GUT FEELINGS: WHAT THE GUT-BRAIN AXIS MAY REVEAL ABOUT DEPRESSIVE SYMPTOMATOLOGY DURING ADOLESCENCE

by

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DISSERTATION ABSTRACT

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Adolescence is a key period of neurobiological and social-affective development. It is also a time of increased vulnerability to psychopathology, such as depression. Growing evidence suggests that the gut microbiome can modify neurobiological and social-affective processes as well as potentiated novel therapies for mental and physical health problems. Despite the promise of these finding, it remains to be tested if these relationships exist during adolescence; a period of increased malleability within several of these systems. In Chapter 1, I first discuss why the gut microbiome has become increasingly relevant to developmental cognitive neuroscience. Specifically, I review the links between the gut microbiome and six overarching domains of change during adolescence: 1) social processes, 2) motivation and behavior, 3) neural development, 4) cognition, 5) neuroendocrine function, and 6) physical health and wellness. Next, I present an empirical study that tests some of the hypotheses put forth in Chapter 1. In a community sample of adolescent girls, I provide the first evidence that the functional composition of the microbiome associate with the amygdala resting state functional connectivity during adolescence. Specifically, these findings suggest the functional capacity of the microbiome associates with functional brain connectivity implicated in cognitive control and flexibility, emotional reactivity, and reward-seeking behaviors. Furthermore, both amygdala resting state functional connectivity and the function composition of the gut microbiome are associated with depressive symptomatology during adolescence. This provides the first evidence that the gut microbiome may be involved central neurobiological and affective behaviors during adolescence. Together, these findings suggest that neurobiological

models of adolescent behavior need to be updated to account for the role of the gut microbiome. This study was not without limitations and requires replication. To conclude my dissertation, I discuss future directions and challenges within this emerging interdisciplinary field; within clinical translation of these findings; and my future programmatic research plans.

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This dissertation is dedication to my mother, Julie Flannery.

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CHAPTER I

IS ADOLESCENCE THE MISSING DEVELOPMENTAL LINK IN MICROBIOME-GUT-BRAIN AXIS COMMUNICATION?

This work was previously published in *Developmental Psychobiology* and was co-authored with B. Callaghan, T. Sharpton, P. Fisher, & J. Pfeifer; therefore, the following chapter is formatted in accordance to the journal's publication standard. I was the first author on this manuscript. I wrote the first draft of the manuscript and had co-authors edit and provide feedback after the full paper was drafted.

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Introduction

Scientific and media coverage on the "Gut-Brain" Axis has dramatically increased in recent years, as we rapidly accrue knowledge about how the gut microbiome and central nervous system (CNS) bidirectionally communicate through neurobiological and immunological pathways to influence behavior. Gut microbial research has opened new frontiers in neuroscience and potentiated novel therapies for mental health disorders (Mayer et al., 2014; Sarkar et al., 2016). The gut microbiome helps cultivate healthy neurobiological programming in early development and continues to associate with brain function and behavior into adulthood (Borre et al., 2014). For example, altering the gut microbiome can change social and affective behavior, and in preliminary rodent and adult studies, reverse psychiatric symptoms (Akkasheh et al., 2016; Dinan & Cryan, 2016). Despite the promise of these dramatic findings, we possess relatively limited insight into the development and function of the microbiome beyond early childhood. This is particularly true of adolescence, which is an important time period of neurobiological and behavioral development, including emergent risk for psychopathology.

Adolescence is increasingly understood to represent more than a transitional period between child and adulthood, but rather a critical period of neurobiological programming, social-affective processing, and explorative behavior that continues to shape our neurobiological and social and emotional processing in adulthood (Dahl, Allen, Wilbrecht, & Suleiman, 2018). This also means that adolescence is a vulnerable period for the heightened risk for the emergence of psychiatric symptoms and disorders that persist across the lifespan. Despite the growing emphasis of adolescence as a "flux" point in development within developmental cognitive neurosciences, and the growing emphasis on the gut microbiome's role in neurobiological, social-emotional, explorative, and psychiatric symptom development, there is a relative dearth of research into the gut microbiome's role during this critical period of developmental programming. In this review, we first highlight why gut microbiome research is becoming increasingly relevant to psychology and the cognitive neurosciences, then focus on the microbiome's specific connections to social-affective processing, and lastly, synopsize six domains of change that are undergoing unique programming during adolescence and that are relevant to the microbiome.

Part I. The gut microbiome's growing connection with psychology and the cognitive neurosciences

The gut microbiome consists of a diverse community of microorganisms ("microbes") and their genes that resides in the gastrointestinal tract, including bacteria, fungi, archaea, and viruses. Our number of microbial cells are now believed to closely match our number of human cells (approximately 1:1 ratio; Ron et al., 2016), with the largest microbial population residing in the gastrointestinal tract. The composition and function of the gut microbiome is dynamic and malleable to the environment across the life span, including changes in diet, geography, stress, illness, or use of antibiotics. The gut microbiome interacts with the Enteric Nervous System (ENS): a branch of the autonomic nervous system (ANS) that can also operate independently to regulate the gastrointestinal system. The ENS contains an estimated 70% of body's immune cells,

and produces an estimated 95% of the body's serotonin (Mayer, 2011). The connections between the ENS and the brain (i.e., the gut-brain axis) were first discovered centuries ago; however, the microbiota's role in this gut-brain communication has only recently emerged (and contributes to the moniker of the "microbiome"-gut-brain axis; Zhu et al., 2017).

The microbiome performs myriad functions. It regulates immune function activity, gut motility, nutrient absorption, fat distribution, and maintains homeostasis of the intestinal barrier (Carabotti, Scirocco, Maselli, & Severi, 2015). The microbiome also communicates with the CNS through bidirectional signaling along the autonomic nervous system, and neuroimmune and neuroendocrine pathways. For example, the microbiome-gut-brain axis operates through microbial sourced metabolites (e.g., short-chain-fatty acids, peptides, neurotransmitters), vagal nerve innervation, hormonal signaling, and immune cells (see Figure 1; see also Sharon, Sampson, Geschwind, & Mazmanian, 2016 for a review of connection mechanisms). Microbiome colonization directly overlaps with the first critical period of neurobiological development, beginning during prenatal development through the mother's placenta and continuing through the first 3-4 years of life (Collado, Rautava, Aakko, Isolauri, & Salminen, 2016). In fact, the microbiome helps form the blood-brain barrier and is necessary for normative brain development, immune function, and hypothalamus pituitary adrenal axis (HPA) axis programming (Borre et al., 2014). Although the mechanistic connections have been predominantly modeled in nonhuman vertebrate animals, these discoveries have raised the potential that the microbiome-gut-brain axis can transform how we understand and approach our models of human behavior.

A Novel Treatment Method. Emergent gut microbial interventions in medicine highlight the microbiome's unique potential to individualize treatment in psychology. One of the most commonly used microbial interventions is probiotic administration. By definition, probiotics are live bacteria that, when administered in precise quantities, provide a health benefit to the host

(Sanders, 2008). Studies have used probiotics to treat a range of both gastrointestinal as well as mental health symptoms. For example, in a population of adults with comorbid Irritable Bowel Syndrome (IBS) and anxiety/depression, a group that took a probiotic for ten weeks had more individuals that showed declines in depression symptoms (64%) than a group that took a placebo (32%; Pinto-Sanchez et al., 2017). A recent systematic review supported the positive effects of probiotics on depression symptoms, but reported that additional randomized control trials are need before clinical recommendations can be made (Pirbaglou et al., 2016; Wallace & Milev, 2017). However, the most recent meta-analysis of those reports only found support for a beneficial effect of probiotics in mildly depressed, compared to not depressed, subjects (there was no effect of probiotics on clinical depression symptoms; (Ng, Peters, Ho, Lim, & Yeo, 2018). No meta-analyses have yet focused on clinical anxiety symptoms.

Another novel treatment that involves an indirect manipulation of gastrointestinal bacteria is administration of prebiotics. By definition, prebiotics are dietary compounds used by bacteria to yield a positive benefit to the host, (e.g., fructo-oligosaccharides and galacto-oligosaccharides; (Gibson et al., 2017). Some studies have administered such prebiotics to either healthy volunteers, showing a reduction in neuroendocrine activity associated with stress regulation (Schmidt et al., 2015) or to rodents, showing positive outcomes on several stress associated outcomes, including anxiety and depression (Burokas et al., 2017). Currently, there are no meta-analyses of prebiotic utility in depression or anxiety.

Microbial interventions have extended to several areas of medicine (Xu et al., 2015). By profiling the gut microbiome, for example, immunotherapies and clinical drug trials are beginning to identify individual differences in the course of optimal chemotherapy and drug metabolism (Petrosino, 2018). Pilot studies in adults have also sparked promise that microbial transplants or "fecal pills" which have been used for GI disorders, may be able to alleviate symptoms of depression or autism spectrum disorder (Kang et al., 2017; Zheng et al., 2016). As the field

expands, it may also mean that psychological interventions could individualize psychotherapeutic treatments based on microbial profiles, or that the microbiome could provide meaningful biomarkers for pre-post psychological interventions. Most of these medical treatments are not yet commercially available, nor sophisticated enough for psychological interventions, but with the rise in technological advancements, they may not be far off.

Advancing Technology. Owing in large part to recent advances in DNA sequencing technology and analytical methodology, our ability to study the gut microbiome's connections to neurobiology and behaviors has dramatically improved. Over the past decade, DNA sequencing procedures have been streamlined and commercialized, resulting in lower costs for generating DNA sequences from a microbiome sample. This has afforded new avenues of discovery. For example, targeted sequencing of the bacterial 16S locus enables relatively rapid, comprehensive, and precise determination of the taxonomic composition of a microbiome sample (Kuczynski et al., 2011). A rapidly growing body of research additionally employs an approach termed shotgun metagenomics, which seeks to sequence the entire genomes of the microbes that comprise a community (Sharpton, 2014). These advanced methods are beginning to uncover the metabolic pathways encoded in these genomes and link these pathways to physiological or behavioral processes, such as CNS functioning, neuroimmunology, and neuroendocrinology (Arnold, Roach, & Azcarate-Peril, 2016).

Innovations in study design are also made possible thanks to initiatives such as AmericanGut, which has created the first open-source, community-generated database of the human microbiome for individuals to participate or analyze the microbiome on a large scale (McDonald et al., 2018). Companies have also developed commercialized collection kits for researchers to conduct their own studies. The advent of such kits and standard operating procedures for microbial collection and analyses (http://www.microbiome-

standards.org/index.html), has opened new avenues for studying high-risk or hard to reach developmental populations (particularly within the United States) by reducing participant burden, providing flexible stool storage options, and enabling the delivery of samples through U.S. mail transport (Anderson et al., 2016). However, online gut microbial repositories are limited in terms of their ability to measure cognitive processes and to provide diagnostic information. As we seek to bridge disciplines, rigorous measures of psychopathology and brain structure, function, or connectivity will still require some portion of in-lab or in-person study design.

Part II. Evidence linking the gut microbiome with social-affective neural processes

The role of the microbiome in social-affective brain function is a relatively recent area of inquiry, especially within human subjects. Non-human studies, however, can provide some initial insight about the microbiome's role in social-affective brain development. We begin by highlighting known links between the gut microbiome and molecular or structural changes in rodent models, focusing on the hippocampus and amygdala – key regions in learning and social-affective processing and potential "hubs" for microbial influence on brain function. Next, we summarize initial work in humans, which also includes other associated regions, such as the paragenual anterior cingulate cortex (pACC), anterior midcingulate cortex (aMCC), insula, and precuneus.

Rodent Models. The hippocampus and the amygdala are two limbic regions centrally involved in social-affective learning and memory that rely on input from the gut microbiome for normative development. Furthermore, the amygdala and hippocampus are probable "hubs" for the gut microbiome's influence on brain function and cognition (for full review of this theory, see Cowan et al., 2018). Briefly, manipulating the presence or composition of the microbiome, changes the molecular and structural development of the hippocampus and amygdala (Foster, Rinaman, &

Cryan, n.d.). For example, the absence of a microbiome (e.g., via animals raised without a microbiome) or the disturbance of the microbiome (e.g., via antibiotic treatment) alters amygdala and hippocampal gene expression involved in neural plasticity (i.e., brain-derived neurotropic factor (BDNF) and Finkel-Biskis-Jinkins murine osteosarcoma viral oncogene homolog B (FosB)), synaptic plasticity involved with learning and memory (i.e., N-methyl-D-aspartate receptor (NMDAR), and the neurotransmission of monoamines, such as dopamine, norepinephrine, and serotonin (Bercik et al., 2011; Neufeld et al., 2011). In addition, the absence of the microbiome results in an enlarged amygdala volume and dendritic hypertrophy in the basolateral amygdala (Luczynski et al., 2016; Stilling, Dinan, & Cryan, 2014). Although these regions are also central in stress regulation, and the pathway may be through reduced glucocorticoids (GC) and proinflammatory cytokines, some evidence suggests these effects can occur independent of GC decreased, or anxiety levels (Gareau et al., 2011; Savignac, Tramullas, Kiely, Dinan, & Cryan, 2015). Such neural alterations to these limbic regions can impair social-affective learning and memory and may influence the later development of the hippocampus and amygdala's functional connectivity to "top-down" regions (e.g., prefrontal cortex) involved in social-affective regulation in adolescence (Tottenham, 2015).

Human Models. Promising work in adults demonstrates the microbiome's role in neural function extends to functional brain reactivity. These connections were first discovered in adults with gastrointestinal disorders, such as IBS, in which the microbiome's composition characteristically differs from that of individuals not manifesting the syndrome (Mayer, Naliboff, & Craig, 2006). Compared to controls, adult patients with IBS displayed altered functional activity in regions involved in attention, sensation, and emotional arousal, including the amygdala, pACC, aMCC, and somatosensory cortex (Tillisch, Mayer, & Labus, 2011), possibly as a function of microbial alterations in the composition and/or stability over time.

The microbiome's link to brain activity has also been observed in healthy adults. For example, in a pre-post fMRI design, compared to baseline activity, women who received a 4-week probiotic treatment had reduced resting-state functional activity in regions involved in emotion and sensation processing, including the insula, somasensory cortex, as well as the prefrontal cortex, parahippocampal gyrus, precuneus, and basal ganglia (Tillisch et al., 2013). In follow up experiments, Tillisch and colleagues (2017) also demonstrated that an individual's resting functional and structural connectivity could be characterized by the type of prominent bacteria in their microbiome (a clustering analysis typically referred to as "enterotyping;" see Costea et al., 2017 for a review on the debate surrounding this methodology). Women in the microbial cluster predominated by *Prevotella* (versus *Bacteroides*) had decreased hippocampal activity and increased functional brain connectivity between brain regions involved in emotional and attentional sensory processing. At the same time, structural connectivity patterns obtained from white and gray matter tracts correctly categorized women into those two predominant bacterial groups with 66.7% and 87.2% accuracy, respectively (Tillisch et al., 2017). Together, these data provide a foundation for thinking about how various sensory, attentional, and emotional processes may be altered by microbiome-gut-brain axis communication.

Gut microbiome development and links to early psychosocial behavior. Currently, the research on gut microbial programming has largely excluded psychosocial development, with some notable exceptions that focus on early childhood. Two foundational longitudinal studies in early childhood revealed that the gut microbiome predicted child cognitive performance at 12 months old (Carlson et al., 2018) and temperament at 24 months old (Christian et al., 2015), suggesting that the gut microbiome is already playing a role in shaping cognition and social-affective behavior from an early age.

There is, however, mounting evidence that the microbiome continues to develop beyond the initially presumed first few years of life. It turns out that children (ages 1-7 years) distinctly separated from adults on a principle component analysis (PCA), showing less diverse microbiomes, and at the genus level, showing higher abundance of *Bifidobacterium*, *Clostridium*, and lower *Prevotella* and *Sutterella* (Hopkins, Sharp, & Macfarlane, 2001). In another study spanning from childhood through pre-adolescence (ages 7-12 years), children also had more similar microbiomes to one another than adults (Hollister et al., 2015). Specifically, children differed in the relative abundance of the taxa that comprise the gut microbiome (i.e., increased *Bifidobacterium* spp*., Faecalibacterium* spp., and members of the *Lachnospiraceae*, while adults harbored greater abundances of *Bacteroides* spp.). Furthermore, the authors concluded that children harbor microbiome metabolic pathways that promote ongoing neural development (e.g., vitamin B12 synthesis, de novo synthesis of folate and oxidative phosphorylation and lipopolysaccharide biosynthesis), whereas adult functional metabolic pathways were more associated with inflammation and obesity (Hollister et al., 2015). These findings suggest that not only does the microbiome continue to change with age, but its constitution and functionality is specifically tailored to relevant developmental processes.

Part III. Adolescence as a sensitive period of microbial change

While human studies of the adolescent gut microbiome are still relatively sparse, rodent models suggest adolescence represents a critical window during which the microbiome's colonization of the gut impacts the wiring of the parts of the CNS linked to stress-associated behaviors (Foster & McVey Neufeld, 2013; McVey Neufeld, Luczynski, Dinan, & Cryan, 2016). Germ-free mice that were colonized with a microbiome either at birth or 3 weeks old showed normalized stress-associated behaviors, whereas germ-free mice that were colonized with a microbiome at 10 weeks old did not show this effect (Heijtz et al., 2011; Neulfeld et al., 2011,

Clarke et al., 2012). Furthermore, emerging evidence demonstrates the gut microbiome plays a critical role in monoamine and neuromodulator function in hippocampus and amygdala specifically during adolescence. For example, antibiotic depletion of the gut microbiome during adolescence led to changed hippocampal monoamine concentrations (i.e., noradrenaline, but not serotonin or dopamine); metabolites, such as increased tryptophan and decreased kynurenine; reduced hippocampal BDNF expression; and reduced neuropeptides (oxytocin and vasopressin) into adulthood (Desbonnet et al., 2015). Furthermore, antibiotic-treated adolescent rats also showed changes in amygdala amino acid (increased levodopa; L-DOPA) and catecholamine metabolite synthesis (increased homovanillic acid; (Desbonnet et al., 2015)). In other words, rodent models suggest changes to the microbiome prior to the end of adolescence can be a unique opportunity to shape CNS development (see Figure 1).

Initial case studies in humans also suggested the microbiome had distinct features in adolescence compared with childhood or adulthood, although the small sample sizes could not rule out that individual variability accounted for the differences (Paliy, Kenche, Abernathy, & Michail, 2009; Schloss, Iverson, Petrosino, & Schloss, 2014). One study confirmed these pilot studies by showing that adolescents' (11-18 years-old) microbiomes were distinguishable from adults (22-61 years-old) by specific taxa and relative abundance of taxa in the gut (Agans et al., 2011). Collectively, these studies provide some of the first evidence that the health and function of the adolescent microbiome is distinct from adults and should therefore be studied and interpreted through a developmental lens. Large-scale studies with the aim to characterize normative developmental changes in the microbiome are currently underway through the NIH initiative human microbiome project (Group et al., 2009).

Uncertainty remains regarding the extent to which adolescence is a unique period of gut microbial development. The environment plays a key role in shaping gut and brain function across development, but it may be particularly relevant in adolescence. Adolescence is arguably

the second largest environmental shift in development (following early life) due to concurrent changes across multiple domains of behavior and neurobiology (Dahl et al., 2018). We therefore propose adolescence reflects a sensitivity along the microbiome-gut-brain axis, in which the microbiome may spark change in developing neurobiological systems and behavior. At the same time the number of changes in environment, neurobiology, and behavior during adolescence may ignite transformation in the microbiome.

Taking a developmental science approach, we argue that several broad domains of adolescent change – specifically 1) social processes, 2) motivation and behavior, 3) neural development, 4) cognition, 5) neuroendocrine function, and 6) physical health and wellness – have clear bi-directional connections to the microbiome. We specifically focus on these domains as they represent a sensitive period of developmental programming that can have long-term implications for functioning into adulthood (Dahl et al., 2018). Below, we briefly summarize how these domains link to the microbiome and the potential implications of such linkages to understanding adolescent psychobiological development (see Figure 2). These categories are not intended to be exhaustive, nor expected to be independent from one another, but are intended to highlight some fruitful directions for future interdisciplinary work.

Social Processes. Adolescence is a time of self-identity development and social exploration both within and outside of one's family (Pfeifer & Berkman, 2018). This also means it is a time of novel microbial exposure to new places and increased direct microbial exchange with others. As social dynamics change within families, friendships, and romantic relationships, peers become more salient, romantic relationships burgeon, and social stressors become particularly potent (Blakemore & Mills, 2014; Spear, 2000). Structurally, adolescence is a time of increased autonomy outside the home and opportunity for new social interactions. Within the US, this typically includes transitioning from grade school into middle and high school, where there is an

increased number of classrooms and peer interactions. At the same time, adolescents spend an increasing amount of time alone, and remain heavily influenced by their parents and their home structure (Larson & Richards, 1994). This period of budding independence sets a unique stage for the existing familial identity and the expanding self and social identities to interact.

These social developmental changes likely have important implications for the developing microbiome. In general, social behaviors can guide the degree of novel microbial environment and microbial exchange that occurs by increasing or decreasing one's level of exploration and exposure to the environment. At the same time, rodent studies suggest the microbiome modifies the initiation of these social behaviors (e.g., altering novel exploration and desire to choose social versus non-social interactions; see Münger, Montiel-Castro, Langhans, & Pacheco-López, 2018 for full review of the bidirectional social-microbiome relationship). Microbial studies of Autism Spectrum Disorder (ASD) demonstrate the reciprocal relationship between social behaviors and the microbiome. Individuals with ASD, traditionally characterized by altered social behavior and communication, show alterations in their gut microbiomes and exhibit significantly higher rates of gastrointestinal problems as compared to neurotypical controls (Strati et al., 2017). For example, germ-free mice showed altered social behavior, characterized as preference for an object over another mouse (Foster et al., 2017); and maternal immune activation (MIA) mice offspring showed disruption to their GI barrier and behavioral features consistent with ASD that could be reversed with probiotic treatment (Hsiao et al., 2013). In preliminary human experimental manipulations of the microbiome (i.e., treated with probiotics or fecal transplants), children with ASD engaged in more social behavior following the interventions (Li, Han, Dy, & Hagerman, 2017). These findings reveal a potential mutually reinforcing relationship between the gut microbiome and social behavior that should be further explored during adolescence.

Motivational and Behavior Change. In addition to the changes that occur in social development, adolescents have unique developmental goals that influence their motivations and explorative behaviors (Crone & Dahl, 2012). One of the developmental goals for adolescents is to prepare for adult roles and relationships. In service of this goal, adolescents demonstrate a greater tolerance of ambiguity (compared to adults) in order to approach novel experiences (Bos & Hertwig, 2017). This pattern of increased exploration inherently involves increased risking-taking and sensitivity to reward (Romer, 2010), and commonly interacts with social development (e.g., influence of peers on risk-taking behavior; (Braams, Duijvenvoorde, Peper, & Crone, 2015). While the microbiome has been linked to behavioral exploration, this connection has not been studied during adolescence. For example, germ-free mice exhibit less environmental exploration and a diminished ability to distinguish novel stimuli (Desbonnet et al., 2015; Heijtz et al., 2011). The microbiome's influence on explorative behavior could have important implications for the type of experiences that adolescents engage in. At the same time, the environments they immerse themselves in also influence their microbial exposure.

Adolescence is also a period of normative change in affective reactivity and regulation, as well as a period of heightened risk for the emergence of mental health disorders. For example, as of 2016 in the United States, 1 in 4 adolescents had a mental health diagnosis and 3.1 million adolescents ages 12-17 had at least one depressive episode, with 70% having severe impairment (NIMH). Although there is mounting evidence that the microbiome is associated with social and affective disorders, and early disruption to the microbiome may lead to greater vulnerability of social and affective disorder later in life (Dinan & Cryan, 2017), the causal mechanisms are still poorly understood and the microbiome-behavior relationship is likely bidirectionally reinforced. As discussed earlier, in adult humans and other vertebrate animals, experimental manipulations such as transplantation and pre/probiotic treatment are being tested for mental health disorders such as depression and anxiety (Sarkar et al., 2016). These studies demonstrate that the gut

microbiome is not just altered with these disorders but prompts these phenotypes. Mental health disorders are also intimately tied to changes in social behaviors (Thoits, 2011), suggesting there is likely a complex interaction between the microbiome, social development, and affective and explorative behaviors. For example, one mechanism could be that a disrupted microbiome profile initiates depressive behavior via encouraging restricted social and explorative behavior. Concurrently, depressive behaviors, such as restricted environment and/or altered diet and weight, may maintain the alteration in the microbiome.

Brain Development. Adolescence is a well-known sensitive period of structural and functional brain development (Dahl et al., 2018). There is limited information, however, on how these neurodevelopmental changes influence microbiome-gut-brain axis communication, particularly regarding known social, motivational, explorative, and affective behavioral changes.

Key brain regions implicated in social-affective processing that also have known connections with the gut microbiome exhibit dramatic changes during adolescence. For example, regions implicated in emotional processing and learning (e.g., amygdala, hippocampus, the dorsolateral prefrontal cortex (dlPFC), and ventral medial prefrontal cortex (vmPFC)) are functionally and structurally changing to reflect the importance of the adolescent environment and developmental stage (Flannery, Giuliani, Flournoy, & Pfeifer, 2017; Mills et al., 2016). Connectivity between limbic regions such as the amygdala, and regulatory regions like the prefrontal cortex, begins to shift to reflect adult-like patterns of top-down regulation (Gee et al., 2013). This period of sensitivity also means greater vulnerability to perturbations. Mental health disorders during adolescence are associated with alterations in brain activity, structure, and connectivity (Lee et al., 2014). Depression during adolescence, for example, is associated with decreased connectivity among regions involved in emotion regulation such as the amygdala, hippocampus, insula, and prefrontal cortex (Connolly et al., 2013; Cullen et al., 2014; Davey et

al., 2015; Pannekoek et al., 2014). Given the gut microbiome's established links to several social-affective processes, it is imperative we understand their relationship during this time of developmental flux in said systems.

Despite the microbiome's link to social and explorative behaviors, the microbiome has not been connected to brain processes or networks centrally involved in social and explorative behaviors, although regions within these networks have been linked to the gut microbiome (e.g., precuneus and ACC). Compared to adults, adolescents show more activity in regions implicated in reward processing when they are making risky choices (e.g., picking an ambiguous option; see review (Sherman, Steinberg, & Chein, 2017; Silverman, Jedd, & Luciana, 2015), and learning a task (e.g., learning pattern to improve; see review (Romer, Reyna, & Satterthwaite, 2017). In addition, regions associated with social processing (i.e., the "social brain network") undergo significant functional and structural changes during adolescence (see review Blakemore & Mills, 2014) as well significant changes in functional activation and connectivity between midline structures involved in self processing (see review Pfeifer & Berkman, 2018). A future avenue of interdisciplinary work should seek to identify gut-brain correlates of social and explorative behaviors during adolescence.

Cognition. Cognitive development heavily intertwines with the adolescent-specific changes in synaptic density in the prefrontal cortex. This time of prolonged maturation through adolescence represents a unique period of developmental programming in higher-order cognitive processes, such as decision-making, working memory, and learning (Juraska & Willing, 2017; Peverill, McLaughlin, Finn, & Sheridan, 2016; Zhou et al., 2016).

Cognitive impairments following alterations in the microbiome have led to the theory of a microbiome-cognition connection. Specifically, the microbiome may be necessary for proper fear learning. Germ-free mice show impaired maintenance of fear stimuli-response associations,

which was partially reversed with microbial colonization and fully reversed with targeted changes to amygdala gene expression (Hoban et al., 2018). These findings suggest the microbiome influences normative gene expression in the amygdala.

Antibiotics can produce decreased spatial learning, increased anxiety (Wang et al., 2015), reduced memory, and impaired novel object recognition (Fröhlich et al., 2016; Vázquez et al., 2015). Antibiotics change BDNF mRNA expression in critical regions of cognition, including the medial prefrontal cortex, hippocampus, and hypothalamus, possibly by altering tight junction proteins and cytokine mRNA expression (Fröhlich et al., 2016) . Alternatively, mice provided with a probiotic (e.g., milk oligosaccharides) or prebiotics showed increased memory and reinforcement learning and cognitive flexibility (attentional set shifting;(Gareau et al., 2011; Wang et al., 2015). These effects were similarly reflected in changes in BDNF, and increased minerocorticoids and NMDA (Gareau et al., 2011; Wang et al., 2015). Notably, there are still inconsistencies in these effects, particularly within humans (Sarkar et al., 2018) that warrants further investigation. Future work should specifically seek to test the microbiome- cognition connection during adolescence when cognition is actively rewiring.

Neuroendocrine Development. One possible mechanism of change in microbiome-gut-brain axis communication during adolescence is neuroendocrine development. Adolescence is typically defined by the onset of puberty, including changes in the hypothalamus pituitary gonadal (HPG) axis, but also in the hypothalamus pituitary adrenal (HPA) axis (Shirtcliff et al., 2015). Although these systems change across the lifespan, adolescence is a well-documented period of developmental programming for the HPA and HPG axis. These neuroendocrine changes are associated and interact with changes in brain structure and function, secondary sexual characteristics, and physiological stress responses, all of which can influence adolescent behavior and the microbiome (Shirtcliff et al., 2015; Siervogel et al., 2003).

Changes in the HPG axis are associated with changes in sex hormones, such as estrogen, progesterone, and testosterone production (which associate with brain structure and function, see Vijayakumar, Op de Macks, Shirtcliff, & Pfeifer, 2018). There is also growing evidence that sex hormones change the vaginal microbiome, in particular that menarche is likely associated with reprogramming of the vaginal microbiome (Brotman, Ravel, Bavoil, Gravitt, & Ghanem, 2014). Further preliminary evidence suggests hormonal changes that occur across a female's menstrual cycle additionally alter the gut microbiome (Chen et al., 2017). Pubertal development, specifically, may represent another sensitive period in reprogramming of both the vaginal and gut microbiome; yet to date, this theory has not been tested. Pubertal development has also been linked to microbial changes in other areas, such as subgingival, skin, and nares, demonstrating it may be a time of widespread change in microbial exposure and malleability (Gusberti, Mombelli, Lang, & Minder, 1990; Oh, Conlan, Polley, Segre, & Kong, 2012).

During adolescence, there are also changes to the HPA axis, the primary stress response system. For example, cortisol normatively changes in adolescence, resulting in a prolonged HPA axis response compared to adults (Bingham et al., 2011). The HPA axis is involved in normative microbiome-gut-brain axis communication (Bailey et al., 2010). For example, the microbiome can shape how an organism responds to stress in early development (O'Mahony, Clarke, Dinan, & Cryan, 2017). Germ-free mice display a hyper-reactive HPA axis response (Sudo et al., 2004), but the introduction of specific microbes such as *Bifidobacterium* can reverse the hyper-reactive HPA axis response (Sudo et al., 2004). Similarly, the HPA axis can change the microbiome. The HPA axis potentiates the release of catecholamines that are metabolized by the microbiome (Moloney et al., 2015). However, under conditions of social and chronic stress, increased cortisol, the end product of the HPA axis, can lead to increased permeability of the intestinal wall (Kelly et al., 2015; Rogers et al., 2016). Stress disruptions to the microbial composition can lead to disruptions in microbiome-gut-brain axis, resulting in low-grade inflammation and depression

(see review Farzi, Fröhlich, & Holzer, 2018) . Adolescence, therefore, provides the opportunity to positively influence the relationship between the HPA axis and the gut microbiome through normative change in HPA axis function and the microbiome's sensitivity to change during adolescence. On the other hand, the increased vulnerability to social stressors and malleability in these systems during adolescence may also lead to disruptions along the microbiome-gut-brain axis.

Physical Health and Wellness. All the changes mentioned above have potential influences on physical health and wellbeing across adolescence, which can culminate in alterations in diet, substance use, body composition, body mass index (BMI), exercise, and sleep (Dahl et al., 2018; Worthman & Trang, 2018). This is particularly critical, given the gut microbiome's primary role and extensive links to these processes across species.

Physically, adolescents are undergoing major changes. In concert with the emergence of secondary sex characteristics, body composition changes, fat distribution changes, and obesity rates can increase (Loomba-Albrecht & Styne, 2009). Exercise habits that are formed in adolescence, however, can promote hippocampal BDNF levels and alter microbial composition (in rodents) in a way that does not occur in childhood or adulthood (Hopkins et al., 2012). Adolescents choose more of their own meals with less parental regulation of their diet. These dietary changes are often associated with an increased percentage of high fat and refined sugars, and heightened risk for increased BMI (Andrade, Previdelli, Cesar, Marchioni, & Fisberg, 2016; Winpenny, Penney, Corder, White, & van Sluijs, 2017). In Western cultures, the school structure can also heavily influence dietary choices (e.g., vending machines and limited healthy options; (Driessen, Cameron, Thornton, Lai, & Barnett, 2014). The school structure is also at odds with the sleep patterns of adolescence, which are associated with a change in diurnal cycling (Colrain & Baker, 2011). Adolescents begin to stay up later, but still need to get up early for school, which

can result in a pattern of inadequate sleep, with periods of "catch up" on the weekends (Carskadon, 2011). Sleep deprivation in adolescence is linked to several behavioral risks, including poor diet; increased stimulant use, such as caffeine; substance use, such as alcohol and cigarette use; lower mood; and high-risk behaviors (McKnight-Eily et al., 2011). Every single one of these risks have been associated with the microbiome.

Alterations to physical health, such as those described above, represent one of the more extensively studied areas in regards to the gut microbiome which suggests adolescence is a particularly critical period of change (see McVey Neufeld et al., 2016). As one of the primary functions, the gut microbiome diversifies in accordance with the nutrients it obtains through diet. For example, individuals with a vegan or omnivore diet need different enzymes to break down their food, and they will produce different nutrients for both the host and the microbes along the gastrointestinal tract (David et al., 2014). The microbiome is also highly sensitive, so changes in diet within an individual will change the individual's microbiome (David et al., 2014). Dietary changes in adolescence, therefore, may have several implications. For example, in rodents, a diet high in fat was associated with elevated anxiety and depression, increased HPA axis response to a stressor, and increased intestinal permeability (de Sousa Rodrigues et al., 2017). There is also preliminary evidence in mice that dietary changes can normalize the gut microbiome following early life stress and altered stress response system, suggesting a change in diet may have a circular effect on said systems (Foster et al., 2017). Thus, one prominent hypothesis is that dietary decisions influence brain and behavior via the gut microbiome.

Food choices are also not the only source of dietary change in adolescence, as this period is partly characterized by the increase in alcohol and drug experimentation. Alcohol use, as well as several recreational drugs, can induce changes in microbial composition and metabolic function, and in some cases can lead to increased intestinal permeability (Gorky & Schwaber, 2016). On the other hand, the microbiome may also influence initial cravings and has been linked

to addictions, though this is still under investigation (de Timary, Leclercq, Stärkel, & Delzenne, 2016) . Lastly, while there is preliminary evidence that the microbiome may be directly altered by changes in sleep profiles (Thompson et al., 2017), the connection between the two may be more easily interpreted within the context of the vast consequences of altered sleep on diet, and digestion. Overall, these data suggest drastic changes can occur during adolescence in several health and wellness domains that are closely linked to the function of the gut microbiome. Therefore, not only is there a strong reason to believe these indices of physical health and wellness change the microbiome, but also that the microbiome is likely a prominent player in the function and changes that occur in this domain during adolescence.

Conclusion.

Adolescence needs to be interrogated as a sensitive period of microbiome-Gut-brain axis communication. We propose that adolescence reflects not only a period of sensitivity to alter the development of the microbiome, but also a period wherein the microbiome may drive other behavioral and neurobiological changes. As such, it is imperative that researchers incorporate the microbiome into conceptual models and measures of adolescent psychobiological development. We wish to emphasize that studying the microbiome in addition to other methodologies, rather than as a replacement for them, should provide maximal insight about adolescent development. As psychologists continue to embrace the gut microbiome as a new methodology to understand psychological development, it is important to understand the causal limitations of any one biological system on development and to avoid bold, over-simplified claims. Multidisciplinary collaborations into the several novel domains summarized here should provide excellent forays to pursue the interactive role of the microbiome-gut-brain axis within adolescent development.

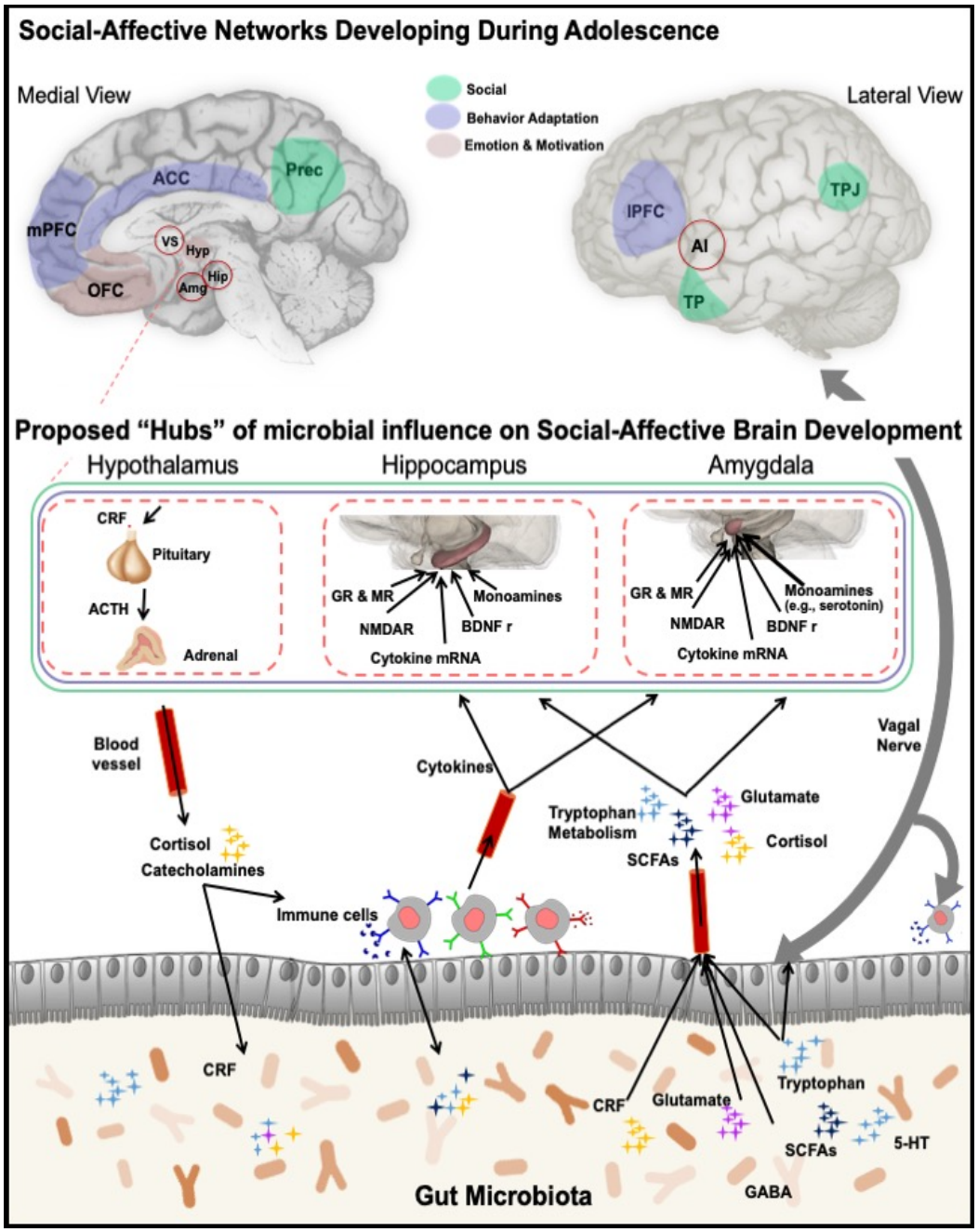

Figure 1. Proposed Critical Hubs of Microbiome-Gut-Brain Axis. Figure 1 depicts the bidirectional pathways between the gut microbiome and both the central and the peripheral nervous systems. Social, Behavioral Adaptation, and Emotion & Motivation are important networks that undergo significant restructuring during adolescence. Proposed brain "hubs" of microbial influence on social-affective brain development include the hypothalamus,

hippocampus and amygdala. Animal literature documents microbial changes influence brain structure and function through changes in MR concentrations, NMDAR, cytokine mRNA, BDNF, and monoamines, such as serotonin in the hippocampus and the amygdala. This is not intended to highlight every region that has been correlated with the gut microbiome, rather we specifically highlight brain regions implicated social-affective, explorative learning and self-processes. Note: Open circle indicates region is subcortical. CRF, corticotrophin releasing factor: ACTH, adrenocorticotrophic releasing hormone; BDNF r, Brain-derived neurotrophic factor receptors; GABA, gamma-aminobutyric acid; GR, glucocorticoid receptors; 5-HT, serotonin; SCFAs, short-chain-fatty acids. Brain regions: Hyp, hypothalamus; mPFC, medial prefrontal cortex; MR, minerocorticoid receptors; NMDAR, N-methyl-D-aspartate receptor; lPFC, lateral prefrontal cortex; AI, anterior insula; TP, temporal pole; OFC, orbital frontal cortex; TPJ, temporal parietal junction; VS, ventral striatum; Hip, hippocampus; Amg, amygdala. Figure partly adopted from Cryan & Dinan, 2012.

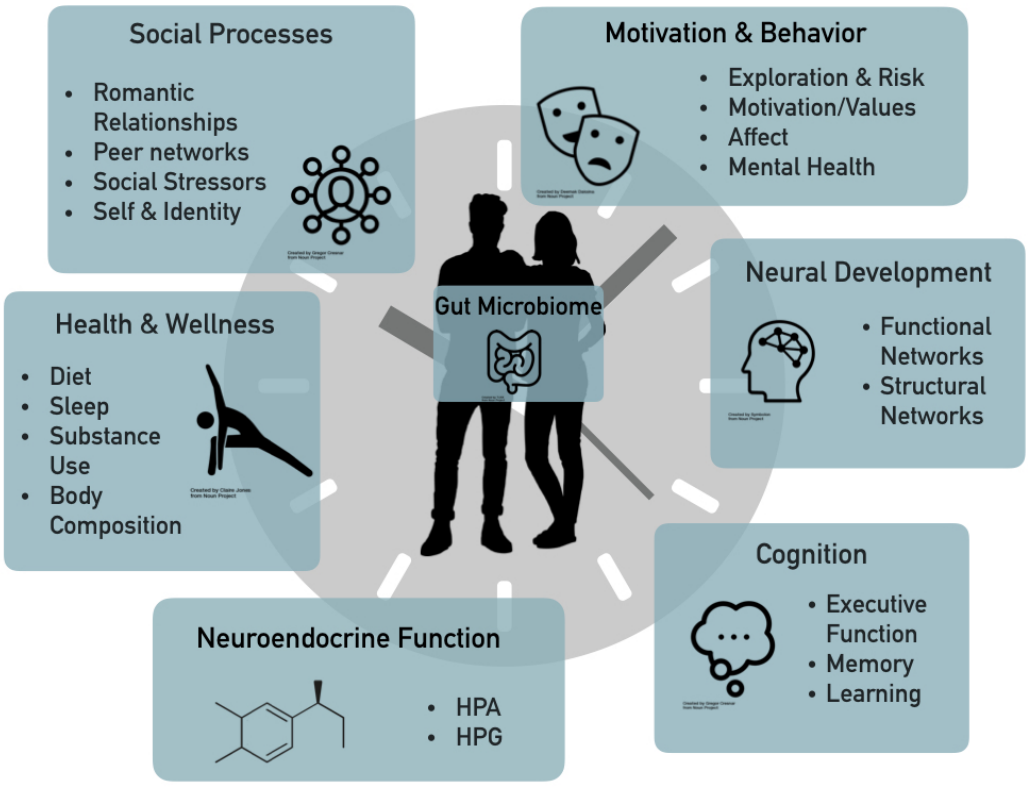

Figure 2. Developmental Timing. Figure 2 depicts the six overarching domains of change that occur during adolescence that we propose are also associated with the gut microbiome. These categories are not intended to be exhaustive, nor expected to be independent from one another, rather they are intended to highlight some fruitful directions for future interdisciplinary work.

CHAPTER II

EMIPIRICAL STUDY INTRODCUTION

Adolescence is a sensitive period of development across indices of neurobiological function, as well as a period of increased socioemotional changes, associated with heightened risk for depressive symptomatology (Crone & Dahl, 2012, Dahl et al., 2018). Despite initial evidence that the microbiome plays a critical role in brain development, socioemotional processes, and depressive disorders, to date no study has assessed these links during adolescence (Flannery, Callaghan, Sharpton, Fisher, & Pfeifer, 2019). In this study, we take the first step toward identifying links between functional brain connectivity, the functional and taxonomic composition of the gut microbiome, and depressive symptomatology during adolescence.

Functional Connectivity & Depressive Symptoms in Adolescence. The beginning of pubertal development typically defines the onset of adolescence, during which many neurobiological systems undergo a sensitive period of development, including changes in cortical-subcortical structural and functional connectivity (Sisk & Zehr, 2005). These neurodevelopmental changes are one proposed mechanism for the heightened rate of depressive symptomatology in adolescence (Andersen & Teicher, 2008).

Depression has been associated with changes in functional brain connectivity, particularly between regions associated with affective processes, such as the amygdala, subgenual anterior cingulate cortex (sgACC), and hippocampus/parahippocampus (Connolly et al., 2013; Cullen et al., 2014; Davey et al., 2015; Dichter, Gibbs, & Smoski, 2015; Pannekoek et al., 2014; Zeng et al., 2012). Amygdala connectivity plays a critical role in emotional regulation (with the sgACC; Etkin, Egner, & Kalisch, 2011; Morawetz, Bode, Baudewig, Kirilina, & Heekeren, 2016; Rey et al., 2016) and emotional memory modulation (with the hippocampus; Phelps, 2004;

Richter-Levin, 2004). However, patterns of amygdala connectivity within adolescence have been inconsistent across studies as to whether depression is associated with increased or diminished positive or negative connectivity (Connolly et al., 2013; Davey et al., 2015; Kaiser, Andrews-Hanna, Wager, & Pizzagalli, 2015; Mulders, van Eijndhoven, Schene, Beckmann, & Tendolkar, 2015). Furthermore, there are inconsistent findings as to whether adolescent depression is associated with differences in amygdala connectivity with the sgACC or the hippocampus (Cullen et al., 2014; Davey et al., 2015).

Recent studies have further highlighted the importance of network-based assessments for understanding the link between depression and brain function. Three networks most commonly implicated in depressive disorders are the salience network (SN), default mode network (DMN), and the frontoparietal network (FPN; Drysdale et al., 2017; Lydon-Staley et al., 2018; Mulders et al., 2015; Pannekoek et al., 2014). The salience network most commonly includes regions such as the anterior insula and dorsal anterior cingulate cortex (dACC), but can also include the ventral striatum, subgenual anterior cingulate cortex (sgACC), amygdala, ventral tegmentum, and substantia nigra (Menon, 2015). Together, the SN is involved in alerting the system of salient stimuli and responding (for review of SN see Menon, 2015). Adolescents with major depressive disorder (MDD), however, demonstrate decoupled or attenuated connectivity within the SN (Jacobs et al., 2016). The default mode network commonly includes regions such as the medial prefrontal cortex, posterior cingulate cortex (PCC), as well as inferior parietal lobule, and is broadly understood to support self-referential processing and episodic memory retrieval (for review of the DMN, see Raichle, 2015). Depression is associated with both hyperconnectivity and aberrant connectivity within the DMN (Figueroa et al., 2017; Mulders et al., 2015; Yan et al., 2019), as well as with altered amygdala connectivity to the DMN (Tang et al., 2018), and hypoconnectivity between the hippocampus and the posterior DMN (Figueroa et al., 2017). Together, these alterations in DMN connectivity are linked with increased cognitive reactivity

and rumination (Figueroa et al., 2017). Last, the frontoparietal network includes regions such as the dorsolateral prefrontal cortex, dorsal premotor cortex and intraparietal sulcus and is broadly understood to support cognitive control and flexibility (Marek & Dosenbach, 2018; Scolari, Seidl-Rathkopf, & Kastner, 2015). Adolescents with MDD show a pattern of hypoconnectivity within the FPN and between limbic regions, associated with decreased cognitive flexibility and increased cognitive reactivity (Sacchet et al., 2016). Together, these networks play critical roles in socioemotional and cognitive functioning during adolescence, yet also appear vulnerable to alterations in functional connectivity associated with depressive disorders (Marek, Hwang, Foran, Hallquist, & Luna, 2015; L. E. Sherman et al., 2014; Solé-Padullés et al., 2016).

Gut Microbiome & Depressive Symptoms in Adolescence. In recent years, growing attention is being paid to the role the gut microbiome may play in depressive behaviors (Dinan & Cryan, 2013). The gut microbiome resides in the gastrointestinal tract and interfaces with the enteric nervous system (ENS). Nonhuman vertebrate models have been instrumental in identifying the gut microbiome's causal role in altering central nervous system (CNS) functioning as well as its role in initiating or reversing depressive symptoms (Dinan & Cryan, 2013; Foster & McVey Neufeld, 2013). In human and animal models, interventions targeting the gut microbiome have been shown to ameliorate or reverse depressive symptomatology (Kelly et al., 2015). However, emerging evidence from rodent models indicate that adolescence is a sensitive period in development of the gut microbiome's communication with the CNS. For example, in rodents, experimental manipulations to the composition and diversity of the gut microbiome during adolescence (but not after) resulted in long term changes in depressive symptomatology (Foster, Lyte, Meyer, & Cryan, 2015; McVey Neufeld et al., 2016). Currently, the majority of human studies have largely focused on either the development of gut microbiome in early infancy and childhood (Bäckhed et al., 2015; Yatsunenko et al., 2012), or in adulthood (Lozupone,

Stombaugh, Gordon, Jansson, & Knight, 2012), with a relative dearth of studies in human adolescence, when the gut is still amenable to long-term alterations (McVey Neufeld et al., 2016).

Functional Connectivity & Gut Microbiome Connection. The ENS operates both independently, as well as in concert with the CNS (Cryan & Dinan, 2012). Communication between the ENS and CNS, known as the gut-brain-axis, has been established through the autonomic nervous system, the immune system, the hypothalamus pituitary adrenal axis, neuroendocrine systems, and metabolic pathways (Carabotti et al., 2015). The amygdala and hippocampus, two key regions involved in social-affective processing, may be two potential hubs of gut-brain axis communication (Flannery et al., 2019; Cowan et al., 2018). For example, gut-brain-axis communication has been shown to influence amygdala and hippocampal neuroendocrine gene expression, GABAergic and serotonergic signaling, synaptic plasticity and development, and brain structure (Carabotti et al., 2015; Cryan & Dinan, 2012; Labus et al., 2017). In adults, the gut microbiome has also been shown to influence both structural and functional connectivity in the brain. Following a 4-week course of probiotics, women showed altered resting state functional connectivity in regions implicated in social-affective processing, including the amygdala (Tillisch et al., 2017). Together, these findings suggest the gut microbiome may play a critical role in brain function implicated in social-affective processing, yet we know very little about these connections during adolescence; a period of increased malleability in these systems.

Based on these purported links between brain function, the gut microbiome, and depressive symptoms, we sought to test these associations in a community sample of adolescent girls through the following three aims.

Aim 1. *Quantify the Significance of Amygdala and Hippocampal Connectivity in Depressive Symptomatology during Adolescence.* This aim seeks to examine how patterns of resting state functional connectivity are associated with depressive symptomatology during an

active phase of brain development. Resting state functional connectivity measures the correlation in the time series of spontaneous fluctuations in bold oxygen level-dependent (BOLD) signal between two or more regions. The strength of these correlated time series of BOLD signal is thought to measure the integrity, or importance of these functional connections. We will specifically use a measure of amygdala and hippocampal resting state functional connectivity to subcortical regions and cortical networks implicated in depressive disorders to provide a measure of intrinsic functional connectivity. Assessing resting state functional connectivity allows us to assess the intrinsic functional integrity of brain function, thought to reflect the importance of experience, while also eliminating task-confounding variables that may otherwise restrict the generalizability of our findings. This aim is the first step toward mapping out the trajectory of change in brain-behavior relationships implicated in the emergence of adolescent depression.

Aim 2. *Characterize the Link between the Gut Microbiome and Depressive Symptomatology during Adolescence*. Research in animal models demonstrate modification of the microbiome can change depressive phenotypes (Foster & McVey Neufeld, 2013). Microbial transplants suggest that the overall composition of the microbiome may be just as important or more important than the presence/absence (i.e., abundance) of a specific microbe or gene pathway (Foster, Rinaman, & Cryan, 2017). Therefore, this aim seeks to test the gut-mood hypothesis put forth by non-human models during adolescence, by first the functional capacity and taxonomic composition of the microbiome, rather than specific functional pathways or microbes. We will test this hypothesis by identifying if adolescents with increased depressive symptomatology have more similar patterns of gut microbial taxonomic structure and functional potential. Using a microbial metagenomic approach will allow us to identify not only which bacteria are present, but understand their functional capacity. Testing this hypothesis during adolescence is particularly significant for clinical translation because the animal studies suggest the adolescent gut

microbiome is still sensitive to long-term intervention effects (Descont et al., 2015), and could provide multiple novel entry points for depression-focused prevention and intervention efforts.

Aim 3. *Explore the relationship between the Gut Microbiome in Amygdala and Hippocampal Connectivity During Early Adolescence.* Despite the established connections between the gut microbiome and specific measures of the CNS, such as neurotransmitters, BDNF, and exploratory correlations with brain structure (Borre et al., 2014; Dinan & Cryan, 2013), and functional brain connectivity in adults (Tillisch et a., 2013; Tillisch et al., 2017), it's unclear how these features may also manifest in patterns of the strength or integrity of the functional connections underlying social and emotional processing during adolescence. This is the necessary next step in understanding how the gut and brain communicate during this sensitive period of development in which experiences, both internal and external to the individual, can have long term implications on the structural and functional integrity of brain connectivity.

The objective of this aim is to identify the relationship between the taxonomic and functional composition of the gut microbiome and functional brain connectivity, specifically between brain regions that have previously been implicated in depression and also shown to be altered by the gut microbiome. Both patterns of resting state functional connectivity and the composition of the gut microbiome are influenced by developmental maturation and experience and presumed to reflect the importance of both internal and external experiences (Stevens, Pearlson, & Calhoun, 2009). There is growing evidence that the functional capacity of the microbiome may provide a greater mechanistic insight into gut-brain axis communication than the taxonomic composition (Sharpton et al., 2014). Therefore, as the first step toward understanding how the gut and brain communicate during adolescence, we used resting state functional connectivity as a task-independent measure of intrinsic functional activity and assessed both the taxonomic and functional composition of the microbiome. Given adolescence is a sensitive period of change in social-affective processing and emergence of depressive disorders,

it's imperative we understand how the gut and brain may communicate to influences the development of these processes.

By taking a multi-modal approach, this study will seek to bridge the two fields of microbiology and developmental cognitive neuroscience, which have until recently operated independently when it comes to understanding mental health outcomes in development. Using task-independent measures of the gut microbiome and functional connectivity is a particularly important first step toward this goal because it provides a foundation to assess how experience and developmental maturation may influence communication between gut-brain axis, independent of task-specific responses. Furthermore, this study represents the first step to fill the missing gap in our understanding of when and how the gut and brain communicate to influence developmental outcomes. This will advance the field forward in its mapping of human sensitive periods in the relationship between the gut microbiome and brain function.

CHAPTER III

METHODS

Participants.

A community age-cohort of female adolescents (ages 11.5-14.5 years old) were sampled from the second wave of the ongoing longitudinal study called the Transitions in Adolescent Girls (TAG) project (for study protocol paper, see Barendse et al., 2019). This study was strategically designed to only include females. This decision allowed us to decrease heterogeneity within our sample and increase the reliability of our findings based on well-documented sex differences in the onset of depression, prevalence rates of depression, and rates of brain maturation in early adolescence (Nolen-Hoeksema & Girgus, 1994; Vijayakumar et al., 2018). The majority of participants were recruited from primary and middle schools in the region, and a small subset were recruited by other methods (e.g., advertising at science fairs, Craigslist). Inclusionary criteria consisted of i) fluency in English, ii) no developmental disabilities (aside from Attention Deficit/Hyperactivity Disorders), iii) no diagnosis of psychotic disorders, iv) no MRI contraindications, v) enrollment in 4th to 6th grade, and vi) an upper age limit of 13.0 years at entry into the study.

We received IRB approval for gut microbial collection and completed collection on 80 adolescent girls for a sub-study of the larger TAG project called the "Gut Mini Study." This study was conducted during active recruitment of wave 2 participants. The option to participate in the Gut Mini Study was offered to every participant that participated in Wave 2 of the longitudinal study. Although Wave 2 is still underway, data collection for my dissertation ended on September 30th, 2018 to provide time for me to complete analyses and write up dissertation for a May 2019 defense. We also had monetary constraints for the gut microbial sample; we had funds allocated

for 80 stool samples to be sequenced for metagenomics, which provides both taxonomic and functionally annotated microbial profiles.

As of September 30th, 2018, 108 participants had completed wave 2 of the longitudinal study. Of the 108 girls, 90 (83%) consented to participate in the Gut Mini Study. Of the 90 girls that consented to the Gut Mini Study, 80 (89%) of girls successfully completed a stool sample. Of the 10 girls that did not complete the sample, only one did not complete due to reported discomfort with collecting a sample. Of the 80 girls with completed stool samples, 9 (11%) did not complete a MRI scan. Of the 108 girls, 94 (87%) completed a MRI scan. Of the 94 girls that completed a MRI scan, 22 (24%) did not complete the Gut Mini Study (14 declined participation; 8 did not complete the stool sample; see Table 1).

Of the 80 participants who completed the Gut Mini-Study, 71 (89%) girls had an MRI scan, but 15 (19%) failed quality control (QC; details listed below). Ten additional scans failed at various steps in our resting state preprocessing pipeline. These errors are being actively investigated to hopefully be included prior to publication. This resulted in three sample sizes for our study: 64 for our resting state fMRI-mood aim; 80 for our gut microbiome-mood aim; and 46 for our resting state fMRI and gut microbiome aim (see Table 1 for details).

Age range. Age was calculated as the difference between the date of Wave 2, Session 2 (date of their MRI scan) and their date of birth. The age range of the Gut Mini Study sample (n = 80) at wave 2 was 11.91-14.66 years old. This tight age range within wave cohort was designed to prospectively follow adolescent girls through pubertal development and the onset of mental health problems as part of the longitudinal study. For this study, we sought to assess a snapshot of these concurrent relationships as the first step toward assessing longitudinal changes. Since the Gut Mini Study occurred at wave 2, this sample was not designed or able to capture early adolescence or early pubertal development. Therefore, age and pubertal stage were not primary

questions within this study. Nevertheless, we assessed the role of age and pubertal stage within the current study in order to characterize their role within our specific aims.

Table 1. Sample Size by Study Completion.

Gut Mini Study Completion	N	%
Total W2S2 Participants	108	
Consent to Gut Study	90	83%
Completed Stool Sample*	80	89%
Completed Stool, but not Scan*	9	11%
Completed Scan	94	87%
Completed Scan, but not Stool	22	24%
Overlap Completed Stool-Scan	71	89%
Scans Eliminated	15	19%
Scans Failed PPC	10	11%
Current Usable Stool-Scan Overlap	46	58%

*Of the 10 incomplete samples, only one participant declined after consent due to the procedure: "tried but felt weird." Three had extenuating family circumstances; two were lost/untracked after reported completion; and four required consistent follow ups with intention to complete but 2 months elapsed and participants did not follow through. Of the 9 girls that completed the stool sample, but did not complete a scan, 7 girls had braces or another medical reason they could not scan, and two opted out of the scan portion of the study.

As part of the larger longitudinal study, pubertal development was assessed through parent and self-report, as well as hormones. For the purposes of this study, we focused on self-reported pubertal development, as measured by the Pubertal Development Scale (PDS; (Petersen, Crockett, Richards, & Boxer, 1988) and the Picture-Based Interview about Puberty (PBIP; Morris & Udry, 1980). PDS scores were transformed to a 5-point scale to map onto Tanner Stages (Shirtcliff, Dahl, & Pollak, 2009). By Wave 2, 60/80 girls had started menstruation and were in late pubertal development (see Table 2).

Table 2. Development Descriptives.

	Age	PDS score	PBIP Score
Mean (SD)	13.52 (.64)	4.06 (.98)	3.80 (.74)
Sample Range	11.91 - 14.66	1 - 5	1.5 - 5

PDS = Pubertal Development Scale; PBIP= Picture-Based Interview about Puberty

Study Procedure.

All families consented to the nature of the longitudinal project at wave 1 and families and participants provided written re-consent at wave 2 (see Table 3). Each wave of data collection consisted of two visits. At the first visit, participants were re-screened for MRI eligibility as per procedures determined by the University of Oregon's Lewis Center for Neuroimaging (lcni.uoregon.edu). After screening, the parent/guardian and participant completed questionnaires and semi-structured interviews regarding current and past mental health history. During the second visit, scheduled approximately one month from the first visit, participants completed the MRI portion of the study as well as additional questionnaires and behavioral tasks. Participants were compensated for their time, and all materials and procedures were approved by the Institutional Review Board at the University of Oregon.

Table 3. Study Design.

Wave 2, Visit 1	At Home	Wave 2 Visit 2
Parent Questionnaires	Stool Sample	Return Stool Sample
Participant Questionnaires	Gut Questionnaires	fMRI scan
Diagnostic Interview		Height/Weight

Gut Microbiome. At wave 2, visit 1, participants were provided the option to consent to a stool sample and parent and self-report questionnaires for an additional monetary compensation ($50). We followed the same stool collection procedures that we successfully implemented in a

prior study with 40 children (Flannery, Stagaman, et al., 2019). Briefly, consenting participants were provided with OmnigeneGUT kits, which have shown to reliably provide usable data and limited burden on the individual to collect (DNA Genotek Inc, 2017). In addition, these kits allow samples to remain at room temperature for up to 160 days (DNA Genotek Inc, 2017), providing ample time to transfer samples to our laboratory, which they were then stored in a -80 C freezer until they were transferred over dry ice to the Sharpton Laboratory at Oregon State University for sample preparation and sequencing. We provided verbal and written instructions for the stool collection, as well as a video explanation (for video, see https://dsn.uoregon.edu/gut_instructions). Parent/guardian and participants also completed questionnaires regarding history of variables known to be associated with alterations in the gut microbiome such as delivery method, prenatal and postnatal development, timeline of anti/pre/probiotic use, and dietary habits. Participants had the opportunity to either mail the samples into the laboratory or bring the sample into visit 2. Stool samples were then delivered to The Sharpton Laboratory at Oregon State University. Participants were instructed to complete the stool sample as close to the scan data as possible. We sent emails to remind families to complete the stool sample within a week of the MRI scan, preferably the day of or day before the scan.

Resting State Functional Connectivity. At wave 2, visit 2, participants returned to the Lewis Center for Neuroimaging (LCNI) for their magnetic resonance imaging (MRI) scan. Two resting state functional MRI (rsfMRI) scans were completed interleaved between a task-based fMRI scan (not included for this study) and following a MP-RAGE for a structural T1 weighted image. A fixation cross was displayed, but participants were instructed to stay still with their eyes closed. This was verified by pupil monitor in the MRI control room. Details of the scan sequences are as follows i) MP-RAGE structural scan: TR = 2500ms, TE = 3.41ms, flip angle = 7, FOV = 256mm, and multi-slice mode = single shot; ii) two rsfMRI scans: TR = 780ms, TE = 32ms, flip

angle = 55, FOV = 210mm, multiband accelerator factor = 6, slice = 60 continuous, and 2.5mm slices.

Preprocessing.

Resting State Functional Connectivity Preprocessing. Raw DICOM image files were converted to the NifTI format with MRIConvert and organized according to the Brain Imaging Data Structure (BIDS) standards that facilitates the use of portable analysis tools called BIDS Apps (https://bids.neuroimaging.io) using the following scripts by DeStasio, K.L., May, E., & Cosme, D., 2018 (https://github.com/kdestasio/bidsQC/tree/v0.3).

Structural Preprocessing. Anatomical scan were processed through the FreeSurfer 6.0 image analysis suite (http://surfer.nmr.mgh.harvard.edu/). Details of motion correction, tissues segmentation, and surface reconstruction are detailed below, as per Freesurfer's method citation resource (https://surfer.nmr.mgh.harvard.edu/fswiki/FreeSurferMethodsCitation). The technical details of these procedures are described in prior publications (Dale, Fischl, & Sereno, 1999; Fischl & Dale, 2000; Fischl, Liu, & Dale, 2001; Fischl et al., 2002; Fischl, Salat, et al., 2004; Fischl, Sereno, & Dale, 1999; Fischl, Sereno, Tootell, & Dale, 1999; Fischl, van der Kouwe, et al., 2004; Jovicich et al., 2006; Reuter, Rosas, & Fischl, 2010; Reuter, Schmansky, Rosas, & Fischl, 2012; Segonne, Pacheco, & Fischl, 2007). Briefly, this processing includes motion correction and averaging (Reuter et al., 2010) of multiple volumetric T1 weighted images (when more than one is available), removal of non-brain tissue using a hybrid watershed/surface deformation procedure (Segonne et al., 2004), automated Talairach transformation, segmentation of the subcortical white matter and deep gray matter volumetric structures (including hippocampus, amygdala, caudate, putamen, ventricles; Fischl et al., 2002; Fischl et al., 2004) intensity normalization (Sled, Zijdenbos, & Evans, 1998), tessellation of the gray matter white matter boundary, automated topology correction (Fischl, Liu, & Dale, 2001; Segonne, Pacheco, &

Fischl, 2007), and surface deformation following intensity gradients to optimally place the gray/white and gray/cerebrospinal fluid borders at the location where the greatest shift in intensity defines the transition to the other tissue class (Dale, Fischl, & Sereno, 1999; Fischl & Dale, 2000). This method uses both intensity and continuity information from the entire three dimensional MR volume in segmentation and deformation procedures to produce representations of cortical thickness, calculated as the closest distance from the gray/white boundary to the gray/CSF boundary at each vertex on the tessellated surface (Fischl & Dale, 2000). The maps are created using spatial intensity gradients across tissue classes and are therefore not simply reliant on absolute signal intensity.

Structural Quality Control. Structural scans were individually coded and rated on a score of 1-3 for motion and surface reconstruction/ boundaries (white and pial surfaces). Scans scored as a 3 for either motion or for cortical reconstruction were excluded. Briefly, a score of a 3 included evidence of either gross motion disturbances (e.g., ringing throughout brain; ghosting etc.) or missing/inaccurate white/pial surface boundaries.

Specifically, motion rating criteria included: 1 = no evidence of motion (clear image, no ringing or blur); 2 = slight motion (some evidence of blur or ringing but constrained to only a few volumes or few areas); 3 = obvious motion (gross disturbances as evident by ghosting, blurred image or ringing throughout the brain that was evident in several volumes or across extensive areas). Surface reconstruction/ boundaries rating criteria included: 1 = corresponded to perfect (white/pial segmentation appropriately defined throughout the brain); 2 = okay (overall good, but a few areas are more poorly defined); 3 = bad (missing or inaccurate surface boundaries). If structural abnormality was deemed benign and did not interfere with cortex, participants were retained (e.g., abnormality in cerebellum structure). This occurred for one participant with a benign mega cisterna magna and pineal cyst. Based on predetermined criteria, this participant was

retained (see Flannery, 2018). A score of 3 in structural quality control resulted in the exclusion of that participant from future analyses.

Following structural preprocessing and quality control, functional processing was performed in AFNI (https://afni.nimh.nih.gov). Anatomical scans that passed structural quality control were run through SUMA to convert surface and volume images to an AFNI compatible format (NIFTI) and Freesurfer automated structural segmentations were labeled.

Functional preprocessing. Steady state was established by removal of the first 5 TRs of each run. Outliers were calculated if > 10% of voxels within a volume were outliers using 3dtoutcount, using the trend and mean absolute deviation (MAD) of each timeseries. Volumes were then registered to the minimal outlier image within the first run, and short, spike-like artefacts due to sudden motion were removed using 3dDespike. Participant's functional volumes were aligned to her T1 image in standard space. Voxels were spatially smoothed to a 2mm full-width at half-maximum Gaussian kernal. Functional images were then normalized to percent signal change (scaled to 200%).

Following tissue segmentation in Freesurfer, white matter and ventricle masks were created and eroded. Nuisance regressors were then created for the localized timeseries of white matter fit over a gaussian curve over a 30mm radius using ANATICOR fast, average time series of the ventricles, and 12 motion regressors per run, which include 6 rigid body corrections (x, y, z, pitch, roll, yaw) and their derivatives. For each time point in the functional series, the square root of the sum of squares across the six motion derivatives (first difference) was calculated with AFNI's enorm function to estimate motion between time points. We censored any time point with greater than 0.2 enorm value as well as the time point preceding, and any volume with more than 10% of voxel outliers. Motion was also censored if >10% of voxels within a volume were outliers. Given our rapid TR (780ms), our bandpass filter was set to a range of .009 to .2 Hz and regressed alongside our nuisance regressors.

Functional Quality Control. Aspects of the functional image processing were individually checked for quality control. To ensure white matter and ventricles were accurately masked, the structural segmentation regions of interest (ROIs; white matter and ventricle) that were created during our functional processing pipeline were visually inspected against the processed anatomical scan for each participant. Structural segmentations ROIs (ventricles and white matter) created in Freesurfer, were coded 0 or 1 as pass/no pass. A score of 1 (no pass) included gross inaccurate coverage (either not enough coverage, or coverage extending into gray matter).

Next, the processed functional image (echo planar image; epi) was visually inspected for proper functional registration to the anatomical scan; field of view (FOV) errors; and functional drop out. A score of 1 (no pass) included inaccurate registration (i.e., epi not aligned to anatomical, epi inflated above anatomical, epi smaller than anatomical, or epi demonstrating abnormal coverage extending outside of brain, such as horns, FOV cut off, or ghosting). A certain degree of drop out is to be expected along edges. If this drop out was extensive, resulting in lack of coverage of an entire region (e.g., medial temporal lobes; medial prefrontal cortex or medial orbitofrontal cortex) it was coded as 1 (no pass). Quality control occurred after motion correction; however, visual inspection was used to confirm we did not see other abnormalities in the functional images (e.g., motion stripes or spikes) and marked as 1 (no pass), accordingly. A score of 1 in functional quality control resulted in exclusion of the participant from future analyses.

Following rigorous structural and functional quality control (QC) measures, 6 participants' scans failed due to excessive motion; 3 failed structural QC; 2 failed functional segmentation QC; 2 failed functional to structural alignment QC (poor functional coverage). In addition, two more participants were excluded: 1 due to a dicom error (unable to retrieve data from scanner) and 1 due to insufficient scan data (only completed one resting state fMRI run). Together, 15 participants were excluded.

Regions of Interest (ROIs). Cortical volumes were extracted based on the Gordon Parcellation (Gordon et al., 2016). The Gordon parcellation includes 333 cortical functionally defined resting-state ROIs that correspond to different functional networks (e.g., salience network, default mode network, frontoparietal network etc.). We chose a parcellation approach, rather than a whole brain voxel-based approach because parcels reduce the number of tests run across the brain, while affording greater signal to noise ratios within selected functional regions or networks of interest. Although there are several parcellation maps, we chose the Gordon parcellation because it was created based on homogeneity boundaries in functional signal, specifically within resting state fMRI and it was shown to have increased homogeneity within parcels, as compared to other parcellation maps (Gordon et al., 2016). Subcortical regions of interest (i.e., amygdala, hippocampus, subgenual anterior cingulate cortex (sgACC), caudate, accumbens) were anatomically defined in Freesurfer. Caudate and accumbens were added in accordance to prior methods (Drysdale et al., 2017), as these regions are implicated in depression and salience-affective networks, but are not captured in the cortical parcellation.

Timeseries were averaged across volumes within region for the Freesurfer subcortical anatomical segmentations and for the Gordon cortical parcels. Timeseries correlations were only conducted on scans that had a minimum of 5 minutes of resting state fMRI data. Extracted timecourses were then used to calculate correlations between each set of ROIs, which were then Fisher-z transformed. Seed-to-subcortical connections of interest (COIs) included bilateral amygdala-hippocampal, and amygdala and hippocampus to sgACC, caudate, and accumbens. Seed-to-network COIs included bilateral amygdala and hippocampus to the Gordon parcels that belonged to either the salience, default mode, or frontoparietal networks.

The scope of seed-to-seed analyses was changed based on prior literature (see Flannery, 2018). *This decision was made prior to conducting any ROI analyses.* COIs still included bilateral amygdala-hippocampal and bilateral amygdala-sgACC connectivity, but they were

expanded to include bilateral caudate and accumbens, as well as three networks: the salience network, default mode network, and frontoparietal network. As detailed in Chapter 2, this decision was based on the prominent role of these three networks in socioemotional processes and their putative association with depression.

We chose our resting state functional connectivity measures based on two reasons. First, our seeds were chosen based on their aforementioned association with the gut microbiome. As discussed in further detail in Chapter 1, the amygdala and hippocampus may serve as "hubs" for gut-brain axis communication (Flannery, Callaghan, Sharpton, Fisher, & Pfeifer, 2019). This study will not be able to disentangle the mechanism of connection (e.g., if links were due to changes in gene expression within the amygdala or hippocampus). Instead, an association would be consistent with animal work that found the gut microbiome is associated with molecular and structural changes in these brain regions. It would further extend these findings to link the gut microbiome with the functional connectivity of these regions during human adolescence.

Second, we chose to look at amygdala and hippocampal connectivity with regions and networks that have been previously implicated in socioemotional processing and associated with alterations in functional connectivity for clinically depressed samples (Mulders et al., 2015). Although a comprehensive network-based approach was beyond the scope of the current study, we chose to focus on the three networks commonly linked to alterations with depressive disorders: the salience network, the default mode network, and the frontoparietal network (Drysdale et al., 2017; Lydon-Staley et al., 2018; Mulders, van Eijndhoven, Schene, Beckmann, & Tendolkar, 2015). In addition to their known associations with depression, these networks are undergoing significant functional changes during adolescence and are associated with several socioemotional processes (e.g., affective reactivity, self-referential processing, affective regulation, and cognitive flexibility). Therefore, if we were to see changes in functional connectivity between our seeds (amygdala and/or hippocampus) and these subcortical regions or

networks, it would suggest that the gut microbiome may not only be associated with functional brain connectivity during adolescence, but may also be specifically associated with aspects of functional connectivity that are still developing during adolescence (Marek et al., 2015; L. E. Sherman et al., 2014; Solé-Padullés et al., 2016).

To further reduce the number of features in our models, subcortical ROIs were averaged bilaterally and cortical ROIs were averaged within networks. Bilateral ROIs were calculated based on the correlation of the Fisher-transformed correlations. Average network connectivity was calculated based on the average correlation of the Fisher-transformed correlations between ROIs that belonged to that network. This resulted in the following 12 COIs: amygdala-hippocampus; amygdala-sgACC; amygdala-caudate; amygdala-accumbens; hippocampus-sgACC; hippocampus-caudate; hippocampus-accumbens; amygdala-salience network (SN); amygdala-default mode network (DMN); amygdala-frontoparietal network (FPN); hippocampus-SN; hippocampus-DMN; hippocampus-FPN.

Microbial DNA Sequence Data Generation and Analysis. Following storage in a -80 freezer at the LCNI, samples were transferred to The Sharpton Laboratory at Oregon State University. Upon receipt, samples were processed using the Center for Genome Research and Biocomputing core facility to prepare NexteraXT libraries for shotgun DNA sequencing (Sharpton, 2014). The CGRB's Illumina HiSeq 3000 sequenced metagenomes at a depth of 20M reads per sample. Samples were sequenced as a group to mitigate batch effects. Our pipeline applied the Human Microbiome Project quality standards, including adapter trimming, sequence quality filtering, and removal of DNA sequences that are highly similar to the human genome (Sharpton, 2014). DNA was extracted using the QIAGEN PowerFecal DNA extraction kit following Earth Microbiome Project guidelines (Earth Microbiome Project, 2018). Metagenomes were functionally and taxonomically annotated, which quantified the presence and relative abundance of the protein families and metabolic pathways that are encoded in the genomes of the

taxa that comprise the microbiome using HUMAnN2 (Franzosa et al., 2018). Please see (Flannery, 2018) for the timestamp change from Shotmap to HUMAnN2.

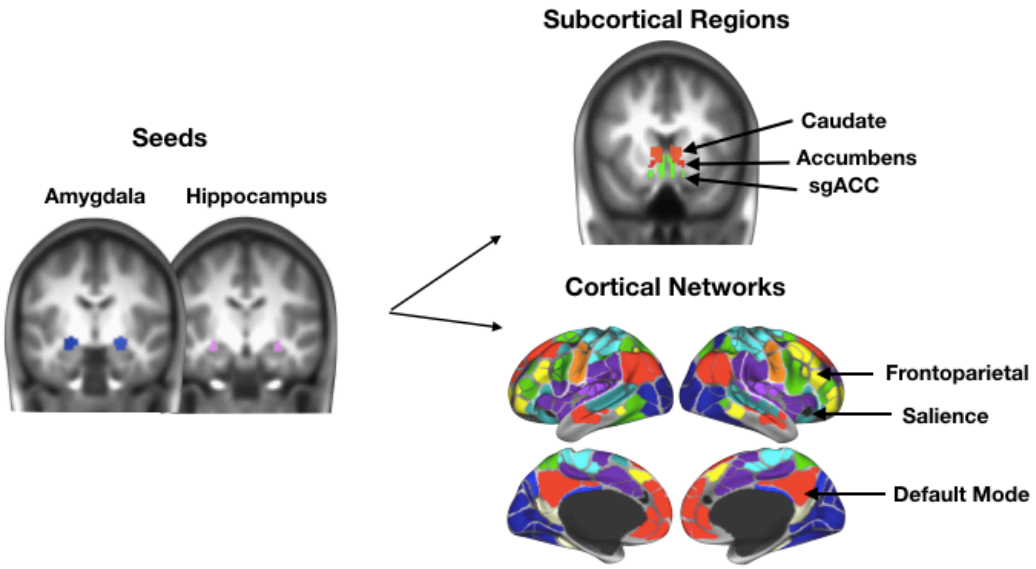

Figure 3. Connections of Interest. Amygdala and hippocampal seeds were anatomically defined in Freesurfer. Subcortical ROIs. Subcortical regions of interest were anatomically defined in Freesurfer and included bilateral caudate, accumbens, and subgenual anterior cingulate cortex. Cortical networks represent the Gordon parcellation. Image adapted from Gordon et al., 2016. Black = parcels belonging to the salience network. Red = parcels belonging to the default mode network. Yellow = parcels belonging to the frontoparietal network. For the purpose of this study, no other networks were examined.

Measurements.

All metadata were assessed for normality prior to assessing correlations or running statistical tests. Any variables with Skew > 2 and/or Kurtosis > 3 were transformed. Due to the different sample sizes across aims, variable distributions were examined within each dataframe.

Mood. Mood was assessed via self-report questionnaires and a semi-structured interview. Due to the high comorbidity of depression and anxiety, we assessed the correlation between depressed mood, anxiety symptoms, and internalizing diagnosis.

We used the Center for Epidemiologic Studies Depression Scale (CES-DC; Shahid, Wilkinson, Marcu, & Shapiro, 2011) as a continuous measure of depressive symptomatology. The CES-DC scores range from 0-60, with a score of 15 or above indicating clinically significant depressive symptomatology. Therefore, in addition to assessing CES-DC symptom score continuously, we also assessed if grouping participants by evidence of depressive symptomatology (score of 15 or higher) or no (score less than 15) correlated with clinical diagnosis of an internalizing disorder. To measure diagnoses, we also administered the Kiddie Semi-Structured Interview of Affective Disorders and Schizophrenia (KSADS; Kaufman et al., 1997) diagnostic interview at all timepoints, including wave 2. Using the Hierarchical Taxonomy of Psychopathology (HiTOP) approach; Kotov et al., 2017), diagnoses were coded for current internalizing disorder versus no disorder. The HiTOP approaches uses a factor approach to account for similar underlying symptomatology and comorbidity between diagnoses (Kotov et al., 2017). Measures of depressive symptoms were also compared against self-reports anxiety from the Screen for Child Anxiety Related Emotional Disorder (SCARED; Birmaher et al., 1997).

Based on the contingency plan for distribution in my preregistration, we did not have enough variability in our depressive symptoms to appropriately test our original depression-behavioral aims. Therefore, depression symptoms were determined by the committee to be looked at as secondary aims. Notably, while prior studies have demonstrated the promise of assessing individual symptomatology (versus global diagnosis or symptom count) with brain function, we did not have the sample size to appropriately statistically model this question (see Xia et al., 2018).

Covariates.

Diet. Typical and average diet information was collected for each participant following their stool sample; the questionnaire was adapted from the Eating Habits Questionnaire (Graham, 2005). This included type and frequency of common food categories participants consumed in the last week prior to stool collection. For each food or drink category, participants were also asked if their diet differed from their typical diet. In addition, participants were asked to categorize their type of diet as raw vegan; vegan; vegetarian; pescatarian; pollo-pescatarian; pollotarian; carnivore/omnivore; or other. For the purposes of this study question, we focused solely on diet categories. Diet category was self-identified. A description of each diet category was provided next to each item to eliminate inconsistency in participant's operational definition of these categories. For example, an item was present as follow: "Vegetarian (e.g., excludes all meat, but includes dairy products, such as eggs, milk, and cheese)."

BMI. Body mass index (BMI) was calculated as 703*(weight/(height_inches^2) (World Health Organization, 2019). As described in the TAG study protocol paper (Barendse et al., 2019), height and weight were obtained in lab during their wave 2, visit 2. Height and weight were measured by a staff member at least twice at the beginning and at the end of the session; height or weight was measured three times, if there was discrepancy between measurements. Height was measured using a stadiometer and recorded in centimeters. Weight was measured using a mechanical column scale with eye level beam and recorded in pounds. Participants were asked to remove shoes, coats, or heavy clothing items prior to weight measurement. BMI calculation used the average of each individual's height and weight values.

Probiotics. Probiotic use was identified by self and parent report. Regular probiotic use was only coded as "yes" if parent reported their daughter had probiotics weekly and this occurred within the last month. In self-reported probiotic use, several participants reported regular probiotic use; however, their reported description of probiotic use included "gogurt" and other

non-probiotic containing foods. Parent-report of participant's regular probiotic use was more consistent with expected probiotics, including cultured yogurt, keifer, kimchi, probiotics pills, and fermented vegetables. Therefore, parent's report of probiotic use was used for future analyses.

Gut Health Concerns. Gut health concerns were coded "yes" if a parent reported the participant experienced any of the following conditions: Crohn's disease, irritable bowel syndrome, gastroesophageal reflux disease (GERD), gallstones, celiac disease, ulcerative colitis, hemorrhoids, diverticulitis, or anal fissures. Importantly, these were not confirmed with medical reports and therefore should not be considered a medical diagnosis.

Recent Gastrointestinal (GI) Concerns. Recent GI concerns were coded "yes" for self-reported GI complaint within the last 7 days. Chronic constipation or chronic diarrhea within the last 14 days were additionally coded separately. Bristol Stool Chart was collected at time of stool sample. Scores of 1 (indication of severe dehydration) or a score of 7 (indicative of severe diarrhea) were coded to assess potential illness at time of sample collection.

Allergies. Allergies were self-reported. Self-reported description of allergies ranged from gastrointestinal (GI), food, to seasonal allergies. Any type of allergy was coded as "yes."

Menstruating last 7 days. Menstruating within the last 7 days was self-reported in relation to the day of stool sample collection.

Sick. Recent sickness was self-defined as feeling sick within the last 2 weeks.

Antibiotics. All participants were instructed to wait to take their sample until they were healthy and at least 2 weeks following the use of antibiotics to ensure we had a measure of their typical stool sample. This information was confirmed in parent and self-reported questionnaires.

Taxonomic and Functional Composition.

Dissimilarity index. Due to the multivariate nature of microbial data (i.e., more than one variable per observation), Bray-Curtis dissimilarity was quantified to define both the taxonomic

and functional beta-diversity between metagenomes. Bray-Curtis dissimilarity index, also known as the Steinhaus dissimilarity or Sørensen index, is an ecological approach to quantify the rank order similarity between samples for multivariate data (Junker, 2018). A distance of 0 indicates identical samples, where a score of 1 indicates orthogonal samples.

Ordination method. Ordination were primarily used to visualize patterns present within the dissimilarity/distance matrix by reducing the number of features visualized. Axis 1 corresponds to the component that explained most variance in the data, where Axis 2 corresponds to the component that explains the second largest amount of variance in the data. There are different ordination methods that are preferred to identify patterns, based on the type of multivariate data (For review, see Ramette, 2007).

We generated a principal coordinate analysis (PCoA), a linear ordination method, based on the Bray-Curtis dissimilarities for both the functional and taxonomic communities using the *ordinate* function from the phyloseq package (McMurdie & Holmes, 2013). In order to determine the variance in microbiome composition (functional and taxonomic) explained by our variables of interest, we used a constrained correspondence analysis (CCA; cca function from the vegan package; Oksanen, 2016). CCA allowed us to quantify the amount of variance explained by our variables of interest, after first accounting for the variance in microbiome composition explained by gut-related covariates, which might otherwise confound our analysis (For information on this strategy, see Oksanen, 2015).

Covariate Reduction. We applied a data reduction technique to minimize the number of covariates considered in our subsequent analyses. This process is important to reduce the potential for model overfit, given the large number of covariates relative to the number of participants in our study. Using the *envfit* function from the vegan package (Oksanen, 2016), we generated a PCoA ordination based on the Bray-Curtis dissimilarities for both the functional and taxonomic communities to identify covariates that explained a significant amount of variation

across individuals. Because *envfit* is sensitive to ordination method, *envfit* results were compared across ordination methods and any significant covariates across ordination methods were included as control variables using the 'Condition' function in our subsequent CCA model.

Significance testing. To assess the relationship between the functional and taxonomic communities and resting state functional (rsFC) connections of interest, all 12 connections of interest (COIs) were put into the CCA model, in addition to the control variables. This CCA object was then subjected to permutation model selection based on the Akaike Information Criterion (AIC) by stepwise addition and subtraction of terms (*ordistep* function; Oksanen, 2016).

The model selected by this method was then analyzed using a permutation ANOVA (PERMANOVA; Anderson, 2017) to determine if there were significant associations between rsFC and the microbiome. This was done for both the functional composition and the taxonomic composition. The same CCA approach was also implemented to assessing taxonomic and functional associations with mood and age.

Resting State Functional Connectivity and Mood and Age Relationships.

Last, we assessed rsFC's relationship with mood and age. Given the prior link between mood and age, we sought to test if rsFC explained additional variance in mood, after accounting for age. However, due to our restricted age range, age may not be the best predictor; therefore, rsFC models were also compared against the null model, without age in the model.

To assess differences in depressive symptomatology, using the "lm" function 'stats' version 3.6.0. To assess differences in internalizing diagnosis as a function of age and rsFC, we fit linear models using the function 'glm' with family set to 'binomial' from the package 'stats' version 3.6.0 that was run in R (R Core Team, 2017). Age was mean-centered (13.51) in all models to facilitate model convergence and intercept interpretation. Age was first tested against the null model. Where age was deemed a better fit above the null, nested model comparison was

assessed by Akaike Information Criterion (AIC). Model selection was determined by lowest AIC value that was significantly different ($p < .05$), as determined from likelihood ratio (LR) tests using 'lrtest' from the 'lmtest' package, version 0.9-36. When age was not deemed a better model above the null, in addition to nested model comparison, rsFC models were also tested against the null to ensure we did not miss a relationship due to poor model fit with age.

Null model: $y = \beta 0 + 1$

Age model: $y = \beta 0 + \beta 1(age)$

rsFC *model:* $y = \beta 0 + \beta 2(coi)$

rsFC *Age Model:* $y = \beta 0 + \beta 1(age) + \beta 2(coi)$

Interaction model: $y = \beta 0 + \beta 1(age) * \beta 2(coi)$

y represents depressive symptomatology or internalizing diagnosis, $\beta 0$ represents the intercept, $\beta 1, \beta 2$represent regression coefficients.

Deviations from Preregistration.

Preregistration details were updated and timestamped to Open Science Framework (OSF) throughout the project. Nevertheless, there were a few deviations from my preregistration that did not get updated online. In Chapter 6, I discuss in more detail some of the difficulties of preregistering this type of study. Here I list the changes and omissions to the preregistration.

First, the Gordon parcellation was used instead of the Power parcellation. This was decided following structural and functioning preprocessing, but prior to extracting timeseries. The Gordon parcellation was preferred because it was designed to account for the heterogeneity with resting state functional MRI scans. The Power parcellation (which uses sphere-defined parcels; for details, see Power et al., 2011) will also be used in subsequent analyses to compare the difference between these parcellation maps. This will ensure are results are not confounded by

parcellation segmentation. Furthermore, it will allow us to directly compare finding to prior studies that used the Power parcellation.

Second, it was decided that specific taxonomic microbes and functional pathway associations with amygdala and hippocampal rsFC was beyond the scope of the current dissertation and will be pursued at a later date. A few methods of combining the data, including Hierarchical All-against-All significance testing (HAIIA; http://huttenhower.sph.harvard.edu/halla) and FDR corrected spearman correlations were attempted, but after further discussion with committee members, it was determined we need a more thorough investigation into the optimal method to accurately accounts for each type of data before we can feel confident in our findings. These findings, therefore, are not included in the current dissertation, but will be discussed in future publications. Therefore, the focus of this study is at the level of taxonomic and functional composition.

Third, due to limited variability in depressive disorders within our sample, we used HiTOP classification of internalizing disorders to compare depressive symptomatology against diagnoses. These analyses were always reported alongside depressive symptomatology and did not replace our primary question of the links with depressive symptomatology.

Last, although I provided a brief description of covariate and model selection for gut microbial beta diversity analyses, the details of these analyses, as well as the modeling strategy for resting state functional connectivity (rsFC) to mood and age were not updated to OSF. This was in large part due to the learning curve associated with working with these data and working within a confined timeframe. Ideally, simulated data would provide the opportunity to troubleshoot and test modeling strategies without running models prior to preregistration.

To help combat the issues that can arise with non-registered analyses, I took an informatically-based approach to model selection, which provided consistent rules for variable inclusion across analyses. Furthermore, to reduce bias of a specific ordination method in the

significance test associated with *envfit* (model used for covariate data reduction), I compared ordination methods and used any significant result as a control variable in my subsequent CCA model. Last, I chose a more conservative approach to assessing rsFC association with mood, by using model fit indices and assessing the relationship after accounting for age.

CHAPTER IV

RESULTS

Demographics.

Overall, the percentage of girls that participated in the Gut Mini Study was higher than originally anticipated for an opt-in study and the discomfort with this type of sample collection for this age range (83%). To ensure participants did not feel pressured into saying yes to the Gut Mini Study, we intentionally did not require girls to explain why they decline participation. We, therefore, do not have information about why girls declined to participate.

However, to see if there were demographic or phenotypic differences between the girls that consented to the Gut Mini Study and the girls that did not, we ran a two-sample t-test on age, puberty, and depressive symptomatology as reported by the CES-DC, and a chi-square test on internalizing diagnosis by group (consented versus did not consent). There was no significant group difference in age, puberty, depression symptomatology, or diagnosis for girls that consented to the Gut Mini Study (n = 90), versus those who did not (n = 18). Since ten girls also consented, but did not complete the stool sample (see Chapter 3, Methods), we also tested if there was a group difference based on completion rather than consent to participate. Similarly, there were no significant differences between groups when comparing girls who completed the Gut Mini Study (n=80) to the girls who did not complete the Gut Mini Study (i.e., girls who did not consent plus girls who consented, but did not provide a stool sample; n = 28). See Table 4. Chi-square results for Internalizing consented: X^2 (1) = .93, p = .33 and Internalizing completed: X^2 (1) = 3.06, p = .08.

Table 4. Consent vs Did Not Consent.

	t	df	*p*	CI	Mean
Age	-.09	23.32	.93	-.41-.38	yes:13.58 no:13.47
Puberty	-.02	25.46	.98	-.46-.46	yes: 4.03 no: 4.03
CES-DC Sx	1.28	22.69	.21	-2.65-11.21	yes: 15.07 No: 19.35

Group comparison between girls who consented to the Gut Mini Study, versus girls who did not consent.

Table 5. Completed Versus Did Not Completed.

	t	df	p	CI	Mean
Age	-.81	36.41	.42	-.51-.22	yes:13.52 no:13.37
Puberty	-.60	47.43	.55	-.53-.29	yes: 4.01 no: 3.94
CES-DC Sx	1.02	41.75	.31	-2.92-8.85	yes: 15.03 No: 18.00

Group comparison between girls who did completed the Gut Mini Study versus girl who not complete the Gut Mini Study (did not consent or did not return a sample).

Next, descriptives are detailed for all the variables within our study. Associations between variables that were not a primary aim of the study are reported in terms of effect sizes, based on Spearman correlation coefficients, not p-values. Coefficients are only described in text if they were above .2 or below -.2 (indicative of at minimum a weak positive or negative relationship).

Development/Maturation.

Although age and puberty were not primary questions for this study, they were assessed to identify if they influenced our variables of interest. Age and pubertal stage were first correlated to identify the strength of relationship within self-reported pubertal stage and between age and pubertal measures. Measures of pubertal stage, including age at menarche, had a medium to strong relationship with age (*r* ranged from .36 to .48). Given the moderate to strong correlation between metrics of pubertal stage (*r* ranged from .6 to .75), PDS score was used for upstream analyses as our index of pubertal stage (See Figure 4).

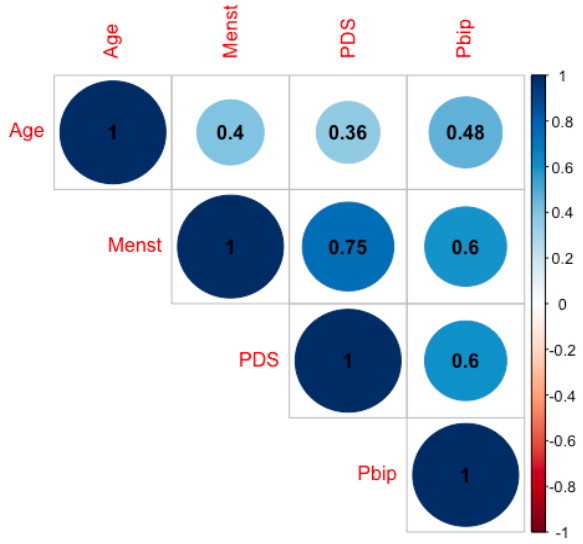

Figure 4. Spearman Correlations of Age and Pubertal Maturation (n = 80). Menst= had started menstruation; PDS= Petersen Developmental Scale; Pbip = Picture-Based Interview about Puberty.

Covariates.

The most prevalent gut covariate was self-reported allergies, followed by parent-reported consistent probiotics use. Out of the eight girls whose parents reported a gastrointestinal (GI) health concern, one individual reported 6/9 GI health concerns. Parent-report GI health concerns

included: 1 Crohn's Disease; 2 IBS; 4 GERD; 1 Gallstones; 3 Celiac; 1 Ulcerative Colitis; 1 Hemorrhoids; 0 Diverticulitis; and 0 Anal Fissures. Note, these were not confirmed by medical diagnoses and therefore cannot conclude evidence of a GI disorder.

Table 6. GI Categorical Covariates.

GI Categorical Covariates	N
Allergies	32
Chronic constipation (with last 14 days)	8
Chronic Diarrhea (within last 14 days)	0
GI concerns (with last 7 days)	13
Sick (within last 2 weeks)	10
Menstruating (within last 7 days)	13
Regular Probiotic Use	20
GI Health Concerns	8

Mean estimated BMI was within the healthy range for adolescent girls for this age range (World Health Organization, 2019), but BMI ranged from underweight to overweight (mean(SD) = 22.39(6.03), range = 13.22 - 44.83). Eight girls had an estimated BMI above 30, which is a strong indication of obesity. Within the gut-brain overlap sample, however, there was only one girl with a BMI above 30 (mean(SD) = 21.04(3.97), range = 15.40 - 36.07). BMI was log transformed due to skew > 2 and kurtosis > 3.

The majority of girls (39) reported a diet consistent with an omnivore diet (defined as no diet restriction). Ten additional girls reported their diet included meat or fish. Eleven girls reported a diet consistent with no meat or fish (See Table 7). Diet category was added to the questionnaires after 15 girls completed the study; therefore, we do not have complete data on this measure. Further interrogation of the diet breakdown from their weekly and normal diet report may reveal more nuanced dietary patterns.

Table 7. Diet Category.

Diet Category	N
Raw Vegan	1
Vegan	1
Vegetarian	8
Pescatarian	2
Pollo-Pescatarian	3
Pollotarian	5
Omnivore	39
Other	6

Antibiotics. Based on parent and self-reported recent antibiotic use, no girls were excluded for antibiotic use. Eight girls self-reported recent antibiotic use; however, when asked to specify the antibiotic, 5 listed over the counter pain medication (e.g., ibuprofen or Advil); 1 listed melatonin and antidepressant; 1 listed over the counter cough medicine and allergy pill; and 1 listed a topical toenail infection cream. This was confirmed with parent-report of antibiotics use (see Table 8). The one report of antibiotics within 7 days was also coded as "sick within the last 7 days." Instead of coding for one participant with recent antibiotic use, that individual was captured under the recent "sick" category. Therefore, no participants were excluded for current antibiotic use.

Table 8. Frequency of Antibiotic Use.

Frequency of Antibiotic Use	N
Within last 7 days	1
Within last month	0
Within last 6 month	2
Within last year	6
Within last year and half	49
Longer than a year and half	71

Covariate Associations. Spearman correlations between covariates showed positive small to medium sized associations between self-reported recent GI health concerns (within the last 7 days) and chronic constipation (within the last 14 days; $r = .39$) or recent sickness (within the last 7 days; $r = .24$). BMI had a positive small-to-medium sized association with chronic constipation ($r = .31$). All other correlation coefficients between GI covariates were .2 or below and are therefore not discussed (see Figure 5).

Covariate Associations with Development/Maturation. Of the GI covariates, the only one with a theoretical link to developmental maturation was BMI, showing a positive small to medium sized association ($r = .32$; see Figure 6).

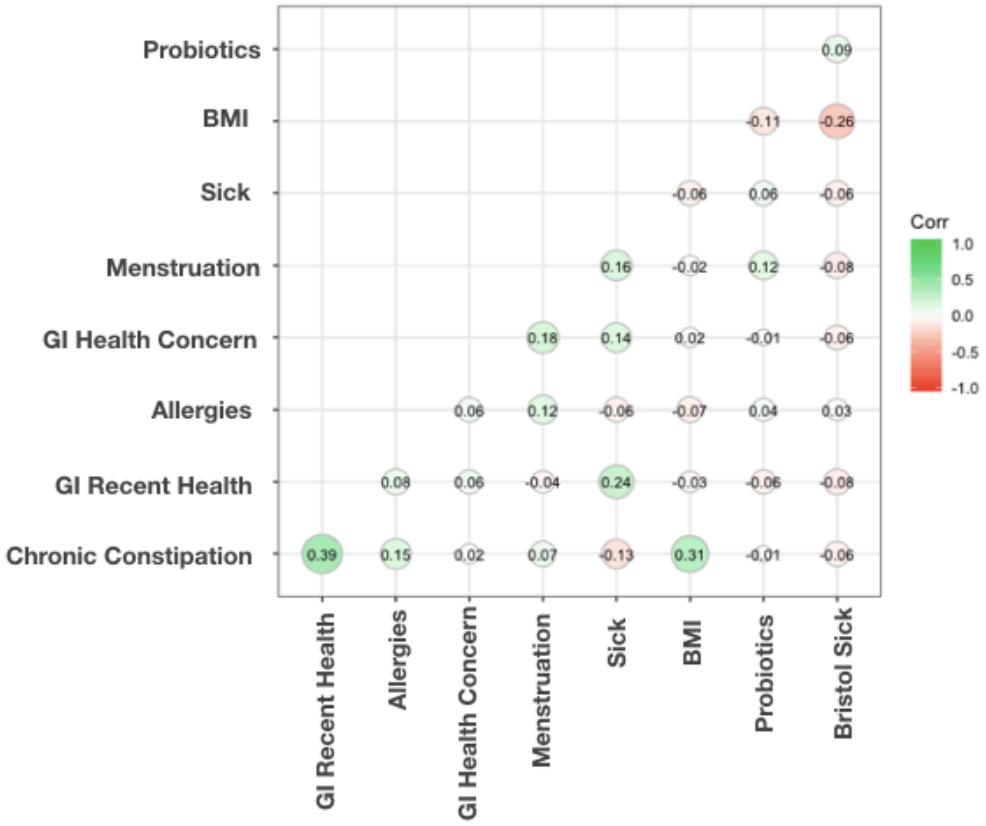

Figure 5. Spearman Correlation of GI Covariates (n=75). Probiotics = weekly probiotic use within the last month. BMI = Body Mass Index, log transformed for skew. Sick = self-reported sickness within 7 days. Menstruation = menstruation within last 7 days. Gut concerns = parent-reported gastrointestinal (GI) concern. Allergies= self-reported allergies. GI recent health = self-report GI discomfort within last 7 days. Chronic Constipation = self-reported chronic constipation within last 14 days. Bristol sick = a score of 1 or 6 on the Bristol stool chart, indicative of either severe diarrhea or constipation at time of sample.

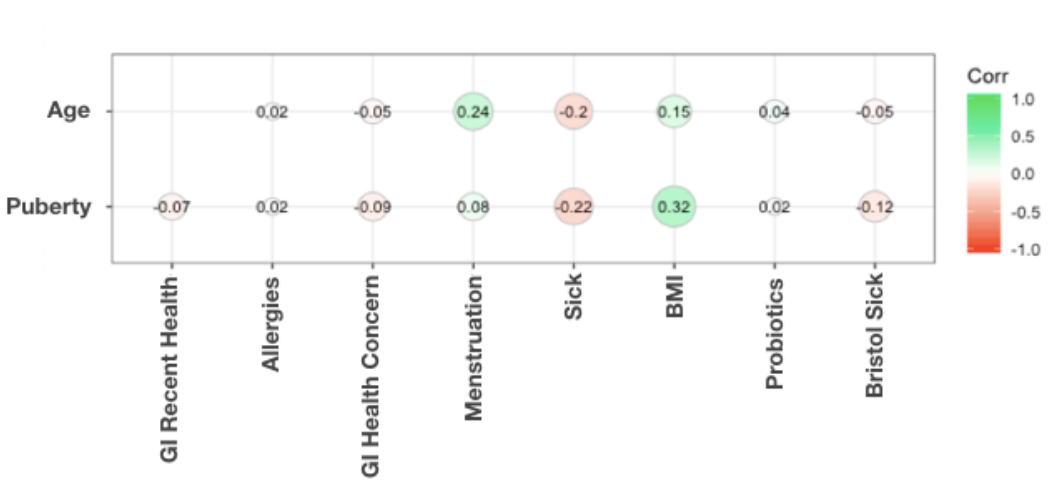

Figure 6. Spearman Correlation of GI Covariates by Age and Pubertal Stage (n=75). Puberty = Petersen development scale score. Probiotics = weekly probiotic use within the last month. BMI = Body Mass Index, log transformed for skew. Sick = self-reported sickness within 7 days. Menstruation = menstruation within last 7 days. Gut concerns = parent-reported gastrointestinal (GI) concern. Allergies= self-reported allergies. GI recent health = self-report GI discomfort within last 7 days. Bristol sick = a score of 1 or 6 on the Bristol stool chart, indicative of either severe diarrhea or constipation at time of sample.

Behavioral Results.

Mood. There was a wide range of self-reported depressive symptoms, as measured by the CES-DC, with the mean score of 15 indicating some depressive symptoms; however, limited participants met for an internalizing disorder. Self-reported anxiety, as measured by the SCARED, was inconsistent with signs of an anxiety disorder. Diagnostic categories, as defined by the HiTOP, indicated 30% of participants met diagnostic criteria for an internalizing disorder. Since this is not a clinical sample, mood associations with resting state functional connectivity and gut microbial composition were tested as secondary aims of this proposal.

Table 9. Self-Reported Mood Descriptives.

	CES-DC	SCARED
Mean (SD)	15.03 (12.71)	2.99 (3.35)
Range	0-49	0-18

Table 10. Diagnosis Descriptives

	N
Internalizing_dx	0=57, 1=23
Distress_dx	0=62, 1=18
Fear_dx	0=66, 1=14
CES-DC_group	0=46, 1=34

0= no, 1 = yes

Self-reported depressive and anxiety symptomatology were moderately correlated with one another (r = .56) and with HiTOP diagnosis of an internalizing disorder (r = .47 and r = .48, respectively). CES-DC grouped by indication of depressive symptomatology (score >= 15) versus no indication of depressive symptomatology (score < 15) showed the lowest correlation with internalizing diagnosis (r = .35). Therefore, depressive symptomatology, as measured by CES-DC continuous, and internalizing diagnosis were used in subsequent analyses as indices of depressive symptomatology and internalizing disorder, respectively.

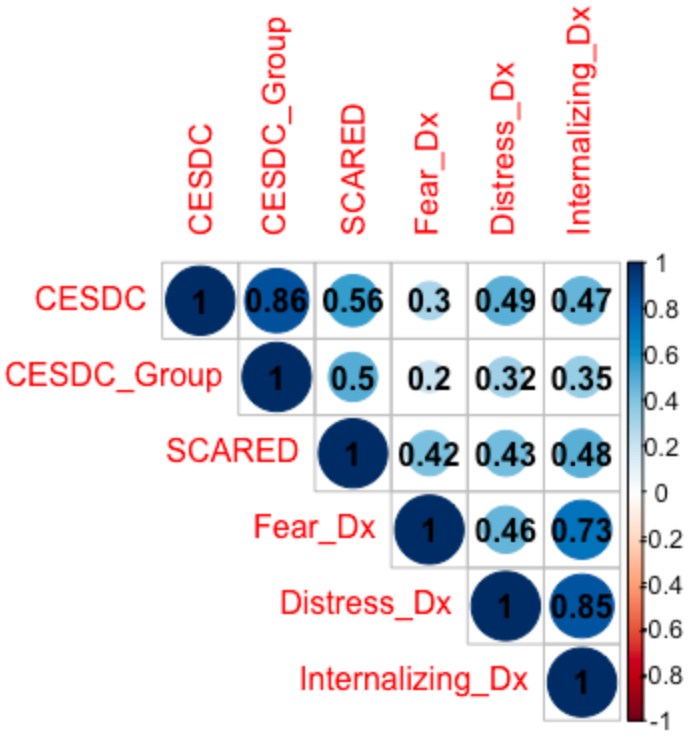

Figure 7. Spearman Correlation of Self-Report Symptoms and Diagnostic Category (n=80). CESDC = Center for Epidemiologic Studies Depression Scale. SCARED = Screen for Child Anxiety Related Emotional Disorder.

Mood Associations with Development. Depressive symptomatology and internalizing disorder were positively associated with age ($F(1,78) = 4.17$ $p = 0.04$, $r = .38$; $t(59.95) = -2.01$, $p = 0.05$, $r = .18$, for symptoms and diagnoses, respectively). On the other hand, at wave 2, depressive symptomatology and internalizing disorder were not significantly associated with puberty, as measured by the PDS score ($F(1,78) = 2.08$ $p = 0.15$, $r = .25$; $t(52.56) = -1.44$, $p = 0.16$, $r = .19$, respectively; See Figures 8-10).

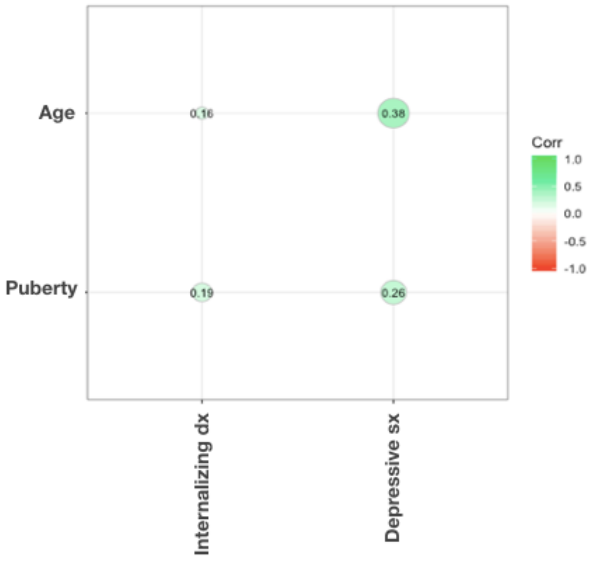

Figure 8. Spearman Correlation of Mood and Development (n= 80).

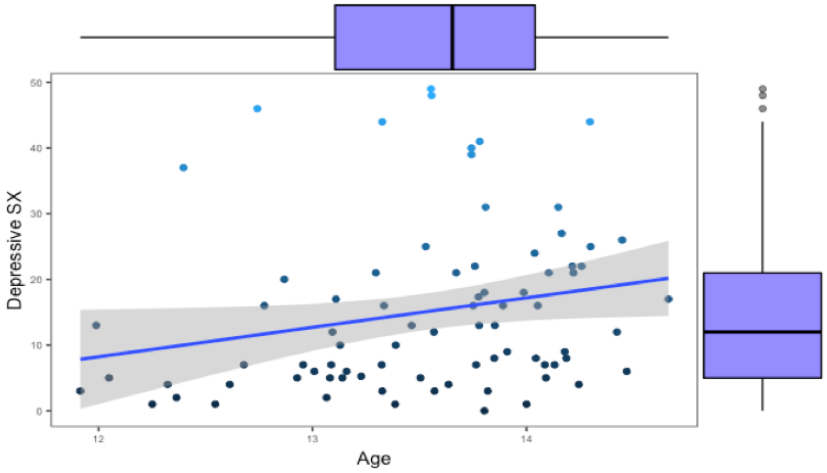

Figure 9. Age by Depressive Symptoms. Correlation between depressive symptoms and age at wave 2 (n =80). Depressive symptoms measured by Center for Epidemiologic Studies Depression Scale.

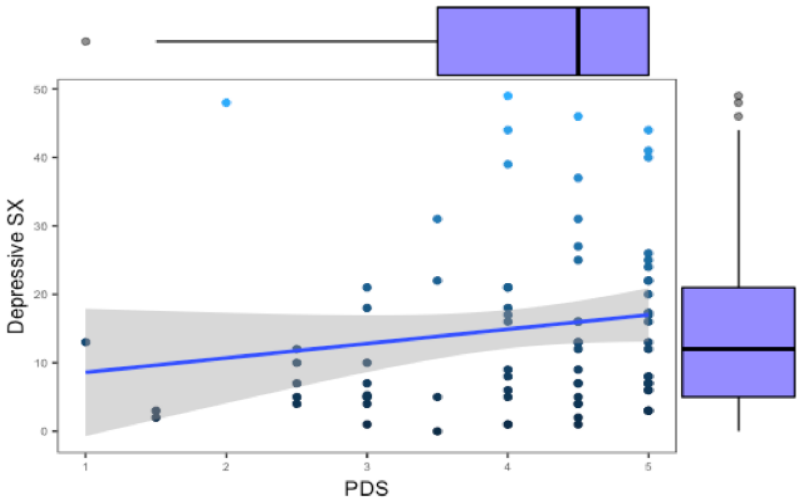

Figure 10. Puberty by Depressive Symptoms. Correlation between depressive symptoms and pubertal stage at wave 2 (n=80). Depressive symptoms measured by Center for Epidemiologic Studies Depression Scale. PDS = Pertersen Development Scale.

Mood Associations with Covariates. Overall, depressive symptomatology and internalizing diagnosis were not strongly correlated with reported GI covariates (*r* ranged from -.15 to .17); however, there was a positive small to medium sized relationship between BMI and both depressive symptomatology (*r* = .26) and internalizing diagnosis (*r* = .3), as well as a positive small sized association between recent menstruation and increased depressive symptoms (*r* = .22; See Figure 11).

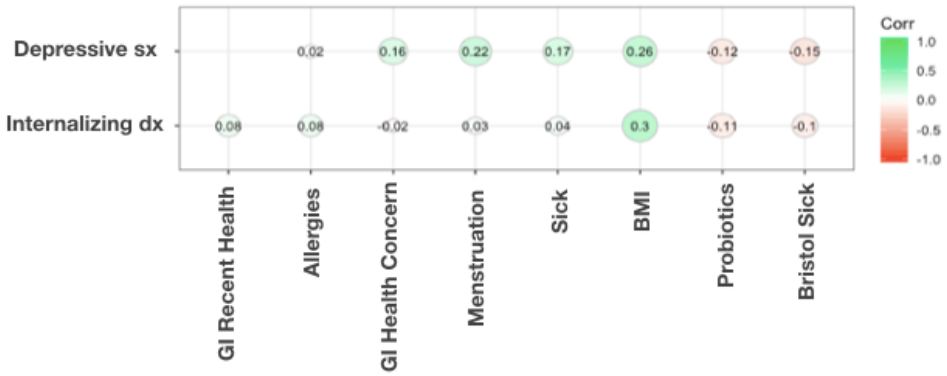

Figure 11. Spearman Correlation of GI Covariates with Mood (n =78). Probiotics = weekly probiotic use within the last month. BMI = Body Mass Index, log transformed for skew. Sick = self-reported sickness within 7 days. Menstruation = menstruation within last 7 days. Gut concerns = parent-reported gastrointestinal (GI) concern. Allergies= self-reported allergies. GI recent health = self-report GI discomfort within last 7 days. Bristol sick = a score of 1 or 6 on the Bristol stool chart, indicative of either severe diarrhea or constipation at time of sample.

Resting State Functional Connectivity Results

Connections of Interest (COIs). Amygdala and hippocampal rsFC to subcortical ROIs showed positive moderate to strong associations (*r*s ranged from .36 to .78). Within networks, both amygdala and hippocampus rsFC to the SN, DMN, and FPN, showed positive moderate to strong associations with each other (*r*s ranged from .38 - .78), except for hippocampus-SN connectivity to amygdala-DMN connectivity, which showed a weak association (*r* = .20). Overall amygdala and hippocampus rsFC to accumbens were weakly associated with other COIs (*rs* ranged from .00 to -.27). Amygdala-hippocampus connectivity, was moderately correlated with

amygdala-sgACC connectivity (r = .37) and amygdala -DMN connectivity (r = .36), but otherwise showed minimal correlation to other COIs (rs ranged from 0 to .24).

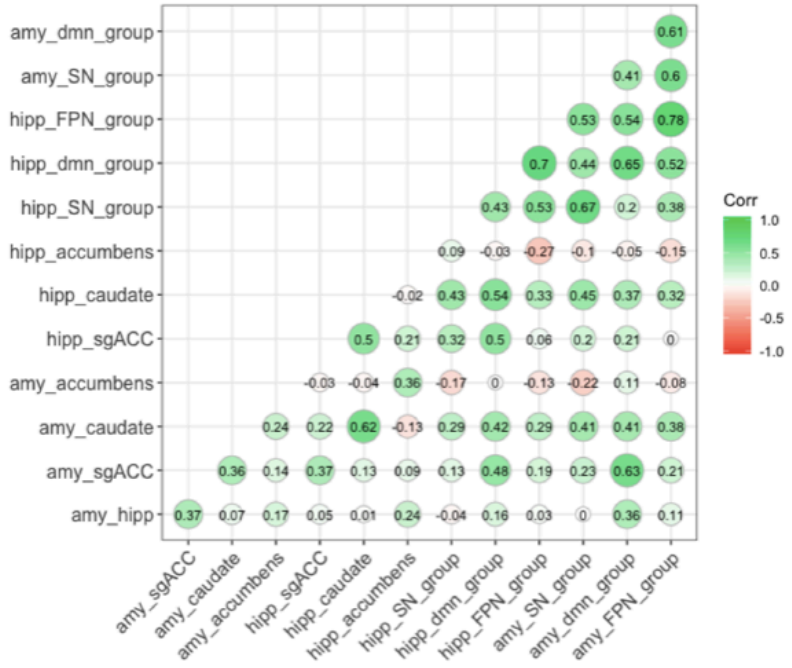

Figure 12. Spearman Correlation of COIs (n= 63). amy= amygdala; hipp = hippocampus; accum= accumbens; caud= caudate; sgACC= subgenual anterior cingulate cortex; SN= salience network; DMN = default mode network; FPN = frontoparietal network.

Subcortical seeds to network connectivity ranged from positive to negative, expect for hippocampal-DMN connectivity, which showed the strongest correlation (r = .25). Amygdala and hippocampal rsFC to SN ranged from negative weak associations to positive weak correlations. Amygdala and hippocampal rsFC to DMN ranged for no correlation to positive moderate correlations. Amygdala and hippocampal rsFC to FPN range from no correlation to positive correlation (see Table 11).

Table 11. Seed to Network COIs.

	Amy-SN	Hipp-SN	Amy-DMN	Hipp-DMN	Amy-FPN	Hipp-FPN
Mean (SD)	-.06(.10)	.09(.13)	.09(.08)	.25(.08)	.02(.08)	.11(.09)
Range	-.28 - .26	-.24 - .36	-.13 - .29	.07 - .41	-.12 - .23	-.04 - .34

amy= amygdala; hipp = hippocampus; SN= salience network; DMN = default mode network; FPN = frontoparietal network. N=63.

Subcortical seeds to subcortical ROI connectivity ranged from positive to negative; expect for amygdala-hippocampus and hippocampus to sgACC. Amygdala-hippocampal connectivity showed the strong strongest correlation. Amygdala rsFC to accumbens and caudate ranged from negative weak associations to positive weak correlations. Hippocampal rsFC to accumbens and caudate ranged from no to moderate correlations. Amygdala and hippocampal rsFC to sgACC ranged from aberrant to strong correlations (see Table 12).

Table 12. Seed to Subcortical ROI

	Amy-accum	Amy-caud	Hipp-accum	Hipp-caud	Amy-hipp	Amy-sgACC	Hipp-sgACC
Mean (SD)	0.11(.12)	0.02(.14)	0.16(.11)	0.13(.13)	0.45(.15)	0.13(.14)	0.41(.14)
Range	-.26 - .33	-.28 - .38	-.09 - .44	-.15 - .35	.19 - .88	-.23 - .50	.01 - .67

amy= amygdala; hipp = hippocampus; accum= accumbens; caud= caudate; sgACC= subgenual anterior cingulate cortex. N=63

Resting State Functional Connectivity with GI Covariates. Resting state functional connectivity for our 12 seed-subcortical connections of interest was not strongly correlated with GI related covariates (*r* ranged from .00 - .45); however, there were a few weak to moderate associations. Regarding seed-to-network COIs, self-reported allergies had a negative association with hippocampal and amygdala-FPN connectivity (r = -.45 and -.31, respectively), whereas

probiotics had a positive weak association with amygdala-accumbens and amygdala sgACC connectivity (r = .26 and .27, respectively; See Figure 15).

Resting State Functional Connectivity Associations with Development. Within the larger sample of scans (n = 63), pubertal maturation (as measured by PDS score) was positively associated with amygdala-hippocampal connectivity (r = .43). Age was not strongly correlated with resting state COIs (rs ranged from 0 to .25). Age had a positive weak association with amygdala-hippocampal connectivity (r = .25). These effect sizes remained in the reduced stool-scan matched sample (n = 46; See Figure 13).

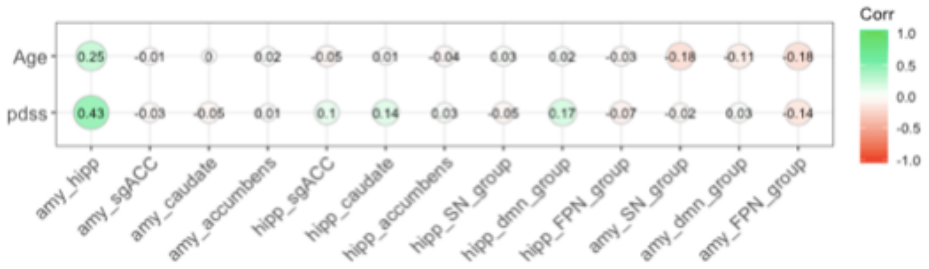

Figure 13. Spearman Correlation of COIs with age and pubertal stage (n=63). amy= amygdala; hipp = hippocampus; accum= accumbens; caud= caudate; sgACC= subgenual anterior cingulate cortex; SN= salience network; DMN = default mode network; FPN = frontoparietal network.

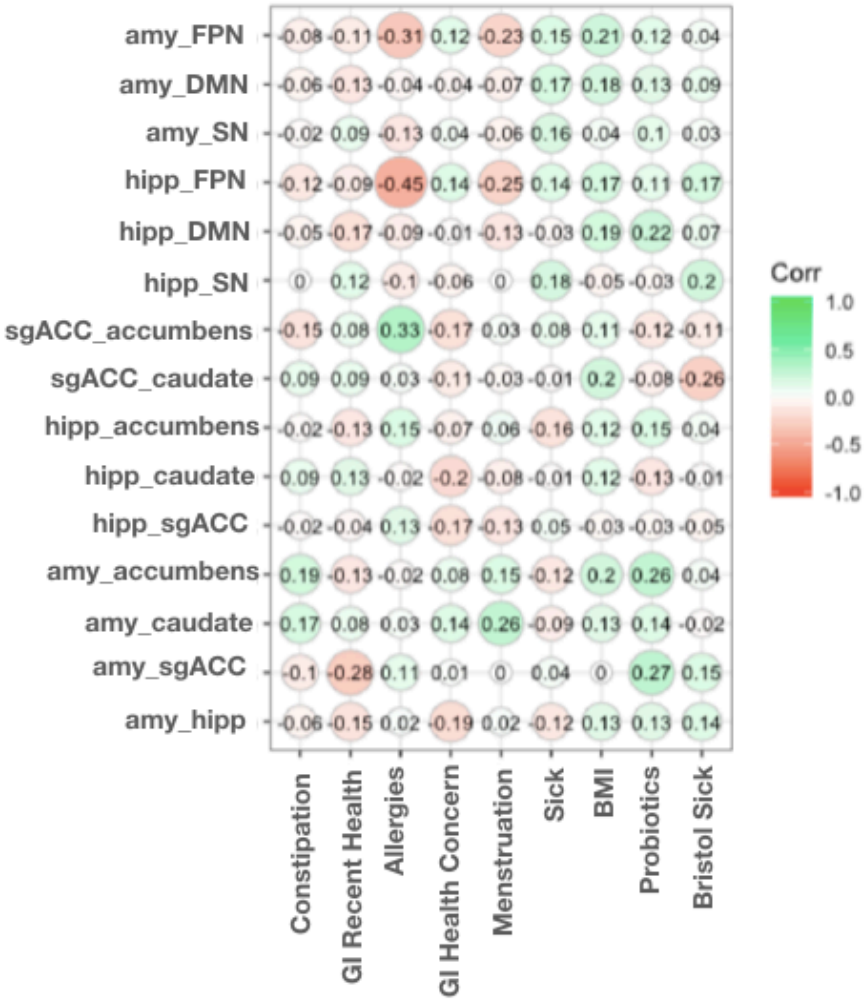

Figure 14. Spearman Correlation of COIs with GI Covariates (n = 44). amy= amygdala; hipp = hippocampus; accum= accumbens; caud= caudate; sgACC= subgenual anterior cingulate cortex; SN= salience network; DMN = default mode network; FPN = frontoparietal network. Constipation = self reported chronic constipation within last 14 days. Probiotics = weekly probiotic use within the last month. BMI = Body Mass Index, log transformed for skew. Sick = self-reported sickness within 7 days. Menstruation = menstruation within last 7 days. Gut concerns = parent-reported gastrointestinal (GI) concern. Allergies= self-reported allergies. GI recent health = self-report GI discomfort within last 7 days. Bristol sick = a score of 1 or 6 on the Bristol stool chart, indicative of either severe diarrhea or constipation at time of sample.

Gut Microbiome Results.

To determine if there was a significant correlation between functional and taxonomic annotation profiles, we conducted a Procrustes analysis (a multivariate correlation method that uses the shape distribution between matrices). Procrustes analysis was performed on the taxonomic and functional PCoA ordinations based on the Bray-Curtis dissimilarities, described in Chapter 3. Taxa and functional pathways were correlated (n = 80; Procrustes $r =\sim 0.78$, $p < 0.001$). This relationship remained similar within our reduced gut-brain dataset (n = 46; Procrustes $r =\sim 0.81$, $p < 0.001$).

Function. To visualize the composition of the functional capacity of the gut microbiome, distances matrices were first compared using various ordination techniques. Several ordination methods revealed potential clustering of microbial composition along axes 1 and 2. For example, PCoA visually appeared to show a possible clustering of microbial composition below 0 and above 0 along axis 1. This was less prevalent with the two non-linear ordination methods: detrended correspondence analysis (DCA) and nonmetric dimensional scaling (NMDS). See Ramette (2007) for details on how these ordination methods are calculated.

We first plotted our gut covariates along PCoA ordination to see if we could visually detect patterns along the axes that were explained by known variants of the microbiome. Visually, we did not detect clear patterns, suggesting patterns in the variance along axis 1 and 2 of the functional composition of the microbiome were not clearly stratified by any single variable, either covariate or variable of interest. See Figure 16 for an example of diet category plotted along the PCoA ordination.

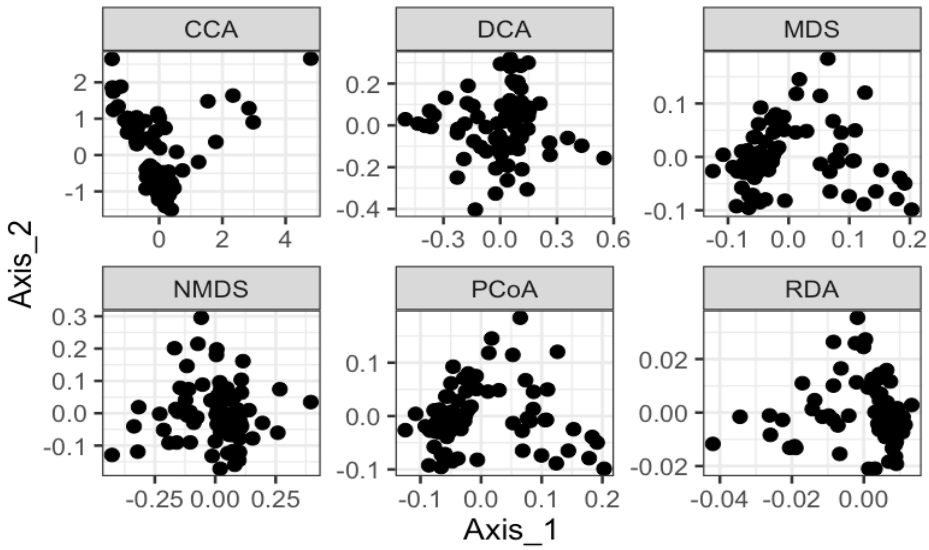

Figure 15. Distance Matrices Plotted by Ordination Method for Functional Microbiome.

Taxa. As with the functional microbiome, to visualize the taxonomic composition of the microbiome, distances matrices were first compared using various ordination techniques. Similar to the functional microbiome, a few ordinations (PCoA, MDS, RDA) visually appeared to show a possible clustering of microbial composition below 0 and above 0 along axis 1. This was less prevalent with CCA and the non-linear ordination methods, detrended correspondence analysis (DCA).

Taxa were also plotted at the level of Phyla, and Order to see if that better explained the compositional distribution along Axis 1 and 2. Descriptively, at the Phylum level it did not appear that the taxa clearly stratified along the axis.

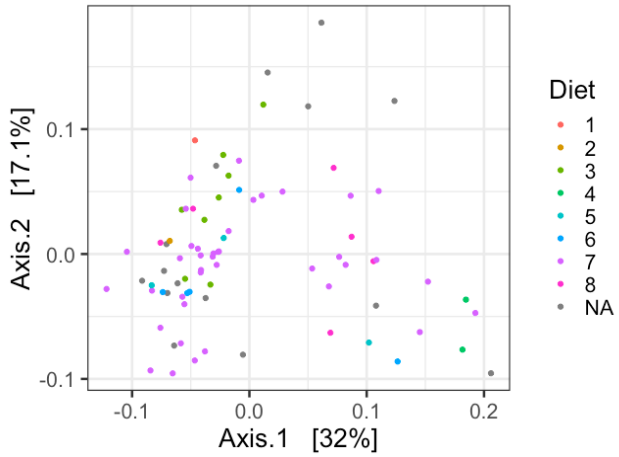

Figure 16. Diet Plotted along PcoA for Functional Microbiome. PCoA = principal coordinate analysis. Diet 1 = raw vegan; 2 vegan; 3= vegetarian; 4= pescatarian; 5 =pollo-pescatarian, 6= pollotarian; 7= carnivore (omnivore); 8 = other.

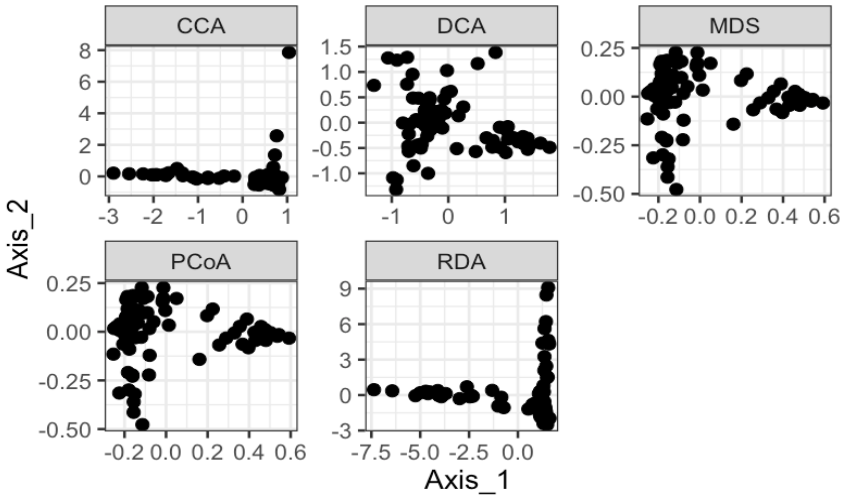

Figure 17. Distance Matrices Plotted by Ordination Method for Taxonomic Microbiome.

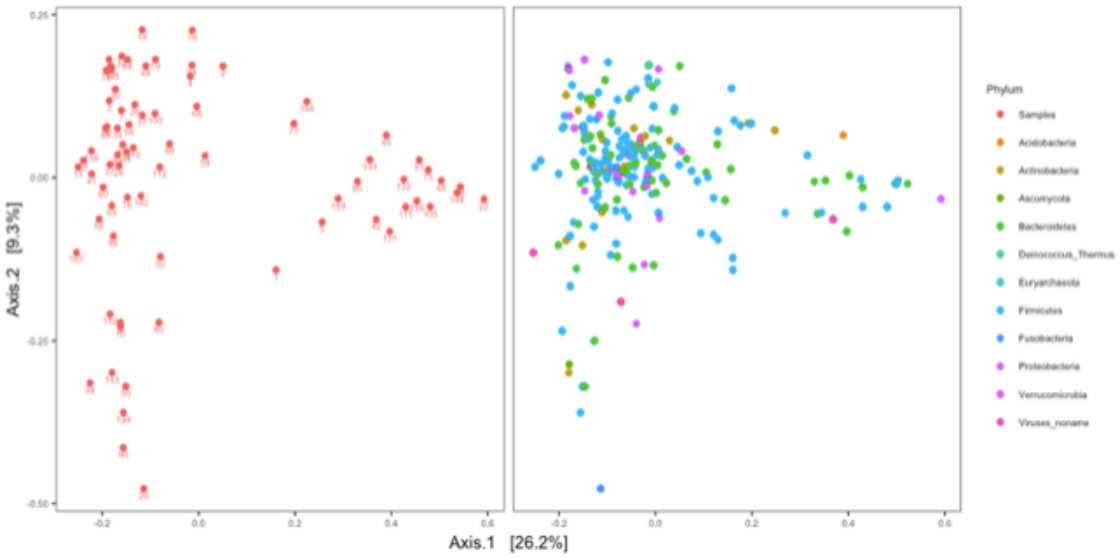

Figure 18. Sample Plotted by Phyla. On the left, the ordination is plotted by individual subject. On the right, the ordination is plotted by taxonomic order within subjects (i.e., each subject has multiple taxa at the phyla level). The right-sided graph depicts each occurrence of specific taxa within individual that is present (i.e., in the case of a rare taxonomic phyla, the order may only be represented a couple times because only a few subjects carry that phyla).

Similarly, at the Order level it did not visually appear that the taxa clearly stratified along Axis 1 or 2.

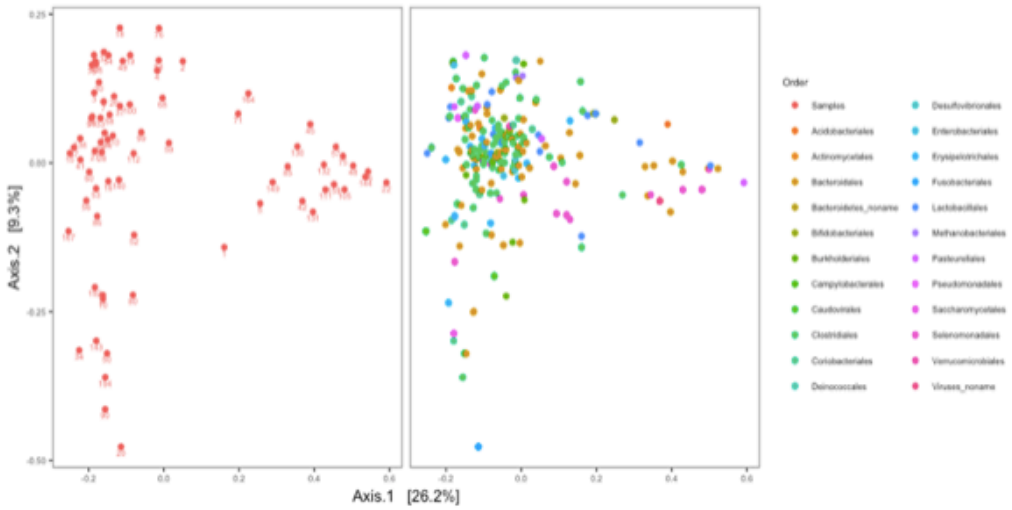

Figure 19. Sample Plotted by Order. On the left, the ordination is plotted by individual subject. On the right, the ordination is plotted by taxonomic order within subjects (i.e., each subject has multiple taxa at the order level). The right-sided graph depicts each occurrence of specific taxa within individual that is present (i.e., in the case of a rare taxonomic order, the order may only be represented a couple times because only a few subjects carry that order).

As with the functional microbiome, we first plotted our gut covariates along PCoA ordination to see if patterns along the axes were explained by known variants of the microbiome. Visually, we did not detect clear patterns, suggesting patterns of the functional composition of the gut microbiome were not clearly stratified by any single variable along Axes 1 and 2. See Figure 21 for an example of diet category plotted along the PCoA ordination.

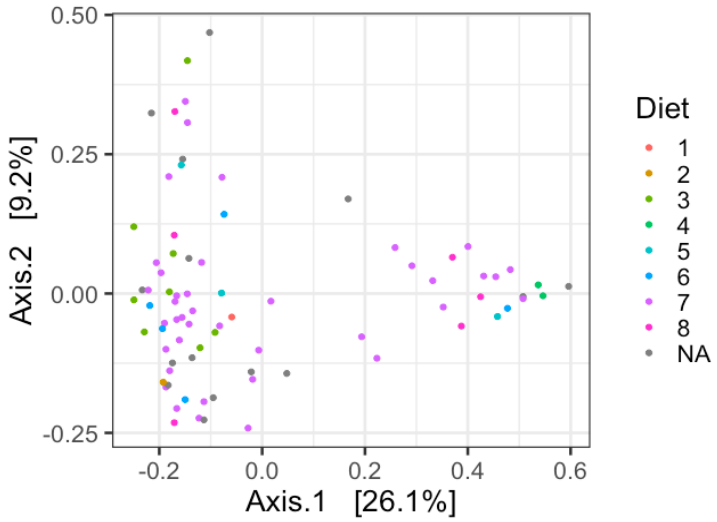

Figure 20. Diet Plotted along PCoA for Taxonomic Microbiome. PCoA = principal coordinate analysis. Diet 1 = raw vegan; 2 vegan; 3= vegetarian; 4= pescatarian; 5 =pollo-pescatarian, 6= pollotarian; 7= carnivore (omnivore); 8 = other.

Covariate Reduction. Although there were no visually strong patterns evident for specific covariates explaining variance along Axes 1 and 2 for the compositional structure of the taxa or functional pathway abundances, we statistically tested this using *envfit* for functional and taxonomic distributions. Since *envfit* is sensitive to ordination method, we ran it across ordination methods. To be conservative, any potential control variable (i.e., gut covariates) that was significant in an ordination method was brought up to be controlled for in the CCA test of beta diversity. This was done in order to reduce the number of features in our statistical models. For the full sample (n = 80), *envfit* did not identify any significant covariates for taxonomic or functional composition. For the gut-brain matched sample (n = 46), *envfit* identified 3 significant covariates across ordinations for taxonomic composition (recent menstruation; self-reported sickness; and chronic constipation within the last 2 weeks), and 3 significant covariates across

ordinations for functional composition (recent menstruation; self-reported sickness; and regular probiotic use).

Resting State Functional Connectivity and Gut Microbiome Results.

Functional Beta Diversity. Constrained correspondence analysis (CCA) was used to determine the additional variance in the functional and taxonomic microbiome composition after controlling for variance explained by gut covariates selected from *envfit*. CCA included all 12 COIs; however, to avoid overfitting, *ordistep* was performed to determine model selection based on AIC. This provided an informatically-driven approach to variable selection for our final CCA model. For functional composition, *ordistep* determined the significant model fit included the gut covariates (recent menstruation; recent sickness; regular probiotic use) and the following three COIs: amygdala-frontoparietal network (FPN); amygdala-caudate; and amygdala-accumbens connectivity. To confirm *ordistep* was not biased toward the order of variables in the model, I ran it with three iterations of COIs re-ordered. The same model was produced each time. Variance inflation factor (VIF) confirmed model selection did not have high multicollinearity (values ranged from 1.05-1.53; values of 1 are considered completely independently and values above 10-20 are considered highly multicollinear). This is consistent with the correlation coefficients reported above. As described earlier, amygdala-FPN and amygdala-caudate connectivity were moderately correlated (r = .34). Amygdala-FPN was not correlated to amygdala-accumbens connectivity (r = -.08), where amygdala-caudate and amygdala-accumbens were weakly associated (r = .24).

A permutation ANOVA (PERMANOVA) on the selected model revealed the overall model was significant ($F(3,37) = 1.90$, $p = .008$), such that after controlling for the variation introduced by the control variables (recent menstruation; recent sickness; regular probiotic use), amygdala-FPN, amygdala-caudate, and amygdala-accumbens connectivity explained a significant

amount of the remaining variation in the composition of functional pathways encoded in the metagenome as measured by PERMANOVA (amygdala-FPN: $F(1,37) = 2.33$; amygdala-caudate: $p = .01$; $F(1,37) = 2.09$; amygdala-accumbens: $p = .03$; $F(1,37) = 3.21$, $p = .002$). Together, the model explained a significant amount of variance along CCA1 ($F(1,37) = 4.79$, $p = .004$. See Figure 22).

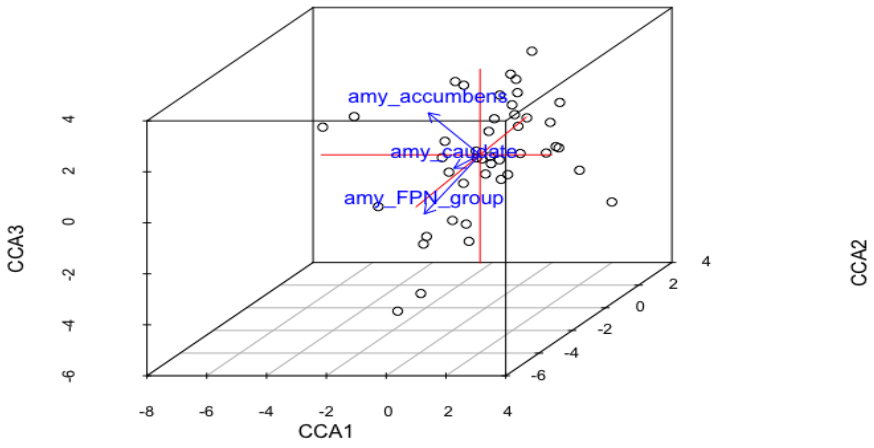

Figure 21. CCA Functional Beta Diversity by Resting State Functional Connectivity.

To understand how these COIs individually contributed to the functional composition of the microbiome, post-hoc analyses were conducted independently for each COI (i.e., 12 models were run with only one COI per model, plus control variable). This approach runs into multiple comparison issues and therefore was not our primary modeling strategy. However, it did allow me to assess if gut-brain associations were missed due to multicollinearity within the original model fed to *ordistep* for model selection (highest VIF score within original model was 6, which is still below scores indicative of high multicollinearity). Consistent with the informatically-driven approach for model selection, amygdala-accumbens connectivity explained significant variance in the functional composition, while controlling for variance associated with gut

covariates ($F(1,39) = 2.36$, $p = .02$). None of the other 11 COIs independently significantly associated with the functional composition of the microbiome, after controlling for the selected gut covariates.

Taxonomic Beta Diversity. The same procedure was done for taxonomic composition; all COIs were included in a single CCA, controlling for significant gut covariates from *envfit* (for taxa, included recent menstruation; recent sickness; and chronic constipation) and run through *ordistep* to determine the best fit model. For taxonomic composition, *ordistep* model selection selected the best model fit as the control variables and the following four COIs: amygdala-SN; amygdala-FPN; hippocampus-sgACC; and amygdala-sgACC connectivity. Permutation ANOVA, however, did not conclude the overall model was significant, and therefore individual terms within the model were not interrogated. This was confirmed with post-hoc analyses where each COI was tested independently; no COIs independently explained significant variance in the taxonomic composition above the variance explained by the gut covariates.

Secondary Analyses with Mood

Although this was not a clinical sample with an equal number of depressed and non-depressed individuals, we conducted follow-up analyses to see how both amygdala and hippocampus rsFC and the gut microbial functional capacity and taxonomic composition associated with depressive symptoms or diagnosis of an internalizing disorder.

Resting State Functional Connectivity with Mood. Spearman correlations suggest there was not a strong relationship with depressive symptoms or internalizing diagnosis with our resting-state functional connections of interest (rs ranged from 0.00 to 0.27; n = 63). Hippocampus-accumbens connectivity had a negative association with both depressive symptomatology and internalizing diagnosis, but these associations were relatively small.

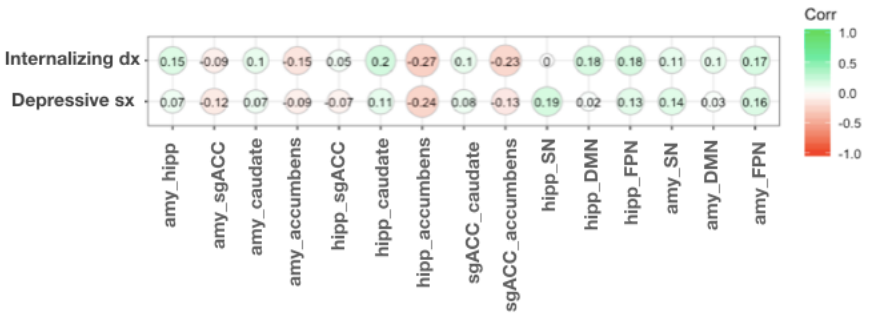

Figure 22. Spearman Correlation of COIs with Mood (n=63). amy= amygdala; hipp = hippocampus; accum= accumbens; caud= caudate; sgACC= subgenual anterior cingulate cortex; SN= salience network; DMN = default mode network; FPN = frontoparietal network.

To statistically test this relationship, our multilevel model revealed minimal relationships between our COIs and depressive symptomatology or internalizing diagnoses. First, linear models were run to see if COIs explained additional variance in depressive symptomatology or internalizing diagnosis above age.

Age was positively associated with depressive symptomatology ($X^2(2) = 12.21$, $p = .0004$); therefore, COIs were assessed to see if they explained additional variance in depressive symptomatology above age. Given the relationship with the 3 COIs (amygdala-FPN, amygdala-caudate, amygdala-accumbens) and the functional composition of the microbiome, we first examined those COIs. Nested model selection indicated the addition of amygdala-FPN connectivity was a better fit to predict depressive symptomatology than age alone ($X^2(4) = 4.77$, $p = .02$; AIC: -242.17, -239.79). Amygdala-FPN connectivity was positively associated with depressive symptomatology $X^2(1) = 4.86$, $p = .03$; amygdala-FPN: $t = 2.17$, $p = 03$; Age: $t = 3.94$, $p = .0002$). The interaction of age and amygdala-FPN, however, did not improve model fit ($X^2(5) = .01$, $p = .91$); suggesting amygdala-FPN connectivity and age have an additive associations with

depressive symptomatology. No other COIs significantly improved model fit to predict depressive symptomatology.

Age, however, did not significantly improve model fit to predict internalizing diagnosis ($X^2(1) = 3.68$, $p = .06$). In addition to nested model comparison, model fit for COIs without age in the model were also tested against the null model. Nested general linear model comparison did not conclude COIs improved model fit to predict internalizing diagnoses above age. COI model comparison to the null model (without age in the model) similarly did not conclude COIs improved model fit to predict internalizing diagnoses.

Gut Microbiome with Mood. We implemented the same CCA approach to test if depressive symptoms or internalizing diagnoses explained variance in the microbiome taxonomic and functional beta diversity. *Envfit* did not identify any significant covariates; therefore, the model was run without controlling for gut covariates. Since there was only one term in the model, it was not appropriate to run *ordistep* for model fit. PERMANOVA results indicated depressive symptomatology explained a significant amount of variance in the functional composition of the microbiome ($F(1,78) = 1.94$, $p = .03$) and significantly explained 18% variance along CCA1 ($F(1,78) = 1.94$, $p = .03$). There was no significant relationship with taxa or internalizing diagnosis. This suggests that increasing symptomatology associated with increased distance moving along CCA1 toward the right.

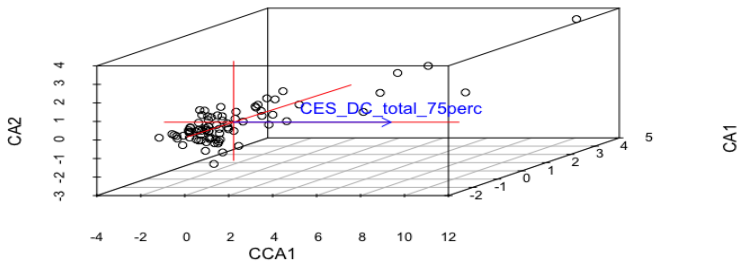

Figure 23. CCA Functional Beta Diversity by Depressive Symptoms (n=80).

CHAPTER V

DISCUSSION

This study represents the first known investigation into functional gut-brain communication during adolescence within a community cohort of girls. Specifically, we used resting state functional connectivity to index amygdala and hippocampal connectivity with other regions and networks implicated in social-emotional development and purported to be disrupted by depressive disorders. We then used shotgun metagenomics to interrogate how both the taxonomic and functional capacity of the microbiome relates to functional brain connectivity and depressive symptomatology during adolescence. Our original aims were to index the association between depressive symptomatology, resting state functional connectivity, and the taxonomic and functional microbiome. However, we found limited evidence of concurrent depressive disorders within our sample, so the reconstituted goals were to first characterize the associations between functional brain connectivity and the taxonomic and functional composition of the gut microbiome, and second, to assess how depressive symptomatology within our sample associated with both functional brain connectivity and taxonomic and functional microbial composition. We compared these findings against the categorization of an internalizing disorder (versus no diagnosis of disorder) to examine if we could detect relationships at subclinical thresholds or if these links to mood were only detectable within clinical samples.

In line with our primary goal, we found evidence that amygdala resting state functional connectivity (rsFC) associates with the functional composition of the gut microbiome during adolescence. In line with our secondary goals, we found evidence that within this cohort of adolescent girls, depressive symptomatology, but not internalizing diagnoses, associates with both amygdala connectivity to the frontoparietal network and the functional composition of the gut microbiome. Notably, this study also provided important proof that this stool sampling

procedure can be successfully implemented within a female adolescent sample, as measured by an 83% opt-in rate and minimal drop out due to measurement discomfort (only one participant). Furthermore, we did not detect group differences in age, pubertal stage, depressive symptoms, or internalizing diagnoses between those who chose to participate in the stool sample collection versus those who did not.

The remainder of this chapter will discuss 1) results from our primary goal of characterizing gut-brain associations, 2) null results and general and specific reasons for these findings, 3) results from our secondary goal of relating both brain function and microbiome composition with depressive symptomatology, 4) immediate next steps within this study, and 5) broader limitations and future directions. Discussion of patterns within functional connectivity and patterns within taxonomic and functional abundance were beyond the scope of the dissertation, but are discussed when directly relevant to the primary goals of the dissertation.

*Gut microbial function and functional brain connectivity***.** We found preliminary evidence that the strength of rsFC from amygdala to reward regions and to the frontoparietal control network (FPN) associate with the functional beta diversity of the gut microbiome (i.e., the compositional dissimilarity between individuals). In particular, female adolescents with increased amygdala rsFC to FPN, accumbens, and caudate tend to carry microbiomes with significantly different functional compositions than those with decreased connectivity between these regions. In other words, within our sample, we found evidence that the overall composition of the functional capacity of the gut microbiome associates with patterns of amygdala rsFC to reward and control-associated regions. Although these networks have not been interrogated in regards to the microbiome, these relationships are consistent with prior reports that the composition of the gut microbiome in adults is associated with changes in functional brain activity involved in emotional reactivity and regulation (Labus et al., 2017; Tillisch et al., 2013).

This suggests that regardless of the specific functional pathways, individuals with more similar functional gut microbial makeups have more similar amygdala rsFC to reward and control related regions and networks. These associations do not allow for causal inferences. It could be that different functional capacities present in the gut microbiome result in changes to functional brain connectivity as seen in probiotic pre-post fMRI studies in adults (Bagga et al., 2018; Tillisch et al., 2017). On the other hand, it could be that patterns of functional brain connectivity result in changes to the functional composition of the microbiome. Yet another possibility, and perhaps the most likely alternative, is that there are bidirectional relationships between the two (see Sharon, Sampson, Geschwind, & Mazmanian, 2016 for review of mechanisms of bidirectional communication).

Given the multivariate nature of the microbiome, patterns in the functional compositions cannot be interpreted as inherently good or bad. Instead, changes in the microbial composition need to be interpreted within what we know about the associated system. Based on what we know about the amygdala rsFC to these regions, it suggests the functional composition of the microbiome may be involved in the manifestation of socioemotional and cognitive control during adolescence (Marek, Hwang, Foran, Hallquist, & Luna, 2015; Sherman et al., 2014; Solé-Padullés et al., 2016). For example, amygdala-FPN network connectivity is associated with cognitive control and flexibility, such that the in healthy communication, the FPN works to down regulate amygdala activity and flexibly reappraise the situation, and communication between limbic and FPN connectivity is linked to self-control later in adolescence (Lee & Telzer, 2016; Marek & Dosenbach, 2018). On the other hand, amygdala-caudate connectivity is linked with emotional valence appraisal, in which the caudate differentiates positive and negative stimuli (Almeida, van Asselen, & Castelo-Branco, 2013). Altered amygdala-caudate connectivity is linked with both depressive disorders and generalized anxiety disorder (Makovac et al., 2016; Ramasubbu et al., 2014). Lastly, amygdala-accumbens connectivity is involved motivation and

reward seeking behaviors (Casey, Heller, Gee, & Cohen, 2019; Stuber et al., 2011), specifically as alterations in amygdala-accumbens connectivity are associated with anhedonia (Bolton et al., 2018). Together, these findings suggest the composition of the functional microbiome may be associated with functional brain connectivity in ways that may influence cognitive flexibility/reactivity, emotional reactivity/salience, and motivation/reward-seeking behaviors. Note, we did not assess these behaviors and these links would need to be confirmed using behavioral assessment.

We also want to be cautious in interpreting patterns of amygdala rsFC as negative or positive, because this is not a clinical sample. In other words, we cannot infer hyper, hypo, or abberant connectivity is between our COIs is a "positive" or "negative" pattern of connectivity. It is nevertheless promising that we found evidence that regions and networks implicated in depressive disorders as well as socioemotional processes associate with the functional microbiome. This suggests that we can begin to map these patterns of functional brain and functional microbial networks in adolescence and then identify how these links are different for clinical samples, or what environmental factors may moderate disruption within this system. For example, within other periods of development, stress, diet, microbial depletion, or microbial environment have all been shown to disrupt gut-brain axis communication (Farzi, Fröhlich, & Holzer, 2018; Foster, Rinaman, & Cryan, 2017; Fröhlich et al., 2016).

One potential mechanism that makes these links particularly important to consider are that the functional composition of the microbiome is influenced by the environmental and developmental needs of the host, and adapt to reflect those needs (Foster et al., 2017). Similarly, resting state functional connectivity is thought to reflect the coactivation of functional activity between networks or regions that are strengthened or weakened based the developmental and environmental needs of the host (Stevens, Pearlson, & Calhoun, 2009). By looking at intrinsic connectivity, versus task specific connectivity or activation, we can start to understand how the

functional and taxonomic microbiome influences the underlying covariation/activation of these functional connections that provide the foundation for how we respond to task-specific stimuli.

Although the mechanism of these connections is still unclear, there are possible pathways by which functional brain connectivity and the functional composition interact with one another as well as the environment. For example, it could be that the composition of the functional microbiome is influenced by changes in the internal environment (e.g., endocrine changes associated with the transition through puberty) and the external environment (e.g., increased exploratory behaviors and changes in peer relationships). The functional composition of the microbiome in turn may influence brain function by changes in functional signaling through SCFA, microbial derived metabolites, or monoamine production, which can alter BDNF or NMDR gene receptor expression. Changes in morphology or gene expression can influence how these regions are functionally responding, adapting and integrating environmental stimuli. For example, altered BDNF expression in the amygdala has been shown to disrupt fear learning, changing how the amygdala functionally couples with other inhibitory or learning-associated regions (Cowan et al., 2018).

Similarly, resting state functional connectivity is maintained or strengthened based on the salience or importance of these connections. Neurobiological models of adolescent behavior have been well documented and reviewed elsewhere (Dahl et al., 2018; Crone & Dahl 2012; Pfeifer & Allen 2012). Briefly, functional brain connections are also undergoing maturational changes in adolescence, which can influence how we respond to our environment. There are several pathways via which changes in brain function and structure may affect the microbiome. A prominent pathway of "top-down" effects of the microbiome is through stress-related responses. The amygdala and hippocampus both play a central role in stress-related disorders (including depression) and have a bidirectional relationship with one of the prominent stress axes, the hypothalamus-pituitary-adrenal (HPA) axis (Herman, & Cullinan, (1997). Exaggerated amygdala

activity (e.g., via decreased connectivity with inhibitory regions), for example, could signal changes along the HPA axis, such as increased corticotrophin releasing factor (CRF) and cortisol, which can increase intestinal permeability (Carabotti, et al., 2015). Intestinal permeability allows bacteria to translocate, or pathogens to secrete into the epithelium, triggering the immune response in the mucosa (Carabotti, et al., 2015).

Furthermore, these brain-behavior links may indirectly influence the microbiome by initiating changes in behavior (e.g., environment, exploration, social relationships, diet) by which the composition of microbiome changes to adapt to the environmental demands of the host. At the same time, these behavioral patterns may be maintained by the functional coupling between the gut microbiome and brain connectivity. Given the complexity of these bidirectional relationships and their interactions with the environment, rather than a specific functional metabolite or specific brain region, it is more likely that both systems operate within a network, by which certain nodes may hold greater weight, but ultimately depend on other network inputs to influence behavioral outcomes.

These results, if replicated, could have several implications for future work and may advance our theoretical model of adolescent development. Current neurobiological models of adolescent behavior emphasize the role of pubertal and neuronal function (Casey, Galván, & Somerville, 2016; Crone & Dahl, 2012), but have neglected to account for the role of the microbiome in these developing neurobiological systems. While our results are correlational and therefore do not reveal directionality, prior work in other populations demonstrates that the manipulation of the microbiome impacts brain structure (Luczynski et al., 2016), neural gene expression (Bercik et al., 2011), and functional activity (Tillisch et al., 2013). It could be that the functions carried by gut microbes link to specific neurological end points, which could reflect pathways via which gut microbes impact these endpoints. Relatedly, it could be that neuronal

activity serves as a mechanism to affect the composition of the gut microbiome. Most likely, these relationships are symbiotic.

Nevertheless, the ability to noninvasively index one marker of the system (i.e., the functional capacity of the microbiome) may provide meaningful diagnostic insight into brain function and behavior. These current finding cannot validate these models, rather this study provides preliminary evidence for future studies to mechanistically model and assess cause and effect.

Null Findings

Within our sample, we did not see evidence that hippocampal connectivity associated with the functional composition of the microbiome or that either amygdala or hippocampal rsFC associated with the taxonomic composition of the microbiome. If replicated, the lack of association may indicate that gut-brain communication may be better indexed by function pathways within the microbiome rather than the composition of taxa. For example, it is possible the composition of specific taxa is less important to overall system health, as long as the metabolic functions of the system are carried out (Selkrig, Wong, Zhang, & Pettersson, 2014). Furthermore, these results may suggest amygdala connectivity serves as a more proximal "hub" of gut-brain communication (Cowan et al., 2018) than hippocampus during this period of development.

Null findings at this stage, however, should still be interpreted with great caution. Both positive and negative results may be due to limits in our power to detect true effects and require replication within a larger, independent sample. We had a relatively small overlapping sample size between resting state functional connectivity MRI scans and stool samples (n = 46). There are a number of steps which can be taken within the current study that will increase our sample size for gut-brain overlap, and we are still collecting stool samples for wave 2 participants.

Ideally, our sample size would still be larger to optimize the power to quantify the relationship between these two high dimensional datasets, although it is unclear what size sample is needed to detect meaningful effects and may in part depend on the level of analysis.

Null results could also be due to a number of reasons more specific to the present study than lack of power. First, it is possible we did not detect taxonomic associations due to the level of taxonomic analysis. We assessed the composition at the level of species. It may be that correlates of brain function are not sensitive to taxonomic composition at the species level, but rather associate with taxonomic clusters or relative proportion taxonomic abundance (as in Tillisch et al., 2017). Second, this study assessed the functional and taxonomic microbiome at the level of beta diversity (i.e., compositional dissimilarity between individuals). This analysis does not provide information regarding the specific functional pathways associated with these microbes. We may be missing important information regarding the potential mechanisms linked to functional brain connectivity that can be uncovered by assessing these links at the level of specific taxa or functional abundance and gene families with our resting state functional connections of interest. It could be that patterns of amygdala or hippocampal rsFC are altered by different functional metabolites or microbial-sourced short-chain fatty acids. Furthermore, our measure of beta diversity was aimed at measuring similarity between individuals based on diversity and relative abundance (i.e., Bray-Curtis distance). It is possible rare taxa or functional pathways significantly contribute to individual differences in the association between taxonomic or functional beta diversity and rsFC.

Fourth, we chose an informatically-driven approach to determine which covariates to include in our models. These covariates were determined based on their prior association with the microbiome. This included variables that may explain significant individual variability in the microbiome (e.g., diet or probiotics), or may confound the sample (i.e., recent sickness or GI concern). These associations are worth explaining in their own right, as they may be symptoms or

indices of depression or other phenomena of interest directly, but these questions were beyond the scope of the current study; therefore, we took a conservative approach to identify which rsFC associations exist after controlling for the variance explained by these covariates. This means we may have missed associations between rsFC and the microbiome by taking such a conservative approach.

Fifth, we only tested twelve resting state functional connections of interest, based on *a priori* hypotheses that the amygdala and hippocampus may serve as potential hubs of gut-brain axis communication and specifically tested rsFC to regions altered with depressive disorders (Flannery, Callaghan, Sharpton, Fisher, & Pfeifer, 2019; Mulders, van Eijndhoven, Schene, Beckmann, & Tendolkar, 2015). Furthermore, to reduce the number of factors we fed into our model, we averaged brain regions bilaterally, as well as across networks. This is an important consideration for generalization or future translation. For example, lateralization of rsFC has been detected between sexes (Gur & Gur, 2017; Kilpatrick, Zald, Pardo, & Cahill, 2006). By averaging bilaterally across our regions, it is possible these results are more generalizable across sexes. On the other hand, we may be missing lateral associations between our seeds and regions or networks of interest. Similarly, by averaging across networks, we could not detect variability in within-network connectivity or between-network connectivity. There is reason to believe that patterns within and between networks may vary in meaningful ways (see Ernst, Torrisi, Balderston, Grillon, & Hale, 2015; Mulders, van Eijndhoven, Schene, Beckmann, & Tendolkar, 2015; Pannekoek et al., 2014; Stevens, Pearlson, & Calhoun, 2009 for a few examples)

Sixth, prior work demonstrates the microbiome can alter amygdala and hippocampus at the structural and gene level (Foster, Rinaman, & Cryan, 2017), but it is possible these links do not directly translate to functional brain connectivity during this period of development or these links are better assessed in relation to specific moderators, such as NMDA or BDNF expression,

within the amygdala or hippocampus (Bercik et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011).

Last, despite a number of measures taken to reduce the number of features in our model and to reduce overfitting, our results may still be biased to model overfitting or instability within model effects. Ideally, these models will be tested against other modeling strategies that are suitable for two high dimensional datasets within our sample constraints.

Mood Correlates with Brain Function and Microbiome Composition.

Resting state functional connectivity with mood. Although this was a community sample and not a clinically depressed one, we still found supportive evidence that greater self-reported depressive symptomatology was positively correlated with increased age and decreased amygdala-FPN connectivity. This is line with prior findings that found these connections are altered in depressive disorders in adolescence (Mulders et al., 2015).

We did not find evidence of associations between depressive symptoms or internalizing disorders and any amygdala or hippocampal rsFC. In addition to similar possible reasons for null hypotheses as it relates to measurement of the gut microbiome listed above, it is possible we did not detect these relationships due to the relatively restricted range of depressive symptomatology and prevalence of internalizing disorders within our sample. Conversely, our categorization of internalizing disorder may be too heterogeneous. Internalizing disorders were collapsed across depressive and anxiety disorders. For example, there is evidence to suggest that neural phenotypes differ within diagnostic category (Drysdale et al., 2017; Xia et al., 2018), and therefore a lack of such a relationship in this sample could be because we are underpowered to detect it, or it could be that functional brain connectivity needs to be studied within more homogeneous groups and at the symptom-level to detect a meaningful signal. Furthermore, patterns may only be present within network that this study was not designed to detect. For

example, Figueroa et al., 2017 demonstrated that depressed adolescents demonstrated hypoconnectivity between the hippocampus and posterior region of DMN.

Gut microbial function with mood. We found supportive evidence that increased depressive symptomatology associates with greater dissimilarity in the functional composition of the microbiome. This is consistent with our prior findings, in which we found the composition of the microbiome was linked to nonclinical levels of depressive symptoms in childhood (Flannery, Stagaman, et al., 2019). This suggests that these gut-mood relationships may be present prior to the onset of a depressive disorder and therefore may serve as early warning signs of a system dysbiosis. This cannot be confirmed within the current sample. However, we have the unique opportunity to test this hypothesis at the next wave within this larger study. Nonetheless, it is promising that initial associations were found in a sample with restricted variability in mental health outcomes. Adolescence is a period of increased rates of developing a psychiatric disorder; therefore, one important goal of this and future studies is to interrogate how gut-brain communication is not only altered by depressive disorders, but how it could provide early markers of a sensitive system and ultimately aid preventive efforts. Due to this study's prospective design, we can already see that depressive disorders and depressive symptomatology increased from wave 1 to wave 2. At wave 3, we will be able to track which girls from this sample developed a depressive disorder, or conversely, enter remission and see what, if any, predictive power is gained by assessing links between gut-brain and gut-behavior communication at wave 2.

Similar to our brain functional connectivity-behavior results, however, we cannot conclude the functional or taxonomic composition of the microbiome associates with internalizing disorder. This further suggests that either we did not have an appropriate sample to test for diagnosis, or that internalizing diagnoses were too heterogenous of a category to detect signal in the relationship between mood and the composition of the microbiome. Last, it does not

rule out these relationships exist, but may be more sensitive to specific taxa, rather than overall composition. For example, several studies have found associations between the relative abundance of specific taxa between depressed and non-depressed adults, although these findings are not consistent (See Huang et al., 2019 for review). The lack of consistency may be also be due to the inherent heterogeneity within depressive symptoms and disorder.

Immediate Next Steps.

Within this specific study, there are a number of immediate next steps we plan to take prior to publication. Overall, our results suggest the compositional structure of functional microbiome, rather than the taxonomic composition, was associated with brain connectivity and mood. This may be consistent with the idea that the functional capacity of the microbiome is a better index of the microbial sourced metabolites (Selkrig, Wong, Zhang, & Pettersson, 2014). In this study, we focused on path abundance as a metric of the functional potential of the microbiome and species as a metric of taxonomic composition of the microbiome; however, we may be missing important information regarding specific gene families or specific taxa at different levels that help explain these preliminary associations with resting state functional connectivity. Our next step will be to understand how functional brain connectivity associates with specific functional path abundances, gene families and taxonomy using compound poisson general linear model (CPGLM) regression with false discovery rate (FDR) correction. This approach uses a distribution with a point mass over zero, which will allow us to more accurately account for the sparseness of community data that we see within microbial data.

In addition, we saw a relatively large portion of variance explained along our first PCA component for both functional and taxonomic composition. This will need further interrogation, as it suggests there is a prominent variable or combination of variables explaining a large portion of variance within our sample. Initial investigation into this pattern was inconclusive. One reason

for this could be that are data are significantly clustered. One of our next steps will be to take a cluster-based approach in order to better tease apart if there are specific metadata factors or taxa or gene families that help to explain the level of variance along our first PCA axis. It is also possible we are losing signal in our overall model because there are associations within the dataset that are specific to these functional or taxonomic clusters.

Furthermore, while we took a number of rigorous quality control steps to ensure the quality of the resting state connectivity data, it is prudent we next assess these relationships controlling for framewise displacement (i.e., motion) to see if any of the relationships we see with our connections of interest are correlated with remaining variability in motion. In addition, we used a minimum number of 5 minutes as out cut off point for feasible scan data. Ideally, if our scan times were longer, we would have liked to have this number be closer to 9 minutes (Birn et al., 2013). We also only used one parcellation for whole brain connections. Parcellation maps are sensitive to sample and may not be equally appropriate for every individual (i.e., not optimally represent the functional parcels for a given individual; Gordon et al., 2016). One way we can address this next is to run a second parcellation and report similarities and discrepancies between methods. Furthermore, it is widely debated if resting state data should apply global signal regression (Li et al., 2019). We chose not to apply global signal regression, as it has been shown to increase the chance of false negative connectivity between regions (Caballero-Gaudes & Reynolds, 2017; J. Li et al., 2019). Prior to publication, we will run our resting state preprocessing pipeline again with global signal regression and results will be reported in supplemental materials (Murphy & Fox, 2017).

Future Directions within the Six Domains of Adolescent Change.

This study focused on assessing functional gut-brain communication during adolescence, but touched on a number of the six domains of change outlined in Chapter 1. Future studies

should seek to further interrogate and disentangle these effects across this developmental period. Below, we highlight a few promising areas of future research.

Mood. While the intent of the larger study was to see which girls go on to develop a diagnosis, it meant we did not have a clinical versus non-clinical sample of adolescent girls. In addition to prospective longitudinal work, these gut-brain-behavior relationships should be compared to that of a clinical population. In this study, we assessed both symptomatology and diagnosis in an effort to better tease apart when the microbiome is involved in shaping mood (i.e., is it altered by a clinical diagnosis, or does it provide early markers of risk for disorder at the early stage of depressive symptomatology?). One way we could address this in future analyses from the larger study would be to see if the composition and specific taxonomic and functional potential gut microbiome at wave 2 predicts symptomatology and diagnoses at wave 3. Future studies should seek to interrogate these links with mood in clinical and nonclinical samples across adolescence.

Social/Exploratory/Brain. We found preliminary evidence that the composition of the microbiome associates with functional brain connectivity implicated in reward processing and learning (as evident by amygdala-accumbens rsFC; Stuber et al., 2011). These data provide some interesting insight into how the gut microbiome may associate with not only emotional reactivity, but motivation and reward seeking behaviors in adolescence. While this study focused on 12 specific connections of interest, it will be prudent to better understand how the microbiome associates with other social and self-processing networks that we know are changing in adolescence (Flannery, Callaghan, Sharpton, Fisher, & Pfeifer, 2019). Future studies should more directly target these associations in relation to exploratory behaviors, and social behaviors.

Developmental Timing and Age. This was a community sample of adolescent females advanced in pubertal maturation and within a tight age range. Within our cross-sectional cohort, age was still positively correlated with depressive symptoms and diagnosis; however, we did not

detect a relationship between mood and pubertal stage. We also did not detect a strong relationship between age or puberty with any of our resting state functional connections or functional or taxonomic composition.

To tease apart the effects of maturational timing on the gut microbiome during adolescence, these findings should be explored earlier in puberty and followed to its conclusion. It is unlikely that puberty does not play a role in these relationships, but that these relationships may be more sensitive to pubertal maturation and timing when there is greater variability. Within this study, this could be assessed by looking at pubertal timing at wave 1, or at the rate of pubertal acceleration from wave 1 to wave 3. Furthermore, we only assessed puberty by using one self-report measure. We have other self-report, parent-report, and hormonal data on puberty. The role of age, puberty, and developmental timing deserves further interrogation to understand these complex relationships. To better test if adolescence is a sensitive period for gut-brain communication, future studies will need to compare functional and taxonomic compositions in adolescence to distributions present in children and adults.

Stress and Neuroendocrine Function. We also know that adolescence is a time of increased social stress and that stress can disrupt the composition of the microbiome and communication along the gut-brain axis (Bingham et al., 2011; Sudo et al., 2004). In addition to self-reported stress the week prior to sample and day of, we have history of early life stress, as well as socioeconomic risk, and social relationships that will be critical to assess in future publications as we continue to assess opportunities and risk factors to target within these populations and biological systems. One of my main future directions will be to understand how stressors may perturbate typical developmental relationships along the gut-brain axis. It was beyond the scope of the current project to include those relationships, but future studies should see if early or concurrent life stressors help explain some of the variance in the microbiome and mood during adolescence or if the number of changes that occur during adolescence act to reset

some of these previously potent predictive factors on gut-behavior or gut-brain relationships. For example, we know that early life stress is tied to early pubertal maturation and purported accelerated brain development (Callaghan & Tottenham, 2016). It is possible this plays a critical moderating role in the relationship between gut-brain axis in adolescence.

Wellbeing. Given the rich dataset that these variables are derived from, there is a lot of potential to refine and optimize our metadata. There was a wide range of estimated BMI and allergies within our sample, which displayed moderate sized effects with puberty and functional brain connectivity. This suggests there may be an immune pathway that may explain this variance. Within this study, diet was grossly defined. We have more detailed data on diet, which may help tease apart meaningful patterns within the microbial composition than is currently visible. For example, "omnivore diet" was defined as no diet restrictions, but that does not mean all girls then ate a balanced diet, or that all girls who self-identified as having an "omnivore diet" consumed similar diets. From what we know in adolescence, it is unlikely that is the case (McKnight-Eily et al., 2011; Worthman & Trang, 2018). While the focus of this study was not on diet or probiotic use, refinement of these variables will be critical in any future study if we want to make claims about their role or lack thereof in these relationships. The current study should not be interpreted as evidence of no impact by these factors. By understanding these relationships in adolescence, we can open new avenues for diagnostic metrics of health into this system.

Conclusion.

Overall, we found preliminary evidence that the composition of the functional capacity of the microbiome associates with amygdala functional brain connectivity and depressive symptomatology in a cross-sectional sample of adolescent girls. These findings suggest the microbiome interacts with brain functional connectivity implicated in mood and cognitive and socioemotional processes during this formative period of adolescent development. We further

found evidence that both functional brain connectivity and the composition of the functional microbiome are associated with depressive symptomatology. These findings provide a foundation for future studies to expand and replicate these findings within adolescence. Building off prior work, the gut microbiome may be one modifiable biological factor that may be amenable to intervention in order to modify not only functional brain connectivity implicated in social emotional learning, but also depressive symptomatology during the formative period of adolescence. Future work will be needed to replicate these findings in order to move the field forward toward developing screening methods that may allow for prevention and intervention efforts to improve adolescent health.

CHAPTER VI

CONCLUSION OF DISSERTATION

There is growing reason to believe the gut microbiome plays an important role in adolescent development. I summarized six domains in which the microbiome interacts with systems known to be developing in adolescence: social processes; motivation and behavior; neural connection; cognition; neuroendocrine function; and health and wellbeing. As a first step toward understanding these relationships in adolescence, we tested how specific aspects of functional brain connectivity, implicated in social-emotional development and altered with depressive disorders, associated with the taxonomic and functional capacity of the microbiome. We did this by focusing on amygdala and hippocampal resting state functional connectivity (rsCF), given their putative role as "hubs" in how the gut microbiome could influence brain function. We found the first evidence that the functional composition of the microbiome associates with amygdala rsFC to two reward/reactivity-associated regions and the frontoparietal network (FPN: a key region implicated cognitive control and flexibility), during adolescence. Within our sample, the functional capacity of the microbiome and functional brain connectivity also associates with depressive symptomatology, despite being a nonclinical sample. Together, these findings provide the first steps toward unpacking these relationships during adolescence and provide supportive evidence that the microbiome is an important, yet overlooked system involved in adolescent development. To conclude, I first discuss four future directions and challenges within this interdisciplinary field, then narrow in on a few future directions within my programmatic line of research. Last, I discuss the potential benefits and limitations of translating these findings into clinical practice.

Future Directions and Challenges within the Field.

We are at the very beginning of understanding the connections between brain connectivity, the gut-microbiome, and mental health during adolescence. There are many future directions and challenges the intersection of these fields will need to confront as it seeks to move forward, including the need for 1) interdisciplinary collaborations, 2) tools for working with high dimensional datasets, 3) incorporation of open science practices, and 4) developmental mapping across and within these biological systems.

Interdisciplinary Collaborations. Interdisciplinary collaborations will be critical for the success of future gut-brain axis investigations and was a critical component of the success of this first investigation. Large-scales studies of this magnitude (i.e., longitudinal and within a developmental population) require coordination and collaboration between numerous staff, laboratory members, and disciplines. For example, the success of this project relied on the coordinated effort between several labs with complementary expertise (i.e., clinical psychology, developmental social neuroscience, translational neuroscience, and microbiology), three lab coordinators, over ten lab members, and numerous research assistants. Collaborations between microbiology and developmental neuroscience, however go beyond the sheer number of people needed to successfully conduct these studies.

Collaborations need to begin during study conceptualization and continue throughout all phases of the research process. There are growing numbers of commercial services that researchers can access for gut microbial sequencing and data analysis. I would argue, however, this level of collaboration occurs at too late a stage to comprehensively and accurately integrate these diverse disciplines. An interdisciplinary team has the ability to ensure the study design is optimized to ask and answer the team's core questions: a critical point as we seek to build translational neuroscience studies. This may mean changing the frequency of variable collection

or adding measurements and questionnaires. Although the current study was built within a larger project, collaborating with a microbiologist on the stool collection study design ensured we collected the appropriate questionnaires (e.g., gastrointestinal history and diet) and additional measurements (e.g., height and weight) that could otherwise confound our results. Ideally, the original study would be designed as a team, so multiple questions could be addressed across disciplines (e.g., microbial environment or toxin).

Beyond study design, the goal of these interdisciplinary collaborations is to add to the scientific body of knowledge, *for both fields*. This feat becomes insurmountable without greater insight into what is meaningful and an appropriate interpretation of the data within each field. This requires learning the "language" of each respective field and appropriately code switching in order to translate research findings to different scientific audiences. For example, gut microbial data is multidimensional and commonly visualized and analyzed within 3-dimensional space with ecologically-based analytic packages to process and interpret the data. Functional brain connectivity is also processed in 3-dimensional space, but it requires an entirely different set up of analytic programs and field-specific knowledge to process data and interpret findings. Therefore, appropriate study design and data interpretation require a) expertise across theoretical and methodological techniques; b) within and between field-specific interpretation of the data and results; and lastly, c) the ability to synthesize the findings between the larger intersection of all respective disciplines.

High Dimensional Data. As we seek to combine these high dimensional data sets, we face new challenges to data analysis and interpretation. Many of the mainstream methods for assessing high dimensional datasets require large sample sizes and/or a high frequency of data sampling. These methodologies pose particular challenges for developmental samples that are constrained to relatively smaller samples sizes than are appropriate for most machine learning or data-driven approaches. Within both developmental neurosciences and microbiology, growing

number of open-access multi-site datasets ameliorates typical samples size restrictions. The use of these databases, however, requires that research question match the publicly available data. This becomes more difficult for special populations.

These challenges are only exacerbated when we seek to combine two high dimensional datasets that do not currently have open access, large-scale databases that contain both neuroimaging and gut microbial data. The current analytic tools that exist to assess two high dimensional datasets are still limited and have been minimally applied to these data (gut-brain data). For this study, I chose to reduce my resting state functional connections of interest to decrease the number of features I was assessing across dataframes; however, this approach inherently meant my questions were limited to what could be answered by assessing overall network patterns between functional connectivity and the gut microbiome. Furthermore, microbial data do not lend themselves to traditional correlation methods with functional brain connectivity measures, but rather require analytic strategies that account for the community sparseness within microbial data and number of features that exceeds the number of subjects.

Last, as studies keep expanding within developmental neuroscience and microbiology, there will exist endless covariates to consider for when studying questions of interest. This poses new challenges, as the inclusion of numerous covariates within a given model also pushes statistical limitations, but at the same time, may be requested/demanded within a group of multidisciplinary reviewers. New methodologies will need to be continuously explored to optimize our ability to assess these systems together. Given the number of modelling constraints inherent with these questions, it is particularly important we strive to describe and interpret results in terms of effect sizes and distribution of results (by running permutation testing; Dinga et al., 2018).

Open Science Considerations. With greater complexity comes new "forks in the road" that inevitably increase researcher degrees of freedom when dealing with two high dimensional

datasets. Luckily, there is a growing movement to help ensure studies are being completed in a transparent, reproducible and replicable manner. For example, there are open software programs, like R (rdocumentation.org), and open access and version-controlled coding platforms, like Github (github.com), to help increase transparency and the ability to find and fix errors. Integrative platforms, such as Center for Open Science (cos.io) have also evolved to support pre-registrations (i.e., time stamp hypotheses and analytic decisions) and registered reports (i.e., peer reviewed study and analytic design before beginning the project).

Despite the growing movement around more reproducible, transparent practices, there are still different challenges that exist with subdisciplines (see Flannery, 2018 for my summary of common issues that developmental neuroscience faces). To help combat some issues, I created a fMRI preregistration template that I used as the template for this study's preregistration (Flannery, 2018). These challenges were only exacerbated, however, when I added metrics of the microbiome. It is particularly hard to preregister all decisions in interdisciplinary fields when researchers in each specialized field are still learning to implement these techniques within their own datasets (e.g., how to best combined methods of microbial and functional brain connectivity). It may be that preregistrations may be premature at this stage in the field.

Alternatively, I have tried to implement an "open notebook" that is publicly available for this study. The open notebook served as a stand-in preregistration by which I tried to document analytic decisions prior to running them. This allowed for greater accountability and transparency, while allowing for the flexibility to change analytic strategies after making them publicly available. I still faced difficulty with this method, however, as I would troubleshoot new problems. For example, as I would learn more about a technique, I often learned it was inappropriate for my data.

Developmental Mapping. Adolescence remains an understudied period of gut microbial development. Thus, it is critical we map these patterns across adolescence before we try to infer

patterns of risk or health. In other words, the presence of certain bacteria or ratio of bacteria, needs to be interpreted within a developmental framework. For example, a high abundance of Bifidobacteria is developmentally expected and appropriate as infants breastfeed, but it would be considered atypical to see relatively high abundance of Bifidobacteria in adults (Arboleya, Watkins, Stanton, & Ross, 2016). It is unclear what relative abundance of specific taxa we should expect to see during different phases of adolescence.

Similarly, we would expect a different abundance of functional pathways to be present during different periods of life, depending on the developmental needs of the host. For example, Hollister et al., 2015, found evidence of increased abundance of functional metabolites associated with brain development for prepubescent adolescents, where adults showed higher abundances for functions metabolites associated with health and obesity (Hollister et al., 2015). One way to test this would be to compare our sample with openly available child and adult microbial taxonomic and functional datasets.

As we seek to characterize brain function, the gut microbiome, mental and physical health, and the intersection of these domains, we must do so from a developmental perspective. Moving forward, researchers should seek to developmentally map the relationship between biological systems, including the microbiome and brain function to ensure we have representative samples and larger sample sizes.

Future Programmatic Research

There are a number of ways I hope to address some of the challenges described above. By implementing a multi-site, prospective, longitudinal design, I ideally hope to better disentangle the questions of developmental timing and to test the predictive value of these biological markers. With larger samples sizes, I plan to divide my sample into a test sample, a holdout sample, and lastly an independent sample. This would help ameliorate some of the

challenges present with preregistration or registered reports by sub-setting a portion of the sample to be exploratory and the second half be subjected to preregistration or a registered report. Multi-site collaborations would increase sample size, allow for more appropriate data-driven analytic approaches, and provide greater representational coverage of the population for which we wish to translate these findings.

Furthermore, one of my main overarching goals is to understand how communication along and between these biological systems is disrupted by psychosocial stressors and what factors may help to protect these developing systems from perturbations. Ideally, I would ask these questions with a similar large-scale prospective longitudinal design, but with a group design: one group of prepubescent adolescents that are at higher risk for mental and physical health problems, such as due to early life adversity or low socioeconomic status, and one group of prepubescent adolescents at low risk, such as having no history of early adversity.

In future studies, I would ideally repeat sampling within a month to assess stability and variability within samples, and implement longitudinal sampling that occurred at the scale of 6 months to a year. For example, patterns of variability within an individual may provide diagnostically important information. What does that tell you about the overall stability of the system? Is that level of variability expected for their developmental age? This would also allow for greater individual variability in measurements and also help reduce the need for a larger sample size, by increasing the number of timepoints per individual.

Potential Benefits and Limitations for Translation.

It is encouraging to find new connections between the gut microbiome and brain function or mental and physical health, but it raises new questions as to the implication of these findings for clinical translation. Understanding the biological mechanisms doesn't always provide translational value. For these findings to be translated, they need to fulfill, at minimum the

following three criteria: 1) the biological system/marker must be disrupted, or modified by adversity, 2) alterations in this biological marker need to link to changes in mental or physical health outcomes, and 3) the biological marker must be modifiable to intervention such that changes to the system can have long term impacts on mental and physical health outcomes.

Promise of the Gut Microbiome. There are several reviews that detail the clinical promise of the gut microbiome (see Dinan & Cryan, 2016; Vargason & Anselmo, 2018). From animal and adult literature, it appears the gut microbiome fulfills all three criteria; 1) the gut microbiome is altered by environmental stressors and this alteration appears to be sensitive to developmental timing (Foster, Rinaman, & Cryan, 2017); 2) the microbiome is linked to mental and physical health outcomes (Dinan & Cryan, 2016; Vargason & Anselmo, 2018); and 3) modification of the microbiome leads to long-term changes in mental and physical health outcomes (Dinan & Cryan, 2016; Vargason & Anselmo, 2018).

The gut microbiome is a noninvasive method that has the potential to provide information into other systems, such as brain function that is hard to index on an individual level. By understanding the gut microbiome's relationship to other systems that are harder to understand on an individual level or to translate into clinical practice, microbial profiles may be able to serve as markers for health of other systems. Here, I briefly highlight a few points as they relate to adolescent development. Within adolescence, research is just starting to uncover the dynamic relationships between the many behavioral and biological systems that are in flux.

Rodent studies indicate that changes to the microbiome during adolescence may have long term implications for future outcomes (Desbonnet et al., 2015). This needs to be replicated in humans, yet suggests that even though the microbiome remains malleable across the lifespan, adolescence may provide an unique opportunity to have long term benefits on mental health outcomes. If we find evidence of causal links, might clinicians be able to impact functional connectivity patterns by modifying the gut microbiome?

In addition to the promise the microbiome has to fulfill these three criteria, it is also a noninvasive measure that has the ability to be incorporated in clinical settings. If we do find the microbiome can serve as a marker of vulnerability or health within the system, measurements could be integrated into healthcare through collaborations between primary care physicians and mental health providers. For example, just as a doctor would do a panel of tests for annual exams, stool samples could be added to their panel of tests. This already occurs if someone expresses a GI concern, but if we were able to incorporate these measures into the primary care setting, we could also help characterize healthy and atypical microbial profiles across development. It could also help provide individually meaningful information on treatment course for medication. For example, could it tell us which medication may be best metabolized for an individual, or provide insight into which cognitive-behavioral strategy to target based on patterns associated to functional brain connectivity (e.g., cognitive flexibility/restructuring, rumination, or emotional reactivity).

These types of personalized medicine approaches may be able to have large-scale impacts for health care, but there are obvious concerns about taking this approach too soon. With the growing public interest in the gut microbiome, several direct-to-consumer companies have started marketing products and personalized medicine promises based on an individual's microbiome before they have been validated by science. These services ultimately hinder scientific progress and can do substantial harm for an individual that does not realize these claims are not backed by rigorous science. My hope is that as we increase the standard of experimental rigor, including reproducible results and representative populations, we will get closer to targetable mechanisms for prevention and intervention efforts. Until then, the primary focus should remain on basic science designs and delineating these complex relationships across development.

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