.600	0.002	0.16
	Turicibacteraceae	EatingC	-0.484	0.018	0.34
Genus	*Anaerostipes*	EatingC	-0.463	0.028	0.47
		ShapeC	-0.491	0.021	0.47
		WeightC	-0.508	0.017	0.47
		EDEQ	-0.505	0.016	0.47
	Bacteroidaceae_genus	EatingC	0.449	0.030	0.47
	Clostridiales_genus	WeightC	-0.453	0.022	0.47
	Clostridium	EatingC	-0.445	0.037	0.47
	Eubacteriaceae_genus	ShapeC	-0.431	0.042	0.47
	Faecalibacterium	EatingC	-0.425	0.029	0.47
		ShapeC	-0.459	0.019	0.47
		WeightC	-0.394	0.046	0.47
		EDEQ	-0.383	0.047	0.47
	Lachnospira	ShapeC	-0.510	0.011	0.47
		WeightC	-0.495	0.015	0.47
		EDEQ	-0.411	0.038	0.47
	Ruminococcaceae_genus	EatingC	-0.433	0.026	0.47
		ShapeC	-0.418	0.032	0.47
		WeightC	-0.422	0.032	0.47
		EDEQ	-0.429	0.026	0.47
	Ruminococcus	EDEQ	-0.390	0.042	0.47
	Turicibacter	EatingC	-0.484	0.018	0.47
	Veillonella	WeightC	-0.512	0.020	0.47

Alpha diversity	# of observed species	ShapeC	-0.515	0.008	0.045
		WeightC	-0.441	0.025	0.045
		EDEQ	-0.429	0.026	0.045
	Chao-1 estimator	ShapeC	-0.476	0.015	0.090
Beta diversity	Weighted (PC 4)	EatingC	-0.394	0.042	0.83
	Unweighted (PC 1)	EatingC	-0.413	0.033	0.58
		ShapeC	-0.437	0.025	0.58
		WeightC	-0.422	0.032	0.58
		EDEQ	-0.448	0.020	0.58

[a] FDR = false discovery rate; PC = principal coordinate
[b] EDEQ refers to the total score on the Eating Disorder Examination-Questionnaire. The following subscales are also included: Dietary Restraint (Restraint), Eating Concern (EatingC), Shape Concern (ShapeC), Weight Concern (WeightC)
[c] Alpha = 0.05; FDR level = 0.1
[d] Associations were examined with the tau-b correlation coefficient.

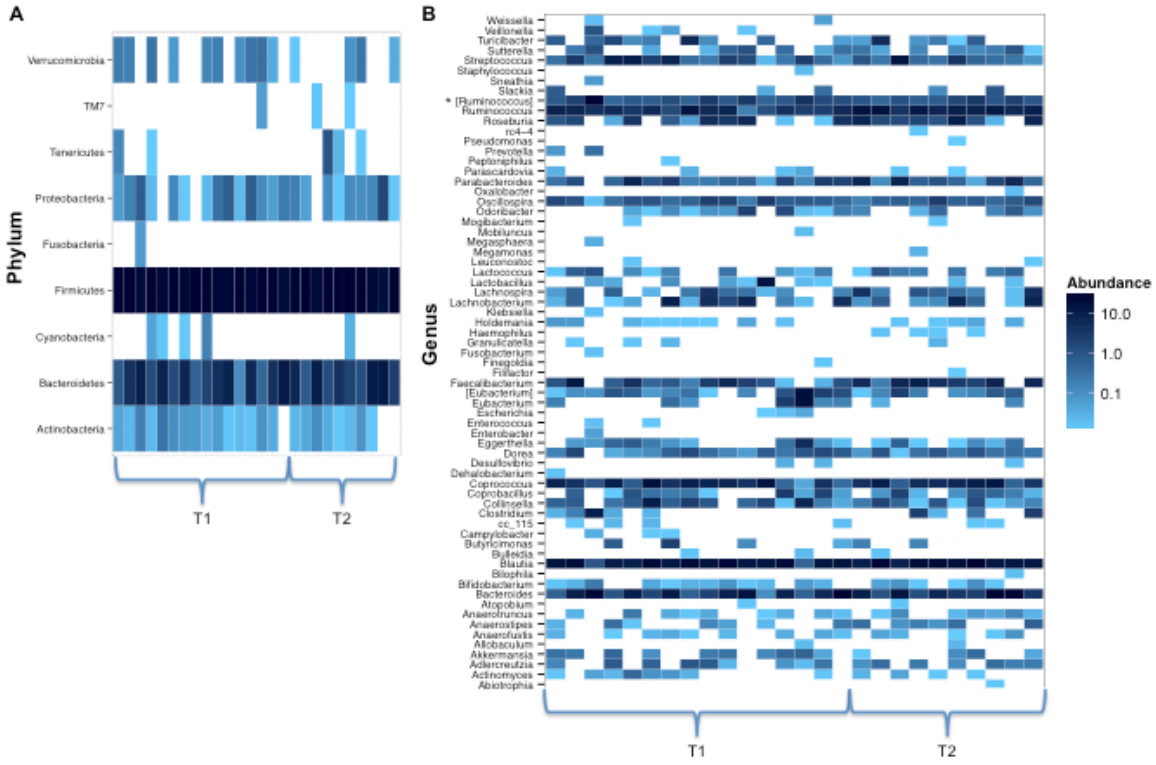

Figure 1. Heatmaps of samples from patients with AN at hospital admission (T1; n=16) and discharge (T2; n=10) at the (A) phylum and (B) genus taxonomic levels. Bacterial composition was characterized by 454 pyrosequencing of the 16S rRNA gene, and sequencing results were processed by the QIIME pipeline. A global, multivariate hypothesis test was used to test for differences in abundance across all bacterial groups at once, per taxonomic level, and indicated significant differences between T1 and T2 at the phylum (p=0.042) and genus (p=0.041) levels. Bacterial taxa are listed vertically, and samples are grouped horizontally by time point (T1 or T2). Greater abundance is designated by darker shading. *[Ruminococcus] indicates unspecified genera in the Ruminococcaceae family. AN = anorexia nervosa; QIIME = Quantitative Insights Into Microbial Ecology.

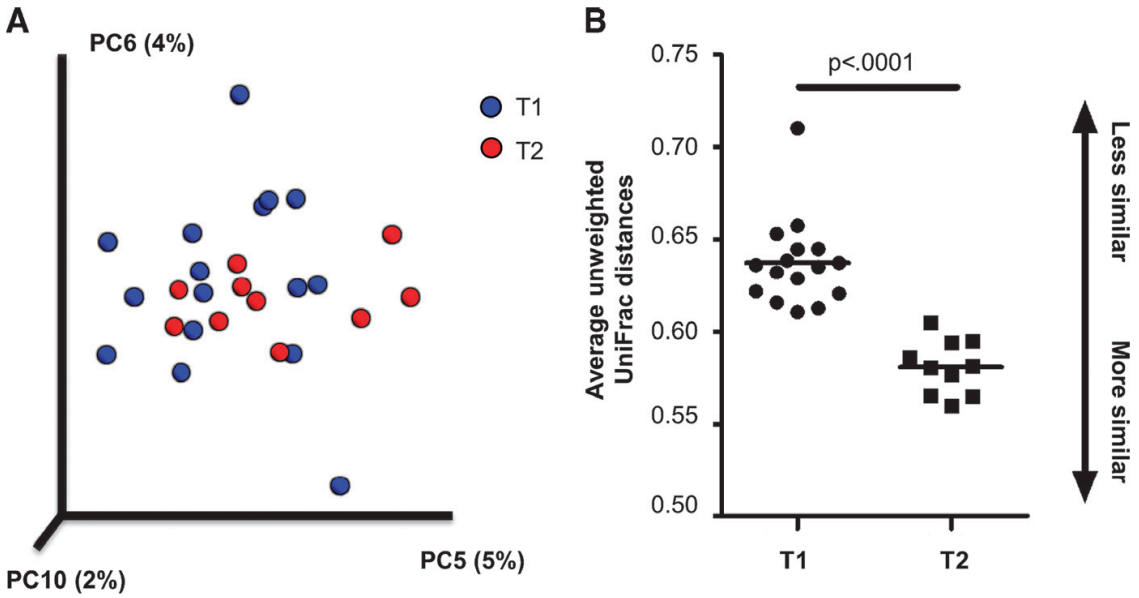

Figure 2. Principal coordinate plot of samples from patients with AN and average unweighted UniFrac distances at hospital admission (T1; n=16) and discharge (T2; n=10). Bacterial composition was characterized by 454 pyrosequencing of the 16S rRNA gene, and sequencing results were processed by the QIIME pipeline. (A) Based on unweighted UniFrac distances, three principal coordinates (PC 5, PC 6, PC 10) were significantly different at T1 versus T2. Percentages indicate the amount of variability in the data explained by each PC. Samples from T1 and T2 are designated by royal blue and red dots, respectively. (B) The average unweighted UniFrac distances were significantly different across groups ($p<0.0001$), with T2 samples showing greater similarity to each other than T1 samples. PC = principal coordinate; AN = anorexia nervosa; QIIME = Quantitative Insights Into Microbial Ecology.

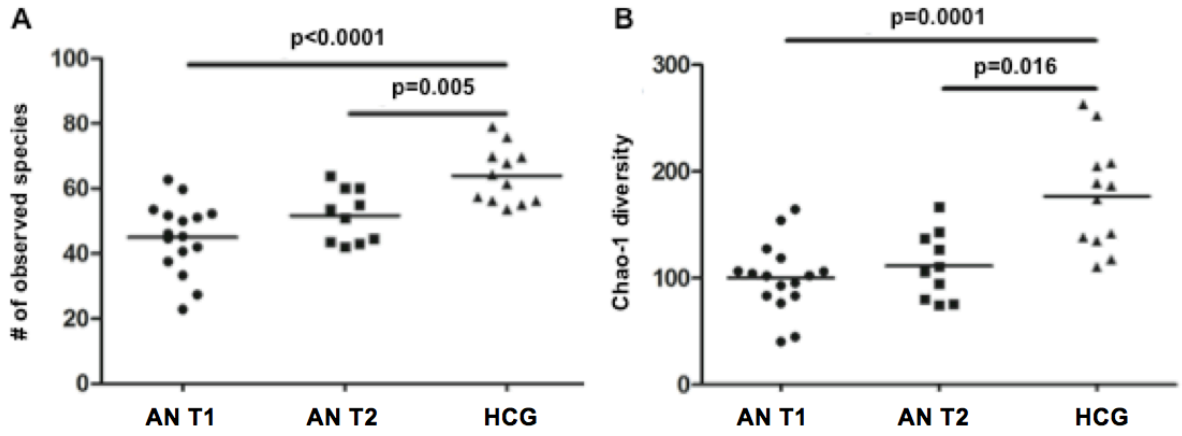

Figure 3. Alpha diversity in samples from patients with AN at hospital admission (T1; n=16) and discharge (T2; n=10) and a healthy comparison group (n=12). Bacterial composition was characterized by 454 pyrosequencing of the 16S rRNA gene, and sequencing results were processed by the QIIME pipeline. Richness was characterized by the number of observed bacterial species in each sample (A) and Chao-1 estimator of diversity (B). Differences in alpha (within-sample) diversity were compared in AN T1 versus AN T2 versus HCG with two-tailed Wilcoxon-Mann-Whitney tests. At both time points (T1 and T2), the alpha diversity remained significantly lower in patients with AN versus HCG, measured as either the number of observed species or Chao-1 estimator. AN = anorexia nervosa; HCG = healthy comparison group; QIIME = Quantitative Insights Into Microbial Ecology.

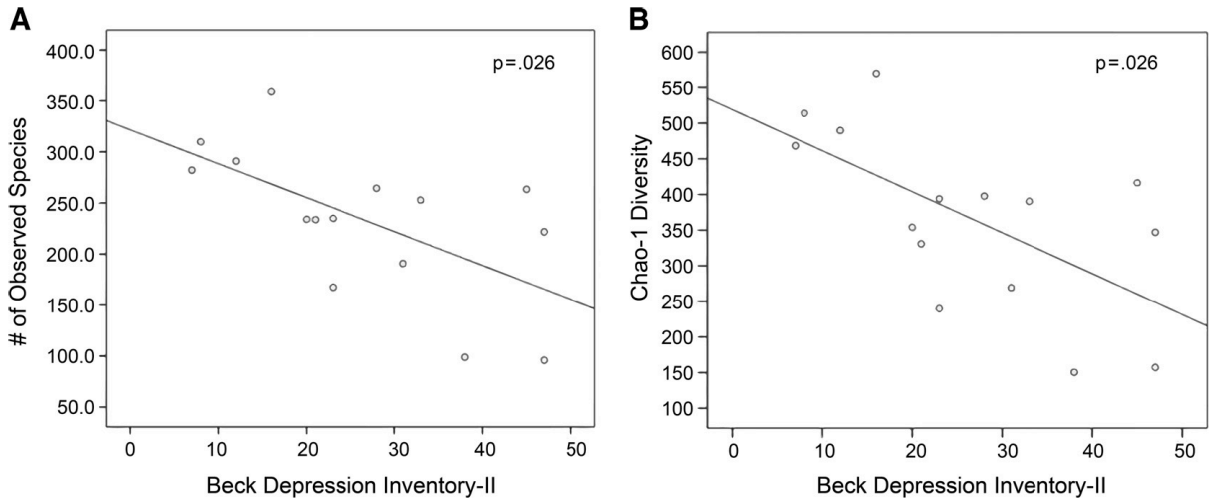

Figure 4. Correlation between depression and alpha diversity in samples from patients with AN at hospital admission (T1). Bacterial composition was characterized by 454 pyrosequencing of the 16S rRNA gene, and sequencing results were processed by the QIIME pipeline. Richness (vertical axes) was characterized by the number of observed bacterial species in each sample (A) and Chao-1estimator of diversity (B). AN patients (n=15; one patient did not complete the surveys) completed the Beck Depression Inventory-II (horizontal axes) within 24 hours of admission. Associations between T1 psychopathology scores and alpha diversity were examined with the tau-b correlation coefficient. Depression was negatively associated with the number of observed bacterial species (p=0.026) and Chao-1 estimator (p=0.026). AN = anorexia nervosa; QIIME = Quantitative Insights Into Microbial Ecology.

CHAPTER 3

ENTERIC MICROBIOME-MEDIATED DIETARY ENERGY EXTRACTION IN ACUTE ANOREXIA NERVOSA: A PILOT STUDY[3]

3.1 Introduction

Treatment of anorexia nervosa (AN) challenges clinicians, scientists, and patients.(115) Variability in response to therapeutic renourishment remains largely unexplained. Speed of weight gain, metabolic fluctuations, physical and psychological distress, and re-loss of restored weight are likely influenced by unknown biological mechanisms.

The intestinal microbiota plays a significant role in metabolic function and weight regulation, and we reported an intestinal microbial dysbiosis in AN.(116) Moreover, obese mice may extract calories from food more efficiently than lean mice, and this "efficiency" can be passed to germ-free mice through fecal microbiota transplantation—causing increased adiposity in obese microbiota recipients, even on isocaloric diets.(27) The intestinal microbiota may mediate increased adiposity via dietary energy harvest. We therefore investigated the relationship between the intestinal microbiota and fecal energy content before and after clinical renourishment in AN.

Fecal energy content provides an indirect measure of energy absorption and proxy for efficiency of microbiota-mediated dietary energy extraction. Overfeeding lean volunteers leads to compositional changes to the intestinal microbiota and less energy lost in feces on a higher-

[3]Kleiman SC, Huh EY, Trillo-Ordonez Y, Bulik-Sullivan EC, Glenny EM, Fodor AA, Bulik CM, Carroll IM. Enteric microbiome-mediated dietary energy extraction in acute anorexia nervosa: a pilot study. *Under Review*.

calorie diet.(117)

Our objectives were to (i) investigate changes in fecal energy content during renourishment; and (ii) examine associations between fecal energy content and composition and diversity of the intestinal microbiota. We hypothesized that the intestinal microbiota in AN, which is altered during renourishment, has adaptively responded to a low-energy environment (i.e., prolonged calorie restriction).

3.2 Methods

The study was approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill. All participants provided written consent. Female inpatients with AN (n=15), representing a subset of participants in a previous study,(116) consented to additional sample analysis. The first fecal sample after admission (T1) was collected for all patients, and when possible, an additional sample was collected before discharge (T2). Samples were mechanically homogenized, aliquoted into 2 mL cryovials, and stored in a −80°C freezer.

To prepare samples (n=24: n=14 for T1, n=10 for T2) for bomb calorimetry, fecal matter from one cryovial was baked in pellet form for 48h at 60°C. Energy content was determined via Isoperibol Calorimeter Model 6200EA (Parr Instrument Co., Moline, IL), calibrated with 10 runs of benzoic acid. Each sample was weighed and placed in an oxygen bomb, which was placed in 2000 mL distilled water inside the calorimeter. Energy content was calculated using heat produced by pellet combustion and corresponding water temperature rise.

Bacterial DNA was isolated, and V1-3 variable region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR).(116) 16S rRNA PCR products were quantified, pooled, and purified for sequencing on a 454 Life Sciences Genome Sequencer FLX machine (Roche, Florence, SC) by the UNC Microbiome Core Facility. Sequence data were processed by

the Quantitative Insights Into Microbial Ecology pipeline,(86) clustered into Operational Taxonomic Units at 97% threshold (using BLAST), and assigned taxonomy using the Greengenes database.(88) Microbial richness was characterized by Shannon diversity index.(118, 119)

Differences in energy intake (kcal/day) and fecal energy content (cal/g) associated with paired T1 and T2 samples (n=7) were compared with paired Student's t-tests (alpha 0.05). Due to significant differences in energy intake between T1 and T2, and the association between energy intake and energy content of fecal samples, we normalized fecal energy content by participant energy intake from the day before sample collection to generate a *relative* measure of fecal energy content. We evaluated differences in fecal energy content using absolute and relative measures.

Mixed linear models examined the relationship between energy content of all available fecal samples (n=24: n=14 for T1, n=10 for T2), Shannon diversity index, and bacterial taxa abundance at the phylum, class, order, family, and genus levels [(Diversity/Taxa) ~ Time + Calorimetry + 1/Participant]. Time (T1 vs. T2) and energy content (cal/g) were included as fixed variables and participant ID as a random variable, capturing variance in outcome explained by each parameter. False discovery rate (FDR) adjusted for multiple comparisons(94) at the recommended level of 0.1(120) using R.(121)

3.3 Results

Participants (n=15), were female, mean age 28.0 (12.1) [mean (SD)] years, with mean BMI 16.3 (1.5) kg/m^2 at T1. A subset of participants (n=10) provided a sample at T2, at mean BMI 17.4 (0.9) kg/m^2 after mean length of stay 24.8 (12.7) days. Participants were Caucasian (n=13) and African American (n=2).

Dietary records were available for n=7 participants and matched to paired fecal samples. Mean (SD) energy intake in these patients increased from 1557 (360) kcal/day at T1 to 2600 (566) kcal/day at T2 over mean length of stay 30.4 (12.6) days, a significant increase ($p<0.001$, paired Student's t-test) reflecting renourishment.

Mean (SD) *absolute* energy content of fecal samples did not differ between T1 [4586 (620) cal/g] and T2 [4591 (500) cal/g] ($p=0.98$, paired Student's t-test). When normalized by participant energy intake, *relative* energy content of fecal samples decreased significantly from T1 [3.1 (1.0) cal/g] to T2 [1.9 (0.7) cal/g] ($p=0.002$, paired Student's t-test) (**Figure 5**).

Mixed linear models of the relation between absolute energy content of fecal samples (n=24) and taxa abundance and diversity of bacterial groups suggest significant but modest associations between phylum-level abundance and energy content for most phyla (**Table 6, Figure 6**), even after FDR correction. Shannon diversity index was significantly associated with fecal energy content ($p=0.042$; adjusted $p=0.063$), with higher energy content associated with greater microbial diversity. Associations at other taxonomic levels were not significant.

3.4 Discussion

We previously described an intestinal microbiota dysbiosis in acute AN marked by lower microbial diversity.(116) Exploring the role of this complex community in dietary energy extraction in these patients, we found evidence of relative but not absolute changes in fecal energy content when comparing T1 and T2, which were also modestly associated with microbial composition and diversity. This supports our hypothesis of microbial adaptation to a low-energy environment and suggests that changes in energy availability may impact mechanisms of dietary energy extraction that are mediated by the intestinal microbiota.

The intestinal microbiota responds rapidly to environmental shifts, including dietary

quality, quantity, and composition.(19, 74, 117) Substantial increases in energy availability associated with renourishment in AN would be expected to influence composition and/or diversity of resident microorganisms. However, we found that absolute energy content did not differ between fecal samples at T1 and T2 despite increased energy intake. This finding could mean that microbial changes during renourishment support the physiologic need for weight restoration in AN by fully capturing additional energy availability for metabolic demands. Alternatively, the absence of a difference could reflect *increased* bioavailability of energy at T1 vs. T2, with the intestinal microbiota at T1 being primed for maximum metabolic efficiency (i.e., extracting as much energy as possible from a low-calorie diet), yet the body is simply unable to take up all available energy—especially since caloric intake in the days after admission may be substantially higher than immediately prior to admission. This inability to fully absorb available energy could be linked to fasting-induced changes in the intestinal epithelium that decrease absorptive capacity.(122)

When considered relative to energy intake, fecal energy content was significantly greater at T1 vs. T2, suggesting that *relative* energy content of fecal samples decreased as caloric intake increased. This pattern suggests that clinically renourished patients both consume and extract more calories from the diet at T2. This parallels a randomized cross-over trial where overfeeding lean males induced rapid compositional changes in the intestinal microbiota that were associated with relatively lower fecal energy content.(117) In addition, a cross-sectional study of AN reported greater abundance of archaeon *Methanobrevibacter smithii* compared with lean controls, which may signal adaptive response to a low-energy environment via increased energy yield from microbial fermentation.(22) Whether changes in relative fecal energy content are fully accounted for by energy increases, or reflect changes in composition and underlying biological

mechanisms of the intestinal microbiota, remains unknown.

Mixed evidence surrounds associations between fecal energy content and microbial biomarkers. In our female patients with AN, greater fecal energy content was associated with greater microbial diversity, increased abundance of phyla Bacteroidetes and Proteobacteria, and decreased abundance of phyla Firmicutes and Actinobacteria. However, degree of overfeeding in healthy males was associated with increased relative abundance of Firmicutes and decreased relative abundance of Bacteroidetes.(117) Moreover, significant increases in fecal energy content on a high-fat diet were not associated with abundance of Firmicutes, Bacteroidetes, or Actinobacteria.(123) Longitudinal studies are required to examine how changes in microbial composition are related to energy balance and biomarkers of metabolism.

Limitations exist in our study. Fecal energy content is an indirect and imperfect measure of nutrient extraction via the intestinal microbiota, and efficiency of energy absorption in patients with AN may differ from healthy individuals due to other variables—e.g., gastrointestinal distress, gastric transit time, etc. This exploratory study was likely underpowered to examine associations between fecal energy content and composition of the intestinal microbiota at other taxonomic levels.

Clinical renourishment in AN induces dynamic shifts in energy intake and, thus, availability of energy to the intestinal microbiota. This change to the enteric environment may be associated with compositional changes that impact efficiency of microbiome-mediated energy extraction to maximize renourishment and minimize energy loss in feces. Longitudinal studies of energy balance in larger cohorts and investigations of functional impact of microbial changes on energy harvest are warranted to refine current therapies by harnessing natural mechanisms to improve efficiency and comfort of therapeutic renourishment.

Table 6. Hospital admission (T1) vs. hospital discharge (T2): associations between microbial taxa and diversity measures in females with AN

Taxonomic level	***p***	**FDR corrected *p***
Firmicutes	0.020	0.063
Proteobacteria	0.038	0.063
Bacteroidetes	0.047	0.063
Actinobacteria	0.053	0.063
Verrucomicrobia	0.768	0.768
Diversity measure	***p***	**FDR corrected *p***
Shannon diversity index	0.042	0.063

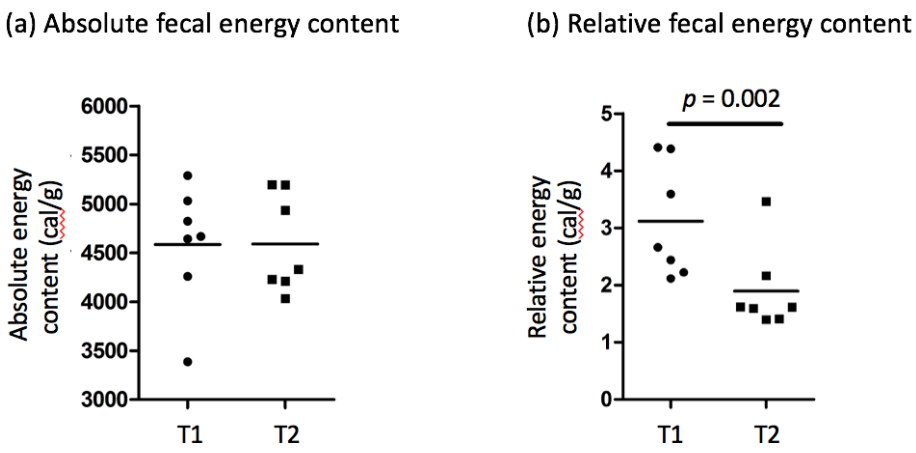

Figure 5. Fecal energy content in paired samples from individuals (n=7) during hospitalized treatment for AN. Differences in fecal energy content were evaluated using absolute and relative measures with paired Student's t-tests. (a) Mean (SD) absolute energy content did not differ between T1 [4586 (620) cal/g] and T2 [4591 (500) cal/g] (p=0.98). (b) Relative energy content of fecal samples decreased significantly from T1 [3.1 (1.0) cal/g] to T2 [1.9 (0.7) cal/g] (p=0.002).

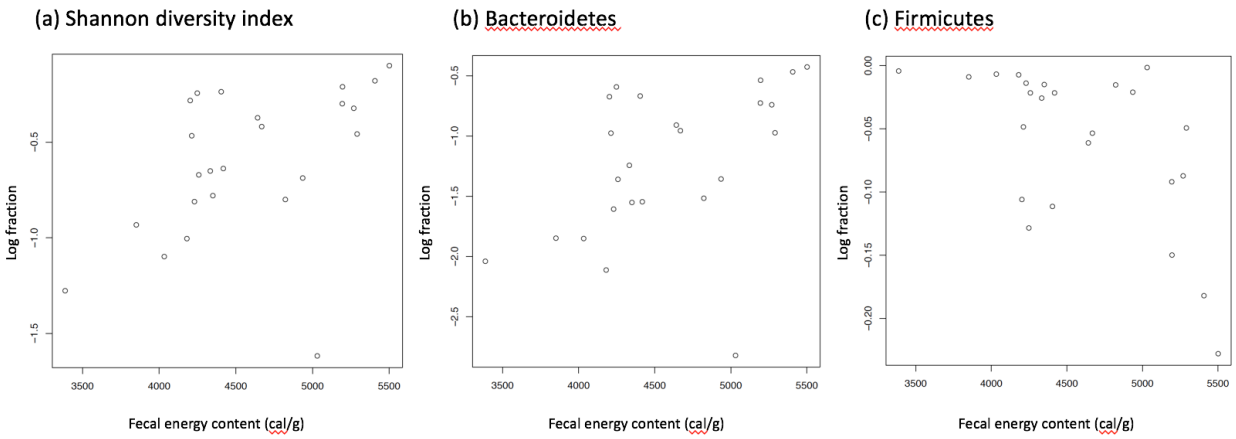

Figure 6. Association between fecal energy content and intestinal microbial measures in samples (n=24) from individuals (n=15) during hospitalized treatment for AN. Fecal energy content was associated with the (a) Shannon diversity index (p=0.042; adjusted p=0.063), as well as phylum-level abundance of (b) Bacteroidetes (p=0.047; adjusted p=0.063) and (c) Firmicutes (p=0.020; adjusted p=0.063).

CHAPTER 4

THE GUT-BRAIN AXIS IN HEALTHY FEMALES: LACK OF ASSOCIATION BETWEEN MICROBIAL COMPOSITION AND DIVERSITY AND PSYCHIATRIC MEASURES[4]

4.1 Introduction

Investigations conducted over the last decade have generated consensus among researchers that the intestinal microbiota plays a vital role in a range of physiologic processes, especially those related to immunologic and metabolic function. A healthy intestinal microbiota is also important for normal brain development and behavioral functions.(108) The enteric microbe-gut-brain axis has garnered increasing attention as a key, bidirectional communication pathway that influences mood, cognition, and behavior.(124-126) In addition to a direct connection via the vagus nerve, gut bacteria may interact with the brain through production of neurotransmitters, hormones, and other metabolites.

Whether a dysbiosis in the intestinal microbiota plays a direct role in the pathophysiology of psychiatric disorders remains to be determined; however, both preclinical animal studies and clinical human studies are actively investigating this question. Numerous studies in animal models have documented behavioral changes following manipulation of the intestinal microbiota, including effects on behavior associated with stress,(111) anxiety,(107, 108, 110, 127) and depression.(41, 42) Validating animal models of the enteric microbe-gut-brain axis in human populations has reported modest associations and has been limited by small sample sizes

[4]Kleiman SC, Bulik-Sullivan EC, Glenny EM, Huh EY, Tsilimigras MCB, Fodor AA, Carroll IM, Bulik CM. The gut-brain axis in healthy females: Lack of association between microbial composition and diversity and psychiatric measures. *Under Review*.

and lack of consistency in assessment of psychiatric and microbial outcome measures. Although underpowered, these studies in human cohorts suggest a potential role for the intestinal microbiota in anxiety, depression, stress, cognitive reactivity, and eating disorders.(45-48, 116)

Given the heightened interest in the enteric microbe-gut-brain axis and accumulating evidence supporting a role for the intestinal microbiota in mood and behavior, we investigated whether such associations would extend to healthy populations. We, therefore, examined associations between the composition and diversity of the intestinal microbiota and measures of depression, anxiety, eating disorder psychopathology, stress, and personality in a group of healthy adult females.

4.2 Methods

The study was approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill (UNC). All participants provided written consent before study participation.

Study Population

Healthy adult females (n=100) ages 15-50 years with BMI 18.5–24.9 kg/m^2 were recruited from central North Carolina via listserv announcements, targeted emails, and social media to serve as controls for ongoing research. Participants were recruited between July 2014 and March 2015. Due to possible impact on the intestinal microbiota, potential participants were excluded for the following reasons: (i) history of gastrointestinal tract surgery (other than appendectomy or cholecystectomy); (ii) history of inflammatory bowel diseases, irritable bowel syndrome, celiac disease; (iii) history of eating disorders (anorexia nervosa, bulimia nervosa, binge-eating disorder); (iv) treatment in the last two months with antibiotics or steroids; (v)

intentional use of probiotics during the last two months (via food or supplement); and/or (vi) abuse of laxatives within the last month.

Body Composition and Assessments

Participants self-reported current height and weight during the screening process. Participants completed an online psychiatric questionnaire that included five widely-used and validated measures: (i) Beck Anxiety Inventory (BAI);(96, 128) (ii) Beck Depression Inventory-II (BDI);(97, 129) (iii) Eating Disorder Examination-Questionnaire (EDE-Q);(130, 131) (iv) Perceived Stress Scale (PSS);(132, 133) and (v) Mini International Personality Item Pool (Mini IPIP).(134)

Sample Collection, Processing, and Storage

During the consent process, participants were provided with an at-home stool collection kit and trained in sample collection procedures. Each kit included: Styrofoam container, disposable collection hat, stool collection tube, biohazard bag, pair of non-latex gloves, two ice packs, and stool collection record sheet. Participants were instructed to return the sample (in the biohazard bag, with ice packs, in the Styrofoam box) to the research office within 24 hours of collection and to keep the sample refrigerated during any interim period. Samples were then immediately transferred to the laboratory, where they were mechanically homogenized with a sterile spatula, aliquoted into sterile 2 ml cryotubes, and stored in a −80 °C freezer for future DNA isolation and molecular microbiological analysis.

DNA Isolation

Bacterial DNA was isolated from collected samples using a phenol/chloroform extraction method combined with physical disruption of bacterial cells and a DNA clean-up kit (QIAmp DNA Stool Mini Kit [Qiagen, Valencia, CA]), as previously described.(79, 85)

Sequencing of 16S rRNA Genes

Bacterial community composition in isolated DNA samples was characterized by amplification of the V4 variable region of the 16S rRNA gene by polymerase chain reaction (PCR) (forward primer 515, 5'-GA GTG CCA GCM GCC GCG GTA A-3'; reverse primer 806, 5'-ACG GAC TAC HVG GGT WTC TAA T-3'). Generation of 16S rRNA sequences consisted of two separate amplifications: (1) 95°C for three minutes, then 10 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by one cycle of 72°C for five minutes using 120 ng of fecal DNA as template, 10 μM of each 16S V4 primer, and the KAPA2G Robust PCR kit (Kapa Biosystems, Wilmington, MA); and (2) 95°C for three minutes, then 22 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by one cycle of 72°C for five minutes using 5 μL of purified PCR product from the first amplification as template, 10 μM of forward and reverse primers that contain Illumina MiSeq adaptor sequences with a 12-base error-correcting Golay barcode incorporated in the reverse primer, and the KAPA HiFi HotStart ReadyMix PCR kit.(135) Purification of PCR products was carried out after each amplification using the HighPrep PCR clean-up kit (MagBio, Lausanne, Switzerland) with a DynaMag-96 side magnet (Life Technologies, Carlsbad, CA). 16S rRNA PCR products were then quantified and pooled for sequencing. Sequencing was performed on an Illumina MiSeq desktop sequencer (Illumina, San Diego, CA) by the High-Throughput Sequencing Facility in the Carolina Center for Genome Sciences at the UNC School of Medicine.

Analysis of 16S rRNA Sequences

16S rRNA sequencing data were processed by the Quantitative Insights Into Microbial Ecology (QIIME) pipeline,(86) with quality filtering as previously described.(79) Forward

sequence reads (250 bp) were clustered into Operational Taxonomic Units based on their sequence similarity at a 97% threshold using BLAST and assigned taxonomy using the Greengenes database.(88) Principal coordinates were generated using unweighted and weighted UniFrac distances.(89-91)

Results were validated using an alternate pipeline, in which forward reads from the 16S rRNA sequencing data were classified with version 2.10.1 of the RDP classifier with a threshold of a 50% RDP score.(136)

Statistical significance was determined using Kendall's tau-b correlation coefficient in R.(121) R scripts are available at https://github.com/afodor/metagenomicsTools/blob/master/src/scripts/IanNovember2015/correlationsOneColumnAtATime.txt

The diversity of the intestinal microbiota was characterized by the Shannon diversity index.(118, 119)

Statistical Analysis

Associations between psychiatric and microbial measures were examined using Kendall's tau-b correlation coefficient, in conjunction with Benjamini and Hochberg's False Discovery Rate (FDR) procedure to correct for multiple comparisons.(94) Psychiatric measures included: BAI (anxiety), BDI (depression), EDE-Q (total + subscales for dietary restraint, eating concern, shape concern, and weight concern), PSS (stress), and Mini IPIP (scales for extraversion, agreeableness, conscientiousness, neuroticism, and imagination). Microbial measures included: alpha diversity (Shannon diversity index) and taxa abundance of bacterial groups at the phylum, class, order, family, and genus levels. Linear models were additionally constructed wherein the first two principal coordinates were regressed against each of the psychiatric measures and other

participant metadata. The FDR procedure was applied to the number of comparisons per outcome and per taxonomic rank. The α level used was 0.05, but for FDR correction, a more lenient criterion of 0.1 was used.(120) All analyses were conducted in R.(121)

4.3 Results

Of 100 participants who consented to participate in the study, 94 completed the psychiatric questionnaires *and* submitted a fecal sample, of which sequencing results from 91 samples met quality control standards for analysis. Demographic and clinical characteristics of the final participant sample (n=91) are shown in **Table 7**. In brief, the participants had a mean (SD) age of 29.0 (7.9) years and were within the normal or healthy weight range for adults.(137) On average, their scores indicate normal or minimal levels of anxiety (BAI), depression (BDI), and stress (PSS) and are in line with, or lower than, those of similar non-clinical samples.(128, 138-140) Total scores on the EDE-Q and its four subscales (dietary restraint and eating, weight, and shape concerns) are lower than norms for U.S. college students and young adult females in Sweden,(99, 141) which is likely a reflection of the participant recruitment and screening process, which eliminated individuals with a lifetime eating disorder history.

Following sequencing of 16S rRNA genes, we had 91 samples with complete data, after excluding those samples with insufficient depth of sequence reads for our downstream analysis. The total number of 16S rRNA sequence reads was 15,408,275, and the mean number of reads was 169,322 per sample (range: 47,709-317,349 sequence reads).

When examining associations between psychiatric measures and the composition and diversity of the intestinal microbiota, there were no associations that met established significance thresholds. We considered 17 different measures from our human participants—the 15 measures in **Table 7** plus participant height and weight. The RDP classifier reported 261 non-rare taxa (13

phyla, 19 classes, 24 orders, 52 families, and 153 genera) that were present in at least 10% of our samples. At each taxonomic level, we also calculated the Shannon diversity index. We evaluated 4,522 hypotheses [17 measures * (261 taxa + 5 Shannon diversity metrics)] using the non-parametric Kendall's tau-b test for association. Histograms of generated p-values across all possible associations (**Figure 7**) are largely uniform, suggesting that the null hypothesis of no association is generally supported across all taxonomic levels. Using FDR correction for all 4,522 hypotheses, there were no significant hits even if the threshold were set to 93% FDR.

We also used a less conservative correction, in which associations between each of the 17 human measurements and each taxonomic level were corrected independently (for example, the comparisons of BDI and the 14 phyla were corrected only for the 14 phyla independent of all the other tests that we ran). Even using this much less stringent threshold, where we might expect some spurious correlations, there were no significant hits at a 5% FDR. We conclude that there is a striking lack of correlation between microbial community composition and the measurements we have gathered from our human cohort.

To further visualize the associations in our data set, we generated principal coordinate plots using unweighted UniFrac distances and colored these plots by quartiles of the main psychiatric measures of interest (BAI, BDI, EDE-Q total, PSS) (**Figure 8)**. These plots are based on the first three principal coordinates, which explain 11.5% (PC1), 5.16% (PC2), and 3.98% (PC3) of the variance in microbial composition. The plots do not show evidence of clustering or segregation based on extreme values on psychiatric measures, which further supports a lack of microbial markers for these psychiatric outcomes in this population. The regression of PC1 and PC2 against each of the psychiatric measures and other participant variables did not indicate any significant linear relationships after FDR correction.

4.4 Discussion

Our results provide evidence for a lack of association in physically and psychologically healthy adult females between microbial markers of gut composition and diversity and a collection of psychiatric measures, including anxiety, depression, eating-related thoughts and behaviors, stress, and personality. No associations between these measures met established significance thresholds in our analysis.

Animal models suggest a role for the intestinal microbiota in anxiety, depression, and stress, and many animal studies have documented behavioral changes following manipulation of the intestinal microbiota using prebiotics, probiotics, antibiotics, infection with pathogenic bacteria, or microbial transfer to germ-free (GF) mice (i.e., mice raised in a sterile environment and lacking an intestinal microbiota). Seminal work by Sudo *et al.* (2004) on hypothalamic-pituitary-adrenal axis activity showed that GF mice have exaggerated stress response when compared to conventionally-raised mice.(111) GF mice also have reduced anxiety-like behavior compared to conventional mice,(107, 108, 110, 127) which can be reversed via early-life colonization with intestinal bacteria.(107, 108) Anxiety-like behavior can also be increased in mice with pathogenic infection(142-144) or transferred between mice with a characteristic anxiety phenotype and non-anxious GF mice using microbial transfer.(40) Probiotic formulations, such as *Lactobacillus rhamnosus* and *Bifidobacterium infantis*, have been shown in animal models to reduce depressive and anxiety-like behavior at effect sizes similar to antidepressant treatment,(41, 42) and prebiotic human milk oligosaccharides may reduce stress-induced anxiety-like behavior and stimulate changes in microbial diversity.(43) Altogether, these findings suggest that changes within the intestinal microbiota may be of central importance to the development or maintenance of depression and anxiety.

Few studies have examined associations of the enteric microbe-gut-brain axis in human samples, and significant results generally lack replication. Mixed evidence has come out of investigations comparing the intestinal microbiotas of individuals with depression to healthy controls, with one study failing to find significant between-group differences in microbial diversity or taxonomic composition,(44) while the other found increased diversity and significant taxonomic differences at the phylum, family, and genus levels.(45) In patients with acute anorexia nervosa, which is frequently comorbid with depression, work from our lab has shown that microbial diversity was both associated with depression and significantly lower than in healthy controls.(116) Composition and diversity of the intestinal microbiota may also be associated with temperament in young children, but how such links may evolve during the development of adult personality is unclear.(145)

Prebiotic and probiotic supplementation have emerged in human clinical studies as potential means for altering mood, but connecting post-intervention changes in mood to differences in microbial composition or diversity is lacking. Studies have reported improvement in measures of depression, anxiety, cognitive reactivity, and stress levels in healthy volunteers after placebo-controlled supplementation trials of prebiotic or probiotic formulas,(46-48) but these supplements may not be associated with observable compositional changes to the intestinal microbiota. In fact, changes in mood or behavior may be mediated by the metatranscriptome (i.e., functional activity of enteric microbes) rather than the intestinal microbiota.(146)

Nevertheless, clinical trials of novel treatments for depression based on manipulation of the intestinal microbiota are underway. Seminal work that used *Lactobacillus rhamnosus* to demonstrate the central importance of the enteric microbe-gut-brain axis(41) is currently being tested in human trials, which are investigating the effects of probiotic supplementation in healthy

volunteers and as an augmentation to antidepressant treatment for individuals with treatment-resistant depression.(125) Another ongoing randomized controlled trial is examining the potential benefit of minocycline (a tetracycline antibiotic) treatment as an adjunctive treatment for individuals with moderate to severe depression.(147) Yet because these intervention studies use microbial strains that are not permanent members of the enteric microbiota, it is possible that any evidence of behavioral change is not due to changes to the intestinal microbiota but rather more direct mechanisms of action.

These results should be considered in connection with several limitations. With respect to psychopathology, our sample was, on average, healthier than other non-clinical samples of young adults and had less variability on psychiatric measures than would have been expected. As such, restriction of range on psychiatric measures may have played a role in the lack of significant associations with microbial markers. In addition, our analysis focused on taxonomic and diversity measures of 16S rRNA sequencing data, which describes microbial composition but does not account for metabolic activity or functional impact of intestinal bacteria. We may have seen different results with RNA-seq or whole-genome metagenomic shotgun sequencing, or had we analyzed microbial communities from intestinal biopsies. Lastly, our sample consisted of adult females (age range: 15-50 years), which may differ with respect to these outcome measures from adult males or individuals in younger or older age brackets.

The enteric microbe-gut-brain axis has attracted considerable attention in recent years, with much focus on the potential role of enteric microorganisms in the development or maintenance of psychiatric illness. Studies involving GF mouse models or clinical populations present extreme cases of psychopathology, which may not reflect microbial mechanisms in a healthy human population. This study was the first to examine associations between composition

and diversity of the intestinal microbiota and psychiatric measures in healthy individuals, and our results do not reveal associations between the intestinal microbiota and low levels of symptomatology in a healthy population. However, the role of the intestinal microbiota in the pathophysiology of psychiatric illness and evidence of the enteric microbe-gut-brain axis may only be observable in the presence of wider variability of symptom measures and more severe psychopathology.

Table 7. Demographic and clinical characteristics of participants (n=91)

	Mean	Standard Deviation
Age	29.0	7.9
BMI	21.7	1.9
BAI	5.0	4.8
BDI	5.2	5.9
EDE-Q Total	0.6	0.5
Dietary restraint	0.4	0.6
Eating concern	0.2	0.2
Shape concern	1.1	0.8
Weight concern	0.7	0.8
PSS	12.4	6.3
Mini IPIP		
Extraversion	12.6	4.1
Neuroticism	9.9	3.4
Agreeableness	16.5	2.6
Conscientiousness	15.2	2.9
Imagination	14.5	3.1

BMI, body mass index; BAI, Beck Anxiety Inventory; BDI, Beck Depression Inventory-II; EDE-Q, Eating Disorder Inventory-Questionnaire; PSS, Perceived Stress Scale; Mini IPIP, Mini International Personality Item Pool.

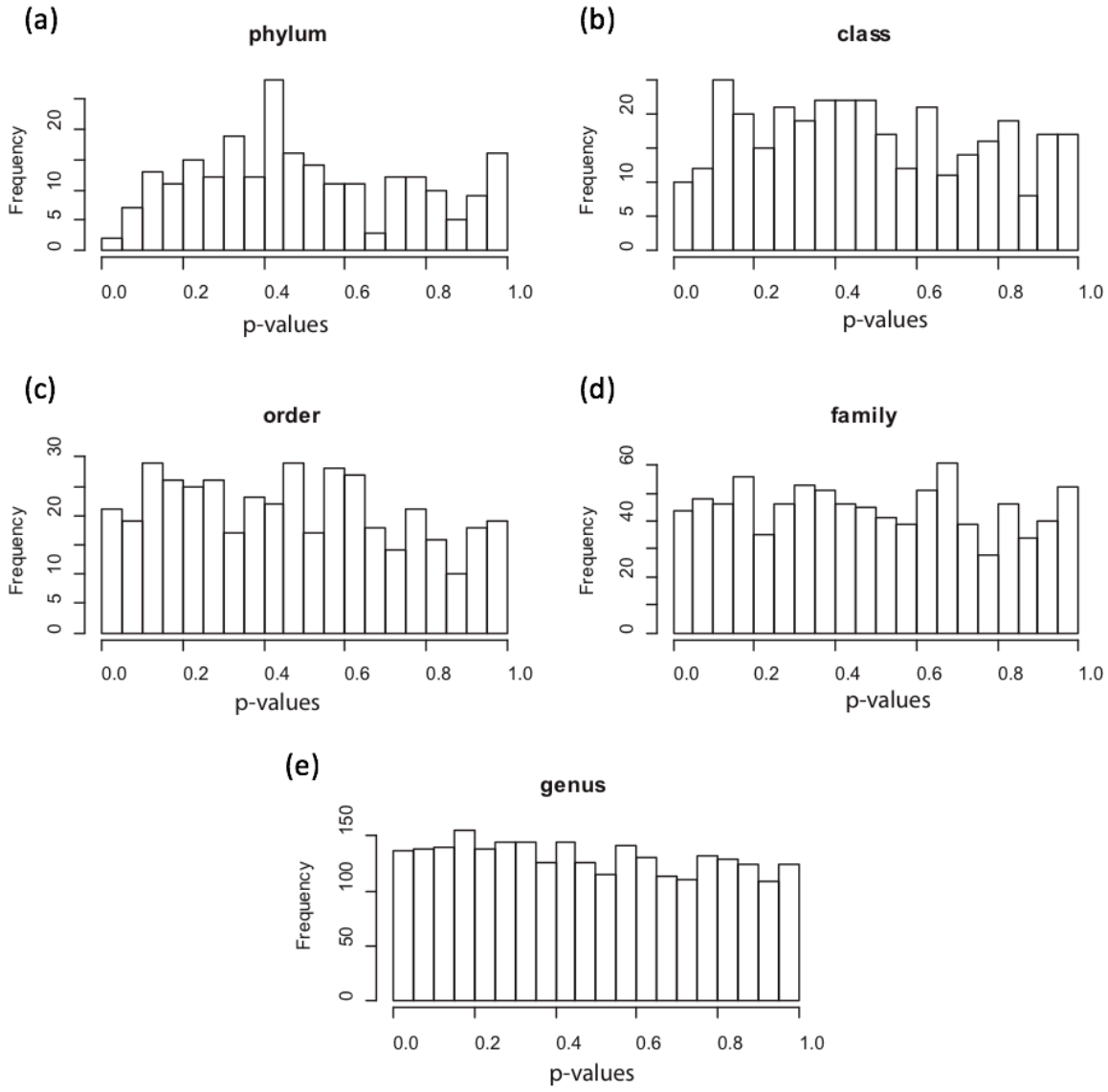

Figure 7. Histograms of p-values for associations with psychiatric measures by taxonomic level. Associations between psychiatric and microbial measures were examined using Kendall's tau-b correlation coefficient, in conjunction with Benjamini and Hochberg's False Discovery Rate procedure, using data generated by the RDP classifier. Psychiatric measures included: Beck Anxiety Inventory, Beck Depression Inventory-II, Eating Disorder Examination-Questionnaire, Perceived Stress Scale, and Mini International Personality Item Pool. P-value frequencies were examined at each taxonomic level: (a) phylum; (b) class; (c) order; (d) family; and (e) genus.

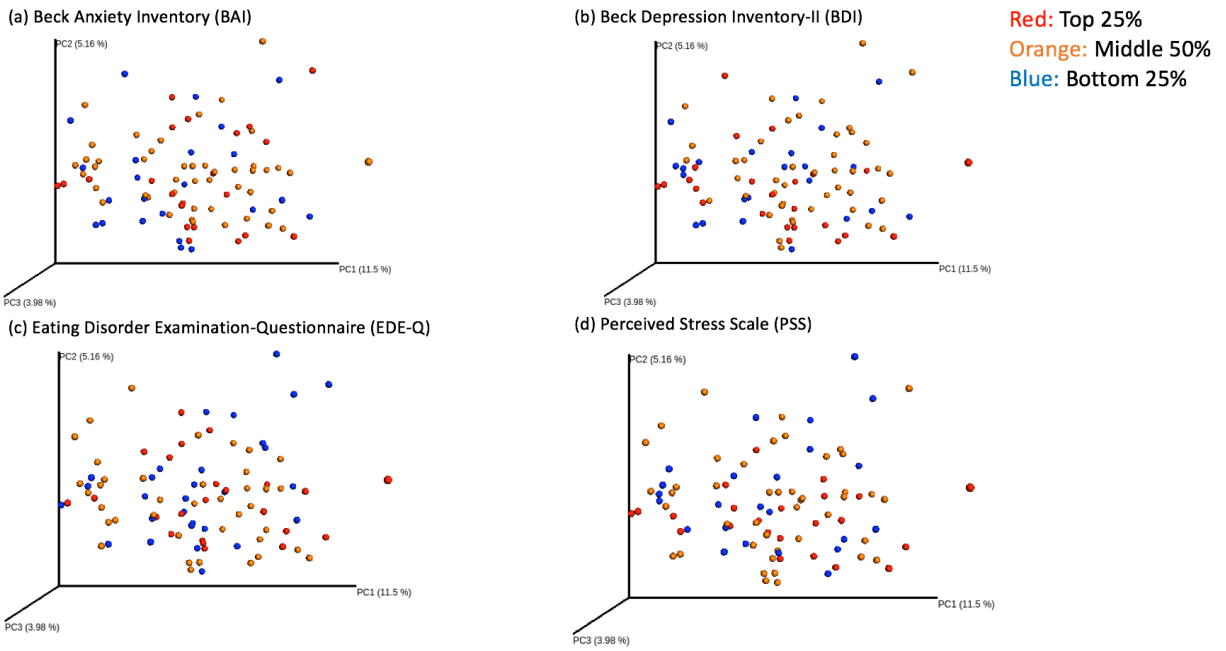

Figure 8. Principal coordinate plots of psychiatric measures by quartile. Principal coordinates were generated using unweighted UniFrac distances from the QIIME pipeline and allocated to quartiles (red: top quartile; orange: middle two quartiles; blue: bottom quartile) based on scores from the (a) Beck Anxiety Inventory; (b) Beck Depression Inventory-II; (c) Eating Disorder Examination-Questionnaire; and (d) Perceived Stress Scale. Plots are based on the first three principal coordinates, which explain 11.5% (PC1), 5.16% (PC2), and 3.98% (PC3) of the variance in microbial composition, and do not cluster by quartile—supporting a lack of association between microbial markers and these psychiatric measures in healthy individuals.

CHAPTER 5

DAILY CHANGES IN COMPOSITION AND DIVERSITY OF THE INTESTINAL MICROBIOTA IN PATIENTS WITH ANOREXIA NERVOSA: A CASE SERIES

5.1 Introduction

Anorexia nervosa (AN) is a severe, often life-threatening illness associated with substantial medical comorbidity and mortality rates among the highest of any psychiatric disorder.(115) The majority of patients with AN present with gastrointestinal (GI) symptoms, including abdominal distention, abdominal pain, and gastroparesis,(148, 149) and the GI-related effects of renourishment are uncomfortable and distressing—resulting in high treatment dropout. Moreover, the evidence base for therapeutic weight restoration is weak, in part because of inadequate understanding of the central biological mechanisms underlying AN.(115) Thus, novel therapeutic approaches that safely and effectively improve outcomes for this disorder are needed.

The intestinal microbiota, which plays a role in gut motility and physiology and energy regulation,(150, 151) warrants investigation for improving treatment for AN. Indeed, AN is associated with an intestinal microbial dysbiosis marked by lower microbial diversity, distinct taxonomic differences compared with healthy controls, and associations with levels of depression and eating disorder psychopathology.(116, 152) Previously, we have documented changes in the intestinal microbiota in patients with acute AN between hospital admission for therapeutic renourishment and discharge,(116) and we now provide a more granular longitudinal exploration of changes over the course of treatment in this unique case series.

Although individual microbial signatures dominate in healthy samples, even over time and under controlled conditions, an intestinal microbial dysbiosis is associated with a range of pathologies, including gastrointestinal disorders, autoimmune disease, and psychiatric illness.(124, 153, 154) Whether the effects of treatment (e.g., clinical renourishment in AN) generate consistent response in the intestinal microbiota, or inter-individual variation predominates, is unknown. Therefore, this study aimed to (i) characterize daily changes in composition and diversity of the intestinal microbiota in three acutely ill patients with AN over the entire course of hospital-based renourishment (73, 58, and 34 days); and (ii) to identify enteric bacterial groups associated with metabolic changes during treatment.

5.2 Methods

The study was approved by the Biomedical Institutional Review Board at the University of North Carolina at Chapel Hill (UNC). All participants provided written informed consent before study participation, and parental permission forms and an age-appropriate assent forms were used for participants younger than 18 years.

Study Population

Females (n=3) admitted for inpatient treatment at the UNC Center of Excellence for Eating Disorders (CEED) participated in the study. Patients ages 15-64 years meeting DSM-5 criteria for AN and presenting at <75% of ideal body weight were recruited from consecutive admissions between May and August 2015. Due to possible impact on the intestinal microbiota, potential participants were excluded for the following reasons: (i) history of gastrointestinal tract surgery (other than appendectomy or cholecystectomy); (ii) history of inflammatory bowel diseases, irritable bowel syndrome, celiac disease; (iii) treatment in the last two months with

antibiotics or steroids; (iv) intentional use of probiotics during the last two months (via food or supplement); and/or (v) abuse of laxatives within the last month.

Body Composition and Assessments

Weight and height were assessed at hospital admission using a calibrated digital scale and stadiometer. Participants were weighed daily (before breakfast, in a gown) as part of standard treatment on the inpatient eating disorders unit and three times per week (before breakfast, without shoes) after stepping down to the partial hospitalization program. Eating disorders diagnosis and psychopathology were established via the Eating Disorder Examination(80) and the Structured Clinical Interview for DSM-IV-TR Axis I Disorders(81) conducted by credentialed members of the UNC CEED Assessment Core. Energy intake was prescribed by registered dietitians, confirmed via clinical consensus, and in line with each participant's target weight gain trajectory.

Participant metabolic rate was measured at admission and weekly thereafter during hospitalized renourishment. Resting energy expenditure (kcal/day) was measured after overnight fast, before breakfast with the MedGem indirect calorimeter (Microlife Medical Home Solutions, Inc., Golden, CO). The same procedure was repeated one hour post-breakfast to measure post-prandial resting energy expenditure and calculate diet-induced thermogenesis (as percentage increase over resting energy expenditure). Daily physical activity expenditure (kcal) was measured using the BODYMEDIA SenseWear armband (BodyMedia, Inc., Pittsburgh, PA), worn on the back of the upper left arm for a 24-hour period each week (with the exception of bathing or any other continuous contact with water). The 24-hour period commenced at the time of resting energy expenditure measurement. Active energy expenditure (min), defined as ≥3.0 metabolic equivalents, was also captured by the armband.

Sample Collection, Processing, and Storage

Fecal samples were collected on a daily basis (or as frequently as possible, if less than daily) from all participants. Input and output are measured as part of routine treatment on the inpatient eating disorders unit, minimizing risk of missing samples, and all samples were collected by unit nurses and nursing assistants trained in collection protocols. After stepping down from the inpatient unit to the partial hospitalization program, participants received training in sample collection procedures and were provided with at-home collection kits for use on-site or at home. Each kit included: Styrofoam container, disposable collection hat, stool collection tube, biohazard bag, pair of non-latex gloves, two ice packs, and stool collection record sheet. All samples were refrigerated after collection and transferred within 24 hours to the laboratory, where they were mechanically homogenized with a sterile spatula, aliquoted into sterile 2 ml cryotubes, and stored in a −80 °C freezer for future DNA isolation and molecular microbiological analysis.

DNA Isolation

Bacterial DNA was isolated from collected samples using a phenol/chloroform extraction method combined with physical disruption of bacterial cells and a DNA clean-up kit (QIAmp DNA Stool Mini Kit [Qiagen, Valencia, CA]), as previously described.(79, 85)

Sequencing of 16S rRNA Genes

Bacterial community composition in isolated DNA samples was characterized by amplification of the V4 variable region of the 16S rRNA gene by polymerase chain reaction (PCR) (forward primer 515, 5'-GA GTG CCA GCM GCC GCG GTA A-3'; reverse primer 806, 5'-ACG GAC TAC HVG GGT WTC TAA T-3'). Generation of 16S rRNA sequences consisted of two separate amplifications: (1) 95°C for three minutes, then 10 cycles of 95°C for 30

seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by one cycle of 72°C for five minutes using 120 ng of fecal DNA as template, 10 μM of each 16S V4 primer, and the KAPA2G Robust PCR kit (Kapa Biosystems, Wilmington, MA); and (2) 95°C for three minutes, then 22 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, followed by one cycle of 72°C for five minutes using 5 μL of purified PCR product from the first amplification as template, 10 μM of forward and reverse primers that contain Illumina MiSeq adaptor sequences with a 12-base error-correcting Golay barcode incorporated in the reverse primer, and the KAPA HiFi HotStart ReadyMix PCR kit.(135) Purification of PCR products was carried out after each amplification using the HighPrep PCR clean-up kit (MagBio, Lausanne, Switzerland) with a DynaMag-96 side magnet (Life Technologies, Carlsbad, CA). 16S rRNA PCR products were then quantified and pooled for sequencing. Sequencing was performed on an Illumina MiSeq desktop sequencer (Illumina, San Diego, CA) by the High-Throughput Sequencing Facility in the Carolina Center for Genome Sciences at the UNC School of Medicine.

Analysis

16S rRNA forward sequence reads were classified with version 2.10.1 of the RDP classifier with a threshold of a 50% RDP score.(136) The diversity of the intestinal microbiota was characterized by the Shannon diversity index.(118, 119)

Following sequencing of 16S rRNA genes, we had 140 samples with sufficient depth of sequence reads for our downstream analysis. The mean number of 16S rRNA sequence reads was 69,967 per sample (range: 12,606-198,025 sequence reads). The RDP classifier reported 185 non-rare taxa (7 phyla, 14 classes, 18 orders, 39 families, and 107 genera) that were present in at least 25% of our samples.

Linear models were constructed with bacterial taxa (or Shannon diversity index) regressed against the interaction of time (i.e., length of hospital stay, in days) and patient, inclusive of their main effects. Taxa abundance and diversity of bacterial groups were considered at the phylum, class, order, family, and genus levels. Additional models that included patient metadata (e.g., BMI and energy intake), in addition to a time-patient interaction, were also explored. Lastly, linear models were constructed to incorporate metabolic metadata of interest (i.e., resting energy expenditure, diet-induced thermogenesis, and active energy expenditure) in addition to a time-patient interaction. The FDR procedure was applied to the number of comparisons per outcome and per taxonomic rank. The α level used was 0.05, but for FDR correction, a more lenient criterion of 0.1 was used.(120) All analyses were conducted in R.(121)

5.3 Results

Demographic and clinical characteristics of patients are presented in **Table 8**. Patient A, age 25, was admitted at BMI 15.6 kg/m^2. She provided 68 fecal samples over 73 days of treatment, reaching BMI 20.2 kg/m^2 at discharge. Patient B, age 29, was admitted at BMI 17.6 kg/m^2. She provided 47 fecal samples over 58 days of treatment, reaching BMI 21.1 kg/m^2 at discharge. Patient C, age 16, was admitted at age-adjusted BMI 13.7 kg/m^2. She provided 33 fecal samples over 34 days of treatment, reaching BMI 15.4 at discharge. Patients A and B were treated on both inpatient and partial-hospitalization units, while Patient C was only followed during inpatient treatment. Initial prescribed energy intake was 1000 (patient A) or 1400 kcal/day (patients B and C) and reached a maximum of 3200 kcal/day prior to discharge in all patients.

Weekly metabolic indicators for participants are presented in **Tables 9-11**. In all patients, resting energy expenditure increased during treatment, and diet-induced thermogenesis reached a peak of 158-181% of resting energy expenditure in the second or third week of treatment, which

is consistent with previous reports.(155, 156) Total physical activity expenditure and active energy expenditure were stable during inpatient treatment but increased substantially in Patients A and B after transfer to the partial-hospitalization unit, likely reflecting unsanctioned increases in moderate-to-vigorous physical activity during time off-unit.

In all three patients, we observed significant changes in the composition and diversity of the intestinal microbiota over the course of hospitalized renourishment at the phylum (n=3), class (n=6), order (n=11), family (n=13) and genus (n=36) levels (**Figure 9**), after FDR correction. In most cases, the magnitude and direction of change were patient-specific.

As patient BMI and energy intake were strongly correlated with length of hospitalization and with each other, similar results were generated by regressing bacterial taxa and diversity against these variables. The high degree of correlation between these variables, and reproduction of results across models using each as the regressand, suggest that increases to model complexity by including BMI or patient energy intake terms to a time-patient interaction model would not yield additional significant findings. This was largely observed in our analysis, and in the small number of cases where significant changes occur in these more complex models, correlation between terms makes biologically meaningful interpretation difficult. Weekly changes in resting energy expenditure, diet-induced thermogenesis, and active energy expenditure were also highly correlated with duration of patient hospitalization. Adding these variables to the time-patient model did not generate any significant associations with composition or diversity of the intestinal microbiota.

5.4 Discussion

These results support earlier assertions that individuals do not share a core microbiota, but rather house unique microbial communities that may experience periods of both high

variability and relative stability over time—due to a variety of intrinsic and extrinsic factors.(19, 157) The Human Microbiome Project underscores the high diversity of "healthy" microbiotas, unexplained by phenotypic differences in participants,(158) and even when controlling both environment and dietary intake, inter-individual differences were the predominant source of variation in the intestinal microbiota during two controlled feeding studies.(18, 159) The observation that this pattern of unique intestinal microbiotas as seen in healthy individuals also occurs and persists in a pathologic state associated with microbial dysbiosis is a novel outcome that warrants replication in other disorders associated with an intestinal microbial dysbiosis, such as gastrointestinal disorders or autoimmune disease. Moreover, although these results add to evidence of high inter-individual variability in the composition of enteric microbial communities, different bacteria can carry out similar metabolic functions. This could generate a similar functional impact from different microbial compositions, which has led researchers to posit that individuals may share a "core *microbiome*" of microbial genes that drive similar functional impact in the host.(160)

Limitations exist in these data. Although a valid design for novel observations, a case series is limited by sample size and lack of control group. Nonetheless, these results remain an important contribution to understanding the biological changes associated with renourishment in AN patients. We may have been underpowered to detect changes in more complex longitudinal models, but this does not rule out the possibility that changes in microbial composition and diversity are associated with changes in BMI, dietary intake, or psychopathology. In measuring metabolic changes on a weekly, rather than daily, basis, we may have also been underpowered to detect associations with microbial measures, despite evidence supporting a role for the intestinal microbiota in metabolic function and dietary energy extraction. Lastly, our analysis focused on

taxonomic and diversity measures of 16S rRNA sequencing data, which describes microbial composition but does not account for functional impact of the intestinal microbiota.

In examining composition and diversity of the intestinal microbiota in three patients undergoing treatment for acute AN, we found significant, patient-specific changes over the course of hospital-based renourishment. Even in a controlled hospital environment, on a stringent refeeding protocol, and in a disease state marked by microbial dysbiosis, individual microbial signatures persisted in accounting for the majority of variation in microbial composition and diversity. Although all three patients experienced peaks in hypermetabolism in the second or third week of treatment, we were unable to detect specific associations between the hypermetabolic state and changes in the intestinal microbiota. It will be important for future work to elucidate temporal changes to the intestinal *microbiome* during renourishment (and associated changes in metabolic activity), which will increase our understanding host-microbial dynamics during treatment and could help explain persistence of weight dysregulation and metabolic changes in recovered AN patients.

Table 8. Demographic and clinical characteristics of patients

		Patient A	Patient B	Patient C
Age (years)		25	29	16
Race		Asian	Caucasian	Caucasian
BMI (kg/m^2)	Admission	15.6	17.6	13.7
	Discharge	20.2	21.1	15.4
Length of stay (days)		73	58	34
Number of fecal samples		68	47	33
Energy intake (kcal)	Admission	1000	1400	1400
	Discharge	3200	3200	3200

Table 9. Weekly metabolic indicators: Patient A

Week	BMI (kg/m^2)	Resting Energy Expenditure (kcal/day)	Post-Prandial Resting Energy Expenditure (kcal/day)	Diet-Induced Thermogenesis	Physical Activity Expenditure (kcal/day)	Active Energy Expenditure (kcal/day)
1	15.6	900	1480	164%	1243	6
2	17.1	1110	1510	136%	1316	17
3	17.4	940	1630	173%	1391	101
4	18.0	1050	1680	160%	1467	120
5	18.4	1100	1590	145%	1453	36
6*	18.6	1120	1510	135%	1671	301
7	19.2	1330	1520	114%	1583	125
8	19.3	1360	1450	107%	1818	387
9	19.7	1260	1540	122%	1747	293
10	20.0	1290	1390	108%	1674	239
11	20.2	1140	1530	134%	1547	66

***** Transfer from inpatient to partial-hospitalization unit

** Diet-induced thermogenesis = post-prandial resting energy expenditure / resting energy expenditure

Table 10. Weekly metabolic indicators: Patient B

Week	BMI (kg/m^2)	Resting Energy Expenditure (kcal/day)	Post-Prandial Resting Energy Expenditure (kcal/day)	Diet-Induced Thermogenesis	Physical Activity Expenditure (kcal/day)	Active Energy Expenditure (kcal/day)
1	17.6	1200	1350	113%	1325	0
2	18.0	1000	1810	181%	1326	0
3*	17.6	1450	1800	124%	1974	636
4	18.2	1580	1810	115%	1774	384
5	18.9	1470	2130	145%	1662	168
6	18.4	1200	1890	158%	1684	283
7	19.5	1450	2030	140%	1843	376
8	19.8	1660	2370	143%	1978	446
9	21.1	1660	2030	122%	1994	472

* Transfer from inpatient to partial-hospitalization unit

** Diet-induced thermogenesis = post-prandial resting energy expenditure / resting energy expenditure

Table 11. Weekly metabolic indicators: Patient C

Week	BMI (kg/m^2)	Resting Energy Expenditure (kcal/day)	Post-Prandial Resting Energy Expenditure (kcal/day)	Diet-Induced Thermogenesis	Physical Activity Expenditure (kcal/day)	Active Energy Expenditure (kcal/day)
1	13.7	990	1250	126%	1462	2
2	14.0	870	1090	125%	1448	4
3	14.4	840	1330	158%	1455	5
4	15.0	1100	1310	119%	1466	8
5	15.4	1310	1380	105%	1465	4

** Diet-induced thermogenesis = post-prandial resting energy expenditure / resting energy expenditure

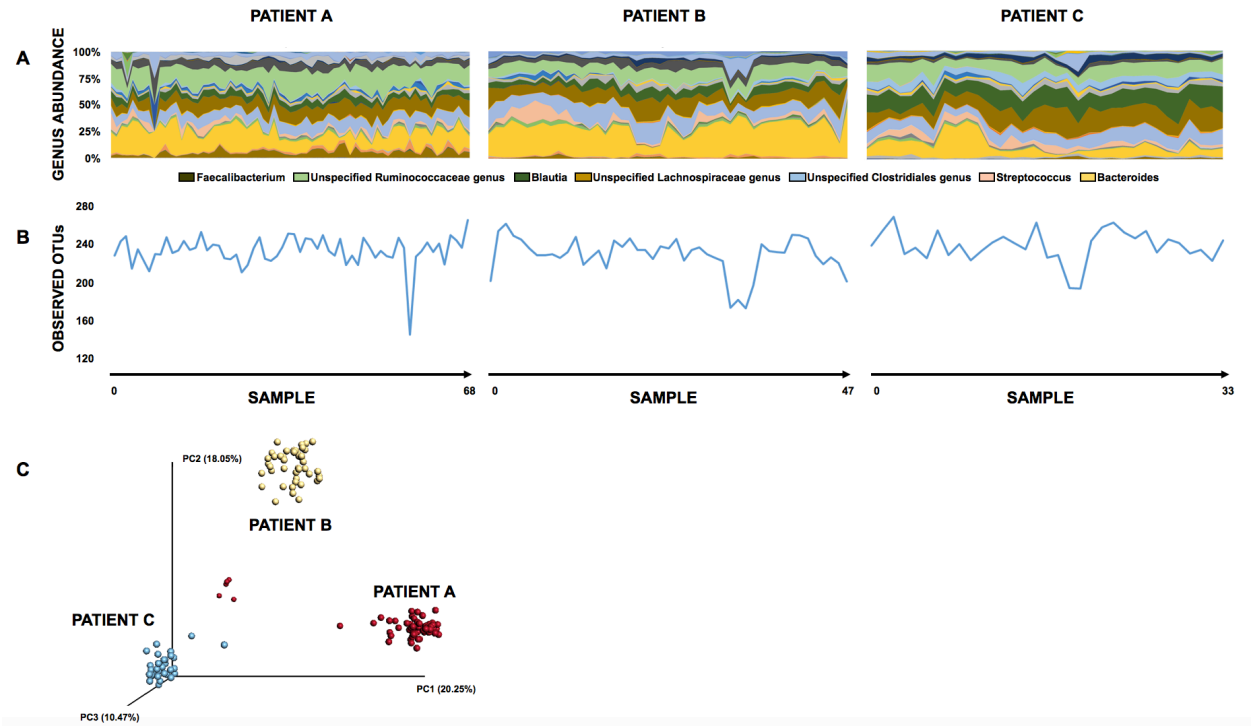

Figure 9. Intestinal microbiotas of three AN patients during therapeutic renourishment. (A) *Taxonomic composition*: abundance of specific genera exhibit significant variability during the course of refeeding. (B) *Microbial richness*: number of observed OTUs in each fecal sample varies over the course of refeeding. (C) *Principal coordinate analysis of unweighted UniFrac distances*: samples significantly cluster by patient (p=0.001, analysis of similarity), suggesting that disease state or clinical refeeding is not sufficient to overpower the unique enteric microbiota composition harbored by each individual.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

6.1 Summary of Findings

This dissertation investigated the role of the intestinal microbiota in the pathophysiology of anorexia nervosa (AN), which we hypothesized would uniquely respond to prolonged starvation, by (i) characterizing the composition and diversity of the intestinal microbiota in acutely ill patients with AN before and after hospital-based renourishment and compared with healthy controls; (ii) examining associations between microbial composition and diversity and measures of depression, anxiety, and eating disorder psychopathology in patients with AN and healthy females; (iii) investigating changes in fecal energy content during hospital-based renourishment and associations with the intestinal microbiota; and (iv) examining longitudinal changes in the intestinal microbiota over the entire course of hospital-based renourishment and associations between these changes and metabolic changes during treatment.

Chapter 2 described an intestinal microbial dysbiosis in patients with acute AN, marked by lower microbial diversity and specific taxonomic differences from a healthy comparison group. We also found specific compositional changes to the intestinal microbiota of these patients over the course of hospital-based weight restoration and associations between microbial diversity and levels of depression and eating disorder psychopathology. *This supports our hypothesis that the intestinal microbiota in patients with AN would differ in measures of composition and diversity from healthy controls, be associated with measures of psychopathology, and change over the course of hospital-based renourishment.* AN now belongs

to an ever-growing list of conditions that are associated with an intestinal microbial dysbiosis and for which microbial mechanisms may play a role in risk, development, or maintenance of pathology. Understanding how the intestinal microbiota in AN differs from that of healthy individuals provides a stepping stone toward designing new therapies, which has been the case for gastrointestinal disorders such as IBD.(64) After replication of initial findings and consensus around the association of IBD with an intestinal microbial dysbiosis, researchers demonstrated in animal models of colitis that gut bacteria are necessary to bring about the disorder's hallmark colonic inflammatory response. Meanwhile, fecal microbial transplantation (FMT), where an entire healthy intestinal microbiota is transferred via enema or capsule, emerged as a novel approach for altering the intestinal microbiota and was shown to be effective in treating *C. difficile*-induced colitis.(51) Thus, it was a natural progression to suggest that IBD patients could benefit from FMT, which has now been tested and shown to be effective in randomized clinical trials.(161-163)

Moreover, finding that levels of depression, anxiety, and eating disorder psychopathology are associated with microbial composition and diversity introduces phenotypic targets for microbial therapies. One such therapy, currently undergoing evaluation for use in treatment-refractory depression,(125) is a novel class of psychotropic medication termed *psychobiotics*—live organisms that, when ingested in adequate amounts, produce health benefits in individuals with psychiatric illness.(164) Some of these probiotic bacteria are capable of producing neurotransmitters and other neuroactive substances, including GABA, serotonin, dopamine, norepinephrine, and acetylcholine, and their effects on the brain may be mediated via the vagus nerve, spinal cord, or neuroendocrine systems. Preclinical animal models have shown that psychobiotics may have antidepressant, anxiolytic, and anti-inflammatory effects, and clinical

studies of probiotic supplementation in humans have shown improvement in mood and reduction of anxiety and psychological distress.(124, 164) Randomized, placebo-controlled clinical trials of psychobiotics as treatment for depression are in progress and similar investigations are warranted for other psychiatric disorders, including AN, which could improve outcome via augmenting the intestinal microbiota.

In the same group of patients, Chapter 3 described relative but not absolute changes in fecal energy content over the course of hospital-based weight restoration, as well as modest associations between changes in fecal energy content and phylum-level microbial composition and diversity. *This supports our hypothesis of microbial adaptation to a low-energy environment, but future work will need to evaluate the biological and clinical significance of this finding.* Both initiation of clinical refeeding in patients with acute AN and overfeeding of lean (but not obese) individuals resulted in a decrease in relative fecal energy content.(117) This runs counter to the microbial "energy extraction hypothesis," which suggests that obese individuals are more efficient at extracting calories from a given amount of ingested food(27)—thus leading to lower fecal energy content. Given the depleted energy state in acute AN, this same "efficiency" is one theory for microbial adaptation, but this would imply greater energy extraction and lower fecal energy content prior to versus post-refeeding, which is not supported by our findings.

Nevertheless, other factors play a role in dietary energy extraction, and may be affected by AN, including absorptive capacity of the intestinal epithelium. Nutrient absorption may be directly influenced by changes in the architecture of the intestinal epithelium (e.g., villi blunting/elongation or altered crypt depth) or indirectly affected by changes in intestinal permeability,(165) though one small study in patients with acute AN found that intestinal absorption was not affected either before or after therapeutic renourishment when measured by a

(13)C-labelled triglycerides digestion test, fecal elastase test, or d-xylose absorption test.(166) Mechanistic studies in GF mice that have been colonized with AN microbiotas, as well as biopsies of the intestinal epithelium from patients with AN, would allow investigation of changes to epithelial structure, epithelial cell damage, and biological markers of permeability (e.g., tight junction-associated proteins). Better characterization of the microbiome-mediated impact of AN on energy uptake may lead to therapies that manipulate microbial composition in order to maximize efficiency of renourishment.

Although we saw evidence of associations between microbial markers and psychopathology in patients with AN, Chapter 4 showed a lack of association between composition and diversity of the intestinal microbiota and psychiatric measures of anxiety, depression, eating-related thoughts and behaviors, stress, and personality in healthy adult females, *which did not support our hypothesis of associations between the intestinal microbiota and measures of psychopathology in healthy females.* Nonetheless, the intestinal microbiota plays a critical role in normal brain development and behavioral functions,(108) and microbial mechanisms are an important component of gut-brain communication. Given evidence of microbial dysbiosis in individuals with psychiatric disorders, including major depressive disorder and AN,(45, 116) it may be the case that compositional changes to the intestinal microbiota only arise in individuals with more extreme psychopathology. However, prebiotic and probiotic supplementation in human clinical studies has led to improvements in measures of depression, anxiety, cognitive reactivity, and stress in healthy volunteers—but without observable changes to composition of the intestinal microbiota.(46-48) Colonization of the intestinal microbiota by probiotic bacteria is transient,(167) suggesting that targeting the intestinal microbiota in order to augment mood or behavior requires a focus on the functional impact of enteric microorganisms

rather than compositional changes.(146)

Lastly, Chapter 5 highlighted patient-specific changes to the composition and diversity of the intestinal microbiota at all taxonomic levels over the entire course of hospital-based renourishment in a case series of three female patients. *Although the intestinal microbiotas in these patients showed longitudinal changes in common taxa, the magnitude and direction of changes were patient-specific, and we were unable to detect significant associations between microbial and metabolic changes during treatment.* High levels of inter-individual variability in microbial composition in patients with AN undergoing therapeutic renourishment mirrors findings from longitudinal and controlled feeding studies in healthy individuals, where individual differences explain the majority of microbial variability.(18, 19, 157, 159) Patient-specific treatment response suggests that we should focus less on the presence or absence of specific enteric taxa and more on enteric microbial function.

Considering the progression of research leading to microbiota-targeted therapies for GI disorders, we are in the early stages of similar findings for AN. We have established that acute AN is associated with an intestinal microbial dysbiosis, which has been replicated in one additional study.(152) Mechanistic studies in GF mice will allow investigation of the specific effects on metabolic function and behavior from colonization with AN microbiotas. FMT may also prove to be a valuable therapy for cases of chronic and enduring AN, where conventional treatments have little efficacy, quality of life is severely impaired, and clinicians and patients alike are desperate for novel treatment approaches.(168) Prolonged exposure to starvation in chronic cases of AN may have intractable effects on the intestinal microbiota that make renourishment more challenging. Although speculative, by introducing a healthy, diverse microbial ecosystem, FMT may improve efficacy and efficiency of therapeutic renourishment

and minimize GI symptoms by reversing microbial dysbiosis in these patients.

6.2 Significance and Limitations

This dissertation has made significant contributions of the eating disorders literature by introducing a novel approach to studying pathophysiology of AN. We were the first to characterize the composition and diversity of the intestinal microbiota in acute AN using high-throughput 16S rRNA sequencing and to examine change after weight restoration, which is a fundamental first step in testing for a causative role of enteric microbes in this disorder. We were also the first to (i) examine changes in fecal energy content during treatment for AN and the role of the intestinal microbiota in dietary energy extraction in this population and (ii) examine longitudinal changes in microbial composition and diversity over the entire course of hospital-based treatment for acute AN and associations with metabolic markers.

In showing that lower bacterial diversity is associated with greater levels of depression and eating disorder psychopathology in patients with AN, we were at the forefront of providing evidence for the enteric microbe-gut-brain axis in a human population. This was followed by an expanded study that was the first to examine associations between composition and diversity of the intestinal microbiota and psychiatric measures in healthy individuals.

This work has initiated the profiling of the intestinal microbiota in individuals with AN, and we hope that by identifying the presence or absence of specific enteric microbes, it may become possible to identify which individuals will experience difficulties with weight restoration and who will succeed in maintaining therapeutically restored weight (versus relapse). Ultimately, we may identify specific bacterial taxa whose promotion or elimination would improve the efficiency of therapeutic weight restoration, as well as the psychological and physical treatment experience of patients with AN.

These results should be considered in light of several limitations. Because this pilot work involved novel exploration of the role of the intestinal microbiota in AN, sample sizes were small, which may have limited our power to detect significant differences in microbial markers at the lowest taxonomic levels, between patients, over time, or in comparison to healthy controls. In addition, all study participants were female and between the ages of 15 and 50 years, reflecting the clinical population at UNC CEED. This may limit the generalizability of results to males, who comprise approximately 10% of individuals with AN,(113) and to those in younger or older age groups.

Moreover, we did not control for dietary intake of either patients with AN or healthy controls in these studies. The composition of the intestinal microbiota is influenced by long-term dietary patterns, as well as short-term shifts in quality or quantity of food intake. This will be important to consider in future work, as the methodology for incorporating dietary data into microbiome research advances and we gain greater insight into the biological mechanisms through which the intestinal microbiota influences nutrient and energy extraction in patients with AN.

As our work examined associations with composition and diversity of the intestinal microbiota in acute illness and differences in these markers between groups or over time, we are unable to distinguish our findings from those that reflect changes in BMI (versus recovery from AN), other factors that influence energy absorption (such as gastrointestinal distress and gastric transit time), or comorbid psychopathology (such as depression or anxiety). In addition, our analysis focused on taxonomic and diversity measures of 16S rRNA sequencing data, which describes microbial composition and diversity but does not account for metabolic activity or

functional impact of intestinal bacteria. Future work using GF mouse models and more advanced sequencing techniques will elucidate the microbial mechanisms unique to AN.

Lastly, a unique challenge for this research is the inability to compare individuals with AN to similarly malnourished individuals who do not have AN or individuals with other medical conditions that result in malnutrition. We are limited by ethical considerations in recruiting from certain "control" groups and in manipulating the physiology of healthy individuals to mimic aspects of AN. As such, we have attempted to account for these challenges by recruiting individuals at a healthy weight and without current or past eating disorder psychopathology; however, we recognize and acknowledge that these individuals remain "imperfect" controls for our patient population. Ongoing work characterizing microbial dysbiosis in children suffering from acute malnourishment and undergoing clinical refeeding may help to distinguish microbial markers of weight dysregulation from those specific to behavioral features of AN.

6.3 Future Directions

Research on the role of the intestinal microbiota in the emergence and maintenance of eating disorders is in its infancy. We have provided evidence for an intestinal microbial dysbiosis in AN, but how do specific microbes affect adiposity, weight regulation, and mood/behavior in this patient population? Future work must advance from observational to functional and mechanistic studies that dissect how these microbes impact host biology. This includes studies examining the enteric microbe-gut-brain axis in GF mice associated with AN microbiotas, which would allow investigating whether colonization with AN microbes induces changes in AN phenotypes, including weight, adiposity, and behavior. Moreover, advances in systems biology modeling will also allow for examining the potential metabolic impact of a defined microbial dysbiosis, which can be validated in human studies. In addition, investigating the role of the

intestinal microbiota in energy balance and gastrointestinal distress can be advanced by characterizing the microbial communities of the small intestine, which can be captured in biopsies of the intestinal mucosa.

The ultimate goal for this line of research is to improve the efficacy and efficiency of treatment for AN and minimize post-renourishment relapse. It is our hope that better characterization of the microbial dysbiosis in patients with AN and its functional impact will lead to interventions that improve treatment outcomes via manipulation the intestinal microbiota, whether via anti-, pre-, pro-, or synbiotic supplementation or FMT. This dissertation has created a solid foundation for future work, which will both advance our understanding of AN and expand to studies of the intestinal microbiota in other eating disorders.

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