Dysbiosis and Host Health: Uncovering the Connection between the Microbiota and Disease

DISSERTATION

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Abstract

The human gastrointestinal tract is heavily colonized by millions of bacteria, termed the microbiota, which is closely involved in host metabolic and immunological processes. This beneficial relationship is dependent upon the community structure of the microbiota, as defined by the relative proportions and diversity of the groups within the community. External or internal factors can impact these population abundances, which can have deleterious effects upon host health. This condition, termed dysbiosis, has been associated with inflammatory bowel disease as well as obesity. The development of these dysbiotic profiles and their involvement in deleterious health outcomes are topics that are not yet well understood. Thus, we developed this study to ascertain factors that influence mammalian hosts to acquire dysbiotic microbiota, and to associate dysbiosis with changes in host health.

Psychological stressor exposure is a primary correlate of symptomatic episodes (e.g., pain, bleeding) in patients with inflammatory bowel disease, though it is not understood how this occurs. Previous studies have shown that exposing mammals to stress can affect the microbiota which are associated with GI mucosal immunity, but the changes have been not been well-defined, particularly in mice. Thus, mice were exposed to a long-term stressor known as restraint in order to evaluate how stress exposure affects the murine gastrointestinal microbiota. In addition, mice were subjected to a social

stressor, social disruption, in order to determine if the type and duration of the stressor affects microbiota community structure differently. Colonic mucosal and luminal contents were collected and 16S rRNA sequences were analyzed. Mucosal and luminal contents had unique community structures that clustered separately on a principle coordinate analysis cluster plot. Restraint stress affected the mucosal associated populations to a greater extent and reduced beneficial groups, including *Lactobacillus*. Likewise, social disruption affected the mucosal-associated microbiota significantly after only 2 hours of exposure, and this effect compounded with repeated exposures. As with restraint stress, *Lactobacillus* was reduced in Social Disruption (SDR)-exposed mice. qPCR analysis indicated that the immunomodulatory species, *Lactobacillus reuteri*, was reduced in absolute abundance in SDR-exposed mice, but not in restraint-exposed mice.

Mice exposed to either restraint or SDR had significant increases in severity of infection by an enteric pathogen, *Citrobacter rodentium*. iNOS, IL-1β, CCL2, and TNFα were significantly increased in mice exposed to the stressors, and overall colitic pathology was also increased in the stress-exposed mice. In order to determine if there is an association between stress-induced changes to the microbiota, and stress-induced aggravation of colitic inflammation during pathogen challenge, germ-free mice were given oral gavage of the fecal slurry from stressor-exposed conventional mice, and then challenged with *C. rodentium*. Mice that received the stress-exposed microbiota had increased pro-inflammatory transcript levels as well as heightened colitic pathology, indicating that stressor-induced changes to the microbiota are associated with changes in gastrointestinal immune function.

Probiotic *Lactobacillus reuteri* can act as an ameliorative agent upon stressor-exacerbated colitis. Since *L. reuteri* can increase colonic diversity, we determined if *L. reuteri* is stabilizing the host microbiota as a primary mechanism in reversing the heightened inflammatory state in stressor-exposed *C. rodentium*-infected mice. SDR-exposed mice that received the probiotic gavage had no change in microbial diversity or composition within the colon after one day of infection compared to control, but stress and infection-associated changes to *Lactobacillus*, *Parabacteroides*, and *S24-7* were observed. Additionally, stress and infection had lasting effects upon the microbiota, which suggests that these impacts can have long-term outcomes upon host health as mediated by the microbiota.

Though these findings make evident that external factors such as psychological stress can disturb healthy microbiota structures, which can then feed deleteriously back upon murine host health, it is not known whether such extrinsic impacts can shift microbiota profiles in human hosts. Host-to-host transmission of microbiota communities is another possible mechanism by which a host might incorporate a dysbiotic profile. Mothers are a primary source of microbes for their offspring in early life, and maternal obesity is a strong antecedent for obesity in later life for the offspring. Thus, we hypothesized that children born to obese mothers will have a unique microbiota structure. Indeed, using UniFrac unweighted distances, children, aged around two years and born to high-income obese mothers clustered separately from children born to high-income non-obese mothers. Clustering was not seen in children born to low-income mothers. These data indicate that both socioeconomic status of the mother, and maternal obesity associate

with the community structure of their children. In sum, these combined data of psychological stress within mice and obese microbiota associations between mother and child in humans lend credence to the overarching hypothesis that external factors can significantly impact microbiota community structure, resulting in changes to health-associated microbial groups and disturbing normal host immune or physiological activity.

I dedicate this dissertation to my wife Dina, daughter Nora, and my parents and brothers.

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Publications

Abdelhamed M, **Galley JD**, Bailey M, Johnston W, Halloway JA, McGlumphy E, Leblebicioglu B. A comparison of zirconia and titanium abutments for microleakage (2015). Clinical Implant Dent Relat Res; 2015 Feb 26. doi: 10.1111/cid.12301.

Christian LM, **Galley JD**, Hade EM, Schoppe-Sullivan S, Kamp Dush C, Bailey MT (2015). Gut microbiota composition is associated with temperament during early childhood. Brain, Behavior and Immunity; Vol. 45: 118-27.

Gutierrez-Orozco F, Thomas-Ahner JM, **Galley JD**, Bailey MT, Clinton SK, Lesinski GB, Failla ML (2015). Intestinal microbial dysbiosis and colonic epithelial cell hyperproliferation by dietary α-mangostin is independent of mouse strain. Nutrients; Vol. 7, no. 2: 764-84

Galley JD, Yu Z, Kumar PS, Dowd SE, Lyte M, Walter J, Bailey MT (2014). The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor. Gut Microbes; Vol. 5, no. 6: 748-60.

Galley JD, Bailey MT, Kamp Dush C, Schoppe-Sullivan S, Christian LM (2014). Maternal obesity is associated with alterations in the gut microbiota in toddlers. PLoS One; Vol. 9, no. 11:e113026.

Galley JD, Nelson MC, Yu Z, Dowd SE, Walter J, Kumar PS, Lyte M, Bailey MT (2014). Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota. BMC Microbiol; Vol. 14: no. 189.

Galley JD, Bailey MT (2014). Impact of stressor exposure on the interplay between commensal microbiota and host inflammation. Gut Microbes; Vol. 5, no. 3: 390-396.

Gutierrez-Orozco F, Thomas-Ahner JM, Berman-Booty LD, **Galley JD**,

Chitchumroonchokchai C, et al (2014). Dietary α-mangostin, a xanthone from mangosteen fruit, exacerbates experimental colitis and promotes dysbiosis in mice. Mol Nutr Food Res; Vol. 58, no. 6: 1226-38.

Allen RG, Lafuse WP, **Galley JD**, Ali MM, Ahmer BM, Bailey MT (2012). The intestinal microbiota are necessary for stressor-induced enhancement of splenic macrophage microbicidal activity. Brain, Behavior, and Immunity; Vol. 26, no. 3: 371-82.

Bailey MT, Dowd SE, **Galley JD**, Hufnagle AR, Allen RG, Lyte M (2011). Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain, Behavior, and Immunity; Vol. 25, no. 3: 397-407.

Bailey MT, Dowd SE, Parry NM, **Galley JD**, Schauer DB, Lyte M (2010). Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by Citrobacter rodentium. Infection and Immunity; Vol. 78, no. 4: 1509-1519.

Fields of Study

Major Field: Oral Biology

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Chapter 1: Introduction

The human body is resident to a multitude of bacteria that colonize niches throughout the host, including the skin, oral, and most numerously, gastrointestinal (GI) compartments (1). Over time, these bacterial populations and their human host have evolved and developed with one another, and as a result, have mutualistic involvement in physiological function (2). Within the GI tract, the bacterial groups that compose the microbiota exist in constant competition for available resources. Regular cross-talk occurs between the microbiota and the host. As a result, microbial populations are modeled by the mucosal immune system and nutrient availability in one direction, while in the other, the microbiota are key actors in immune development and regulation, as well as host digestion and nutrition (3-8). The microbiota forms a community structure that is generally stable over the human adult life-span (9). As external factors, such as dietary shifts or the use of antibiotics that target microbial populations, cause significant disruptions to GI microbiota community structure, dysbiotic profiles can be established (10, 11). Dysbiotic community structures can have shifts in diversity or blooms in certain bacterial groups that are pathogenic, changes that have been associated with increased disease pathology (12, 13). However, the relationship between the bevy of elements that

can induce dysbiosis and how those profiles can then impinge upon host health is not yet well-delineated, and is a major focus of this study.

Causative Factors of Dysbiosis

Among the factors that can affect microbiota community structure that have been characterized to a considerable extent are antibiotics and dietary composition. *Clostridium difficile* infections have been associated with antibiotic use due to a reduction in pathogen defense, a benefit of a whole and healthy GI microbiota (14, 15). Certain diets, such as those high in fat and sugar can shift microbiota dominance to bacterial groups that have increased capacity for energy extraction, which then results in higher adiposity and weight gain (16). Studies on these effectors have been able to highlight particular changes to the microbiota, including alterations in the *Firmicutes:Bacteroidetes* ratio in obese subjects (17). Another event that can affect the microbiota, psychological stress exposure, has not had the same depth of analysis performed.

Psychological Stress

A psychological stressor is any stimuli, real or imagined, that disrupts systemic homeostasis and activates an organism's stress response to maintain this homeostasis. As an organism perceives a threat (through the memory and sensory centers of the cortex and subcortex), its systems undergo significant changes to manage the threat through the action of a cascade of hormones and neurotransmitters. These changes include altered cognition, elevated core temperature, increased arousal, and large-scale changes to

energy and oxygen partitioning (18-21). The changes are far-reaching, affecting circulation, respiration, and gastrointestinal function (22-24).

The Hypothalamic-Pituitary-Adrenal Axis

There are two major pathways that control and mediate the mammalian stress response: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). The HPA axis, as well as the SNS, can be activated through projections from the amygdala (where emotional memory is stored), hippocampus (where context of events are processed), and the prefrontal cortex (assists in coping with stressful stimuli) (25-27). These sections project into the paraventricular nucleus (PVN), located in the hypothalamus, via noradrenergic neurons from the ventrolateral medulla and the nucleus of the solitary tract. Upon activation, the parvocellular cells of the PVN releases corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). Both of these hormones act upon the pituitary gland, which secretes adenocorticotrophin-releasing hormone (ACTH) from the anterior section of the gland (28). ACTH traffics to the adrenal gland, where it will activate the production and release of glucocorticoids (GCs) from the adrenal cortex (29).

While a portion of the released GCs will negatively feedback upon the PVN and the pituitary gland and shut down the pathway, the secreted systemic GCs have far-reaching effects. These effector molecules act upon both intracellular GC receptors (GCRs) that are involved in stress function and non-stress related mineralocorticoid receptors. The GCR, composed of two subunits, is bound to heat shock proteins -70 and -

90 until it comes in contact with GC, at which point the receptor dimerizes and the complex translocates to the nucleus, where it will bind to glucocorticoid response elements on the DNA. At these sites, gene transcription is inhibited or activated. Often, these effects are anti-inflammatory and immunosuppressive, as primary targets are NFκB and AP1 (30). GCs also act upon cellular proliferation as well as metabolism pathways, by modulating gluconeogenesis in the liver, as well as overall mitochondrial oxidation (31, 32).

The Sympathetic Nervous System

The SNS, one half of the autonomic nervous system in addition to the parasympathetic nervous system, is activated through the locus coeruleus, located in the brain stem (33, 34). The locus coeruleus sends signals to pre-ganglionic neurons via the spinal column. These neurons release acetylcholine (ACH), the primary pre-ganglionic neurotransmitter of the autonomic nervous system, which activates post-ganglionic neurons throughout the periphery. The primary mediators of the SNS are the catecholamines, norepinephrine (NE) and epinephrine (EPI), which bind to adrenergic receptors, of which there are two types: alpha and beta. The adrenal medulla is the primary source of EPI as well as a smaller proportion of NE (~20% of the medulla's total catecholaminergic output). The majority of NE comes from post-ganglionic neurons that have been signaled by ACH. NE and EPI have major effects on host physiology. Often associated with the 'fight-or-flight' response, they can mediate heart rate and blood flow by controlling vasoconstriction and vasodilation of blood vessels within brain, muscles

and heart. NE and Epi can also upregulate gluconeogenesis and increase oxygen intake (35). In sum, the activity of both the SNS and HPA axis increase and re-direct available resources in order to better cope with the stressor.

Stress and Fecal Microbiota Abundance

Exposure to psychological stressors has been previously shown to affect the gastrointestinal microbiota. Non-human primate studies have exhibited that an acoustic startle stressor as well as maternal separation were sufficient to significantly affect broad bacterial groups cultured from fecal matter (36, 37). Similar results extend to humans, wherein Knowles et al showed that fecal lactic acid levels were reduced in medical students during exams (38). Likewise, fecal culturing techniques enabled researchers to report that stressor exposure increased aerobic and facultative anaerobic bacterial counts in shed mouse feces. However, only ~10% of total bacteria within the colonic tract are cultivable. Thus, the quantification of bacterial groups from fecal matter or even tissue on broad agars such as brain-heart infusion agar or tryptic soy agar only gives partial insight into how overall populations might be affected by stress.

Pyrosequencing

Recent advances in microbiota analysis have led to the advent of 16S sequencing. Prior technologies including denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism gave researchers a ‘fingerprint’ of a microbial community composition. These methods use DNA from whole bacterial communities,

and after enzyme treatment to fragment the DNA, fragment composition is measured via electrophoresis or fluorometry (39, 40). However, this level of analysis does not provide for ease of identification of bacterial groups or in-depth analysis of how community abundances might be affected. While other methods such as shotgun sequencing have been around for decades, 454 pyrosequencing is a much more recent addition to genomic analysis. With this approach, genomic DNA is isolated from a bacterial community (e.g., wastewater, colonic tissue, stool), and affixed with both adaptor and barcode identification sequences (41). Affixed to beads, the template DNA is sequenced via addition of pyrophosphate-tagged nucleotides. Upon binding to the complementary base, the pyrophosphate is unbound, and light is released via a luciferin-based reaction (42). Consecutive like bases result in increased light intensity (43). Sequence lengths vary, but can reach ~500 nucleotides. Sequencing depth, or the amount of sequences obtained per sample, can also vary from just a few to over a million. Increased sequence length corresponds with increased accuracy upon taxonomic classification (44). Recently, the 454 sequencing method has been phased out for cheaper technologies that support deeper sequencing, such as Illumina. Both 454 and Illumina target pre-determined hypervariable regions of the 16S ribosomal RNA. The bacterial 16s rRNA is highly conserved, making it an ideal target in total bacteria analysis, but the hypervariable regions within are distinct and can be used to classify operational taxonomic units (OTU) based on taxonomic classification (e.g., genus, family). Because of unique loops and hairpins in the 16S structure, the hypervariable regions can have biases, resulting in artificially inflated OTU counts based entirely on the targeted region (45).

Sequencing Analysis

Using sequencing platforms that allow for quality filtering, OTU clustering, and taxonomic assignment such as Quantitative Insights Into Microbial Ecology (QIIME) gives researchers simple tools for personal analysis of the attained sequences (46). With QIIME, users can filter out sequences based upon read quality and even truncate sequences to remove poor quality ends. Many databases and programs have been implemented into QIIME, including taxonomic databases (e.g,. GreenGenes) used for OTU picking, statistical programs (e.g., R package vegan), and graphic visualization software (e.g., Emperor) that in total, can produce in-depth analysis of whole microbiota communities (47-49). Depending on the methodology used within QIIME, sequences are typically clustered based upon percent similarity to one another (i.e., de novo clustering), to a reference database (i.e., closed-reference) or a combination of the two (i.e., open-reference) into OTU bins, which will then be classified taxonomically using either readily available public databases like GreenGenes or a database that can be privately maintained and curated. In order to compare community structure, a number of distance metrics can be used. An example is UniFrac, which uses the branch lengths on a phylogenetic tree to calculate similarity (50). In short, all operational taxonomic units for multiple samples are compared on the tree, and shared and unshared branch length are measured between communities, resulting in a metric that increases with higher levels of dissimilarity.

Stressors

Rodents have become a common animal model in the field of stress biology due to the well-characterized stressors that can be applied and the highly-reproducible results. One such stressor is social disruption, in which a retired breeder mouse is placed in a cage with the younger test mice. Over the course of two hours, the retired breeder will attack and defeat the test mice, disrupting the established social hierarchy in the test cage. The stressor is applied to the mice for six consecutive evenings, beginning at the start of the mouse active cycle (~1700 hours) (51). As little as two complete cycles of SDR are necessary for a significant increase in corticosterone (CORT) to be observed in the test mice. This increase lasts through the entire six-cycle stressor (52).

Concurrent with the spike in CORT levels, SDR mice exhibit glucocorticoid insensitivity, an effect that is associated with reduced capacity for the GC-GCR complex to translocate to the nucleus in the SDR-exposed mice (52, 53). This is a phenomenon that is generally associated with high levels of systemic CORT, and a resultant desensitization of cells to the molecule (54). CORT induces apoptosis in LPS-stimulated spleen cells, but this effect is abrogated in ex-vivo cells from SDR-exposed mice, as overall cell viability is increased (55). Mice that have been exposed to SDR also exhibit higher levels of systemic inflammation, as measured with IL-6, despite the typically immunosuppressive function of CORT. Physical contact between aggressor and test mouse is required for the insensitivity effect to be observed (56). In total, SDR-induced effects in mice bear many of the same characteristics as patients with major depressive disorder, including increased levels of IL-6 and the GC insensitivity (57, 58).

In addition to the insensitivity, there are whole-scale changes in the murine immune system after SDR exposure. Increased abundance of monocytic and granulocytic progenitors (identified by CD34+) as well as immature Ly6C^{hi} monocytes and Ly6C^{int} granulocytes within the bone marrow and spleen (resulting in increased organ mass, termed splenomegaly) is a major characteristic of SDR (59, 60). CD11b+ macrophages are also increased within the spleen, and show an increased ability to kill pathogens, with concurrently amplified levels of TLR2/4 expression (61). During SDR, CD11b+/Ly6C^{hi} monocytes traffic to the regions of the brain involved in threat appraisal and stress, particularly the amgydala and PFC, mediated in part by amplified expression of the chemokine, CCL2 (62). Abrogating the transit of these cells from the spleen via splenectomy or the use of CCR2 knockout mice abolished anxiety-like behavior, indicating a close association between these cells, their increased movement through the periphery, and the SDR-induced behavioral changes. Interestingly, many SDR-induced effects can be abrogated with the use of propranolol, a β2-adrenergic receptor antagonist, which indicates that the SNS also plays a major role in the SDR response (63, 64).

Other cell types undergo significant changes in activity and abundance, including CD8+ T-cells, which were increased in the lungs of SDR-exposed influenza-infected mice, and natural killer cells (65, 66). In addition to systemic increases in inflammation and innate immune cell trafficking, SDR can exacerbate allergy-like symptoms, and elevate the immune response to other pathogenic challenges, including herpes simplex virus, and *Porphyromonas gingivalis* (51, 67, 68). The microbiota have also been associated with SDR's physiological effects. A recent study demonstrated that SDR-

induced increases to macrophage killing was abolished in germ-free mice; peroxynitrite, a reactive nitrogen species that is used in bacterial killing, was reduced, and overall inflammatory cytokine release (i.e. TNF-α and IL-6) was down (69). Similar findings were reported in mice administered an antibiotic cocktail (70). SDR also alters the diversity of the cecal microbiota in mice (70).

Another group of frequently used stressors are the physical restraint stressors. One of the oldest stressors in the study of stress biology and physiology, restraint consists of closely constraining mice within a tube or along a surface for a set amount of time. Repeated application of restraint stress increases both anxiety-like and depressive-like behavior in mice (71, 72). As with SDR, repeated restraint exposure leads to increases of systemic CORT (71). Overall immune function is suppressed in restraint, with reduced cellular infiltration and inflammation (73, 74). The process of wound healing is also greatly slowed, and IL-1β levels are dysregulated throughout (75, 76). As with SDR, restraint can affect microbial diversity within the murine cecum (77).

The GI Tract

The GI tract is a dynamic system, due in large part to the large surface area and direct contact with the intestinal lumen, in many cases separated from the host by a single cell layer. The majority of the digestive activity occurs in the stomach and small intestine, while water re-uptake and excretion are primary functions within the colon. As a major component in digestion, immunity, and neuroendocrine activity, the tract has a collection of cells with particular functions to assist in these processes. The most abundant cell type

is the absorptive enterocyte, which acts as a major barrier against bacterial invasion. Through these cells, water, ion, and the macromolecules from digested food pass through to the host interior. Much of the control of digestion and motility is modulated by the enteroendocrine cells, which can detect ion levels in the digesta and produce a vast number of hormones including gastrin, which increases motility and stimulates gastric acid secretion, and cholecystokinin which can stimulate fat and protein digestion and inhibit gastric emptying and gastric acid secretion. Acidity is also tightly controlled by a certain cell type. The majority occurs in the stomach, with release mediated by the parietal cells. The activity of these cells is activated by gastrin, histamine, and muscarinic agonists, like acetylcholine. In addition to these GI physiological function hormones, the GI tract is also the primary source of some hormones that have activity elsewhere in the human body. The majority of systemic serotonin (5-HT) is produced from enterochromaffin cells within the GI tract, which are activated upstream by the ventromedial nucleus. 5-HT does have digestion-centric functions, including mediating motility (by increasing contractions around food), mucus secretion, and ion and fluid secretion (78, 79).

If intestinal epithelial cells do not form into enterocyte or enteroendocrine cells, some might differentiate into goblet cells, the primary mucous producers, if activated by the Spdef transcription factor (80). These goblet cells secrete mucous from apically-located villi, in a constitutive and consistent release. However, in the event of a large release, mucin is stored for compound exocytosis (81). Other major cell type can be found in the gut-associated lymphoid tissue (GALT). These areas are key for luminal

sampling and microbiota modeling. The Peyer's patches of the small intestine, mesenteric lymph nodes and lymphoid follicles are all examples of GALT. Because of the close proximity of bacteria and antigen, a strong defense is absolutely necessary. This is partially monitored by maintenance of the tight junctions between the intestinal epithelial cells. Tight junctions are regulated by multiple proteins including claudin, occludin, and zonula occludens (ZO1) (82). Claudins are transmembrane proteins that can attach to other claudins from adjacent cells, controlling the paracellular space. Occludin are also transmembrane proteins that bind to intracellular ZO1, which further binds to F-actin, tightening the junction. Inflammation can increase barrier permeability. One method by which this happens is through TNF-α mediated activation of NFκB, which feeds into transcription repression of occludin and ZO1 (83). TNF-α can also increase myosin light-chain kinase (MLCK) levels, which phosphorylates myosin light chain (MLC), a mechanism that also increases permeability via the endocytosis of occludin (84, 85).

In addition to the cells that modulate GI function, there are also regular physiological processes that are unique to the GI tract. Motility, for example, is the activity of the GI tract to move fecal boluses and digesta from stomach to excretion via the action of the longitudinal and circular muscles, modulated by the myenteric and submucosal plexuses. Movement of a single fecal mass in the human colon can take between 24-48 hours, while in the small intestine, it is around 2-3 hours. This movement is controlled by a number of mechanisms, including the parasympathetic nervous system and acetylcholine, catecholaminergic activity, hormones released from the

enteroendocrine cells, and the interstitial cells of Cajal which maintain a steady pace via action potentials.

Absorption is another function closely managed by the GI tract. In order for this to be completed efficiently, the tract has an incredible expanse of area for larger access to luminal bio-molecules. This massive surface area is due to its cellular structure. New cells are formed in crypts, and divide via signaling from the Wnt pathway (86). Over time, cells move vertically up a villus structure as more cells are produced from the bottom of the crypt. During this period, cells will differentiate into the distinct types dependent upon cell signals. Villus project into the extracellular space, expanding total area for the purpose of increased luminal sampling and molecular uptake, be it water, ions, nutrients or otherwise. Furthermore, microvilli cover the villus, increasing this surface area to an even greater extent.

The Microbiota

The human GI tract is colonized by 10^{14} bacteria, outnumbering total human cells throughout the body by a factor of 10 (87). The vast majority of the GI microbiota are located within the distal portion of the colon, though large communities are still established in both the stomach and small intestine of most mammals (88). The resident microbiota have unique properties depending on the location. For example, small intestinal bacterial groups are key in immune sampling, due to the high density of Peyer's Patches and resident immune cells (89). Similarly, it has been recently posited that the microbes that adhere to the mucosa are more closely involved in immunomodulation,

while luminally-associated groups participate in digestion and metabolism function (90). In the colon, *Firmicutes* and *Bacteroidetes* are often the dominating phyla, with the minor phyla being prominently comprised of groups from *Actinobacteria*, *Verrumicrobia*, *Proteobacteria*, and *Cyanobacteria* (91). Around 400 to 500 unique species can be found in the colon (92). During infancy, the microbiota is characterized by extensive volatility and predominance of groups like *Lactobacillus* and *Bifidobacterium* due to the effect of maternal contact and breastmilk, but as the child begins an adult-like diet, the microbiota also begins to resemble adult profiles (93-95).

Germ-free studies have demonstrated the importance of the microbiota in host physiology and immunity. Using these mice, researchers have shown that the microbiota are involved with vasculature development within the GI tract, affecting epithelial cell differentiation (96, 97). Adiposity and metabolism are closely modulated by the microbiota, as germ-free mice do not respond to high-fat diets with increased weight gain as conventional mice do (98). Glucose tolerance is also increased in germ-free mice, indicating the involvement of the microbiota in metabolism, both health and disease-related (99). Germ-free mice have immature immune responses, with reduced antibody responses, under-developed GALT, lower overall adaptive cell counts, and altered macrophage activity to name just some of the ways in which the presence of the microbiota assist host GI immunity (7, 100-103). Systemic issues also arise in germ-free mice, including increased liver fibrosis and reduced lung inflammatory response to ischemia and reperfusion injury (103, 104).

Individual bacterial groups have been identified in mediating some of these functions. Some groups, including members of the *Lachnospiraceae* family, particularly the *Roseburia* genus, produce short-chain fatty acids (SCFA) that include butyrate, propionate, and acetate, that have major effects on host physiology (105, 106). SCFAs can be anti-inflammatory, repressing NFκB activity (by inhibiting the degradation of IκB) and TNFα transcription, while also increasing MUC2, a component in intestinal mucus, and tight junction proteins (107-111). Changes to diet can lead to shifts in *Bacteroidetes* and *Firmicutes* abundance (112). Alterations to these bacterial groups could affect their ability to modulate physiology, and thus affect host health. This has been corroborated in a recent study in which mice were given antibiotics at birth and had increased prevalence of obesity and changes to overall metabolism (113).

The mucus layer upon which a wide assortment of the microbiota reside is comprised of mucin proteins bound to multiple O-glycan saccharides that are capable of binding water in high concentrations, which gives the mucin a gel-like structure. The primary mucin type depends upon the region of the organism. For example, Muc2 is the primary component within the colon, and Muc5AC predominates within the stomach (114, 115). After translation, Muc2 subunits are linked together via disulfide bonds in the endoplasmic reticulum, and then bound with O-glycans (mainly N-acetylgalactosamine) at the Golgi apparatus, before being stored in granules for secretion from goblet cells (116, 117). The mucosal barrier is also made up of immunoglobulins and Fc-binding proteins for the purpose of commensal monitoring (116). Depth and thickness of the layer varies throughout the GI tract. From the stomach to the jejunum of the small

intestine, the mucus layer does not eclipse 200 uM in total thickness, and in many places, only has a single layer. However, within the colon, two layers exist, a dense, but shallow inner layer, and a much deeper, but diffuse outer layer (118). This outer layer is much less dense due to proteolytic breakdown of the mucin from host enzymes and the microbiota, which uses the mucus as a major food source (119, 120). Bacteria that are able to adhere to the outer mucus layer can attach to clusters of mucus, loose epithelial cells, and residual food, while others adhere directly to the mucus layer. The adherent microbiota can subsist upon these assemblages, using the glycans as a food source (121, 122).

The microbiota are associated with host behavior and brain activity, through the established gut-brain axis. Commensal bacteria can also mediate ACTH release, as illustrated in a study that reported that germ-free mice released higher levels of the hormone upon exposure to a stressor (123). The presence of the microbiota can alter behavior, and appears to have mouse strain-specificity, as switching microbiotas from one strain of mice to another led to shifted outputs in behavioral testing (124). Individual microbial groups can also impact host behavioral outputs. Pathogenic bacteria have been shown to be able to increase brainstem activity and anxiety-like behavior (125). Mice infected with *Campylobacter jejuni* had higher levels of anxiety-like behavior in the elevated maze test as early as two days post-infection (126). *Bifidobacterium longum* and *Lactobacillus helveticus* are able to reduce brain cell apoptosis, and abolish colitis-induced reductions in brain-derived neurotrophic factor (BDNF) through the vagus (127,

128). This same microbe is also able to reduce anger and depression readouts in humans (129). Likewise, some lactobacilli can normalize CORT levels in stress models (130).

The Effect of Stress upon the GI Tract

The GI tract is home to the enteric nervous system (ENS), second only to the central nervous system in total innervation, with around 100 million unique neurons. A primary function of this system is to control intestinal motility in union with the parasympathetic nervous system, while also monitoring digestion, appetite, and absorption. However, exposure to external stressors can have considerable direct effects on the ENS, which can then influence GI function, including motility (131). The HPA arm of the stress response has also been shown to be able to impact GI physiology, through the activity of ACTH, CRH, and urocortin (22, 132, 133). CRH, a 41 amino-acid peptide, can bind to two different receptors, CRHR-1 and CRHR-2. In the colon, the former can be found on goblet and crypt cells, while the latter is predominantly located on crypt cells and blood vessels (134). When CRH binds the receptor, adenylate cyclase is activated and pkA levels are increased. pkA will then phosphorylate downstream targets. CRH can also increase gut permeability, as well as mediate increases in both mucus secretion and motility (132, 135, 136). NE, of the SNS response, can have direct effects upon bacteria. Gram-negatives were able to bloom after 6-OHDA, which destroys noradrenergic neurons and releases NE, was used (137). Also, *Salmonella* was able to grow better in the presence of NE after previous passage through a monkey (138).

The effect of psychological stress on the GI tract depends upon the location. In the upper bowel, stress slows gastric emptying (139). Motility, which is controlled by both the ENS and the sacral parasympathetic preganglionic neurons, is generally increased in the lower bowel (22). Stressor exposure also increases barrier permeability, which leads to leaky gut and the passage of antigen and bacteria into the lamina propria (140). Mucus secretion is increased in short-term stressors, but can be reduced over long-term stressors (132, 141), and ion secretion can also be affected (142). Pain sensitivity is also increased, and in some stressors, base-line inflammation can be elevated (143, 144). A number of chronic bowel illnesses, like the inflammatory bowel diseases, have been associated with stress. Relapses in IBD have been correlated with stressful life events (145). Pain perception in irritable bowel syndrome, which does not have inflammatory pathology, is associated with stress as well (146). Patients exhibiting these illnesses often have altered microbial profiles (147, 148).

Gastrointestinal Immunity and Inflammation

Due to the close proximity to a vast reservoir of bacteria and possible immunogens including foods and parasites, it is necessary for the host GI immunity to be active and far-ranging. The GALT, consisting of Peyer's patches and lymphoid follicles, as well as the mesenteric lymph nodes, are key centers for antigen procurement, processing, and cross-communication between antigen-presenting cells and B/T cells. Within the small intestine, where GI antigen sampling is at its height, microfold (M) cells that are located on the apical surface of the Peyer's patches (PP), take up antigen from the

lumen via endocytosis. The antigen is transported across the cellular space to basolaterally-located dendritic cells and naïve T-cells. Though there are no PP's within the colon, CX3CR1+ macrophages and CD103+ dendritic cells can extend processes into the luminal space for direct sampling (149), and non-PP-associated M cells as well as greater follicular lymphoid cell complexes are dispersed throughout. Monitoring dendritic cells and infiltrating monocytes and macrophages also travel into the lamina propria, which lies adjacent to the basolateral surface of the epithelial cell layer, allowing for extensive immune monitoring and communication throughout the tract.

Within the GALT, intestinal T-cells are primed, activated, and then differentiate into Th1 or Th17 lineage cells, depending on certain signals. Beyond IL-2, which is a primary autocrine stimulator of T-cell activation, other cytokines will induce differentiation into the Th1 lineage, including IL-12 and IL-15. Additionally, co-stimulation is required, as the B7 cell marker of B-cells or antigen-presenting macrophages must attach to the CD28 T-cell marker. The Th17 lineage is further induced when a T-helper cell is activated by IL-6, IL-23 and TGF-β. These cells protect against invading pathogens, in part through the release of inflammatory cytokines IL-17 and IL-22, which can further activate the secretion of antimicrobial peptides as well as increase chemokine-mediated trafficking of inflammatory cells to the site of infection. Interestingly, the presence of segmented filamentous bacteria is reported to induce differentiation of Th17 cells, demonstrating the importance of microbiota structure in immune development (150). Another group of T-cells common to the GI tract is regulatory T-cells (T-regs). These T-regs are defined by early transcription of Foxp3,

which will induce differentiation in an immature T-cell into the T-reg lineage. These cells are involved in the gut tolerance system, as well as overall regulation of T-cell activation throughout the GI tract (151).

Other major activators of the intestinal immune response are the TLRs and NLRs, or NOD-like receptors, which acts as intracellular equivalents of Toll-like receptors. These are pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns. Two of the primary bacterial byproduct-recognizing TLRs, TLR-2/4, which recognize peptidoglycan and lipopolysaccharide respectively, are down-regulated apically in order to avoid overactivation, but are still present basolaterally. NOD2 can recognize muramyl dipeptide, found in both gram positive and gram negative bacteria while NOD1 recognizes mesodiaminopimelic-1 acid, a component of chiefly gram negative cell walls (152, 153). Both PRR types, upon agonist activation, will signal the NFκB and AP-1 pathways. NFκb, formed by p50 and p65 protein subunits, is bound by IκB in its inactive state, but upon phosphorylation, becomes unbound and further ubiquitinated, translocating to the nucleus. The now active NFκB transactivator will bind to a leucine-rich region of the host genome, and activate transcription. NFκB is associated with pro-inflammatory cytokines, chemokines and mediators, the hallmark pro-inflammatory markers in the gut being TNF-α, IL1β, and iNOS. TNF-α increases adhesion molecules and overall barrier permeability (154), and can activate NFκB. IL1β activates neutrophils and T-cells and can increase inducible nitric oxide synthase (iNOS) transcription and nitric oxide production, which is involved in bacterial cell killing. iNOS is an enzyme that catalyzes the conversion of L-arginine and oxygen to L-citrulline and

nitric oxide in the presence of NADPH. Nitric oxide is one of the volatile reactive nitrogen and oxygen species that can disrupt both bacterial and epithelial cells.

Major mediators of GI inflammation are the innate immune cells, including macrophages, monocytes, neutrophils and dendritic cells. These cell types can be identified with various cell markers, including CD11b+ among most of these innate myeloid cells, CD11c+ on dendritic cells, and Ly6G+ on neutrophils. While resident versions of these cells are present during healthy periods, bone marrow-derived inflammatory cells will traffic in upon signal from pro-inflammatory chemokines. Chemokine ligands binding the chemokine receptor on the immunocyte causes actin polymerization, pulling the cell along the chemokine gradient. These trafficking cells adhere to endothelial cells using CD31/PECAM1, and migrate to the site of infection via diapedesis by the combined activity of epithelial selectins (e.g. E-selectin) and cellular adhesion molecules (e.g. ICAM) and receptors like LFA1 upon the leukocyte. Once the cell has arrived, it can act in a variety of different ways, including phagocytosis of pathogens and the subsequent breakdown in lysosomes using reactive oxygen species and other corrosive enzymes. Macrophages, monocytes and dendritic cells might also present antigen to T and B cells within the GALT, or release pro-inflammatory cytokines to increase vasculature size, blood-flow, barrier permeability, and adhesion molecules (154). Neutrophils can also degranulate, releasing a cascade of damaging enzymes. Other methods of bacterial control and mucosal immunity within the GI tract include secretory IgA, a major factor in microbiota modulation. It is produced by the GALTs and can coat various antigens in the GI tract, before eventually being taken up by the sampling

antigen-presenting cells. Paneth cells can also release antimicrobial peptides like cryptdin, RegIIIγ, and lysozyme (155-157), some of which will embed in the mucosal layer (158).

IBD is characterized by a heightened state of inflammation, as well as bleeding, ulceration, diarrhea, and nociception in the GI tract (159). The root of these illnesses is not known, but Crohn's Disease, which afflicts the ileum and colon, is associated with mutation to NOD2, of the NLRs (160). Stressful life events have also been correlated with the diseases (145). Disrupted microbial community structure is also observed in patients with IBD (148), but whether the dysbiotic profile is a cause or effect of the inflammatory microenvironment has not yet been elucidated.

Citrobacter rodentium

The study of GI inflammation is a prospering field and is necessary due to the prevalence of colitis in human hosts through infectious disease like enteropathogenic *Escherichia coli* (EPEC) and *C. difficile*, to the inflammatory bowel diseases. The use of animals such as mice to model mucosal immune and inflammatory responses is commonplace. There are a number of methods by which researchers can induce colitis in murine hosts, be it chemically with dextran sodium sulfate, or with the use of a pathogen. In particular, *Citrobacter rodentium* is a widely used mouse pathogen that has genetic homology to EPEC, and induces an immune response that is similar to some cases of IBD (161). Thus, it makes for an ideal means to model both infectious and non-infectious colitis.

C. rodentium and EPEC both share a pathogenicity island within their genome, named the locus of enterocyte effacement, which allows for the use of a Type-3 secretion system to inject intimin into neighboring epithelial cells (162). Intimin induces actin re-polymerization for the purpose of assembling a pedestal, termed an attaching/effacing lesion, upon which the *C. rodentium* pathogen can better evade immune detection (163). Colonic hyperplasia is induced in *C. rodentium*-infected mice, leading to an increase in colon mass (163). Infection in C57Bl/6 and CD1 mice generally is self-limiting, and clears within 3-4 weeks, peaking by 12-14 days (164, 165).

The typical inflammatory response to *C. rodentium* is Th1 mediated, and includes the release of inducible nitric oxide synthase (iNOS), TNF-α, IL-1β and IL-6 (161, 166). This response is mediated by the NLRs and TLR2, as well as TLR4 to a lesser extent, which will activate the production of CCL2 (167-169). CCL2, a pro-inflammatory chemokine, recruits inflammatory monocytes and macrophages through agonism of CCR2, a cell surface receptor on the leukocytes. These monocytes and macrophages can be identified via cell markers CD11b+/Ly6c^{hi}/F4-80+ and will traffick to the lamina propria (167). Local inflammation is elevated by these trafficking cells through the activity of the transcription factors, NFκB and AP-1. Infectious clearance is resolved with the activity of CD4+ Th17 and T-Reg cells (170). The presence of the mucus layer within the colon acts as a partial defense against the invasion of *C. rodentium*, as Muc2 knockouts have increased severity of infection comparatively (171). Likewise, a healthy microbiota community structure has been shown to be protective against *C. rodentium* infection (172).

Probiotics

Probiotics are living microorganism that can confer a health benefit upon the host. In human hosts, the primary activity of probiotic bacteria is to antagonize enteric pathogens, modulate both innate and adaptive immunity and strengthen mucosal barrier function. Numerable probiotic strains of bacteria are known to exist, with varying degrees of efficacy in affecting host health beneficially. Many lactobacilli, such as *L. rhamnosus*, *L. reuteri*, and *L. paracasei* are probiotic, as well as species within *Bifidobacteria* and individual species like *Escherichia coli* Nissle strain (97, 165, 173-175). General effects can include increasing secretory IgA levels, mediating TNF-α production, and amplifying the adaptive immune response (174, 176). Probiotics can reduce the severity of colonic infection, including *C. rodentium*, *C. difficile*, and *H. pylori* (165, 177, 178). There are also prebiotics, molecules that can aid commensal and probiotic bacteria in growth (179). Common prebiotics include fructo- and galacto-oligosaccharides.

Lactobacillus reuteri

Lactobacillus, a member of the *Firmicutes* phyla, is typically microaerophilic and is a gram-positive bacterium. Members of these genus ferment hexose and other sugars to produce lactic acid, a defining characteristic of this particular group of bacteria. As a result, many lactobacilli within the GI tract prefer acidic microenvironments and can use this as a competitive advantage. Beyond their importance in lactic acid production, lactobacilli are often used as probiotics. *Lactobacillus* species can reduce pro-

inflammatory gene transcription in multiple infection models (178, 180). They can downregulate a number of transcriptional pathways, including IKKα, MAPK, and NFκB by inhibiting ubiquitinylation and affecting phosphorylation (180-182). While it is believed that secreted peptides are responsible for these activities, specific effector molecules have not yet been identified.

Dozens of lactobacilli species have been identified for their probiotic effects. One leading species is *Lactobacillus reuteri*, which can be found throughout mammals in varying concentrations (183). It can also be found consistently in human mother's milk (184). The genome of *L. reuteri* varies based upon host strain, as rodent and human strains have major contrasts. Rodent strain genomes have a urease cluster, as well as a system called SecA2 that gives these strains the ability to export adhesin proteins. Further, rodent-strain *L. reuteri* are able to break down plant sugars using a xylose operon not found in human strains. Many human strain genomes contain the pdu-cbi-cob-hem cluster which is involved in the production of the bacteriocin, reuterin. This cluster also allows human strains to utilize propanediol and glycerol, a key survival tactic due to lower nutrient availability in human colons compared to rodent colons (184). Universally, *L. reuteri* is capable of utilizing multiple electron acceptors (185). Plasmids are present based on strain; F-275, a human strain, does not contain any plasmids, while 100-23, a mouse strain, contains two at a size of around 500 kilobases apiece. *L. reuteri* plasmids can contain genes for the function of antibiotic resistance (186). *L. reuteri* is a natural colonizer of the murine forestomach, but is regularly shed and can be found throughout the GI tract (184, 187). It makes use of mucous binding proteins, called MUBs, that

allows for transient colonization to colonic mucous layers, as well as bacterial aggregation (188, 189).

As with many lactobacilli, *L. reuteri* is anti-inflammatory. It can stimulate IL-10 secretion by driving the development of IL-10-secreting T-regulatory cells, as well as from dendritic cells, and has been shown to be protective in mice deficient in IL-10 (190-192). Under certain growth conditions (i.e., grown anaerobically, in the presence of glycerol), *L. reuteri* can produce reuterin, a bacteriocin (193). Possibly through the use of reuterin, *L. reuteri* is capable of inhibiting invasion by numerous pathogens, including *Salmonella*, *Shigella*, and *Helicobacter* (194, 195). The probiotic can also inhibit *Candida* infection by reducing pH and interfering with *Candida* DNA replication and translation (196). *L. reuteri* has also shown the capability of transiently increasing the diversity of a microbial community, though the mechanism has not yet been uncovered (197).

Obesity and the Microbiota

Microbiota structure is influenced by a variety of factors, not the least of which is available nutrients and resources. Carbohydrate levels are one of the main determinants of the community structure, as large levels of substrate are needed to offset the fact that the basic method of energy generation among the microbiota, fermentation, results in low ATP yield (198). Transit time through the colon can also impact the microbiota, as differential rates of motility can lead to shifts in available nutrients for local bacterial groups (199).

Commensal microbes can influence fat accumulation, as has been demonstrated with germ-free mice. Germ-frees conventionalized with normal microbiota have increased epididymal fat deposition, despite reduced food intake (8). The mice with conventional or conventionalized microbiota exhibited increased lipoprotein lipase, which increases fatty acid uptake, and decreased Fiaf, which monitors the lipoprotein lipase. The production of short-chain fatty acids by the microbiota, such as butyrate, has also been associated with obesity (200, 201). However, while obese subjects exhibit reductions in microbiota diversity and alterations in the *Bacteroidetes:Firmicutes* ratio is commonly seen (17, 201, 202), specific changes to bacterial abundances are often inconsistent from study to study.

Maternal Obesity and Transmissibility

Early dogma regarding the microbiota and the fetus was that the fetus was sterile in utero. This concept is now currently under debate, but it is well known that a large bloom occurs rapidly in the GI tract within hours of birth for the newborn (203). These bacteria are transmitted from mother to child via skin-to-skin contact, breast-milk, and through the vaginal canal. In fact, children born vaginally have a unique microbial structure when compared to children born via caesarean (204). Environmental microbes also make up a portion of the early microbiota. However, the importance of the mother in early microbiota transmission cannot be undervalued. Studies have shown that administering antibiotics to the mother prior to birth can affect the microbiota of the offspring (205). The influence of the mother upon the child microbiota continues until the

child begins to follow a more ‘adult-like’ diet, sometime after the first year of life (95, 203). Parental obesity is a major predictive factor in offspring obesity (206), and while variables like diet and environment are deviated in households with obese parents, the involvement of the microbiota has not been determined as it pertains to mother-child obesity.

Study Hypothesis

The colonic microbiota co-exist with the mammalian host, aiding in many host physiological and immunological processes. Disruptions in the microbiota can feed deleteriously into these processes, disrupting homeostasis. Defining how dysbiotic profiles come to predominate within a GI microbiota is significant in the development of preventative measures and treatments for downstream illnesses. While a number of factors can disturb microbiota structure and induce dysbiosis, the effect of psychological stressor exposure on the microbiota has not been as well defined using modern sequencing methods. Thus, Chapter 2 will evaluate how RST, a repeated stressor, affects the microbiota, and in particular describes how a stressor can affect luminal- and mucosal-associated microbial populations differentially. Chapter 3 looks at how a single cycle of SDR, a less severe social stressor, can affect the microbiota in comparison. Both stressors resulted in reductions of mucosa-associated *Lactobacillus*, a finding corroborated frequently in popular research (36, 207). It is also known that stressful life events are a primary correlate of symptomatic episodes of pain and bleeding in patients with IBD, but there is currently a lack of knowledge into how this association might be

connected to stress-induced changes to the microbiota (145). Previous studies have also shown that exposing mice to RST stress can increase the severity of enteric infection (77). Thus, in Chapter 4, we showed how mice exposed to SDR had a more severe infection and resultant inflammation compared to non-stressed mice. We also hypothesized that stress-induced microbiota community structure was directly associated with the increases in colitic severity, and used germ-free mice and oral gavage of stress-exposed microbiota to demonstrate this correlation. Chapter 5 highlights a viable treatment for stress-induced aggravation of colitic severity. *Lactobacillus reuteri*, a probiotic that can reduce colitic severity in SDR-exposed mice challenged with an enteric pathogen, can also affect microbial diversity in a community (197). Therefore, we examined how this probiotic can affect the microbiota, using Illumina sequencing. While probiotic treatment did not affect microbiota diversity, stressor exposure and infection had lasting effects beyond the peak of infectious colitis and physical application of the stressor. Though this research lends credence to the hypothesis that external factors inducing dybiosis can have significant health effects for the host, application to human hosts is a necessity. Thus, in Chapter 6, we studied the effect that human maternal obesity can have upon the offspring microbiota, in order to analyze a novel extrinsic pathway by which a dysbiotic profile may be established within a host. Because the mother is a main source of microbial transmission, it is feasible that dysbiotic profiles could be passed via this route (204, 208). As hypothesized, children born to obese mothers had a significantly different microbiota in comparison to children born to non-obese, and alterations in health- and diet-associated microbial groups. Chapter 6 makes evident that similar

concepts that compose the murine chapters apply to humans: the influence of external impacts upon the microbiota and their propensity to induce dysbiotic changes.

This thesis demonstrates how external factors can upset equilibrium within a normal microbiota, and in particular, focuses on two under-represented elements in the current literature, psychological stress and maternal influence. We show that stress-induced dysbiotic profiles can have significant effects on host immune and inflammatory responses in murine infection. The association of maternal obesity and offspring microbiota structure indicates both that human microbiotas can be shifted by extrinsic sources much as mice in stress studies can, as well as how early alterations can occur in the microbiota. Together, these seemingly disparate aspects of this thesis highlight universality in the concept of microbiota structure transformation, and open the door to deeper translational analyses into how factors like stress and maternal obesity can affect the microbiota and what the downstream effects on host health might be due to such alterations.

Chapter 2: The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor[1]

Introduction

There are extensive bidirectional interactions between the brain and the gut, and it is well recognized that during a stress response, the central and enteric nervous systems have a large influence on gastrointestinal (GI) motility, secretion, blood flow and immune reactivity (209-211). Thus, it is not surprising that stressor exposure has been linked to exacerbations of intestinal diseases, like the inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), and irritable bowel syndrome (IBS) (145, 212). The mechanisms through which the stress response impacts these diseases are not yet completely understood, but it is possible that stressor-induced changes to the intestinal microbiota are involved. Altered profiles of intestinal microbiota have been identified in both IBD and in IBS patients (88, 213). Thus, understanding factors that maintain microbiota community stability as well as those that can disrupt the structure could lead to a deeper understanding of how factors such as stress can affect intestinal disease.

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The microbiota that colonize the human body collectively outnumber cells of the body by a factor of 10 (87). The vast majority of these microbes are bacteria that reside within the intestines as part of the intestinal microbiota, with microbiota levels ranging from $< 10^5$ bacteria per gram of digesta in the upper parts of the small intestine, to $> 10^{12}$ bacteria per gram of digesta in the large intestine (88). The microbiota reside as a largely stable community that develops as a result of a series of ecological successions involving the selection of species best adapted for the available niche (214). This climax community is relatively resistant and resilient to long-term disruptions in community structure (215), but many factors, such as antibiotic use or colonic inflammation can cause alterations in their community structure (10, 216-218). Whether these types of alterations impact the function of the microbiota is not completely understood, but it is recognized that disrupting the microbiota through the use of antibiotics can enhance pathogen colonization and proliferation (172).

Exposure to stressors has been shown to significantly change microbial populations in the gastrointestinal tract of both humans and laboratory animals. This was first demonstrated in Russian cosmonauts preparing for space flight, who were found to have significantly different levels of shed microbiota, with later studies suggesting that the changes could be due to the stress of confinement (219, 220). In a more recent study, it was demonstrated that lactobacilli levels shed in the stool of college students were lower during final examinations than during quiescent periods, suggesting that the stress of the examinations affected the microbiota (38). These findings are consistent with findings in laboratory animals, where stressor exposure has been shown to significantly

impact the populations of certain bacteria shed in the stool of nonhuman primates, as well as rodents (36, 37, 70, 77, 220). For the most part, these studies have relied on culture-based techniques and have only examined bacteria in the lumen of the intestines or shed in the feces. Although this approach is informative, there are several disadvantages. Approximately 90% of the intestinal microbiota are strict anaerobes that have not been characterized by traditional culture-based methods due to undefined culture conditions (221). In addition, the diversity of the communities present in the lumen of the intestines is different than those found in the mucous layer (222, 223). Thus, it is possible that the stressor-induced alterations to the microbiota might differ between GI compartment.

A recent study using culture-independent 16S rRNA based sequencing has shown that only two hours of acute exposure to the social disruption stressor (SDR) altered the mucosa-associated microbiota of C57BL/6 mice (187). Acute and chronic stressors can have distinct effects upon gastrointestinal physiology, particularly in mucus secretion, wherein acute stressors tend to increase mucus production, while repeated chronic stressors can reduce mucin gene expression (132, 141, 224). It is unknown if chronic stress might also have unique impacts upon the colonic microbiota, or if such impacts might be specific to luminal or mucosa-associated microbial niches. Prolonged restraint (RST) is a widely used chronic murine stressor that has been extensively characterized in the literature and is the most commonly used murine stressor in biomedical and biobehavioral research (225). This stressor involves both physical and psychological components. While the physical components are obvious (i.e., physical confinement), the psychological components are more complex and are thought to reflect the animal's

perception of burrow collapse and confinement (226). The autonomic nervous system and the hypothalamic-pituitary-adrenal axis are stimulated during the stressor as part of a robust physiological stress response (75, 227, 228). SDR and RST, two prime representatives of acute and chronic stressors respectively, have contrasting effects on host immunity and cellular stress responses (51, 55, 67, 75, 228-230), so it is important to evaluate how these stressors might differentially affect the gastrointestinal microbiota. In the present study, it was hypothesized that exposure to the prolonged stressor, RST, would have a unique impact on the colonic mucosa-associated microbiota in comparison to the luminal microbiota. To test this hypothesis, bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed in this study to comprehensively characterize chronic stressor-induced changes to community structure in both lumen and tissue of the mouse colon.

Materials and Methods

Mice: All experiments were performed using male CD-1 mice, 6-8 weeks of age, from Charles River Laboratories. Upon arrival, mice habituated to surroundings for 1 week. Vivarium was AALAC-approved. Food and water provided *ad libitum*, unless experimentation was being performed (food and water deprivation or restraint). Mice were kept on a 12:12 hr light:dark schedule (lights on at 0600). All procedures were approved by The Ohio State University's Animal Care and Use Committee.

Stressor Paradigm: Mice were placed in restraint stress (RST Stressor), non-stressed home cage control (HCC Control) or non-stressed food and water deprivation control (FWD Control) groups. For restraint stress, mice were placed inside ventilated 50-ml

conical tubes beginning at 1800 (start of mouse active cycle) and removed at 0900 the following morning. This was repeated for a total of 7 consecutive overnight restraint sessions. During the stressor period, the HCC Control had full access to food and water and were not handled, and the FWD Control had access to food and water removed to control for the RST Stressor diet conditions. During non-stress periods, all mice were allowed full access to food and water and were not handled.

Tissue Removal: Immediately following the seventh and final cycle of RST Stressor, all mice were humanely euthanized by CO_2 asphyxiation. Colons were sterilely removed with forceps, and all fecal contents were so that mucous layer was not disturbed. Both the colonic contents and tissue were snap-frozen and stored at -80C.

DNA extraction: Samples were centrifuged at 14000 rpm for 30 seconds, then resuspended in 500µL of RLT buffer with β-mercaptoethanol (Qiagen, Valenica, CA). Samples were further lysed in a QIAgen TissueLyser (30 Hz for 5min) with the use of a single sterile steel bead (5-mm) and 500 µL of glass beads (0.1-mm) (Scientific Industries, Inc., NY, USA). After the samples were quickly centrifuged, a 100 µL aliquot of sample was combined with 100 µL of 100% ethanol. This combination was added to a Qiagen DNA spin column, and the QIAamp DNA Mini Kit Tissue Protocol was followed, beginning with Step 7. Elution was performed using 30 µL of water. Samples were quantified with a Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Samples were then diluted to 20 ng/µL.

Pyrosequencing: bTEFAP was performed as described previously at the Research and Testing Laboratory (Lubbock, TX) (77).

Sequence analysis- The Quantitative Insights Into Microbial Ecology (QIIME) software package was used to analyze the sequences obtained from bTEFAP pyrosequencing (46). In summary, sequences were grouped by unique barcode labeling. The mean of all high-quality sequences among all samples was 9119. Lower bound on sequence length was 200 bases and upper-bound was 600 bases. Barcode lengths were 8. Maximum homopolymer run and ambiguous bases allowed were set at 6. No mismatches were allowed in primer sequences. 89.47% of obtained sequences were selected for analysis based upon these thresholds. The analysis was performed on tissue and lumen samples combined as well as both groups separated. Operational Taxonomic Units (OTUs) were made using the pick_otus.py command pipeline. UCLUST was used in OTU-picking at a threshold level of 0.97 in order to cluster like-sequences (231). A representative sequence was obtained from each OTU and aligned to the GreenGenes reference database of sequences using PyNAST (48, 232). Taxonomy was then assigned to each representative sequence using the Greengenes taxonomy database at a minimum confidence threshold of 0.8 (233). A phylogenetic tree was constructed using these aligned sequences for downstream analysis using UniFrac (234).

Statistical Analyses: Non-parametric Kruskal Wallis Tests were used for comparison of HCC Control, FWD Control, and RST Stressor group abundances. Non-parametric Mann-Whitney U Tests with modified Bonferroni corrections were used as post-hoc tests and were performed using SPSS for Windows (v.21, Chicago, IL). The alpha diversity measurement, Shannon Diversity Index (SDI) was computed by QIIME. Shannon, equitability and Chao1 significance was calculated using a parametric T-test at a depth of

7402 sequences for mucosal samples and 5589 sequences for luminal samples with a modified Bonferroni correction, and at a depth of 6177 sequences for combined alpha-diversity comparisons of mucosal and luminal microbiota (235). Unweighted UniFrac principal component analyses and dendograms were created using a rarefaction depth of 8500 for tissue samples alone and 6303 for luminal samples alone and both groups combined. The beta-diversity analysis, analysis of similarity (ANOSIM), was used to detect significant differences in the distance matrices between groups. This was performed using the vegan package of R, implemented in QIIME (47, 236).

Sequence Availability: Sequences were deposited on the Metagenomic Analysis Server MG-RAST and are identified as MG-RAST 4573854.3 through 4573884.3.

Results:

The colonic mucosal microbiota and the luminal microbiota are significantly different

Initial analysis of 16s rRNA gene pyrosequencing data showed that regardless of exposure to the stressor, food and water deprivation, or control conditions, colonic mucosa and the lumen each have a distinct microbiota (Fig. 1). This was first manifest as a significant increase in alpha diversity in mucosa-associated microbial communities. Rarefaction using the observed_species analysis showed that sufficient depth was reached in the sequencing for both luminal and tissue-associated samples (data not shown). Shannon Diversity Index (SDI) was significantly different in the lumen vs. the mucosa ($p<.05$) demonstrating that the colonic mucosa has significantly higher overall alpha diversity than in the lumen (Fig. 1A). Microbial richness, assessed using Chao1, was

similar in the luminal and mucosa-associated microbiota (Fig. 1B). However, there was significantly higher evenness in the mucosa-associated microbiota compared to the luminal-associated microbiota ($p < .05$ as assessed using the equitability measurement) (Fig. 1C).

In addition to the changes in the alpha diversity, the beta diversity of luminal and mucosa-associated microbiota communities were significantly different independently of whether the mice were exposed to the stressor, food and water deprivation or control conditions. Luminal samples clustered separately from the mucosa-associated samples on a principal coordinate analysis (PCoA) plot of the unweighted UniFrac distance matrix for all samples combined (Fig. 1D). The clustering was statistically significant ($p < 0.0005$) based on ANOSIM indicating the mucosal and luminal microbial populations are significantly different. These overall differences extended to significant differences in *Actinobacteria*, *Acidobacteria*, *Deferribacteres*, and *Proteobacteria* at the phyla level (Fig. 1E and 1F). *Actinobacteria* was significantly increased in the luminal group over the mucosal group (1.31% vs. 0.48%) ($p<0.05$), while *Acidobacteria* (0.01% vs. 0.05%) ($p<0.05$), *Deferribacteres* (0.03% vs. 1.00%) ($p<0.001$) and *Proteobacteria* (0.11% vs. 0.20%) ($p<0.05$) were all significantly enriched in the mucosal group (Fig. 1F). The two colonic compartment's contrasting profiles provided the rationale for investigating the effect of stressor exposure on the two microbiotas independently of one another.

Restraint Stress alters the beta-diversity, but not the alpha-diversity of the luminal-associated microbiota

Upon separating the luminal-associated microbiota from the tissue-associated microbiota, the effect of restraint-stress exposure on luminal populations alone was examined. SDI was used to discern changes in overall alpha diversity as a product of stressor exposure, but was unchanged in the luminal microbiota due to restraint stress (Fig. 2A). This was further confirmed by measuring evenness (using the equitability measurement) and richness (using Chao1) in which there no changes due to restraint stress (Figs. 2B, 2C).

The major phyla, *Firmicutes* and *Bacteroidetes,* were unaffected by either restraint stress or food and water deprivation in the luminal-associated microbiota (Fig. 3A). However, restraint-stressor exposed groups (RST Stressor) had significantly lower levels of the phylum *Actinobacteria* compared to both the food and water deprivation control group (FWD Control) and the undisturbed control group (HCC Control) ($p < 0.05$). *Deferribacteres*, another phylum was significantly increased in the RST Stressor group compared to both control groups in the luminally-associated populations ($p < 0.05$) (Fig. 3B). HCC Control and FWD Control were not significantly different among the phyla abundances in the luminally-associated microbiota. In beta diversity analysis, the PCoA of the unweighted UniFrac distance matrix for the luminal samples showed significant clustering of the RST Stressor group compared to the FWD Control group ($p<0.01$), but RST Stressor did not cluster separately from the HCC Control group. In addition, FWD Control and HCC Control groups did not cluster significantly differently from each other (Fig. 3C). Though RST-5 was separated from the other samples, it was

not an outlier using the 2x standard deviation method when the absolute abundance of Firmicutes and Bacteroidetes in RST-5 were compared to other RST samples.

Restraint Stress shifts both alpha and beta diversity of the mucosa-associated microbiota

In contrast to the lack of alpha diversity changes in the luminal associated microbiota, restraint stress altered both the alpha and beta diversity in the mucosa-associated microbiota. Separate analysis of the mucosa-associated microbiota made evident significant shifts in the RST Stressor group compared to FWD Control and HCC Control groups. RST Stressor had a significant reduction compared to FWD Control in alpha diversity using the SDI ($p<0.01$). SDI was not different between RST Stressor and HCC Controls or between FWD and HCC Control groups (Fig. 4A). Further alpha diversity analysis revealed that there were no differences between any groups in richness estimated using Chao1 (Fig. 4B), but the RST Stressor group was associated with a reduction in equitability in comparison with FWD Control ($p<0.05$) but not HCC Control (Fig. 4C). HCC Control and FWD Control groups did not differ in either Chao1 or equitability.

At the phyla level in the mucosa-associated populations, the *Firmicutes* and *Bacteroidetes* were unaffected by exposure to the restraint stressor (Fig. 5A). *Actinobacteria* was significantly reduced in the RST Stressor group compared to both FWD Control and HCC Control mice, while *Deferribacteres* was significantly increased in RST Stressor mice over HCC Control only (Fig. 5B) ($p < 0.05$). There were no significant differences between FWD Control and HCC Control in phyla abundances in

the mucosa-associated microbiota. PCoA of the unweighted UniFrac distance matrix for the mucosa-associated samples illustrated the clustering of the RST Stressor group from the FWD Control and HCC Control groups (Fig. 5C). The clustering was significant, using ANOSIM (HCC Control vs. RST Stressor, $p<0.01$) (FWD Control vs. RST Stressor, $p<0.01$). HCC Control and FWD Control also clustered separately (HCC Control vs. FWD Control, $p<0.05$). Overall, these data highlight that restraint stress as well as food and water deprivation cause changes in the microbiota community membership and composition at the mucosal tissue level.

Restraint Stress alters the relative abundance of colonic microbiota bacterial groups

Larger taxonomic comparisons were performed to distinguish stressor- induced shifts in the microbial groups associated with the mucosa and lumen. In the lumen, the family *S24-7* of the order *Bacteroidales* was significantly reduced in RST Stressor mice ($p<0.05$) compared to HCC Control mice, but not FWD Control mice (Table 1). At genus level, *Adlercreutzia* was decreased in RST Stressor mice ($p<0.05$) in comparison to HCC Control and FWD Control mice (Table 2). Additionally, an unclassified genus in the family S24-7 was significantly decreased in RST Stressor mice compared to HCC Control mice only ($p<0.05$)

In the tissue, the family *Ruminococcaceae* were also increased in RST Stressor over HCC Control ($p<0.05$), and the family S24-7 of the order *Bacteroidales* was reduced in RST Stressor compared to both HCC Control and FWD Control ($p=0.05$). The family *Lactobacillaceae* was also significantly reduced in RST Stressor-exposed mice ($p<0.05$) (Table 3) compared to both FWD Control ($p<0.05$) and HCC Control groups ($p=0.05$).

When genera were examined in the *Lactobacillacea* family, *Lactobacillu*s spp. was the only genus significantly reduced in stressor exposed mice compared to both HCC Control ($p=0.05$) and FWD Control ($p<0.05$) (Table 4); HCC Control and FWD Control were not significantly different from one another. The effects of the stressor on the lactobacilli were limited to mucosa-associated populations, and RST Stressor exposure did not significantly reduce the relative abundance of luminal-associated *Lactobacillus*. The relative abundance of the genus *Oscillospira* was also significantly increased in the mucosa of RST Stressor-exposed mice and FWD Control mice compared to HCC control ($p<0.05$). There were not significant differences in *Oscillospira* relative abundance in the mucosa of RST Stressor and FWD Control mice (Table 4). Food and water deprivation also had effects on bacterial abundances that were independent of stressor exposure. In the lumen, *Candidatus Arthromitus*, a group of segmented filamentous bacteria, and the genus *Ruminococcus* of the *Ruminococcaceae* family were decreased in FWD Control compared to HCC Control ($p<0.05$) (Table 2). The relative abundances of bacterial families and genera were not significantly different between the FWD Control and HCC Control mice when assessed on the mucosal tissue.

Discussion

Exposure to psychological stress has been associated with exacerbation of IBD and heightened immune responses to enteric pathogens (77, 165, 237). Some studies suggest that changes to the microbiota could be involved in stressor-induced GI immune dysfunction, but stressor-induced changes in gut microbiota have not been well-characterized (144, 238). In this study, mice were exposed to a widely used and well-

validated murine model of chronic stress to elucidate the effects of a long-term stressor upon the colonic microbiota. The data show that microbial communities associating with colonic tissue and found in the lumen of the colon have unique community structures that are differentially impacted by psychological stressor exposure. In particular, the restraint stressor significantly reduced the alpha diversity in the mucosa-associated compartment of stressed mice compared to controls, but did not affect the alpha diversity in the luminal populations. Furthermore, stressor-induced alterations in beta-diversity were demonstrated when compared with both food and water-deprived controls and undisturbed HCC Control mice in the mucosa, but stressor-associated changes were only identified between RST Stressor and FWD Control groups in the lumen. The mucosal community-wide effects were partially due to a significant reduction in the relative abundance of bacteria in the immunomodulatory genus *Lactobacillus.* Stressor exposure also affected luminal microbiota groups, such as the genus *Adlercreutzia* that was significantly reduced, but the relative abundance of *Lactobacillus* was not significantly reduced in the colonic lumen of stressor-exposed mice.

The lack of a stressor effect on the relative abundance of luminal lactobacilli was surprising, because previous studies have shown that stressor exposure can reduce fecal lactobacilli. These studies, however, have primarily involved higher mammals. For example, rhesus monkeys that experienced a chronic prenatal stress or maternal separation stress had reductions in *Lactobacillus*, with similar effects of stress upon lactobacilli being reported in college students during exam periods (36-38). However, our current study is in agreement with several other studies involving laboratory rodents

that have failed to find a significant effect of stressor exposure on fecal or luminal lactobacilli levels (70, 144, 239). However, the finding that colonic mucosa-associated lactobacilli are reduced in mice exposed to the chronic restraint stressor is consistent with a previous study in as little as one two-hour exposure to a social stressor significantly reduced the abundance of colonic mucosa-associated *Lactobacillus* in C57BL/6 and CD-1 mice (187). Thus, it is apparent that stressor exposure reduces mucosa-associated, but not luminal, lactobacilli in laboratory mice. Additional studies are needed to confirm and extend these findings in higher mammals.

It is important to note that true stratification of luminal and mucosal populations does not exist, because there is substantial crossover between microbes associated with the lumen and those that can adhere to the mucus layer of the gastrointestinal tract. In this study, we analyzed the majority of the luminal population separately from the mucosa-associated populations. The finding that stressor exposure has strong effects on mucosa-associated populations is important, because gut microbes that adhere to the colonic mucosa can have different effects on the host than luminal populations (90). Mucosa-associated lactobacilli may be particularly important, because this bacterial group is well known for its ability to impact mucosal immune responses. Probiotic *Lactobacillus*-mediated interventions have been shown to downregulate TNF-α in the colon, enhance gut barrier activity, and reduce overall reductions in colitis-related pathology (176, 181, 240, 241). Additionally, treatment with probiotic *Lactobacillus reuteri* in mice abrogates the stressor-induced increases in the severity of the inflammatory response to a colonic pathogen (165). Interestingly, though previous studies

have found that *Lactobacillus* is reduced in the stool (36-38, 207), this study showed a reduction in mucosal epithelium-associated *Lactobacillus* but not in luminal lactobacilli, implying that psychological stressor exposure can have a distinct effects upon groups of mucosal microbiota compared to their luminal counterparts. Since *Lactobacillus* and other commensals can mediate host gut health, this specific reduction might be associated with stressor-induced increases in colitic inflammation (242-244).

Despite the constant crossover between luminal and mucosa-associated populations, unique properties and diversity levels can be associated with each niche (90, 222, 223, 245). Descriptive analyses often concentrate on fecal or luminal contents as a read-out for the entire community structure of the gastrointestinal tract, but the current data emphasize the importance of a targeted approach. In this study, stressor-induced changes to bacterial groups and the extent to which stressor-exposure affected diversity were different based upon the colonic compartment that was analyzed. Bacteria in the colonic lumen and associated with the colonic mucosa have evolved to their present relationship symbiotically and as a result have different biological function. For example, it is thought that the luminal-associated microbiota are more heavily involved in metabolism and digestion, while the microbes of the mucosal epithelium assist the host in immunomodulation (90). Thus, characterizing how factors that cause dysbiosis, like psychological stress, affect microbes in these niches can have different implications.

The current study indicates that chronic stress affects the composition of the colonic mucosa-associated microbiota and thus may influence colonic immunity. Many colonic pathogens, like enteropathogenic or enterohemorrhagic *Escherichia coli* (EPEC

or EHEC, respectively), which are often modeled by murine challenge with *Citrobacter rodentium*, colonize the intestinal epithelium and can cause severe colitis along the epithelial barrier (246, 247). The colonic microbiota are known to influence susceptibility and resistance to these attaching and effacing pathogens (248, 249), since disrupting the mucosa-associated populations with antibiotics increased disease severity upon pathogen challenge (14, 15). Stressor induced shifts to luminal populations might also have downstream effects upon host metabolism. The changes to these niches could be inter-related, wherein possible mechanisms such as stressor-induced reductions or exhaustion of mucous secretion, as well as increased motility could affect the composition of both compartments. Increased shedding of mucosal populations into the lumen would also alter overall community structure. As these populations shift during stressor exposure, it is likely that overall microbiota function is also being impacted in regards to how the microbiota interact with the host and with each other. Proper analysis of these compartment-specific changes could be performed in future studies using whole genome sequencing and metagenomic approaches, as well as metabolomic analyses in order to investigate if the unique stressor-induced changes to lumenal and mucosal population structure extends to distinct changes in functional output.

We have previously shown that exposure to prolonged restraint stressor increases the severity of *C. rodentium* infection upon oral challenge (77, 165). It is not known whether stressor-induced changes to the microbiota were solely responsible for the increased disease severity, but orally treating the mice with probiotic *L. reuteri* reduced the severity of *C. rodentium* infection in stressor-exposed mice (165), suggesting that

preventing stressor-induced reductions in lactobacilli might attenuate colonic inflammation. However, the effects may not be specific to lactobacilli. This study, along with our previous study, demonstrated that prolonged restraint decreases alpha diversity in the colonic mucosa, as well as in the cecal lumen. Reduced diversity has previously been associated with increased cecal colonization by *Campylobacter jejuni* (250), marked by marginally increased pro-inflammatory marker (e.g. IL-1β, IL-6, TNF-α, IFN-γ, IL-10) levels, while mouse strains susceptible to *C. rodentium* infection have lower fecal bacterial counts (particularly in *Bacteroidetes*) than those that are resistant (251). Thus, it is possible that the shifts in certain bacterial populations evident in the microbiota of stressor-exposed mice in this study contribute to enhanced colonic inflammation. Indeed, a preliminary, unpublished work by Galley et al. indicate that RST-induced changes to the microbiota are directly associated with an colonic inflammatory response to a pathogen challenge, likely due to shifts in microbiota function. Further studies are already underway to identify how compositional changes in the microbiota are related to functional shifts that affect host health.

It is currently not known whether stressor-induced changes to the microbiota contribute to exacerbation of human colonic inflammation, but it has been well-documented that patients with IBD and IBS often have an altered microbiota compared to healthy subjects. Such differences include a reduction in overall diversity, and shifts in major phyla like the *Firmicutes* and *Bacteroidetes*, as well as commensals like *Bifidobacterium, Faecalibacterium prausnitzii* and *Roseburia* (13, 252-255). It is not yet clear whether the changed microbiota is the cause or the effect of the increased

inflammatory flare-ups and sensitivity to pain, but studies are beginning to demonstrate a role of the microbiota in the exacerbation of these diseases (256, 257). Of particular importance, exposure to psychological stress often precedes the exacerbation of IBD (145, 212). In our experience, the effects of stressor exposure on microbial community structure are more consistently manifest on the mucosal surface versus the luminal or fecal compartments. While it is a goal to understand how changes to microbial community structure in the feces predicts changes to community structure at the mucosal surface, until these relationships are well understood, mucosa-associated populations should be assessed in studies of the brain-gut microbiota axis during periods of health and disease.

In the current study, mice were exposed to repeated daily exposures to the prolonged restraint. Interestingly, this results in partial habituation; corticosterone levels after 1 day of the stressor are higher than corticosterone levels after 6 days of the stressor (even though corticosterone levels are higher than baseline levels on all days of stressor exposure) (258). Despite this habituation, past studies show that the effects of stressor exposure upon the microbiota are additive, with significant effects of the stressor on microbiota composition, including absolute levels of *Lactobacillus reuteri*, only being evident after repeated cycles of the stressor (187). It is not yet known whether alterations in the microbiota contribute to this stressor-induced corticosterone profile, but it is known that microbiota do significantly impact hypothalamic-pituitary-adrenal activity and corticosterone levels (123, 259). Thus, it is possible that differences in corticosterone levels and in microbial communities over repeated cycles of stressor exposure are inter-

related. However, repeated cycles of stress are not always needed in order for changes in the microbiota to manifest. For example, a single 2-hr exposure to a social stressor was sufficient to significantly shift the mucosa-associated microbiota (187). Further research is needed to determine why a single exposure to some stressors is sufficient to change the microbiota, whereas others require repeated exposures.

Prolonged restraint is a widely used murine stressor that has been extensively characterized in the literature and is one of the most commonly used murine stressors in biomedical and biobehavioral research (225), in part because it reliably induces a physiological stress response that results in the elevation of endogenous glucocorticoids (75, 227, 228). Because the strong glucocorticoid response can suppress multiple components of the immune response (228, 260), it seemed plausible that stressor-induced suppression of the mucosal immune response led to the observed changes in microbiota community structure. However, RST did not induce changes in TNF-α, iNOS, or IL-6 message levels in the colonic tissue (data not shown). Moreover, in the absence of pathogen challenge, stressor exposure had no effect on other measures of mucosal immunity (e.g., secretory IgA, β-defensin-1, and leukocyte infiltration into the colon) in previously published work (77). Thus, it is unlikely that RST-induced suppression of immune activity strongly affects the composition of the gut microbiota. However, immunomodulation is not the only way in which stressor exposure may impact the composition of the gut microbiota. In an olfactory bulbectomy model of stressor sensitization, Park et al. (2013) demonstrated that stressor-induced increases in colonic motility impact microbiota structure through a corticotrophin-releasing hormone (CRH)-

dependent mechanism (261). Because CRH-induced alterations in colonic motility can be disrupted by cutting the vagal nerve(135), and because the vagus nerve is considered a primary route through which the brain and gut microbiota interact (130, 262, 263), it is possible that the observed stressor-induced changes in the colonic microbiota are vagally-mediated. This hypothesis warrants further attention.

Results from studies involving behavioral stressors in laboratory animals must be cautiously extrapolated to humans due to differences in host perceptions to stressful stimuli, the physiological stress response, and available coping mechanisms. However, microbiota community structure alterations that consistently occur in response to stressors of different type (e.g., physical vs. social), different duration (e.g., a few hours vs. a few days or weeks), and in different host species (e.g., rodents vs. non-human primates) can be predicted to occur in human populations. Although the prolonged restraint stressor used in the current study is considered a severe, chronic stressor, results obtained with prolonged restraint are consistent with an increasing number of studies indicating that stressor exposure changes gut microbiota community composition (70, 77). This was previously demonstrated with exposure to as little as 2 hrs of a social stressor(187), and now has been demonstrated with a prolonged exposure to a physical stressor. In both cases, exposure to the stressor reduced lactobacilli. Stressor-induced reductions in lactobacilli have also been observed in non-human primates (36, 37), suggesting that reductions in lactobacilli are a conserved response to stressor exposure that is likely to occur in human populations. In support of this contention, stressor-

induced reductions in lactobacilli have been observed in college students during examination periods (38).

Diet can greatly affect the GI microbiota (11, 264). The data here demonstrate that food and water deprivation is able to affect the community structure of the mucosal associated microbiota in comparison with undisturbed controls, while also affecting the abundances of select bacterial groups in the lumen. This confirms a previous study that showed separate clustering of control mice from FWD mice after a single 16-hr period of food and water deprivation (77). Thus, it is difficult to fully control for the effects of food and water deprivation in stress studies. However, samples from RST Stressor mice consistently cluster separately from samples from FWD Control (and HCC Control) mice indicating that the psychological and physical aspects of the prolonged stressor have unique effects on the microbiota, including a reduction in *Lactobacillus* and overall alpha diversity. These changes can not simply be accounted for by the fact that mice in restraining tubes do not consume food or water, since the effects are not evident in animals deprived of food and water. We have not found that the overall quantity of food consumption is affected by RST, as the stressor-exposed mice consume an equivalent amount of food as HCC mice, despite having reduced access (unpublished observations). However, since mice are restrained during the dark (i.e., active) cycle, which is when mice typically consume their food, exposure to the RST stressor undoubtedly skews feeding patterns while leaving overall food quantity unchanged. This likely affects overall metabolism, since mice exposed to the RST stressor lose weight with repeated exposures to the stressor. Thus, it is clear that further studies are needed to understand

the individual contributions of the stress response and food and water deprivation on stressor-induced alterations of the GI microbiota.

Dysbiotic microbial profiles are associated with inflammation and increases in disease severity. Stressful periods, which can alter microbial community structures, can worsen the pathology of colonic pathogens (e.g., EHEC) and inflammatory disorders (e.g., IBD) (145). Thus, investigations into the disruptions in the community structure of beneficial GI microbes as a result of external effectors like psychological stress are especially important. These results further develop the relationship between psychological stress and the microbiota by demonstrating that restraint stressor-induced dysbiosis in the colonic microbiota changes the community composition of both the luminal and mucosa-associated populations, but that the stressor-induced disruptions upon each niche are distinct.

	HCC	FWD	RST		
Lactobacillaceae	30.06 ± 8.32	37.56 ± 8.19	20.50 ± 5.27		
S24-7	22.54 ± 2.82	18.81 ± 2.30	13.16 ± 2.25	*	RST vs. HCC
Clostridiales;f__	12.41 ± 1.50	14.25 ± 2.83	20.75 ± 6.60		
Lachnospiraceae	12.80 ± 4.79	9.21 ± 1.70	17.41 ± 3.83		
Rikenellaceae	4.52 ± 0.43	4.71 ± 0.80	7.18 ± 0.70		
Bacteroidaceae	3.56 ± 0.92	2.20 ± 0.29	7.74 ± 2.13		
Ruminococcaceae	2.42 ± 0.49	3.19 ± 0.53	5.04 ± 1.32		
Unassigned	3.32 ± 0.38	3.74 ± 0.27	2.57 ± 0.20	*	RST v FWD
Clostridiaceae	4.36 ± 0.94	1.23 ± 0.43	1.64 ± 0.51	*	HCC v FWD
Prevotellaceae	0.38 ± 0.19	0.90 ± 0.30	2.04 ± 1.28		

Data are the mean relative abundance ± standard error.
* $p < .05$

Table 1: Top 10 Most Abundant Colonic Luminal-Associated Bacterial Families, Restraint

	HCC	FWD	RST	
Lactobacillus spp.	30.06 ± 9.61	37.56 ± 8.19	20.50 ± 5.27	
S24-7, g__	22.54 ± 3.26	18.81 ± 2.30	13.16 ± 2.25**	RST vs. HCC
Clostridiales; f__; g__	12.42 ± 1.73	14.25 ± 2.83	20.75 ± 6.60	
Lachnospiraceae; g__	9.90 ± 4.55	7.11 ± 1.34	15.57 ± 3.61	
Rikenellaceae; g__	4.52 ± 0.50	4.71 ± 0.80	7.17 ± 0.70	
Bacteroides spp.	3.56 ± 1.06	2.20 ± 0.29	7.74 ± 2.13	
Unassigned	3.32 ± 0.43	3.74 ± 0.27	2.57 ± 0.21**	RST vs. FWD
Candidatus Athromitus	4.19 ± 1.08	1.02 ± 0.40	1.50 ± 0.51**	HCC vs. FWD
Oscillospira spp.	1.06 ± 0.22	0.88 ± 0.15	2.10 ± 0.61	
Prevotella spp.	0.38 ± 0.22	0.90 ± 0.30	2.04 ± 1.28	
Adlercreutzia spp.	2.11 ± 0.38	1.61 ± 0.30	0.20 ± 0.02**	RST vs. HCC/FWD
Ruminococcaceae; g__	0.89 ± 0.20	1.23 ± 0.28	1.90 ± 0.56	
Lachnospiraceae; Ruminococcus	1.62 ± 0.81	1.08 ± 0.30	0.63 ± 0.25	
Lachnospiraceae; Other	0.75 ± 0.29	0.48 ± 0.06	0.64 ± 0.25	
Ruminococcaceae; Ruminococcus	0.21 ± 0.09	0.91 ± 0.21	0.55 ± 0.11**	HCC vs. FWD

Data are the mean relative abundance ± standard error.
** $p < .05$, * $p = .05$

Table 2: Top 15 Most Abundant Luminal-Associated Bacterial Genera, Restraint

	HCC	FWD	RST		
Clostridales; f__	25.67 ± 6.27	31.03 ± 3.58	34.92 ± 5.49		
S24-7	30.74 ± 7.85	20.94 ± 4.22	9.85 ± 2.02		
Lachnospiraceae	8.68 ± 1.62	13.15 ± 2.82	16.49 ± 4.35		
Rikenellaceae	8.54 ± 1.60	8.74 ± 2.20	11.23 ± 1.75		
Ruminococcaceae	5.23 ± 1.26	7.88 ± 0.84	10.95 ± 1.39	*	RST vs. HCC
Bacteroidaceae	5.51 ± 1.59	3.24 ± 0.63	7.98 ± 2.01		
Lactobacillaceae	6.75 ± 0.32	5.46 ± 1.39	1.25 ± 0.43	*	RST vs. FWD
Unassigned	3.64 ± 0.63	3.44 ± 0.15	0.23 ± 0.12	*	RST vs. FWD
Prevotellaceae	0.98 ± 0.56	0.72 ± 0.23	1.77 ± 1.26		
Deferribacteraceae	0.14 ± 0.05	2.08 ± 1.71	0.74 ± 0.12	*	RST vs. HCC

Data are the mean relative abundance ± standard error.
* $p < .05$

Table 3: Top 10 Most Abundant Colonic Mucosal-Associated Bacterial Families, Restraint

	HCC	FWD	RST		
Clostridiales; f__; g__	25.67 ± 6.27	31.03 ± 3.58	34.92 ± 5.49		
S24-7; g__	30.74 ± 7.85	20.94 ± 4.22	9.85 ± 2.02		
Lachnospiraceae; g__	6.64 ± 1.15	10.60 ± 2.60	14.36 ± 4.02		
Rikenellaceae; g__	8.54 ± 1.60	8.73 ± 2.20	11.22 ± 1.75		
Bacteroides spp.	5.51 ± 1.59	3.24 ± 0.63	7.98 ± 2.01		
Lactobacillus spp.	6.75 ± 3.20	5.46 ± 1.39	1.25 ± 0.43	*	RST vs. FWD
Oscillospira spp.	1.75 ± 0.32	3.44 ± 0.36	4.69 ± 0.69	*	HCC vs. FWD/RST
Unassigned	3.64 ± 0.63	3.44 ± 0.15	2.33 ± 0.12	*	RST vs. FWD
Ruminococcaceae; g__	1.98 ± 0.54	2.78 ± 0.18	3.81 ± 0.79		
Prevotella spp.	0.98 ± 0.56	0.72 ± 0.23	1.77 ± 1.26		
Mucispirillum spp.	0.14 ± 0.05	2.08 ± 1.71	0.74 ± 0.12	*	RST vs. HCC
Ruminococcaceae; Other	0.51 ± 0.11	0.73 ± 0.25	1.58 ± 0.52		
Ruminococcaceae; Ruminococcus	0.99 ± 0.45	0.91 ± 0.18	0.85 ± 0.17		
Lachnospiraceae; Ruminococcus	0.87 ± 0.32	0.93 ± 0.16	0.82 ± 0.19		
Clostridiales; Other; Other	0.69 ± 0.04	0.89 ± 0.16	0.56 ± 0.09		

Data are the mean relative abundance ± standard error.
* $p < .05$

Table 4: Top 15 Most Abundant Mucosal-Associated Bacterial Genera, Restraint

Figure 1: The mucosa-associated and luminal-associated microbiota communities are significantly different from each other. Alpha diversity measurements,including Shannon Diversity, Chao1, and equitability were calculated using QIIME and compared. A.) The Shannon Diversity Index was significantly increased in mucosal samples over luminal samples as measured with QIIME and averaged by group. B.) Richness, estimated with Chao1, was unchanged between both compartments. C.) Evenness, using the equitability measurement, was also increased in the mucosal samples over luminal samples. All data in A-C are mean ± S.D. All alpha diversity measurements were analyzed using a parametric T-test on QIIME at a sequence depth of 6177. D.) Luminal samples and mucosal samples clustered independently of one another based upon unweighted Unifrac distances on a Principle Coordinate Analysis using the ANOSIM statistic ($p<0.0005$). E.) Major phyla, including Firmicutes and Bacteroidetes, were unchanged when compared between luminal and mucosal-associated samples. F.) There were significant differences in the lesser phyla when compared between mucosa-associated microbiota and luminally-associated microbiota. The relative abundance of *Actinobacteria*, *Deferribacteres*, *Proteobacteria*, and *Acidobacteria* were all significantly different when compared between both gastrointestinal compartments. Phyla-level relative abundance was compared using Mann-Whitney U non-parametric tests. Mucosal-associated samples n=15; luminal-associated samples n=14.

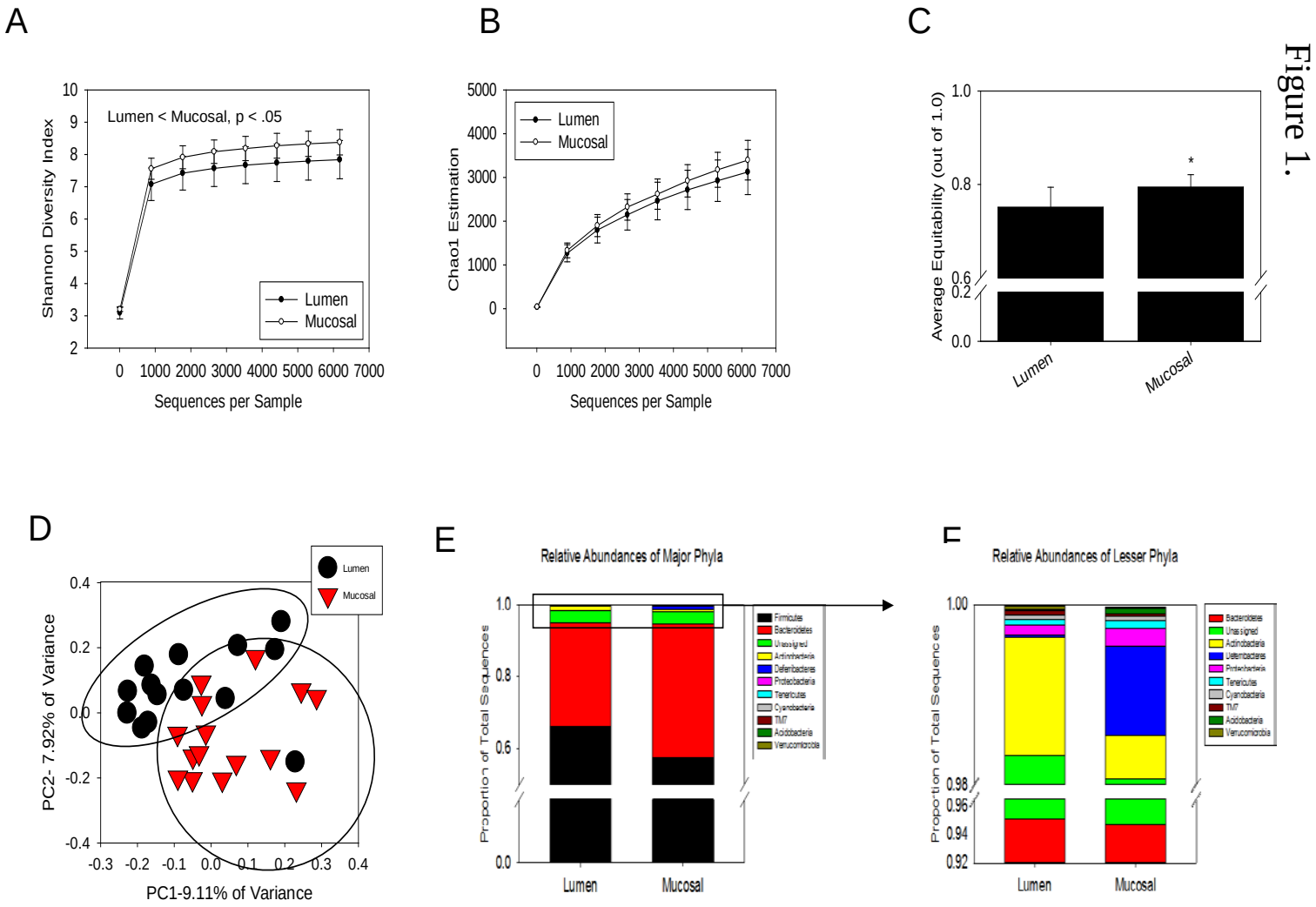
A
Lumen < Mucosal, p < .05
Shannon Diversity Index
Sequences per Sample
Lumen
Mucosal
B
Chao1 Estimation
Sequences per Sample
Lumen
Mucosal
C
Average Equitability (out of 1.0)
Lumen
Mucosal
D
PC2- 7.92% of Variance
PC1-9.11% of Variance
Lumen
Mucosal
E
Relative Abundances of Major Phyla
Proportion of Total Sequences
Lumen
Mucosal
Relative Abundances of Lesser Phyla
Proportion of Total Sequences
Lumen
Mucosal

Figure 1.

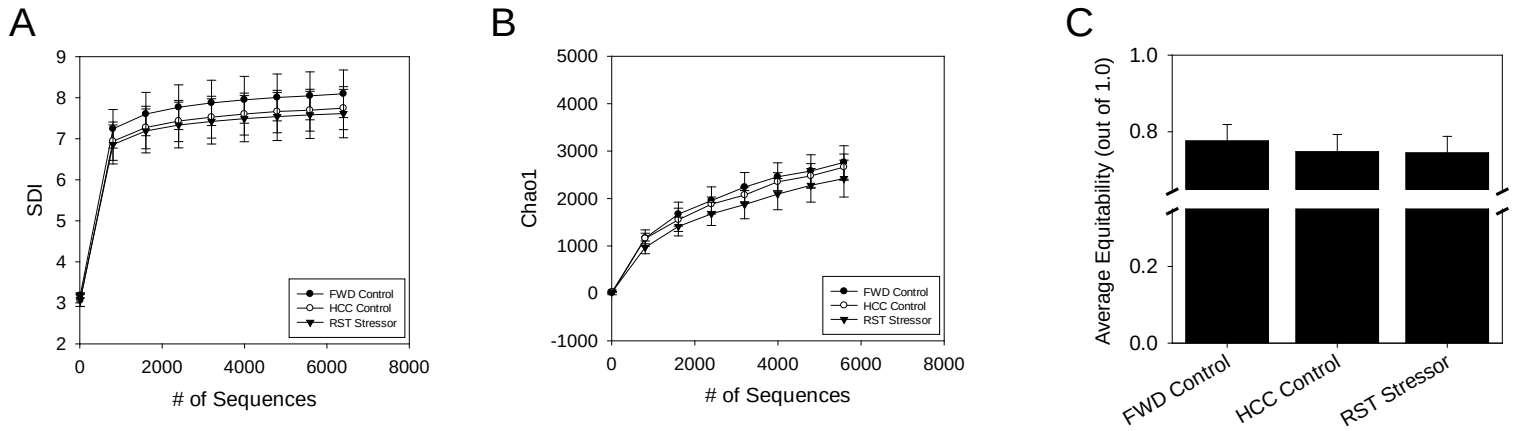

Figure 2: Stressor exposure was not associated with shifts in alpha diversity in the luminally-associated microbiota. A.) The Shannon Diversity index (SDI), B.) richness (using Chao1) and C.) Evenness (using equitability measurement) was measured for each sample using QIIME and averaged by group. None of the alpha diversity measurements were affected by stressor exposure. Data are mean ± S.D. N=5 for all groups, with the exception of HCC-Control, which had n=4. Groups were compared using parametric T-tests on QIIME with a modified Bonferroni correction for multiple comparisons.

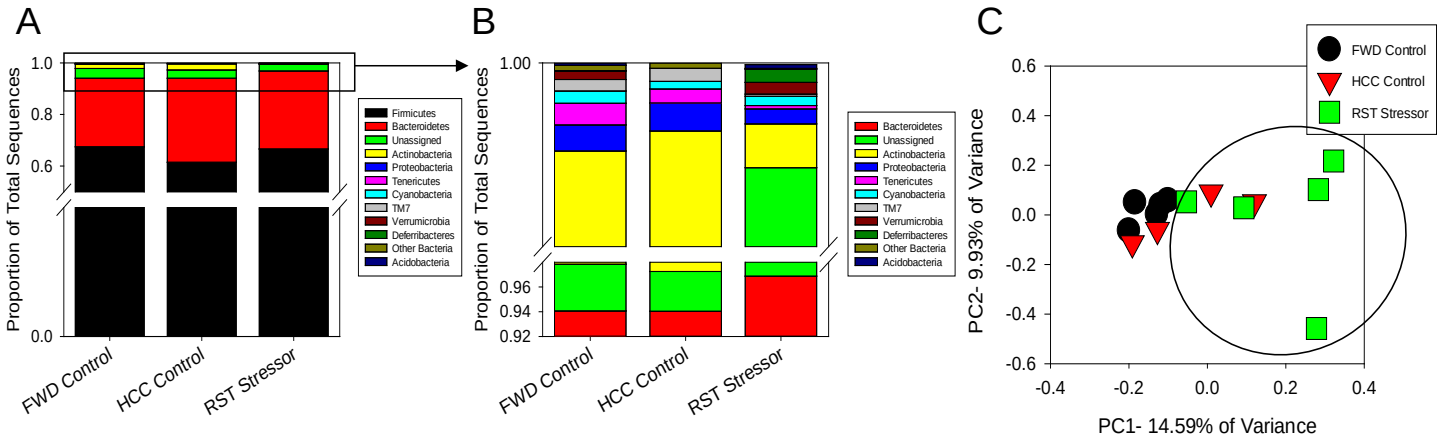

Figure 3: Stressor exposure significantly affects the community structure of the luminally-associated microbiota. A.) Major phyla were unchanged between any group. B.) Among the lesser phyla, *Actinobacteria* was significantly reduced in RST Stressor mice compared to both HCC Control and FWD Control, while *Deferribacteres* was significantly reduced in RST Stressor mice compared to HCC Control and FWD Control mice. Abundances were compared using non-parametric Kruskal-Wallis tests, and post-hoc testing was performed with non-parametric Mann-Whitney U tests. C.) Principle coordinate analysis was used to visualize stressor exposure-induced community profile clustering based upon unweighted Unifrac distances. RST Stressor shifted the community structure of the luminally-associated microbiota compared to FWD Control mice ($p < 0.01$) significantly using the ANOSIM statistic, but not significantly compared to HCC Control mice. FWD and HCC Controls were unchanged compared to each other. Data are from n=5 for RST Stressor and FWD Control groups, and n=4 for HCC Control.

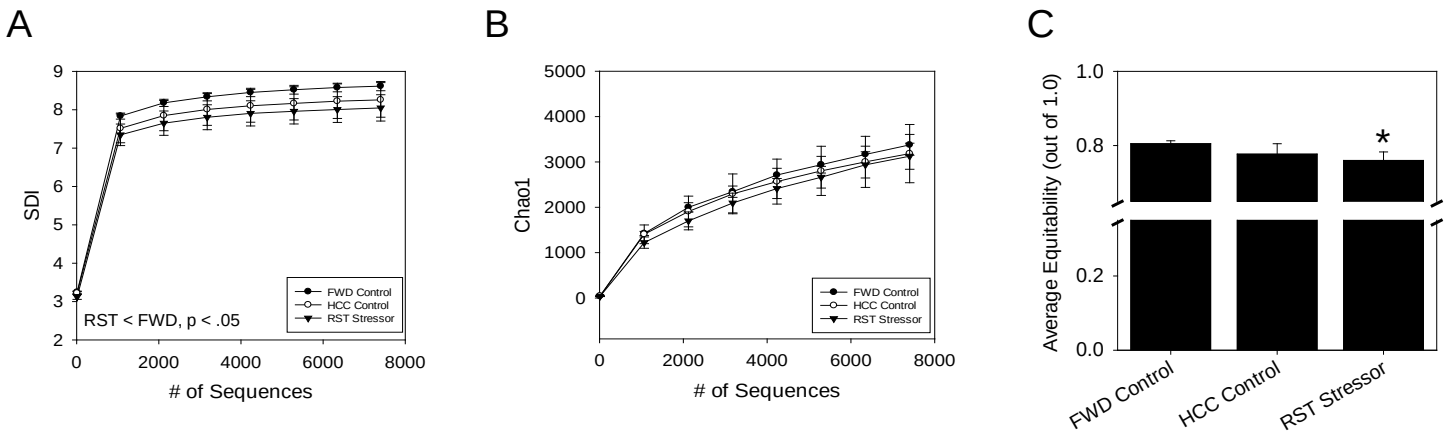

Figure 4: Stressor exposure significantly reduces alpha diversity in the mucosa-associated microbiota. A.) The Shannon Diversity index (SDI) was measured for each sample and averaged by group. RST Stressor was significantly lower than FWD Control mice ($p<0.05$), but was not significantly different than HCC Control SDI values. B.) Richness (by Chao1 measurement) and C.) evenness (by equitability) were then calculated using QIIME and averaged by group. RST Stressor did not affect overall richness, but evenness was reduced in RST Stressor-exposed mice when compared to FWD Control mice ($p<0.05$). Data are mean ± S.D. for n=5 for all groups. For alpha diversity, groups were compared using parametric T-tests on QIIME with a modified Bonferroni correction for multiple comparisons.

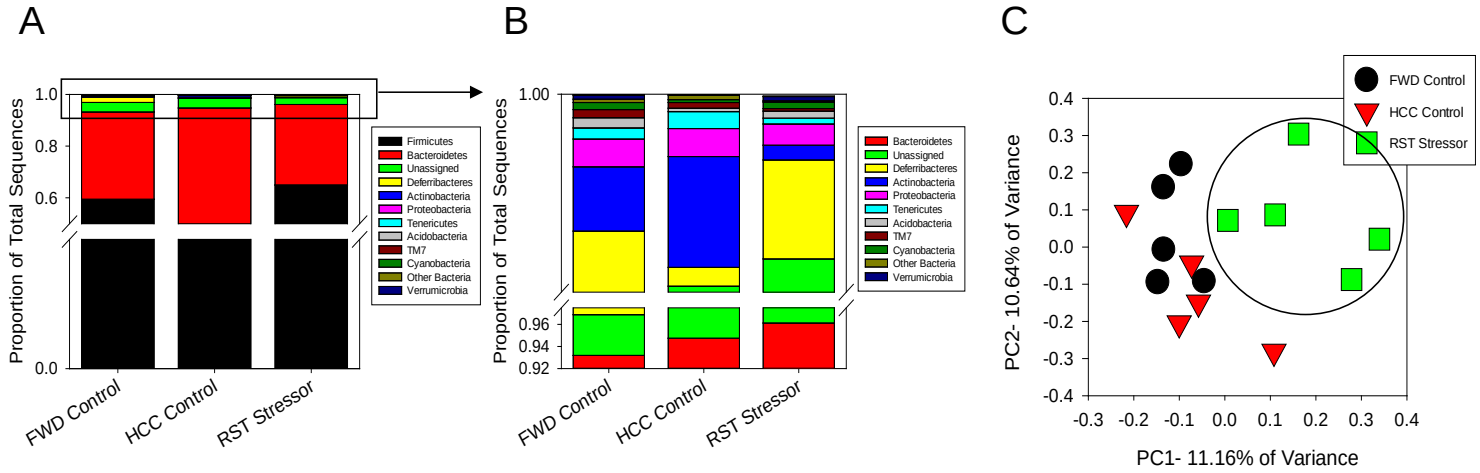

Figure 5: Stressor exposure significantly alters the community structure of the mucosa-associated microbiota. A.) *Firmicutes* and *Bacteroidetes* were unchanged between any of the groups in the experiment. B.) Upon examining the lesser phyla, *Actinobacteria* was significantly reduced in the RST Stressor group compared to FWD Control and HCC Control mice. *Deferribacteres* was significantly increased in RST Stressor mice compared to HCC Control mice, but not FWD Control mice. Phyla abundance data were compared first using non-parametric Kruskal-Wallis test, and Mann-Whitney U tests were used post-hoc. C.) Principle coordinate analysis was used to visualize clustering of similar community profiles. Restraint stressor-exposed mice significantly clustered using the ANOSIM statistic (HCC Control vs. RST Stressor, $p < 0.01$) (FWD Control vs. RST Stressor, $p < 0.01$). FWD Control and HCC Control also clustered significantly apart from each other ($p < 0.05$). Data are for N=5 for all groups.

Exposure to the prolonged stressor, restraint, significantly disrupted the colonic mucosa-associated microbiota community structure, as well as the structure of the luminally-associated populations. The specific effects upon the two unique niches differed, as restraint stress reduced the commensal genus, *Lactobacillus*, in the mucosal compartment, but not in the lumen. The food and water-deprivation control group also displayed significant shifts in colonic mucosa community structure, indicating that the dietary shifts associated with the restraint stressor may partially explain the results. Further, restraint stress induces physiological and immunological impacts that are distinct from other stressors due in part to the long-term repeated nature of the stressor. Thus, the well-characterized stressor, social disruption, which induces heightened immune cell trafficking and increased phagocytic activity among other hallmarks, was used in order to examine if stressor-induced effects upon the microbiota are an artifact of restraint stress or an outcome associated with psychological stress as a whole.

Chapter 3: Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota[2]

Introduction:

The human body is colonized by an enormous array of microbes collectively referred to as the microbiota. It is estimated that there are approximately ten times more bacterial cells than there are human cells in the human body with microbiota being found in various receptive niches such as on the skin, in the oral and respiratory tracts, reproductive tract, and most numerously, in the gastrointestinal (GI) tract (87, 265). In the GI tract, the microbiota form a community that fully interacts with one another and with the host. The GI microbiota have multiple beneficial effects on their hosts (8, 266, 267) and the structure of the GI community can impact these functions. Of the beneficial interactions between microbiota and host, those that involve the immune system are some of the most intensively studied, and several lines of experiments have demonstrated that the GI microbiota and the host immune system are deeply intertwined in each other's development (268). For example, germ-free mice, which have never been colonized by any type of microbe, have diminished immune responses compared to mice colonized with a healthy microbiota (269, 270). Mice deficient in Nod2, a gene that encodes for a

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receptor that is involved in immune recognition of bacterial muramyl dipeptide, develop a unique microbiota that can lead to colonic inflammation when transmitted to wild-type mice (5, 271). It is now well recognized that the GI microbiota have strong effects on immunoregulation, and immune system activity, in turn, helps to shape the GI microbiota (268-271).

It is estimated that over 500 different species of bacteria can colonize the GI tract (272). Despite this enormous species-level diversity, only a small number of bacterial phyla are represented. The vast majority of bacteria in the human GI tracts belong to the phyla *Firmicutes* and *Bacteroidetes*, with bacteria in the phyla *Actinobacteria* and *Proteobacteria* also comprising a smaller portion of the overall microbiota as well (91). The GI microbiota of the murine GI tract is similar to that of humans, with the majority of microbes belonging to the major phyla, *Firmicutes* and *Bacteroidetes* (70, 273, 274). These populations shift slowly over time, but their general stability is important to the health of the host. Abrupt changes to the gut microbiota have been shown to potentially lead to serious negative host health outcomes, including diarrhea, opportunistic infections, and obesity (275-277). Changes to the microbiota can be caused by factors such as antibiotic use and severe enteric infection. Data from this laboratory, as well as others, indicate that exposure to either physical or psychological stressors can also alter intestinal microbe profiles. Stressor exposure early in life has been demonstrated to alter the types and abundance of bacteria found in the intestines. Separating infant monkeys from their mothers to induce a physiological stress response resulted in a significant reduction in the number of total lactobacilli that could be cultured from the stool (36).

Reductions in lactobacilli are meaningful as certain species, including *L. reuteri*, are involved in immunomodulation (165, 278). In rats, separating the pups from their mothers during the first 14 days of life led to an altered GI microbiota (279). Stressor exposure during adulthood can also impact the stability of the intestinal microbiota. For example, exposing adult mice to a prolonged restraint stressor was shown to significantly alter microbial profiles in the cecal contents (77).

Similar findings have been associated with a social stressor called social disruption (SDR) (70) that involves repeated social defeat as a result of inter-male aggression over a 2 hr period. When repeated over 6 consecutive nights, this stressor induces a physiological stress response marked by the activation of the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system (63). The study by Bailey et al demonstrated that this week-long exposure to the stressor can alter the cecal luminal microbiota in outbred CD-1 mice (70). However, it is not known whether a single 2 hr exposure to the stressor could induce similar alterations, or whether other mouse strains, such as inbred C57BL/6 mice that are widely used in infectious disease research, are affected by stressor exposure. Knowing whether short-lasting stressors can impact the microbiota has translational importance particularly for the inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). Patients with IBD often report stressful periods preceding symptom flares (145). It is not clear whether acute stress can exacerbate IBD symptomatology, but it is recognized that acute stressor exposure is associated with increased visceral sensitivity in IBS (280). Because alterations in gut microbiota community structure are evident in IBD and IBS (88, 213), and because these

alterations are thought to possibly contribute to these diseases (281, 282), we determined whether a short-lasting stressor was sufficient to impact gut microbiota community structure.

Previous studies assessing the effects of stress on the microbiota have relied on assessment of intestinal lumen or fecal populations (37, 70, 77). Studies in healthy individuals as well as patients with IBS, IBD, or hepatic encephalopathy demonstrate that luminal/fecal microbiota is significantly different than mucosa-associated microbiota (283, 284). Because shifts in microbial populations that are in close proximity to the intestinal epithelium are thought to have the strongest effects on host immunity (90), an additional aspect of this study was to determine whether stressor exposure could impact tissue-associated microbial community profiles.

Stressor-induced changes in lactobacilli have reliably been found in laboratory animals as well as in humans (36, 38, 207). However, stressor-induced changes in other taxa have not been widely reported. Thus, 454 pyrosequencing was performed on the colonic tissue of inbred C57BL/6 mice to determine whether a single 2 hr stressor exposure could change colonic tissue-associated microbial community profiles. Quantitative PCR (qPCR) was then used to determine whether stressor-induced alterations in relative abundances evident with the 454 pyrosequencing were also evident as alterations in absolute abundance in both inbred and outbred mice.

Materials and Methods:

Mice: Male CD-1 mice and male C57BL/6, aged 6-8 weeks, were ordered from Charles River Laboratories (Wilmington, MA; Raleigh, NC). The mice were housed in groups of

2 or 3 mice per cage in an approved vivarium and were allowed to habituate to the vivarium for one week prior to testing. The cages were kept in an approved vivarium with a 12:12 hour light-dark schedule with lights on 0600 to 1800. Food and water was available *ad libitum*. The Ohio State University's Animal Care and Use Committee approved all experimental procedures (Protocol 2009A0235-R1). CD-1 mice were used in cytokine and bacterial qPCR experiments. C57BL/6 mice were only used in the 454 pyrosequencing analysis and the cytokine and bacterial qPCR experiments.

Social Disruption Stressor: The SDR experiments were performed as described previously (285). Briefly, an aggressive male CD-1 retired breeder, termed the aggressor, was placed into a cage with younger C57BL/6 resident mice at 1700 hours, which represents the beginning of the mouse active cycle. The aggressor repeatedly attacked and defeated the C57BL/6 test mice over the course of the 2 hour SDR cycle. If the aggressor did not begin to attack the test mice within 10 min of placement in the cage, it was removed and another aggressor was put in. Wounding was monitored throughout the SDR cycle. Only slight superficial wounds were allowed. If wounds that penetrated the cutaneous layer developed over the course of SDR, those test mice were removed from the study. While it would be desirable to test the effects of the stressor on both male and female mice, female mice do not fight in this paradigm. Thus, all analyses were limited to male mice.

*Tissue Removal***:** After exposure to the SDR stressor (a single 2 hr cycle for pyrosequencing, a single 2 hr cycle or 2 hr cycles repeated on 6 consecutive days for qPCR), or at the equivalent time in non-stressed HCC mice, mice were euthanized using

CO_2 asphyxiation. Colons were removed aseptically. Luminal contents were removed by cutting into the tissue where liquid and/or solid contents rested, and gently extracting with forceps. This represented the non-mucosa associated populations. Scraping/tissue squeezing was not performed so as not to disturb the mucosa-associated populations, which are adhered to an easily disturbed mucous layer. The colonic tissue and remaining adherent microbiota populations were placed into a microcentrifuge tube and snap frozen in liquid nitrogen. All samples were stored at -80°C until processing. Studies involving 454 pyrotag sequencing utilized the C57BL/6 colonic tissue that was collected after a single 2 hr cycle of SDR, with n=5 per group (HCC vs. SDR). Animals were kept 3 and 2 mice/cage to minimize cage effects. Studies involving bacterial quantification PCR utilized colonic tissue collected at 0 days of SDR (n = 16 C57BL6, n = 21 CD-1), following a single 2 hr cycle of SDR (n = 7 C57BL/6, n = 15 CD-1) as well as samples collected after SDR repeated on 6 consecutive days (n = 8 C57BL/6, n = 6 CD-1). All experiments were n = 3 or 4 per group. The single 2 hour cycle of SDR in C57BL/6 was replicated once, and six repeated exposures of SDR on C57BL/6 was replicated twice. The single 2 hour cycle of SDR in CD1 mice was replicated four times, and six repeated exposures of SDR on CD1 mice was replicated once. Successive replicates were performed to reduce high variability between samples in bacterial qPCR. No-SDR-exposure groups were sacrificed alongside both single 2 hr cycle of SDR and 6 repeated cycles of SDR groups and combined during statistical analyses, resulting in an increased number of replications over test groups.

bTEFAP pyrosequencing: : Amplicon pyrosequencing (bTEFAP®) was originally described by Dowd et al. on C57BL/6 mice colonic samples has been utilized in describing a wide range of environmental and health related microbiotas including the intestinal populations of a variety of sample types and environments (41, 286, 287). In this protocol, a 1-mm segment was used from the center of the entire colonic length. In a modified version of this process, 16S universal Eubacterial primers 530F 5'GTGCCAGCMGCNGCGG and 1100R 5'GGGTTNCGNTCGTTR were used in a single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA). The following conditions were used: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds; 53°C for 40 seconds and 72°C for 1 minute; after which a final elongation step at 72°C for 5 minutes was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing Roche 454 FLX titanium instruments and reagents and following manufacturer's guidelines.

Microbial community analysis: The returned sequences were processed and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) (version 1.4.0) (46) according to standard protocols. Filtering passed sequences based on: length between 200 and 1000bp, zero ambiguous bases or primer mismatches, and no homopolymer runs greater than 6. 97.9% of the sequences passed quality filtering for further analysis. After filtering, there was an average of 10496 sequences per sample. Sequences with 97% similarity were grouped into OTU using UCLUST (231). Representative sequences from

each OTU were aligned against the Greengenes core reference alignment using PyNAST (48, 232). The RDP Classifier was used to assign taxonomy to each representative sequence against the RDP database using standard options (44). The minimum confidence threshold for taxonomic assignment was 0.80. OTUs were considered unclassified if there was not a strong match within this confidence interval for the representative sequence within the RDP database. A phylogenetic tree was built using FastTree from the aligned OTU representative sequences for determining UniFrac distances between samples (234).

Quantification of Bacterial Groups Using Quantitative Polymerase Chain Reaction (qPCR): DNA Extraction: 50 mg of tissue, taken from the ascending colon portion of the colonic tract, were homogenized in liquid nitrogen in a mortar and pestle and transferred to a microcentrifuge tube. The samples were incubated for 75min in lysozyme buffer (20 mg/mL lysozyme, 20 mM Tris-HCl, 2mM EDTA, 1.2% Triton) at 37°C to weaken the cell wall of Gram-positive bacteria. Following this, DNA was isolated using a modified version of the Qiagen DNA Mini Isolation Kit protocol (Qiagen, Germany). In short, 100 µL of Buffer ATL and 20 µL of proteinase K were added to the microcentrifuge tube and incubated for 3.5 hr at 56°C, with manual vortexing at 30 minute intervals for the duration of the incubation. The samples were heated at 85°C for 10 min to inactivate proteinase K and then transferred into a new tube containing 20 µL of proteinase K and 200 µL of Buffer AL. The samples were incubated at 56°C for 30 min followed by 95°C for 10 min. 200 uL of absolute ethanol was then added to the sample and the sample was added to a QIAamp spin column. After sample washing, the DNA from each sample was

recovered into 100 µL of Buffer AE using a QIAamp spin column per manufacturer's protocols. The DNA extracts were stored at -80°C until analysis was performed. Genomic DNA from pure bacterial cultures was isolated essentially as described above, but using a Qiagen protocol for the isolation of genomic DNA from Gram-positive bacteria and shorter (45 minutes instead of 75 minutes) incubation in the lysozyme buffer.

*Sample Derived Standards***:** The method of Chen et al. (2007) was adapted for the generation of sample-derived qPCR standards (288). Briefly, equivalent mass amounts of colonic DNA from each sample were pooled as the template for PCR to obtain even bacterial representation in order to generate standards for bacterial copy number quantification. DNA was quantified using the Quant-it PicoGreen kit (Life Technologies, Grand Island, New York). Amplification PCR targeting specific genera or species-level 16S rRNA was performed to obtain standard amplicons based upon the representative populations within each sample. PCR was achieved using an ABIPrism 7000 thermocycler. The primer sets are shown in Table 1. The standard amplification PCR thermoprofile for the genus *Lactobacillus* consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 40 sec, and final extension at 72°C for 10 min. For *L. reuteri* PCR, the thermoprofile used was the same as those for the genus-specific except for a higher primer annealing temperature (60°C instead of 58°C). The *Bacteroides-Prevotella-Porphyromonas* standard amplification thermoprofile was 1 cycle at 95°C for 5 min, followed by 35 cycles of 95°C for 20s, 68°C for 30s, and 72° for 55s, with 1 final annealing cycle of 72°C for 5 min.

The *Parabacteroides distasonis* standard amplification thermoprofile was 1 cycle at 94°C for 5 min, followed by 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 40s, with 1 final annealing cycle at 72°C for 8 min. After the amplification PCR, the PCR product was purified using the Qiagen PCR Purification Kit and quantified by Quant-it PicoGreen kit to determine copy number of the community standards.

A standard curve for absolute quantification was created from the previously amplified standards to encompass 10^1-10^8 copies per reaction. The population of total lactobacilli and *L. reuteri* was separately quantified using respective specific primers and SYBR Green against these dilution series as described previously (289). qPCR thermoprofiles for *Lactobacillus* and *L. reuteri* were 1 cycle at 94°C for 5 min, followed by 40 cycles of 94°C for 30s, specific annealing temperature for 30s, and 72°C for 40s. Annealing temperature for *Lactobacillus* was 58°C and *L. reuteri* was 60°C. qPCR thermoprofile for *P. distasonis* was 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, then 40 cycles at 95°C for 15s and 60°C for 60s. qPCR thermoprofile for *Bacteroides-Prevotella-Porphyromonas* was 1 cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 15s, 68°C for 20s, and 72°C for 30s. Abundance of bacterial groups (copies of 16s rRNA gene per gram/sample) was computed based on the copies of qPCR reaction and the number of reactions that could be performed with the DNA derived from 1 g of each tissue sample. The detection limit were as follows: total lactobacilli and *Bacteroides-Prevotella-Porphyromonas* was ~4.0 log copies/gram of wet tissue ($\log_{10}$), *L. reuteri* was ~4.5 log copies/gram of wet tissue ($\log_{10}$), *P. distasonis* was ~5.0 log copies/gram of wet

tissue. Primer sequences have been previously published and are listed in Table 1(290-294).

*Quantification of Colonic Cytokine and Inflammatory Mediator mRNA using quantitative RT-PCR***:** Total RNA was isolated from whole colonic tissue samples (~120 mg) using TRI-zol (Invitrogen, Carlsbad, California) according to manufacturer's instructions. The extracted RNA was quantified via spectrophotometry, and converted to cDNA using the Reverse Transcription System (Promega, Madison, WI). qPCR was then completed using a master mix containing 2× Universal TaqMan master mix (Life Technologies, Grand Island, New York), 0.9 μM (each) forward and reverse primers (see Table 5), and 0.250 μg sample cDNA. 18S was used as the housekeeping gene. The PCR was performed using a Prism 7000 sequence detection system with the following thermoprofile: 2 min at 50°C, 10 min at 95°C, and then 40 amplification cycles of 15 s at 95°C and 1 min at 60°C. The relative amount of mRNA was determined using the comparative cycle threshold (C_T) method as previously described (295). Non-stressed HCC control samples were used as baseline controls and were set at a value of 1. All other samples are based on a fold change from these control samples. Primer sequences have been previously published and are listed in Table 1 (69). All groups were n = 3 – 5, using a single experiment without replicates.

Statistical Analyses: Alpha diversity measurements, including Shannon diversity index, equitability (evenness) and Chao (richness) were computed using QIIME on the groups (HCC control vs. SDR Stressor) (235). Non-parametric T-tests with 999 Monte Carlo

permutations were used on these alpha diversity measurements to test for significance at a sampling depth of 4805 sequences per sample.

Taxonomic abundances attained from QIIME were compared between the two groups of mice using non-parametric Mann-Whitney U Tests. PCoA of unweighted UniFrac distance matrices was used to determine clustering between the two groups (HCC control vs. SDR Stressor) (50). Analysis of Similarity (ANOSIM), a beta-diversity statistic that is available through the vegan package of R and accessible with QIIME, was used to calculate statistical significance between the distance matrices of groups at 999 permutations(47, 236). All of these analyses were performed in QIIME.

Differences in bacterial abundances were determined using non-parametric Kruskal Wallis tests with the cycle of SDR (i.e., 0, 1, or 6 cycles) as the single factor. Mann-Whitney U tests were performed *a priori.* Changes in cytokine gene expression were determined using a two-factor ANOVA with group (HCC control vs. SDR Stressor) and Day (1 Day vs. 6 Days) as the two independent factors. Protected means comparisons were used as post-hoc tests. In all cases, α was set at 0.05, while tendency was declared at $0.05<p<0.10$. These tests were performed using SPSS v.21 (IBM, Chicago, IL).

Availability of Supporting Data: The sequences supporting the results of this article are available in the NCBI Sequence Read Archive under the study accession number SRP035598 (http://www.ncbi.nlm.nih.gov/sra/?term=SRP035598).

Results:

Social stress affects the community structure of the colonic mucosa-associated microbiota- We analyzed the effect of a short term SDR stressor on the colonic mucosa-associated microbiota using 16S rRNA gene pyrosequencing of samples from both SDR C57BL/6 stressor-exposed mice and non-stressed HCC control mice. After normalization of the datasets by rarefaction, we observed no statistical difference in the total number of operational taxonomic units (OTU) between the SDR and control mice (data not shown). Additional analysis of the Shannon, equitability and Chao1 alpha diversity metrics showed no statistical difference between the two groups (Figure 6A-C). Thus, a single two-hour cycle of exposure to the social stressor did not affect the alpha diversity of the mucosa-associated microbiota.

While we did not observe any statistically significant differences in the alpha diversity of the microbiota between the SDR and control group, beta diversity analyses did reveal differences in composition. A principal coordinates analysis (PCoA) of the UniFrac distances between samples showed that they clustered into separate groups according to treatment (Figure 7A). An unweighted pair group with arithmetic mean (UPGMA) hierarchical clustering dendrogram confirmed this clustering (Figure 7B). Statistical analysis of the UniFrac distances using the analysis of similarity statistic (ANOSIM) indicated that colonic mucosal microbiota from SDR stressor-exposed mice were significantly different from those of the home cage control (HCC) mice ($p < 0.05$).

Populations of the genus *Lactobacillus* and *L. reuteri* are reduced in mice exposed to a social stressor- The taxonomic profile of the microbiota at the phylum level showed no significant differences between the two treatment groups (Figure 8). Analysis of relative

abundances at lower taxonomic levels showed a reduction in the family *Porphyromonadaceae* in SDR stressor-exposed mice compared to non-stressed HCC mice ($p<0.01$) (Table 6). Exposure to the SDR stressor also reduced the relative abundance of bacteria in the family *Lactobacillaceae* ($p < .05$) (Table 6). This reduction in the *Lactobacillaceae* was reflected by a reduction in the relative abundance of bacteria in the genus *Lactobacillus* ($p < .05$) (Table 7). In addition to the reduction in the abundance of lactobacilli, it was evident that exposure to the SDR stressor significantly reduced the relative abundance of the genus *Parabacteroides* ($p < .01$), as well as an unclassified group within the phylum *Firmicutes* ($p < .05$) and an unclassified group within the class *Bacilli* ($p < .05$) (Table 7).

Many bacteria in the genus *Lactobacillus* have been shown to have immunomodulatory functions in the colon, and can be used as a probiotic to treat inflammation. Thus, we used qPCR to quantify the effect of the SDR stressor on the abundance of this group of bacteria (165, 278). When the a priori hypothesis that repeated administration of the SDR stressor would reduce the abundance of lactobacilli was tested, it was determined that the absolute abundance of *Lactobacillus* spp. was significantly lower ($p < .05$) after 6 cycles of the stressor in comparison to baseline levels in C57BL/6 mice (Figure 9A). This reduction in lactobacilli abundance was also observed in outbred CD-1 mice ($p < .05$), with the largest reduction in mean levels of lactobacilli also occurring after 6 days of the SDR stressor (Figure 9B). In addition, qPCR analyses also revealed that exposure to the SDR stressor significantly reduced the abundance of the immunomodulatory species *L. reuteri* over the course of six cycles of

SDR ($p < .05$). This difference was only evident in outbred CD-1 mice (Figure 9C). L. *reuteri* levels were below the qPCR limit of detection of 4.5 copies/gram of wet tissue (log_{10}) in inbred C57BL/6 mice.

Relative bacterial levels differ by mouse strain- In order to confirm that stressor exposure was reducing the absolute abundance of other bacterial groups that were reduced in relative abundance in the pyrosequencing analysis, qPCR was performed on colonic tissues from both inbred C57BL/6 mice and outbred CD1 mice. Primers targeting the 16s ribosomal RNA genes of *Parabacteroides distasonis*, a member of the *Parabacteroides* genus, and *Bacteroides-Prevotella-Porphyromonas* were used. Stressor exposure did not significantly affect the absolute abundance of either *P. distasonis* or *Bacteroides-Prevotella-Porphyromonas* genera (Table 8). In addition, levels of *Bacteroides-Prevotella-Porphyromonas* group and *P. distasonis* were similar in the two strains of mice.

Colonic inflammatory cytokine mRNA levels are not affected by social stressor exposure- The colonic mRNA levels for IL-1β, TNF-α, and iNOS were unaffected by exposure to the SDR stressor, suggesting that exposing C57BL/6 and CD-1 mice to the SDR stressor did not result in detectable increases in colonic cytokines or inflammatory mediators (Table 9).

Discussion:

Exposure to physical and psychological stressors has been shown to impact the gut microbiota of both laboratory animals and humans (36, 38, 279). However, the majority of the previous studies have utilized repeated and prolonged stressors, and have

assessed microbial populations in the lumen of the intestines or present in the fecal matter. Whether stressor exposure has different effects on the microbiota of different strains of mice is also poorly understood. Because gut microbes that adhere to the colonic mucosa can have different effects on the host (90), this study assessed whether stressor exposure could alter the community structure of mucosa-associated microbes. This study demonstrated that as little as 2 hrs of stressor exposure is enough to significantly change the structure of the microbiota associated with the colonic mucosa. This effect was not manifest as alterations in alpha diversity, but rather as alterations in beta diversity. The ANOSIM distance matrix analysis and cluster analysis based on unweighted UniFrac demonstrated that microbiota within the samples from stressor-exposed mice were significantly different than those from the non-stressed HCC control mice. In addition to significantly changing beta diversity, exposure to the stressor significantly changed the relative abundance of 2 bacterial genera, namely *Parabacteroides* and *Lactobacillus,* and one bacterial family, *Porphyromonadaceae*.

This study targeted mucosa-associated populations, but true stratification of luminal and mucosal populations does not exist. There is much crossover between microbes often associated with the lumen and those that can adhere to the mucus layer of the GI tract, as the former becomes trapped in the mucus layer and the latter sheds into the lumen. Steps were taken to remove the majority of the fecal matter from tissue samples, wherein lie the majority of the luminal portion of the GI microbiota. This study extends previous studies that indicated that luminal and fecal microbiota can change as a consequence of stressor exposure to now include mucosa-associated populations.

To determine whether the stressor effects only encompassed changes to bacterial relative abundance or whether changes in absolute abundance (as assessed by determining copies of 16s rRNA gene per gram of sample) may also result from stressor exposure, qPCR was used to further investigate the effects of the stressor on bacterial abundance. This was performed in both an inbred (i.e., C57BL/6) and an outbred (i.e., CD-1) mouse strain, because studies consistently show that microbiota composition is associated with mouse strain (296, 297). In addition, different mouse strains can also have different physiological and behavioral responses to stressor exposure, including changes to anxiety-like behavior, as well as diarrhea output and colonic serotonin concentration (298, 299). Thus, it was important to determine whether stressor effects were conserved across mouse strains. While 2 hrs of stressor exposure was enough to reduce the relative abundance of the genus *Lactobacillus*, a reduction in the absolute abundance was only evident after repeated exposure to the stressor. This indicates that some of the effects that stressor exposure has upon the microbiota are additive. Both pre- and post-stressor lactobacilli levels were similar in outbred CD-1 mice and inbred C57BL/6 mouse strains, demonstrating that the effects of the stressor are consistent across mouse strains. This finding was not surprising given that stressor-induced reduction in lactobacilli levels have been documented in other host species, including human and non-human primates.

The finding that stressor exposure reduced the relative and absolute abundance of lactobacilli is consistent with previous studies demonstrating that stressor exposure can reduce the lactobacilli (36-38, 207). To date, however, the *Lactobacillus* species that can

be reduced by stressor exposure has not been addressed. Many species of bacteria in the genus *Lactobacillus* are known to have health promoting effects, and studies from this laboratory, as well as others, indicate that administering probiotic *L. reuteri* to mice can reduce colonic inflammatory responses (165, 181, 278). Because stressor exposure can exacerbate colonic inflammation (300), we focused on whether indigenous *L. reuteri* was reduced in stressor-exposed animals. As predicted, *L. reuteri* was significantly reduced in colonic mucosa from outbred CD-1 mice exposed to the SDR stressor. Interestingly, however, *L. reuteri* was not consistently detectable in inbred C57BL/6 mice. Thus, it is apparent that *L. reuteri* levels on the mucosa of C57BL/6 mice are lower than levels observed in outbred CD-1 mice and below the detection limit (4.5 log copies/gram of wet tissue).

Although it is difficult to compare deep sequencing results across different experiments that assess different body niches and encompass different stressors, the use of 454 pyrosequencing demonstrated that the effects of stressor exposure are not confined to effects on the lactobacilli. The relative abundance of bacteria in the family *Porphyromonadaceae* was found to be significantly reduced by exposure to the SDR stressor. This finding is consistent with a previous study demonstrating that exposure to a prolonged restraint stressor in oubred CD-1 mice was sufficient to reduce the relative abundance of cecal *Porphyromonadaceae* (77). However, it was not previously known whether stressor exposure could reduce the absolute abundance of the *Porphyromonadaceae* family. Thus, qPCR was used to determine whether this stressor also affected the absolute abundance of *Porphyromonadaceae* by assessing levels of

bacteria in the *Porphyromonas-Bacteroides-Prevotella* group as a surrogate marker. This was performed because of the problems designing qPCR primers specific for the *Porphyromonadaceae* family. The absolute abundance of the *Porphyromonas-Bacteorides-Prevotella* group was similar in CD-1 mice and inbred C57BL/6 mice, and stressor exposure had no effect on the absolute abundance in either mouse strain. Thus, although this study and others [23] have found stressor-induced reductions in the relative abundance of *Porphyromonadaceae*, these results indicate that absolute abundance of the *Porphyromonadaceae* is unaffected by stressor exposure. Stressor exposure also did not affect the absolute abundance of *P. diastonosis* in either strain of mice, even though the relative abundance of *Parabacteroides* was reduced as detected by 454 pyrosequencing.

Colonic inflammation is well known to impact the gut microbiota, and studies have found that intestinal inflammation can also reduce the abundance of the family *Lactobacillaceae* (164, 301) and *Porphyromonadaceae* (302). Given the bidirectional interaction between the microbiota and intestinal inflammation and increased intestinal physiological inflammation upon exposure to stressor (303), it is often suggested that increases in intestinal inflammation during stressful periods could be responsible for changes in gut microbiota. However, inflammatory cytokine gene expression was not significantly changed by either 2 hrs of the SDR stressor or 2 hrs of the stressor repeated on 6 consecutive days. Thus, the results of this study indicate that it is not likely that stressor-induced colonic inflammation is responsible for altering the abundances of the indigenous colonic mucosa-associated microbiota.

The effects of SDR on male mouse immunological, physiological, and behavioral functioning have been well characterized over the past fifteen years (53, 65, 304). The consistent activation of the HPA axis and the SNS makes SDR an ideal method of inducing an acute stress response in male mice. While it would be desirable to determine the effects on female mice as well, aggression in female mice is too low to induce a physiological stress response in this paradigm. Other stressor paradigms, however, have shown that stressor exposure in a lab setting can also affect the microbiota composition of female mice (238, 305). Thus, it is likely that a social stressor in female mice would also impact the lactobacilli, as well as *Porphyromonadaceae* and *Parabacteroides*. Interestingly, studies have shown that male and female microbiota can evoke different levels of host sex hormone release and immunological outputs that can then feedback and alter microbial composition (306). Future studies comparing the microbiota, mucosal immunity and endocrine responses in both male and female mice are likely to demonstrate additional multidirectional interactions between host physiology and the microbiota. This requires further study.

Gut microbes have important effects on the host especially when they are associated with the mucosa. Previous studies have found that stressor exposure can affect the microbiota, in particular the lactobacilli. However, many of these studies focused on microbes that are shed in the stool during prolonged or chronic stressors (36, 37, 77, 305). It was not previously clear whether the effects of stress on the microbiota were limited to changes in the number of bacteria shed in the stool, nor whether short lasting acute stressors could also impact the microbiota. The present study demonstrates that

exposure to as little as 2 hrs of a social stressor is sufficient to significantly affect some populations of the colonic mucosa-associated microbiota. This builds upon previous work by showing that exposure to the social stressor affects multiple regions (e.g. colon, cecum) and niches (e.g. lumen, mucosa-associated) of the GI tract, as well as two different strains of mice, suggesting that the effect of social stresss exposure upon the microbiota is a universal process, as opposed to either an artifact or isolated finding. The mechanisms by which this occurs are not yet understood, but are not likely to involve stressor-induced inflammation in the colon. Future studies should assess the impact of stressor-associated hormones and gut functioning since others have demonstrated that activation of the stress-associated hormone corticotrophin releasing hormone (CRH) alters gut microbiota, an effect that was associated with alterations in intestinal motility (261). Because the gut microbiota has been associated with diverse diseases that are known to be exacerbated by stressful situations, such as the inflammatory bowel diseases, irritable bowel syndrome, and even multiple sclerosis, it is becoming increasingly important to understand the impact that stress can have on the microbiota and whether stressor-induced alterations in the microbiota are involved in stressor-induced disease exacerbation (237, 307-309).

Lactobacillus genus	Forward	AGCAGTAGGGAATCTTCCA
	Reverse	CACCGCTACACATGGAG
Lactobacillus reuteri	Forward	CAGACAATCTTTGATTGTTTAG
	Reverse	GCTTGTTGGTTTGGGCTCTTC
Parabacteroides distasonis	Forward	TGCCTATCAGAGGGGGATAAC
	Reverse	GCAAATATTCCCATGCGGGAT
Porphyromonas -Bacteroides -Prevotella	Forward	GGTGTCGGCTTAAGTGCCAT
	Reverse	CGGA(C/T)GTAAGGGCCGTGC
TNF-α	Forward	CTGTCTACTGAACTTCGGGGTGAT
	Reverse	GCTCTGGGCCATAGAACTGATG
	Probe	ATGAGAAGTTCCCAAATGGCCTCCCTC
IL-1β	Forward	GGCCTCAAAGGAAAGAATCTATACC
	Reverse	GTATTGCTTGGGATCCACACTCT
	Probe	ATGAAAGACGGCACACCCACCCTG
iNOS	Forward	CAGCTGGGCTGTACAAACCTT
	Reverse	TGAATGTGATGTTTGCTTCGG
	Probe	CGGGCAGCCTGTGAGACCTTTGA

Table 5: PCR Primers and Probes

	HCC	SDR
Lachnospiraceae	38.71 ± 5.22	54.10 ± 5.81
Clostridiales; Other	9.01 ± 0.56	10.14 ± 0.96
Bacteroidaceae	10.77 ± 1.49	6.67 ± 2.11
Lactobacillaceae	13.66 ± 4.81	3.13 ± 1.00*
Ruminococcaceae	5.22 ± 0.36	4.35 ± 0.62
Acetobacteraceae	0.01 ± 0.004	8.86 ± 8.83
Clostridiaceae	6.34 ± 3.62	0.92 ± 0.78
Porphyromonadaceae	2.67 ± 0.20	1.59 ± 0.25*
Bacteria; Other	2.21 ± 0.25	1.84 ± 0.46
Peptostreptococcaceae	1.81 ± 0.35	0.94 ± 0.40

Data are the mean relative abundance ± standard error. * $p < .05$ vs. HCC

Table 6: Top 10 Most Abundant Colonic Mucosal-Associated Bacterial Families, SDR

	HCC	SDR	
Lachnospiraceae; Other	37.61 ± 5.23	53.02 ± 5.74	
Clostridiales; Other; Other	9.01 ± 0.56	10.14 ± 0.96	
Bacteroides spp.	10.77 ± 1.49	6.67 ± 2.11	
Lactobacillus spp.	13.53 ± 4.76	3.11 ± 1.00	*
Rosemonas spp.	0.00 ± 0.00	8.83 ± 8.83	
Ruminococcaceae; Other	3.81 ± 0.44	2.95 ± 0.45	
Clostridium spp.	5.86 ± 3.37	0.82 ± 0.70	
Unclassified Bacteria	2.21 ± 0.25	1.84 ± 0.46	
Parabacteroides spp.	2.25 ± 0.12	1.35 ± 0.20	**
Peptostreptococcaceae; Other	1.80 ± 0.35	0.93 ± 0.40	
Unclassified Firmicutes	1.25 ± 0.20	0.69 ± 0.13	*
Marvinbryantia spp.	0.79 ± 0.11	0.81 ± 0.24	
Turicibacter spp.	0.94 ± 0.30	0.52 ± 0.20	
Oscillibacter spp.	0.62 ± 0.29	0.65 ± 0.28	
Asaccharobacter spp.	0.81 ± 0.25	0.39 ± 0.12	
Akkermansia spp.	0.73 ± 0.32	0.39 ± 0.22	
Unclassified Bacteroidetes	0.60 ± 0.05	0.33 ± 0.16	
Butyricicoccus spp.	0.40 ± 0.03	0.52 ± 0.19	
Unclassified Alphaproteobacteria	0.62 ± 0.40	0.14 ± 0.11	
Erysipelotrichaceae; Other	0.47 ± 0.14	0.24 ± 0.07	
Butyricimonas spp.	0.43 ± 0.09	0.22 ± 0.07	
Hyphomonadaceae; Other	0.01 ± 0.00	0.58 ± 0.55	
Clostridiaceae; Other	0.48 ± 0.26	0.10 ± 0.08	
Alistepes spp.	0.38 ± 0.09	0.18 ± 0.05	
Unclassified Cyanobacteria	0.01 ± 0.00	0.45 ± 0.42	
Chitinophagaceae; Other	0.39 ± 0.35	0.00 ± 0.00	
Bacillariophyta; Other	0.06 ± 0.05	0.28 ± 0.17	
Anaerotruncus spp.	0.15 ± 0.04	0.17 ± 0.02	
Coprobacillus spp.	0.15 ± 0.05	0.14 ± 0.06	
Mucispirillium spp.	0.17 ± 0.10	0.11 ± 0.05	
Ruminococcus spp.	0.22 ± 0.08	0.05 ± 0.03	
Lactobacillales; Other; Other	0.21 ± 0.07	0.06 ± 0.02	
Blautia spp.	0.19 ± 0.05	0.07 ± 0.02	
Unclassified Bacilli	0.18 ± 0.04	0.05 ± 0.02	*
Anaerostipes spp.	0.16 ± 0.05	0.07 ± 0.01	
Ponticaulis spp.	0.00 ± 0.00	0.22 ± 0.21	**
Anaerovorax spp.	0.14 ± 0.05	0.08 ± 0.02	
Holdemania spp.	0.16 ± 0.06	0.04 ± 0.01	
Bacillus spp.	0.19 ± 0.19	0.01 ± 0.00	
Roseburia spp.	0.09 ± 0.03	0.10 ± 0.04	

Data are the mean relative abundance ± standard error.
* $p < .05$ vs. HCC; ** $p < .01$ vs. HCC

Table 7: Top 40 Most Abundant Mucosal-Associated Bacterial Genera, SDR

		C57BL6	CD-1
Parabacteroides distasonis	Day 0	6.27 (6.02 – 7.42)	6.65 (6.53 – 6.78)
	Day 1	6.94 (6.66 – 7.23)	7.54 (7.30 – 7.77)
	Day 6	6.52 (6.10 – 6.94)	7.00 (6.26 – 7.74)
Porphyromonas -Bacteroides -Prevotella	Day 0	9.10 (8.94 – 9.26)	7.20 (7.02 – 7.39)
	Day 1	9.50 (9.33 – 9.67)	7.37 (6.93 – 7.82)
	Day 6	8.98 (8.78 – 9.18)	6.97 (6.44 – 7.50)

Data are the mean (standard error) of the copy number (log10) of target 16s rrn per gram of tissue.

Table 8: Real-time PCR Assessment of Bacterial Group Abundances

		C57BL6		CD-1	
		Day 1	Day 6	Day 1	Day 6
IL1β	HCC	1.00 (0.57 – 1.75)	1.00 (0.55 – 1.82)	1.00 (0.37 – 2.68)	1.00 (0.64 – 1.57)
	SDR	0.95 (0.49 – 1.84)	1.37 (1.00 – 1.37)	3.18 (2.92 – 3.47)	1.11 (0.59 – 2.11)
iNOS	HCC	1.00 (0.56 – 1.78)	1.00 (0.50 – 2.01)	1.00 (0.56 – 1.78)	1.00 (0.69 – 1.46)
	SDR	0.62 (0.46 – 0.83)	2.50 (1.92 – 3.25)	0.32 (0.24 – 0.43)	0.23 (0.14 – 0.37)
TNFα	HCC	1.00 (0.74 – 1.35)	1.00 (0.51 – 1.98)	1.00 (0.76 – 1.32)	1.00 (0.61 – 1.65)
	SDR	0.50 (0.35 – 0.73)	1.03 (0.71 – 1.49)	0.92 (0.69 – 1.22)	0.92 (0.63 – 1.33)

Data are the mean (standard error) of the fold change in cytokine gene expression over non-stressed HCC controls.

Table 9: Real-time PCR Assessment of Colonic Inflammation

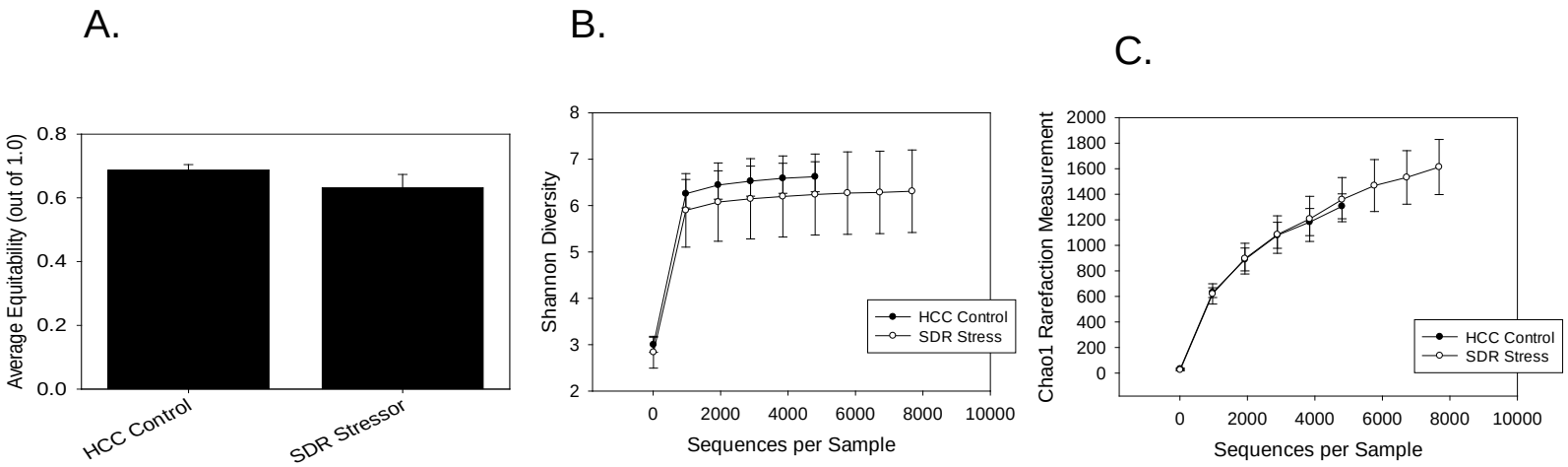
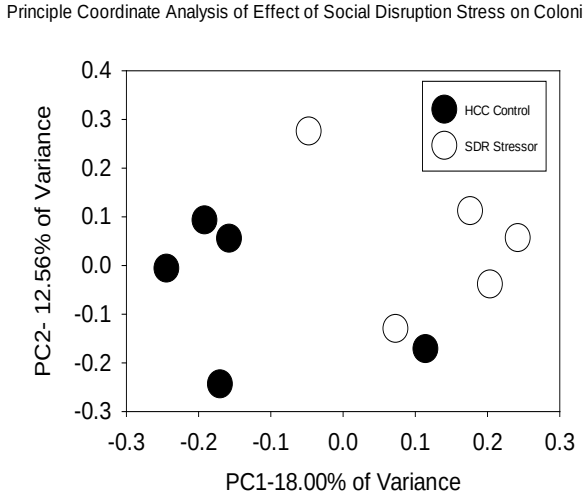


Figure 6: Exposure to the SDR stressor did not impact alpha-diversity. Mice were exposed to the SDR stressor, or were left undisturbed as non-stressed Home Cage controls (HCC control). After 454 pyrosequencing, three measures of community alpha-diversity were calculated using QIIME. A. Equitability Index, B. Shannon Diversity Index, and C. Chao1 Rarefaction Measurement were unaffected by exposure to the SDR stressor. Data are from n = 5 mice per group.

A.



B.

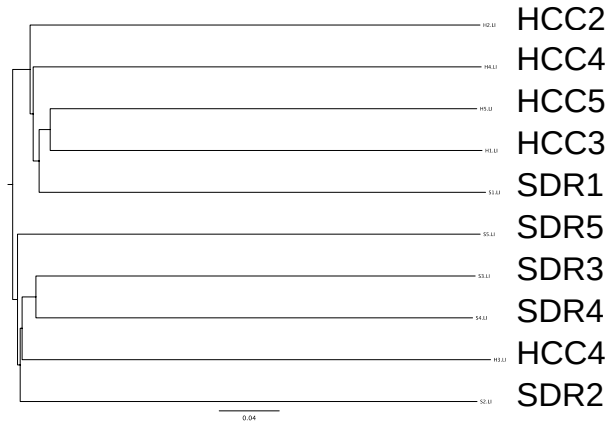


Figure 7: Exposure to the SDR stressor significantly changes beta-diversity. After 454 pyrosequencing, UniFrac distances were calculated using QIIME and plotted on a 2-D principal coordinates graph (A). Exposure to the SDR stressor shifted the colonic microbiota, with 4 of 5 SDR stressor samples clustering apart from HCC controls. UniFrac distances were also used for construction of an unweighted pair group method by arithmetic mean (UPGMA) dendrogram, in which 4 of 5 SDR stressor samples clustered together. The clustering in Figures 2A and 2B was statistically significant ($p<0.05$) after performing the ANOSIM test to measure differences between distance matrices of SDR stressor and HCC control groups. Data are from n = 5 mice per group.

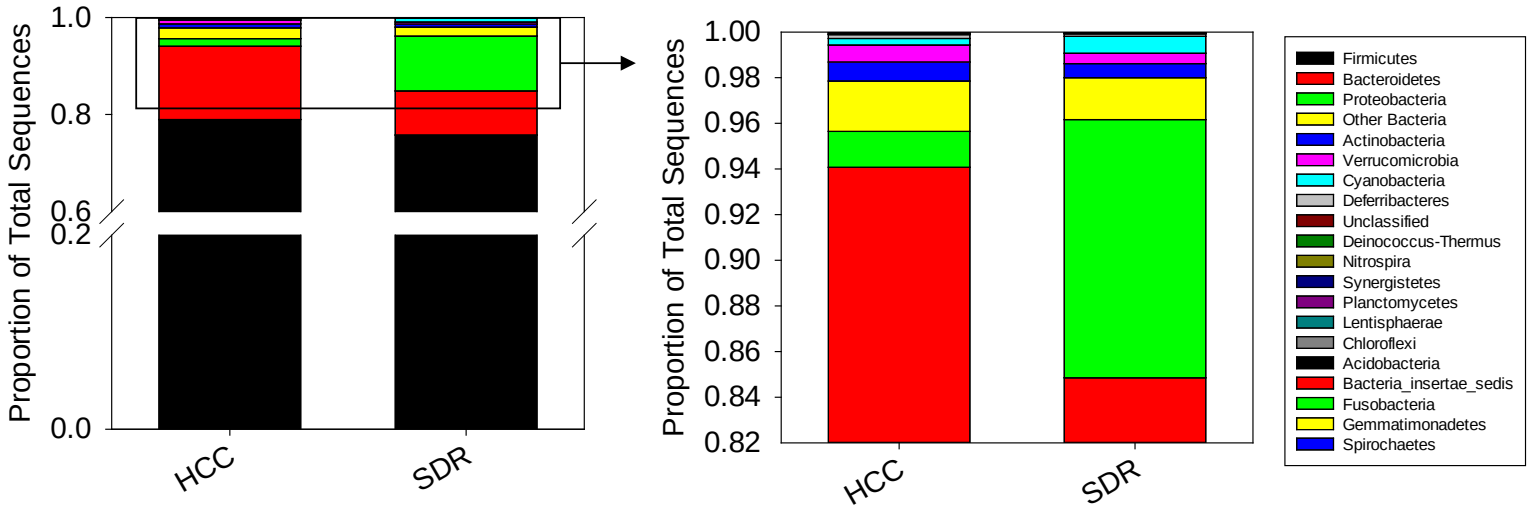


Figure 8: The relative abundances of mucosal-associated bacterial phyla were unaffected by exposure to the SDR stressor. Data are the mean+/-S.E. of the relative abundances calculated from 454 pyrosequencing data and are from n = 5 mice per group.

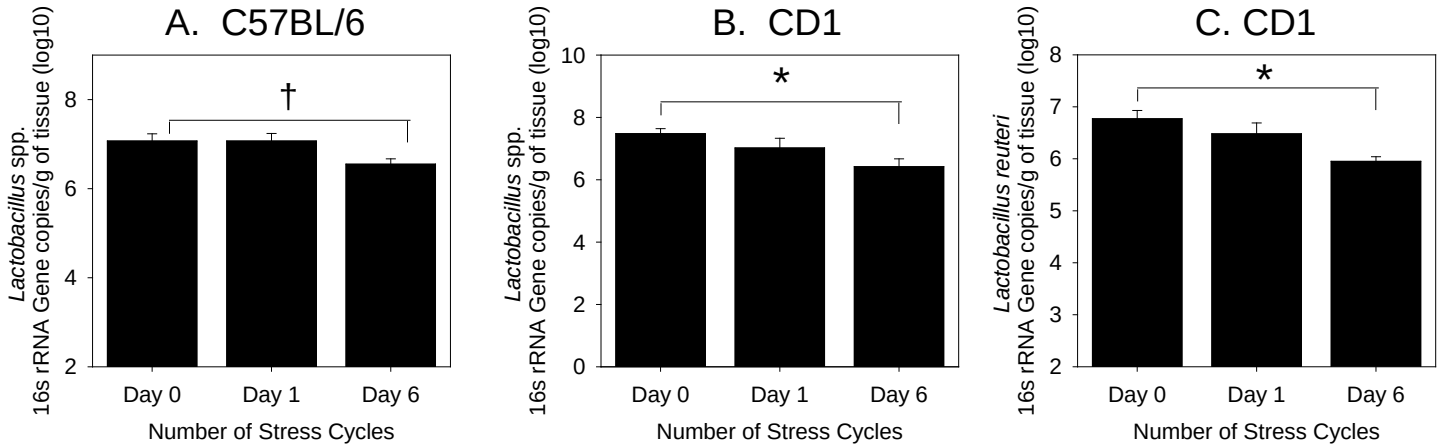


Figure 9: The absolute abundance of bacteria in the genus *Lactobacillus* is reduced by exposure to the SDR stressor. Mice were exposed to either 0, 1, or 6 consecutive days of the SDR stressor and qPCR used to quantify *Lactobacillus* group bacteria (A and B) or L. reuteri (C) in the colon. * indicates $p < .05$ vs. 0 cycles of SDR (0 cycles is equivalent to HCC controls). Inbred C57Bl/6- n = 16 at Day 0, n = 7 on Day 1, and n = 8 on Day 6. Outbred CD-1- n = 21 at Day 0, n = 15 at Day 1, and n = 6 on Day 6.

Both restraint and SDR alter the community structure of the colonic mucosa-associated community profile, while also reducing the abundance of the *Lactobacillus* genus, demonstrating ubiquity in the effects of stress upon the colonic microbiota. In addition, C57BL/6 and CD-1 mice were affected by stressor exposure, demonstrating that the effect of these stressors is not strain-dependent. However, it is not known if these changes in community structure are dysbiotic, and have downstream effects upon host health. Previous research has shown that exposing mice to either social disruption or restraint results in increased severity of enteric infection. Since restraint and social disruption both reduce health-associated groups like *Lactobacillus*, we hypothesized that alterations to the colonic mucosal microbiota are directly associated with the elevations in colitic inflammation reported in mice exposed to a stressor and challenged with an enteric pathogen. Thus, in Chapter 4, germ-free mice were gavaged with a slurry of the microbiota of conventional mice exposed to either restraint or social disruption, and then challenged with *Citrobacter rodentium* in order to evaluate if stressor-induced disruptions in microbiota community structure is associated with changes in inflammatory severity.

Chapter 4: Stressor-induced disruptions in colonic microbiota community structure is associated with elevations in inflammatory response to an enteric pathogen

Introduction:

The inflammatory bowel diseases (IBD), ulcerative colitis and Crohn's disease affect an estimated 1.17 million Americans (310). These diseases are characterized by pain, inflammation, bleeding, and diarrhea (311, 312), and have also been associated with altered community profiles among the microbiota that colonize the lumen and mucosa of the colon (13, 148, 253). Disrupted microbial communities, a state termed dysbiosis, can negatively feedback upon the host, and have been associated with dysfunction in host physiology and immunology (313-316). A causative role between dysbiotic profiles and IBD has not been assigned to this point. Thus, deeper investigation into how dysbiosis affects host immunity and whether these effects are associated with IBD pathology and symptomology is required.

Dysbiosis can be induced through a number of external effectors, including antibiotics and diet (264, 317, 318). Recent research has demonstrated that perception of a psychological stressor can also shift microbiota community structure. In murine studies, exposure to both chronic, long-term stressors and short-term social stressors significantly

altered the mucosal-associated microbiota and reduced the relative abundance of the immunomodulatory genus, *Lactobacillus* (187, 319). Similar findings have been extended to human and non-human primate hosts (36, 38). Exposure to stressors has been associated with symptomatic episodes in IBD patients, as well as with increases in the inflammatory response to enteric pathogen challenge in animal models (77, 145, 237). Though whether stressor-induced shifts in gut microbiota can affect host function is unknown, such a connection could explain the relationship between stress and IBD. Thus, this study aimed to determine if there was a direct association between the stressor-induced microbiota disruptions and the elevated inflammatory state in stressor-exposed mice challenged with colitis-causing pathogen, *Citrobacter rodentium*.

Germ-free (GF) mice are a commonly used model for examining the effect the microbiota might have upon host physiology and immunity. Researchers have been able to demonstrate the involvement of the microbiota in weight gain, as conventionalized mice had increased fat deposition than GF mice (275). These studies have also established that the microbiota engages in mucosal immune cell maturation, abundance and/or activation, including Th17 cells and macrophages (103, 320, 321). Additionally, gavaging GF mice with the microbiota of NOD2-/- mice or obese humans can indicate how the host modeling of the microbiota, genetic or otherwise, can further feedback upon host function (5, 322). We hypothesized that stressor-induced alterations to the microbiota were directly associated with a heightened inflammatory response to colitic pathogens, and we tested this hypothesis by gavaging GF mice with the microbiota from stressor-exposed mice and challenging the mice with *C. rodentium*. Multiple stressors

were also used in order to examine the differential effects each unique stressor might have on dysbiosis and gastrointestinal inflammation.

Materials and Methods:

Mice: Conventional male CD-1 mice and male C57Bl/6 mice (aged 6-8 weeks) were ordered from Charles River Laboratories (Raleigh, NC). Mice were housed 3 per cage and acclimated for a week in the vivarium before stressor exposure. Germ-free (GF) Swiss Webster (aged 6-40 weeks) and C57BL/6 (aged 22 weeks) mice were obtained from Dr. Brian Ahmer at The Ohio State University and were kept in a fully decontaminated BSL-2 level biosafety cabinet for the duration of the study. All mice were given food and water ad libitum and kept on a 12-hour light:dark cycle (Conventional mice- 0600 to 1800 lights on, Germ-free mice- 7 am to 7 pm on). All stressors, infection, and sacrifice protocols were approved by the Ohio State University Animal Care and Use Committee. CD-1 and Swiss-Webster mice were used in the restraint experiment, while C57BL/6 mice were used in the SDR experiment.

Stressor: Social disruption (SDR) and restraint (RST) stress were administered to conventional mice as previously published (187, 319). In short, for SDR, an aggressive retired breeder was placed in the home cage for the test mice, wherein he repeatedly attacked and defeated the mice over the course of 2 hours. The stressor was performed from 1700 to 1900 every evening for six consecutive evenings. Wounding was monitored in order to prevent heavy wounds. In addition, there was an un-disturbed control group kept in a separate room from the fighting (HCC). For RST, mice were placed in a 50-mL conical tube for 16 consecutive hours, beginning at 1700 and concluding at 0900 the

following morning. The RST stressor was continued each night for a total of seven cycles. Two control groups were used: a food and water deprivation group that had food and water removed during the restraint period, but were not otherwise restrained (FWD), and a control group that was undisturbed and kept in a separate room (HCC).

Germ-Free Reconstitution Experiments: Immediately following the final cycle of the respective stressors, conventional mice were euthanized via CO2 asphyxiation. Colons were aseptically removed, and the tissue was bisected using sterile forceps and scissors. An equivalent amount of fecal pellets for each group was pooled in 3 mL of anaerobically pre-reduced PBS, in addition to a mucosal scraping from each colon. Each donor group was comprised of pooled fecal and mucosal slurry from three total mice per experiment. The PBS slurry was immediately placed in an anaerobic canister with a BD GasPak EZ until gavage into the GF mice. GF mice were gavaged within two hours of conventional mouse sacrifice. GF mice were gavaged with 200 uL total of slurry, and then were food and water deprived for two hours. Groups are labeled RST-GF, HCC-GF, and FWD-GF for the Restraint Experiment, and SDR-GF and HCC-GF for the SDR Experiment. The slurries were kept at -80°C until sequencing via Illumina at a later date.

Bacteria: *Citrobacter rodentium* strain DBS120 was grown overnight at 37°C in tryptic soy broth. After growth, the pathogen culture was brought up to $1x10^9$ cfu/mL and 100 uL was given to each GF mouse 24 hours after fecal microbiota transplant with donor slurries, resulting in a total inoculum of $1x10^8$ cfu/mouse. Mice were monitored and sacrificed at days 6 and 12 post infection. Total infectious burden was measured by

plating shed fecal pellets from the GF mice on MacConkey agar supplemented with 20 mg/mL kanamycin.

qRT-PCR: Colons were collected and bisected. Half was used for RNA isolation using the previously published TriZOL method (165). Briefly, isolated RNA was normalized to 1 μg per sample and then reverse transcribed to cDNA via Promega Reverse Transcription System. Multiplex qRT-PCR was performed on the ABI Prism 7000 system using primers targeting mRNA for interleukin-1β, tumor necrosis factor-α, CCL2, and inducible nitric oxide synthase. Murine 18S was used as a housekeeper gene. The comparative threshold cycle method was used for data analysis as previously described, with HCC-GF set as baseline (77).

Histopathology: The other half of the collected GF colon was fixed in neutral buffered formalin until paraffin embedding and hemotoxylin and eosin staining by the Ohio State University Comparative Pathology and Mouse Phenotyping Shared Resource. Total colitic pathology was scored by a board-certified veterinary pathologist. Each section was scored by the pathologist based upon hyperplasia, dysplasia, edema, epithelial defects, and inflammation. Every category had a scale of 0 to 4, with 0 indicating not present and 4 indicating severe. All categories were totaled for a maximum score of 20.

Sequencing: Illumina sequencing was performed by the Molecular and Cellular Imaging Center, located at the Ohio State University Ohio Agriculture Research and Development Center. 2x300bp paired end sequencing was achieved using Illumina MiSeq. Upon completion of sequencing, sequences were demultiplexed using Sabre (website:http://github.com/najoshi/sabre), and joined on Quantitative Insights Into

Microbial Ecology (QIIME) 1.8.0 using fastq-join (46, 323). Closed-reference OTU picking was performed against the GreenGenes 13_8 database using UCLUST, set at 97% identity, which added another quality filtering step (231, 233). Quality filtering was performed at a qual score of 20, with 0 allowed N characters, 1.5 allowed barcode errors (G to T, A to C count as 1 error, all other mismatches are 0.5 errors), and 3 consecutive low quality bases allowed before sequence truncation. After OTU picking and taxonomic assignment with GG_13_8, an average of 2693 sequences/samples remained, in a total of 561 unique OTUs. Relative taxonomic abundances were calculated in QIIME.

Results:

***Bifidobacterium* was not present in the Restraint Donor Slurry-** Donor microbiota transplant pools were pooled by experiment, resulting in two donor pools per group (RST, FWD, HCC). The experiment was repeated once, for a total of two experimental runs. The microbiota composition of each donor pool was analyzed in QIIME after Illumina sequencing. After quality filtering and closed reference OTU picking, a mean of 2694 sequences remained, spread among 561 unique OTUs. Considerable variation existed between the experimental runs. RST1 was predominantly *Firmicutes* (98.5%), while HCC1 was 67.8% *Firmicutes* to 31.0% *Bacteroidetes* and FWD1 had 54.5% *Bacteroidetes* and 39.6% *Firmicutes*. *Actinobacteria* made up 5.6% of the FWD1 composition, but was <1% of total relative abundance of the other two groups in Run 1. In the second run, RST2 had 62.3% *Firmicutes* to 36.8% *Bacteroidetes,* FWD2 had 70.4% *Firmicutes* to 27.4% *Bacteroidetes,* and HCC2 had a higher abundance of *Actinobacteria* at 38.0%, while *Firmicutes* comprised 50.7% and *Bacteroidetes* 11.1% of

the total abundances (Table 10). Trends related to stress or food and water deprivation were not evident between runs. Genus abundances varied as well between donor groups. The most abundant genus on average was *Lactobacillus*, which ranged from 5.54% of total sequences to 65.03% of total sequences. *Bifidobacterium*, the sixth most abundant genus group, was not present in the Restraint pools (Table 11).

***Citrobacter rodentium* burden was not increased in RST-GF mice-** The level of *Citrobacter rodentium* burden within the colons was measured by fecal pellet culturing on MacConkey Agar. A two-factor ANOVA for days-post-infection (DPI) and group was performed. Both DPI ($p<.05$) and Group ($p<.05$) were significantly associated with increased *C. rodentium* burden. In order to evaluate how the restraint-stressed microbiota or FWD-GF microbiota affected *C. rodentium* burden by DPI, post-hoc LSD tests were performed. Levels were unchanged at d3, d4, d6, or d12. FWD-GF levels were significantly reduced at 5 DPI ($p<.05$). At 9 DPI, HCC-GF levels were significantly higher than both RST-GF and FWD-GF ($p<.05$) (Figure 10).

Colitic pathology and colon mass was increased in RST-GF mice- Colonic tissue mass during *C. rodentium* infection is associated with the colonic hyperplasia induced by *C. rodentium* pathogen and is indicative of a heightened colitic state. Analysis of colon masses revealed that there was an interaction between DPI and Group ($p<0.05$). RST-GF mice had significantly increased colonic mass over both control groups at 6 DPI ($p<0.05$), but not at 12 DPI. Overall, 12 DPI mice had significantly greater colonic mass than 6 DPI colonic mass using a post-hoc T-test ($p<0.001$). Due to the increased colonic mass, colitic pathology was examined using H&E staining. At 6 DPI, GF mice that

received microbiota from RST-exposed conventional mice showed a trend towards increased colitic pathology scores over both FWD-GF and HCC-GF groups (p=.061), indicating that the stress-exposed microbiota was marginally associated with the heightened colitic pathology (Figure 11).

Pro-inflammatory mRNA transcripts were increased in RST-GF mice- The mRNA levels of pro-inflammatory markers was examined in the GF mice that received the transplanted microbiota at days 6 and 12 post infection. IL-1β, a pro-inflammatory cytokine, was associated with an interaction between DPI and Group ($p<.05$). IL-1β levels were significantly increased in the RST-GF group over HCC-GF at 6 DPI ($p<.05$), but not FWD-GF. IL-1β was significantly increased at 12 DPI compared to 6 DPI ($p<.001$) (Figure 12A). Inducible nitric oxide synthase (iNOS) was increased at 12 DPI over 6 DPI using a post-hoc T-test ($p<.05$). There was no significant difference in iNOS levels among the groups (Figure 12B). CCL2, a pro-inflammatory chemokine that draws trafficking monocytes and macrophages from the bone marrow so they can migrate to the site of infection, was associated with an interaction between DPI and Group ($p<.05$). Post-hoc testing with LSD indicated that there was no change at 12 DPI, but RST-GF was significantly increased over both HCC-GF and FWD-GF at 6 DPI. A post-hoc T-test showed that 12 DPI was marginally increased over 6 DPI ($p=0.072$) (Figure 12C).

Mice that received slurry from SDR mice did not exhibit any change in inflammation upon pathogen challenge: After fecal transplant from conventional mice that were exposed to SDR to the GF mice and infection with *C. rodentium*, infectious burden was measured in the shed fecal pellets. There was no change detected between GF

mice that received HCC or SDR-exposed microbiota, but overall *C. rodentium* was significantly increased at d5 over d2 using LSD post-hoc testing ($p<.05$) (data not shown). At 6 DPI, mice were sacrificed and colon masses were taken. There was no significant difference between colons of GF mice that received HCC microbiota and those that received SDR-exposed microbiota. Likewise, pro-inflammatory markers (CCL2, TNFα, IL1β, and iNOS) were also unchanged between the HCC-GF and SDR-GF groups at 6 DPI (data not shown).

Discussion:

In this study, conventional mice were exposed to psychological stress and the microbiota from these mice was transferred via fecal microbiota transplantation by oral gavage to GF mice. Two stressors, SDR and RST, were used. Interestingly, the two different stressors, SDR and RST, had contrasting results. These stressors have unique impacts upon murine immunity. RST is immunosuppressive, slowing wound healing, and reducing the memory response of the adaptive arm, particularly among T-cells (75, 228, 229). Studies characterizing SDR have demonstrated how this social stressor is associated with increased cellular trafficking to the secondary lymphoid organs, such as the spleen, as well as into the brain (64, 324). Mice that have been exposed to SDR have a rapid response of T-cell memory, which stands in contrast to RST, as well as increased macrophage killing of bacteria (61, 65). Both stressors have been associated with behavioral changes in the affected mice, with increases of anxiety-like behavior in SDR mice and anxiety-like and depressive-like behavior in RST mice (304, 325). Previous studies have shown that both stressors affect immunomodulatory groups among the

microbiota, including *Lactobacillus*, and can disturb the mucosal-associated and fecal-associated microbiota in the colon and the cecum (70, 77, 187, 319). In studies that evaluated the effect of these stressors upon host immunity during *C. rodentium* challenge, mice exposed to RST had increased colitic pathology (165), while SDR was associated with a less severe colitis (Mackos et al, Under Review). Thus, these results concur with previous studies that indicate that RST has a greater effect upon host inflammation and pathology, a finding that could be associated with differential alterations to the microbiota.

At 6 DPI in mice gavaged with RST-exposed microbiota, effects of the stressor were observed, particularly on pro-inflammatory chemokine and cytokine transcription and colonic mass. However, at 12 DPI, differences were no longer evident. At this point, control group colonic mass and mRNA transcription levels had reached a similar level as the RST group. These data indicate that transplantation with microbiota from RST-exposed mice promotes a more rapid innate immune response in comparison to the microbiota from the control groups. The microbiota is normally protective and can shape the immune response, and GF mice are severely underdeveloped in their own immune activity (269, 270, 326). Thus, as the infection proceeds to 12 DPI, it is likely that the infection overcomes the GF mouse's diminished ability to fight the infection.

Illumina sequencing was performed on the RST donor stool and mucosal scraping slurry used in the oral gavage to the GF mice. Sequencing did not identify significant differences between the RST slurry and the control groups in beta and alpha diversity. The RST1 donor slurry contained a high abundance of *Firmicutes*, which made up 98.5%

of the total sample. This value is different than typically observed *Firmicutes* abundances, but could be due to the RST stressor-induced shifts to the microbiota. Major groups including *Lactobacillus, Clostridiales,* and *Parabacteroides* showed considerable variability by group. *Bifidobacterium* was absent in the stressor-exposed donor microbiota. Species in *Bifidobacterium* are associated with reductions in GI inflammation, and are involved with host physiology, particular in obesity and insulin and glucose regulation(175, 327-330). The data suggest that *Bifidobacterium* is necessary for early regulation of the immune response to the *C. rodentium* infection. Additionally, functional changes within the microbiota can still exist, though a metatranscriptomic approach would need to be taken to evaluate how the stressor is affecting microbiota function.

The practicality and applicability of microbiota transplants is both a recent advent into gastrointestinal illness treatment and a hotly-debated methodology. Though patients with inflammatory bowel disease or *Clostridium difficile* infection that have received fecal microbiota transplant from 'healthy' donors often report abrogation of symptoms (331, 332), the long-term effect of the transplanted microbiota upon the new host over time is not yet understood. Microbiota community structure has been associated with obesity and diabetes, as well as altered inflammatory responses (4, 201, 333). Previous studies have demonstrated that psychological stress can impact microbiota profiles (187, 319), and here the data indicate that community profiles that have been exposed to stress can further affect the inflammatory response to an enteric pathogen. The microbiota can

affect host physiological and immune function negatively as well as positively, and this study highlights the importance of caution in fecal microbiota transplantation procedures.

This study follows previous work that showed that stressor exposure was associated with both alterations to the mucosal-associated microbiota of murine hosts, as well as increases in inflammatory severity in response to an enteric pathogen. Transplanting the microbiota from stressor-exposed conventional mice to GF mice allowed for examination of how these altered microbiotas may affect the host directly. Psychological stress can have a variety of effects upon host health, especially in the gastrointestinal tract. Motility and mucus secretion can be disrupted and altered during a stress period (22, 141, 224), which can affect the microenvironment that the microbiota reside in, which could change microbiota structure based upon nutrient and niche availability. Furthermore, stress has been correlated with symptomatic flare-ups in patients with IBD (145). The reason for this association is unknown, but since patients with IBD often have altered microbiotas (13, 253), and because the microbiota can feed into host immune function, it is possible that stress-induced shifts in microbial populations of IBD patients could augment the inflammatory spikes observed in flare-ups. The results herein demonstrate how the microbiota can steer the inflammatory response within the GI tract, and implicate psychological stress as a means by which the microbiota can be altered to an inflammation-inducing structure. Future studies must continue to characterize the particular structures that associate with inflammation, as well as the groups and abundances that comprise these profiles.

	HCC Run 1	FWD Run 1	RST Run 1	HCC Run 2	FWD Run 2	RST Run 2
Firmicutes	67.82	39.59	98.46	50.65	70.44	62.34
Bacteroidetes	31.02	54.52	1.36	11.10	27.40	36.83
Actinobacteria	0.88	5.61	0.00	37.98	1.08	0.14
Verrucomicrobia	0.08	0.27	0.02	0.00	1.04	0.00
Deferribacteres	0.08	0.00	0.15	0.00	0.00	0.64
Tenericutes	0.00	0.00	0.00	0.26	0.00	0.05
Proteobacteria	0.12	0.00	0.02	0.00	0.04	0.00

Data are percentage of total attained sequences

Table 10: Major Phyla Abundance in Donor Fecal Slurries

	HCC Run 1	FWD Run 1	RST Run 1	HCC Run 2	FWD Run 2	RST Run 2
Lactobacillus	5.54	12.60	65.08	23.69	51.83	22.76
Unclassified Clostridiales	30.58	14.25	25.51	17.04	8.53	21.80
Unclassified S24-7	0.00	44.79	0.00	6.85	0.00	25.55
Parabacteroides	30.10	0.27	1.22	0.07	2.64	1.01
Unclassified Lachnospiraceae	19.58	3.84	3.86	1.63	4.82	45.70
Bifidobacterium	0.84	4.93	0.00	30.94	1.08	0.00
Unclassified Rikenellaceae	0.92	7.40	0.14	3.92	1.04	8.68
Ruminococcus	3.79	1.10	1.16	1.63	0.62	1.37
Unclassified Erysipelotrichaceae	2.00	3.15	0.15	2.09	0.23	1.37
Oscillospira	2.67	0.55	1.12	0.85	0.35	3.25
Lachnospiraceae; Ruminococcus	1.20	0.96	0.50	0.52	0.39	4.30
Adlercreutzia	0.00	0.68	0.00	7.05	0.00	0.05

Data are percentage of total attained sequences, >1% total sequences

Table 11: Most Abundant Genera in Donor Fecal Slurries

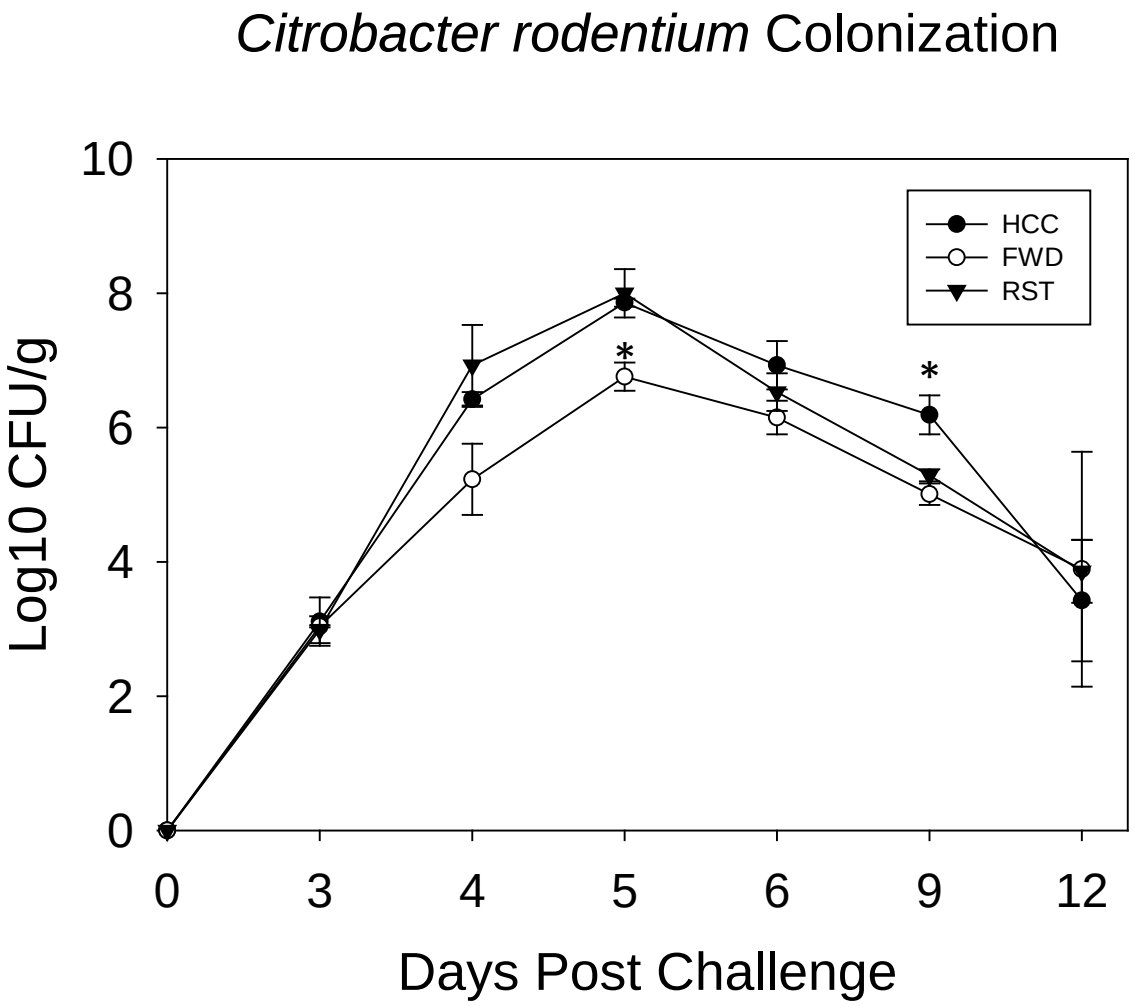


Figure 10: *Citrobacter rodentium* colonization in germ-free mice colonized with microbiota from restraint-exposed mice. Mice gavaged with FWD microbiota had significantly less *C. rodentium* burden at 5 DPI ($p<.05$), while mice gavaged with HCC microbiota had significantly higher *C. rodentium* burden at 9 DPI ($p<.05$), using ANOVA with post-hoc LSD testing. 3, 4, 5, 9, and 12 DPI n=2-4 for all groups; 6 DPI n= 6 or 7 for all groups

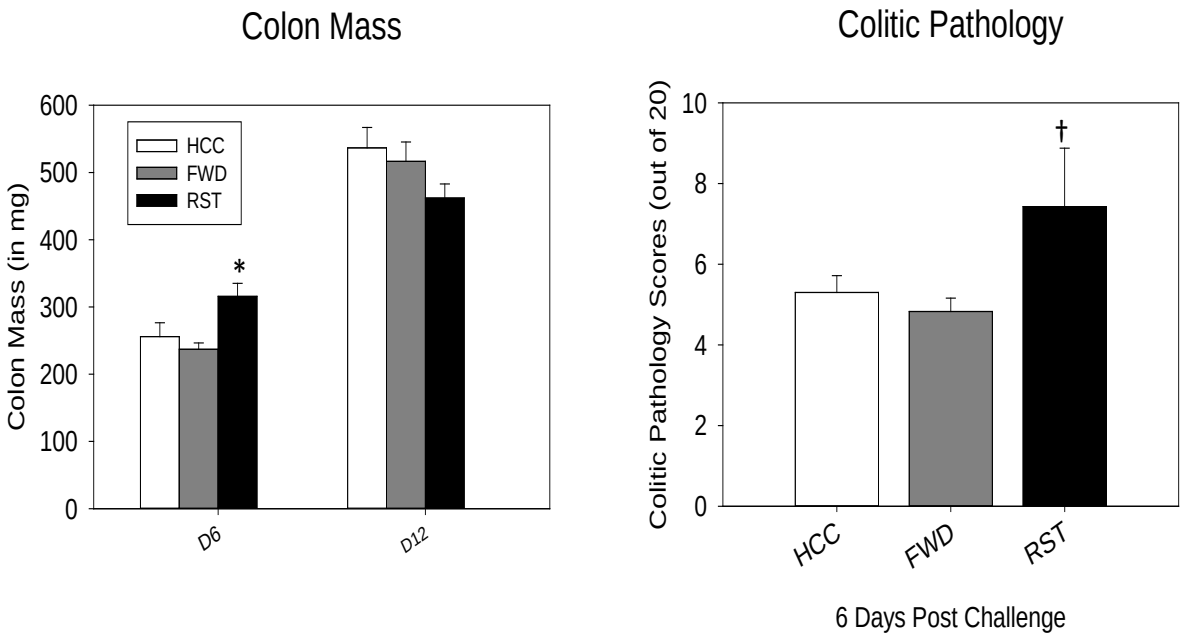


Figure 11: Restraint stressor-exposed microbiota is associated with increased colon mass and colitic pathology in re-conventionalized germ-free mice challenged with *Citrobacter rodentium*. A.) Germ-free mice gavaged with the microbiota from stressor-exposed mice had increased colon mass at 6 DPI compared to germ-free mice gavaged with control microbiota ($p<.05$). There was no change at 12 DPI. B.) Colitic pathology, measured on hemotoxylin and eosin stained colonic tissue sections, was also marginally increased in germ-free mice that received restraint-exposed microbiota over controls ($p=0.061$). 6 DPI n=6 per group, 12 DPI n=3 per group.

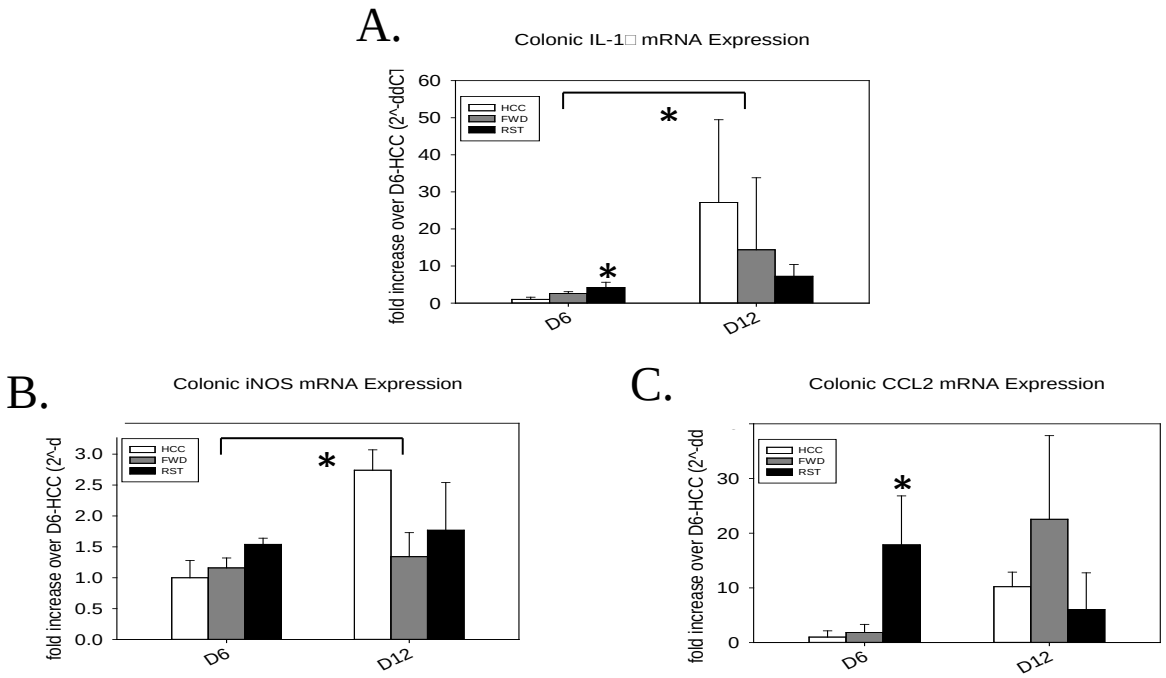


Figure 12: Pro-inflammatory marker transcript levels are increased in *C. rodentium*-infected germ-free mice that have been re-conventionalized with stressor-exposed microbiota. A.) Pro-inflammatory cytokine IL-1β was significantly increased at 12 DPI over 6 DPI ($p<.001$) over all groups, and was increased in germ-free mice that received the restraint-exposed microbiota over the HCC control group ($p<.05$) at 6 DPI. B.) Inducible nitric oxide synthase (iNOS) was significantly increased at 12 DPI over 6 DPI ($p<.05$) over all groups. iNOS was unchanged between stress and both control groups. C.) CCL2, a pro-inflammatory chemokine, was significantly increased at 6 DPI in the germ-free mice that received the restraint stress exposed microbiota over both control groups using ANOVA with post-hoc LSD testing ($p<.05$). 6 DPI n=6, 12 DPI n=3.

The results in Chapter 4 showed that stressor-induced shifts to the microbiota are directly associated with increases in the resultant inflammation from challenge by *Citrobacter rodentium*. Since these results implicate the dysbiotic alterations in community structure with dysregulation in the inflammatory response, treatments that target the microbiota community structure restoration may help ameliorate dysbiosis-associated exacerbation of inflammation and other negative health outcomes. *Lactobacillus reuteri*'s probiotic utility in colitis treatment has been widely-reported, but the mechanism by which it can abrogate inflammatory outputs during colitis is unknown. One possible mechanism by which *L. reuteri* may aid host immunity is by rectifying stressor- and infection-induced changes to the overall microbiota structure. Thus, in Chapter 5, we used Illumina sequencing to evaluate how the probiotic treatment affects community structure, while also examining the long-term effects of stress and infection upon the microbiota. The SDR model was used in this chapter since probiotic *L. reuteri* has increased effectiveness in infected mice exposed to SDR over mice exposed to restraint, and thus may have a greater measurable effect upon the microbiota.

Chapter 5: Probiotic *Lactobacillus reuteri* does not normalize stressor-induced alterations to colonic microbial community structure

Introduction:

In humans, the gastrointestinal tract (GIT) is the site of chronic and acute inflammatory illnesses that include the inflammatory bowel diseases (IBD), ulcerative colitis and Crohn's disease (334). Though the exact causative agents of these illnesses have not been fully explicated, it is known that the GIT has a particular micro-environment that contains immune and epithelial cells in close proximity to a constant source of external stimuli and luminal antigens, which can stem in part from the expansive intestinal microbiota that co-exists adjacently (335). There is normal bi-lateral communication between host immune cells sampling the periphery and the microbiota, but disruptions in the microbiota have been associated with negative health outcomes (14, 15, 264). As such, the conditions that skew the types and quantity of luminal antigen or the activity and response of resident host GI cells could be factors that associate with IBD. Psychological stress is one such factor.

Psychological stressor exposure corresponds with elevated inflammation, bleeding, and pain in both inflammatory bowel disease and enteric infection (77, 145, 165). Though the mechanism by which psychological stress exposure induces this

heightened activation of mucosal immunity is unknown, previous studies have shown that stressor exposure can affect the GI microbiota in a number of different mammalian hosts, including humans and non-human primates. Affected bacterial groups included lactic acid bacteria and other health-promoting groups, which were reduced after exposure to stress (37, 38). Recently, we have shown that mice that have undergone social disruption (SDR), a social stressor that involves aggressive interactions between retired breeder mice and test mice, have significant changes to the mucosa-associated colonic microbiota community structure after only two hours of exposure to the stressor (187). The stressor also reduces the absolute abundance of beneficial groups like *Lactobacillus* and *Parabacteroides*. SDR has also been associated with elevated inflammatory severity during challenge with *Citrobacter rodentium*, which can induce an IBD-like colitis (Mackos et al, Under Review). Specifically, mice that have undergone SDR exhibit increased pro-inflammatory marker mRNA levels, including CCL2, TNF-α, and iNOS, in addition to significant increases in the overall *C. rodentium* pathogenic burden.

Probiotic bacteria are microbes that can enhance host health when sufficiently given to the host. *Lactobacillus reuteri* is an immunomodulatory probiotic that can ameliorate the severity of colonic infection (278) and can down-regulate CCL2, TNF-α, and iNOS mRNA levels in SDR-exposed *C. rodentium*-infected mice, as well as abrogate the heightened colitic pathology that is endemic to these mice (165). Probiotic treatments like *L. reuteri* can act directly upon host immunity, such as by modulating phagocytosis and cytokine release by macrophages and monocytes, but they can also affect overall microbiota diversity, which can be associated with host health (197, 336-338). The

mechanism by which *L. reuteri* modulates host immunity is not currently known, but a possible pathway could be through prevention of stressor-induced dysbiosis. Normalizing microbiotas via fecal microbiota transplant has been received as a viable treatment against *Clostridium difficile* infection and inflammatory bowel disease, and the effect *L. reuteri* has upon the microbiota could work in similar fashion. Thus, this study set out to determine if *L. reuteri* treatment affects the colonic mucosa-associated microbiota diversity in *C. rodentium*-infected mice that have been exposed to a stressor, through the use of Illumina sequencing, in order to better evaluate the ameliorative effects of *L. reuteri* in stress-exacerbated colitis.

Materials and Methods:

Mice- Male C57Bl/6 mice (age 6-8 weeks) were ordered from Charles River (Raleigh, NC), housed three to a cage, and allowed to habituate in an approved Ohio State University vivarium for one week upon arrival. Mice were given food and water ad libitum and kept on a 12:12 hour light dark cycle, with lights on from 0600 to 1800 hours.

Bacteria- Citrobacter rodentium strain DBS120 was grown for 18 hours at 37°C in Luria-Bertani broth. Prior to infection, *C. rodentium* was diluted to a final stock concentration of 3-5x10^7 cfu/mL in PBS. To measure *C. rodentium* in shed stool pellets, stool was homogenized in a slurry in Dulbecco's PBS with calcium and magnesium, then plated in serial dilutions in MacConkey Agar with 20 ug/mL of kanamycin added.

Lactobacillus reuteri ATCC 23272 was grown for 18 hours at 37°C at 5% CO2 in MRS

agar. *L. reuteri* was prepared to a stock concentration of $1x10^9$ cfu/mL. Each mouse received a total inoculum of $1x10^8$ cfu of *L. reuteri*.

Stress and Infection Study- Test mice were exposed to social disruption stress (SDR), wherein a retired breeder mouse is placed in a cage with younger test mice. The retired breeder attacks and defeats the test mice over the course of two hours. This process is repeated for a total of six evenings, from 1700 to 1900 hours, the beginning of the mouse active cycle. A group termed home cage control (HCC) mice were kept separately and left undisturbed for the duration of the stressor. All SDR and HCC mice were infected with *C. rodentium* immediately following the first cycle of SDR. Each mouse received 100 uL of the *C. rodentium* stock for a total of $3\text{-}5x10^6$ colony-forming-units (cfu)/mouse. All infected mice had food and water removed for two hours post infection. In addition, following each of the six cycles of SDR, half of the SDR and HCC mice received $1x10^8$ cfu of *L. reuteri*, while the other half of the SDR and HCC mice received PBS vehicle. In sum, there were four experimental groups: HCC-Vehicle, HCC-*Lr*, SDR-Vehicle, and SDR-*Lr*. Lastly, a final group was maintained that was not stressed, infected, or treated with probiotic *L. reuteri*, termed Uninfected.

Sacrifice- Mice from the four experimental groups (HCC-Vehicle, HCC-*Lr*, SDR-Vehicle, and SDR-*Lr*) were sacrificed at 3, 6, 12, and 24 days post infection. Colons were collected for Illumina sequencing analysis, while stool was collected for the purpose of C. rodentium quantification. Colons were snap frozen in liquid nitrogen and stored at -80°C until DNA was isolated for sequencing. An initial experiment was performed, as well as three experimental repeats, for four total experimental runs. Total sample sizes at

the four time points (3, 6, 12, 24 DPI) varied from 9 to 12 for each experimental group after combining the four experimental runs. There were a total of 5 Uninfected mice.

Sequencing- 2x300 bp paired-end sequencing (V1-V3) was performed by the Molecular and Cellular Imaging Center (MCIC) in Wooster, Ohio on an Illumina MiSeq. DNA was extracted from the proximal section of the colon using a QIAgen DNA Mini Kit using the manufacturer's instructions with slight modifications. In summary, tissues were incubated for 45 minutes at 37°C in lysozyme buffer (20 mg/mL lysozyme, 20 mM TrisHCl, 2mM EDTA, 1.2% Triton-X, pH 8.0), then beadbeat for 150 seconds with 0.7mm zirconia beads. Samples were incubated at 56°C for 2 hours with Buffer ATL and Proteinase K, then incubated at 56°C for 30 mins and 95°C for 10 mins upon addition of Buffer AL. From this point, the Qiagen DNA Mini Kit isolation protocol was followed from the ethanol step forward. Samples were quantified with the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) using the dsDNA Broad Range Assay Kit. Samples were standardized to at least 5 ng/uL before being sent to the MCIC for sequencing.

Data Analysis- Forward and reverse ends were demultiplexed using Sabre (website: http://github.com/najoshi/sabre), with 1 allowed barcode mismatch. Barcodes were removed and sequences were trimmed for equal lengths using FastX Trimmer (website: http://hannonlab.cshl.edu/fastx_toolkit). Sequences were joined with Fastq-Join, with 10% allowed differences within the overlap region. Quality filtering was performed with the following parameters: quality score of 20, 0 allowed N characters, 1.5 allowed barcode errors, 3 consecutive low quality bases allowed. qiime_tools (website: http://github.com/smdabdoub/phylotoast) was used for closed reference OTU picking

against the 13_8 GreenGenes database (339). Briefly, the complete dataset was split into smaller .fasta files, and OTUs were picked in parallel on the Ohio Supercomputer using parallel BLAST OTU picking (340).

Statistical Analysis- Alpha diversity was measured using the Shannon Diversity index metric, which estimates richness and evenness. Beta diversity was measured with the unweighted UniFrac distance metric (50). Alpha and beta diversity were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) (46). Differences in alpha diversity were calculated with parametric t-tests, while beta diversity shifts were calculated with adonis, which permutationally analyzes variance in distance matrices (47). Taxonomic abundances at the phyla and genera levels were normalized by finding the arcsin of the square root of the proportion for each taxonomic classification. Groups were compared using SPSS v.21 (IBM, Chicago, IL). Days post infection (DPI) was compared between groups using ANOVA with post-hoc LSD tests, while the effect of stress, probiotic and infection was compared with Independent Samples T-tests.

Results:

Infectious burden was increased in SDR-exposed mice at 12 DPI. The infectious burden was examined by measuring the amount of shed *C. rodentium* in the infected mouse stool. There was no three-way interaction between Stress, Treatment and DPI. There was an interaction effect between Stress*DPI ($p<.05$), regardless of treatment with the probiotic or vehicle. *C. rodentium* burden levels were significantly increased at 12 DPI in SDR mice over HCC ($p<.005$) (Figure 13A). Likewise, an interaction effect existed between Stress*Treatment ($p=.05$). Over the course of infection, SDR mice given

Vehicle treatment were significantly increased in pathogenic burden over HCC-Vehicle ($p<.005$), while SDR mice that were given probiotic *L. reuteri* had marginally increased pathogenic burden over HCC mice that received the probotic ($p=0.06$).

SDR-induced increase in colon mass was abrogated by probiotic L. reuteri treatment. Colon mass was used to measured colitis, as *C. rodentium* induces colonic hyperplasia, a hallmark of the colitic pathology associated with *C. rodentium* infection. There was no three-way interaction between Stress, Treatment, and DPI. However, there was an interaction effect between Stress*DPI ($p<.001$, $F=16.80$). Mice exposed to SDR had significantly greater colon mass at 12 DPI ($p>.005$, $t=3.08$), and marginally increased colon mass at 1 DPI ($p=.069$, $t=-1.89$) and 24 DPI ($p=.064$, $t=1.93$) (Figure 13B). There was also an interaction effect between Treatment*DPI ($p<.001$, $F=14.94$). Mice given Vehicle treatment were had significantly higher colon mass over probiotic-treated mice at 12 DPI ($p<.005$). With the a priori hypothesis that probiotic treatment reduces colitic pathology and colon mass as has been reported in previous studies, a t-test was performed comparing SDR-Vehicle and SDR-*Lr*-treated mice. At 12 DPI, SDR-exposed mice that received Vehicle treatment had significantly higher colon mass than SDR mice that received probiotic treatment ($p<.001$).

Probiotic L. reuteri treatment was associated with changes in microbiota community structure at 1 DPI, but not beyond, while Stress and Infection were both associated with significant alterations to the microbiota. Initially, the entire sample was examined for changes in alpha diversity, using the Shannon Diversity index, as well as the evenness metric and the Chao1 estimation of richness, as well as changes in beta

diversity, ascertained with the unweighted UniFrac distance metric. There were no changes evident in any of the measured variables (probiotic gavage, infection, days post infection, or exposure to stress). The study was comprised of four runs, which had a significant impact upon mucosal microbiota community structure ($p = .001$, $R^2=0.02366$). In spite of this, the adonis statistic showed that psychological stress exposure ($p = 0.001$, $R^2=0.01731$) (Fig. 14A) and *C. rodentium* infection were significantly associated with altered microbial profiles ($p=0.003$, $R^2=0.01253$) (Fig. 14B), while probiotic gavage with *L. reuteri* did not affect the microbiota structure in the overall community ($p=0.258$, $R^2=0.00779$). In order to visualize the clustering on a PCoA, a custom axis for Run was added at the expense of the third PCoA axis. Clustering can be observed in the 2nd, 3rd, and 4th Runs as a function of Stress in the overall sample, while clustering is not present in any of the four runs as a function of probiotic Treatment.

A principle coordinate analysis produced with QIIME indicated that throughout the timecourse, the dispersion of the samples increased as a function of DPI (Fig. 15). In order to examine how stress and probiotic use affected the microbiota as the infection progressed, samples were filtered based on DPI. At 1 DPI, the *L. reuteri* probiotic had a significant effect upon the structure of the mucosal-associated microbiota ($p=0.005$, $R^2=0.05243$) (Fig. 16), while stressor exposure did not. As the infection proceeded to 6 DPI, SDR now had a significant effect, as by this point, the mice had undergone six consecutive cycles of the stressor ($p=0.013$, $R^2=0.0478$). Probiotic gavage no longer significantly associated with changes in the microbiota. The effect of the SDR stressor could continue to be observed at 12 DPI ($p=0.001$, $R^2=0.05282$) and 24 DPI ($p=0.029$,

R^2=0.05725) (Fig. 17). *L. reuteri* treatment did not have an effect at either of these two timepoints. The mucosal microbiota also shifted as the infection progressed (p=0.001, R^2=0.02405).

Major taxonomic changes were associated with stress and infection. *Firmicutes* were reduced at 6, 12, and 24 DPI compared with 1 DPI (p<.01). *Bacteroidetes* was increased at 6, 12, and 24 DPI compared with 1 DPI (p<.01). *Actinobacteria* was increased at 12 DPI compared with 1 and 6 DPI (p<.05). *Verrucomicrobia* were significantly reduced in infected mice compared to uninfected mice (p<.001). It was also reduced in mice at 1 DPI compared with mice at 12 and 24 DPI (p<.001) (Table 12).

At the genus level, S24-7 (p<.05) was significantly increased in infected mice, while *Oscillospira* (p<.001), *Bacteroides* (p<.005) and *Parabacteroides* (p<.005) were reduced in those mice. *S24-7* was also reduced at 1 DPI compared with all following days (p<.001). *Bacteroides* was increased at 24 DPI compared to 6 DPI and 12 DPI (p<.005), and 1 DPI was increased over 12 DPI. Likewise, *Parabacteroides* levels were elevated at 24 DPI in comparison with 6 and 12 DPI (p<.001) and 1 DPI was increased over 6 DPI only. *Prevotella* levels were significantly reduced at 12 DPI (p<.001), while *Enterobacteriacae* levels increased at 12 DPI compared to 1 and 24 DPI (p<.005) (Table 13).

Mice given L. reuteri treatment had increases in *Clostridiales* (p<.05), and *Ruminococcus* at 1 DPI (p<.01). *S24-7* (p<.05) was decreased. Interestingly, there was no change in *Lactobacillus* in these mice. At 24 DPI, *S24-7* was increased (p<.05) (Table 14). There were also considerable changes as the infection progressed as a function of

stressor exposure (Table 15). While no changes were detected at 1 DPI, at 6 DPI, *Lactobacillus* ($p<.01$), *Bacteroides* ($p<.01$), and *Parabacteroides* were all decreased ($p<.005$), while *S24-7* increased ($p<.005$). *S24-7* stayed at an elevated level through 12 DPI ($p<.005$) in SDR exposed mice, though no other changes were detected. At 24 DPI, *Clostridiales* ($p<.05$), *Parabacteroides* ($p<.05$), *Ruminococcus* ($p<.05$), and *Ruminococcaceae* ($p<.05$) were all reduced in SDR-exposed mice, while *S24-7* was increased in the SDR mice ($p<.005$). Redundancy analysis (RDA) using CANOCO further highlighted these results. OTUs with <10 total observations were removed for RDA analysis. DPI ($p<.01$, $F=4.2$), Stress ($p<.01$, $F=3.4$), and Infection ($p<.05$, $F=1.8$) were all significant on the RDA plot. OTUs that accounted for greater than 20% of the variance explained by the two axes were included on the RDA triplot, wherein SDR associated with *S24-7* OTUs, and *Bacteroides, Parabacteroides*, and *Adlercreutzia* OTUs identified with no stress. *Clostridiales* had a negative association with the temporal variable of DPI (Fig. 18).

Discussion:

Psychological stress exposure can alter microbial community structures, turning a normally stable microenvironment into a dysbiotic profile of volatility (36, 164, 187). Disruptions in the microbiota can have serious consequences on host physiology and immunity, and those induced by stress may be associated with aggravation of symptomology in IBD patients. Probiotic *Lactobacillus reuteri* can increase microbial diversity within the GI tract and a normalization of dysbiotic community structures may explain how *L. reuteri* has been associated with reductions in stressor-induced elevation

of inflammatory marker mRNA levels (e.g., CCL2, TNF-α, iNOS) and colitic pathology in mice that have been infected w/ *Citrobacter rodentium*. This study aimed to determine if *L. reuteri* ameliorates the stressor-induced increases in colonic inflammation by directly affecting colonic mucosal-associated microbiota community structure.

In this study, probiotic *L. reuteri* treatment only affected microbial populations at 1 DPI, despite repeated gavages up to 5 DPI. This result is surprising, as inflammation within the GI microenvironment is a factor that can induce dysbiosis on its own, therefore, the inflammatory reduction caused by *L. reuteri*, by a yet unknown mechanism, would be expected to result in changes to microbial abundances. One consideration is that SDR induces a moderate colitis during low grade infectious challenge with *C. rodentium*, which is reduced by *L. reuteri* to a mild state (Mackos et al, Under Review). The reduction in inflammation achieved by the probiotic may not be sufficient to affect microbial populations after 6 DPI on, as even probiotic-treated mice have marginally increased colitis scores at 12 DPI compared to control. Further, the magnified effect of the stressor by the sixth cycle could mask any effect the probiotic might have on commensal populations. However, these data do not support the hypothesis that probiotic *L. reuteri* amelioration of pathogen-induced inflammation is mediated through manipulation of the commensal microbiota.

The SDR stressor can impact colonic mucosa-associated microbial profiles after only one stressor cycle in uninfected mice, and can also disrupt cecal profiles immediately following the sixth cycle of SDR, but long-term effects of the stressor upon the microbiota had not been elucidated (70, 187). Here, the residual impact of the stressor

upon the microbiota is evident, as the effect of SDR upon the microbiota can be observed up to 24 DPI (19 days after cessation of SDR). It is possible that the effect of stressor at 12 and 24 DPI is associated with the infection or confined to the colonic mucosa, as previous studies have shown that the effect of stress upon the microbiota dissipates by 15 hours after the end of the sixth cycle exposure in the cecal-associated microbiota. The physiological and behavioral effects of SDR, including inducing glucocorticoid resistance, anxiety-like behavior, and monocyte trafficking, also have lasting effects beyond the scope of the stressor, out to 8 days after the final cycle (55, 59). These results emphasize how stressor exposure can have continuing impact upon the microbiota, which could perturb the GI immune response well after exposure to the stressor has abated. It is likely that the repeated exposures to stress compound the impacts upon the microbiota. *Lactobacillus* levels were reduced to a greater extent in mice exposed 6 cycles of SDR compared to mice exposed to only one cycle (187). Thus, within the colon where altered motile function and mucus secretion as a result of stress might not allow for normalization of the community structure of the microbiota, stress-associated impacts linger (22, 132).

SDR exposure was also associated with reductions in *Parabacteroides* and *Lactobacillus*, confirming previous research. Both genera have anti-inflammatory properties (165, 337, 341), and their reduction by stress may be associated with stressor-induced increases in colitis during *C. rodentium* infection. To better understand how SDR-induced disruptions in the microbiota adversely affect host immune responses, the involvement of these microbial groups in immune maintenance and why they are

specifically targeted by social stress must be explicated. In addition, the genus *Bacteroides* was significantly decreased in SDR-exposed mice and unclassified members of the family *S24-7* were increased.

SDR is associated with elevated colitic pathology and increased pro-inflammatory marker transcript levels in mice infected with the enteropathogenic *Escherichia coli* homolog, *C. rodentium* (Mackos et al, Under Review). A healthy microbiota is protective in *C. rodentium* infections, and antibiotic perturbation of the microbiota results in increased colitic severity (172, 251). The same concept applies to psychological stress. Stressor-induced alterations in the microbiota could be responsible for the elevated *C. rodentium* burden and resultant inflammation observed in previous studies (77). *C. rodentium* infection in relation to immune activation and clearance has been well studied, as has its effect in remodeling the microbiota during infection progression (164, 342, 343). In particular, *Firmicutes*, *Lactobacillus*, *Lachnospiraceae* and *Parabacteroides* have been shown to be reduced in the colon during *C. rodentium* infection, particularly around the peak of infection, at 10 DPI. This study adds to previous research by demonstrating the effects the *C. rodentium* infection can have on the microbiota in stressor-exposed mice. We report reductions in the phylum *Firmicutes*, and the genera *Oscillospira* and *Parabacteroides* in infected mice, and increases in the phylum *Bacteroidetes* and in unclassified members of the family *S24-7*. Gastrointestinal inflammation is often associated with dysbiotic profiles, observed clinically in IBD patients, and experimentally using dextran sodium sulfate (344-346). Thus, reductions reported here in *Prevotella* and *Parabacteroides* in SDR-exposed, infected mice at 6 and

12 DPI could be in direct response to the heightened inflammatory state within the colon at the peak of infection. Further, these data suggest that *S24-7* and *Parabacteroides*, bacterial groups affected by both stress and infection, could be health-associated and the stressor and infection-induced effects upon their abundance may be inter-related.

Dysbiotic profiles can have significant effects upon host immunity and inflammation. Studies using mutant mice deficient in various facets of the immune response, including NOD2, an intracellular pathogen recognition receptor, and Lyn, a kinase involved in pro-inflammatory gene transcription, have altered microbial profiles that are transmissible to wild-type mice, leading to increased levels of inflammation (4, 5). These studies make evident the interplay between the microbiota and inflammation, and how disruptions in one aspect can incite the other in a positive loop. Treatments that attempt to normalize dysbiotic profiles may be an effective method of reversing the symptomology observed in GI illnesses like IBD and *C. difficile* infections, without the long-term unknowns inherent to fecal microbiota transplantation. A major goal of this study was to examine the effect that probiotic *L. reuteri* had upon the microbiota in stressor-exposed mice challenged with *C. rodentium*. While the probiotic had no discernible effect upon microbiota community structure beyond 1 DPI, the effect of the stressor and *C. rodentium* upon the microbiota was apparent up to 24 DPI, and specific bacterial groups were identified as being affected by these factors, including *Parabacteroides*, *S24-7*, and murine residential *Lactobacillus*. Identifying how particular bacterial groups are affected by both social stress and enteric infection can allow researchers to shift the focus to the functional influence these bacterial groups have with

the host, and continue to develop methodology like prebiotic formulations that may revert dysbiotic communities to a healthier state.

	Uninfected	1 DPI	6 DPI	12 DPI	24 DPI
Firmicutes	77.86 ± 2.38ab	85.35 ±1.94a	75.43 ± 2.41b	73.05 ± 2.91b	71.96 ± 2.69b
Bacteroidetes	14.86 ± 2.07ab	7.88 ± 0.86a	14.44 ± 1.98b	13.75 ± 1.61b	19.53 ± 2.54b
Proteobacteria	4.28 ± 1.80	5.64 ± 1.33	8.34 ± 2.15	10.60 ± 1.98	6.52 ± 0.90
Deferribacteres	1.90 ± 1.80	0.83 ± 0.32	1.39 ± 0.52	0.47 ± 0.13	0.65 ± 0.25
Actinobacteria	0.17 ± 0.06abc	0.17 ± 0.05a	0.19 ± 0.03ac	1.69 ± 0.74b	0.77 ± 0.23bc
Verrucomicrobia	0.83 ± 0.29*	0.05 ± 0.01a	0.11 ± 0.04a	0.20 ± 0.05b	0.18 ± 0.06b

Data are mean of percentage of total attained sequences ± standard error
*- p<.05 vs. Infected Mice

Table 12: Major Phyla Abundance over Course of Infection

	Uninfected	1 DPI	6 DPI	12 DPI	24 DPI
Unclassified Clostridiales	33.19 ± 6.33	40.33 ± 2.58	37.77 ± 2.54	33.10 ± 2.39	38.76 ± 2.84
Lactobacillus	24.43 ± 9.80	26.97 ± 3.51	21.28 ± 2.99	22.01 ± 3.09	16.15 ± 2.05
Lachnospiraceae	5.21 ± 1.09	6.92 ± 0.45	6.50 ± 0.54	6.79 ± 0.53	5.95 ± 0.39
S24-7	0.01 ± 0.01*	0.52 ± 0.30^{a}	8.17 ± 1.79^{b}	6.76 ± 1.21^{b}	9.14 ± 2.22^{b}
Bacteroides	8.90 ± 1.44*	4.32 ± 0.48^{ac}	3.25 ± 0.56^{c}	3.17 ± 0.55^{b}	5.10 ± 0.84^{a}
Oscillospira	5.41 ± 0.87*	3.04 ± 0.23	2.78 ± 0.27	2.57 ± 0.21	2.47 ± 0.17
Shewanella	1.47 ± 0.66	2.11 ± 0.53	2.31 ± 0.70	2.27 ± 0.45	2.37 ± 0.36
UnclassifiedRuminococcaceae	2.80 ± 0.50	2.44 ± 0.36	2.02 ± 0.17	2.02 ± 0.17	2.37 ± 0.27
Lachnospiraceae; Ruminococcus	1.62 ± 0.38	2.09 ± 0.25	2.06 ± 0.20	1.87 ± 0.23	1.81 ± 0.14
Unclassified Halomonadaceae	1.38 ± 0.55	1.78 ± 0.41	1.66 ± 0.39	1.87 ± 0.35	1.91 ± 0.24
Halomonas	1.27 ± 0.54	1.66 ± 0.39	1.81 ± 0.58	1.80 ± 0.35	1.94 ± 0.30
Parabacteroides	3.02 ± 0.66*	1.39 ± 0.17^{ab}	0.89 ± 0.23^{c}	1.17 ± 0.19^{b}	1.81 ± 0.33^{a}
Prevotella	1.72 ± 0.27	1.17 ± 0.16^{a}	1.40 ± 0.26^{a}	0.72 ± 0.12^{b}	1.47 ± 0.24^{a}
Enterobacteriaceae	0.01 ± 0.01	0.01 ± 0.00^{a}	1.31 ± 0.79^{ab}	2.48 ± 1.01^{a}	0.07 ± 0.05^{b}
Ruminococcus	1.93 ± 0.64	1.14 ± 0.12	0.89 ± 0.08	0.84 ± 0.07	0.94 ± 0.08

Data are mean of percentage of total attained sequences ± standard error
Filtered to genera that comprise>1% total sequences
*- p<.05 vs. Infected Mice

Table 13: Most Abundant Genera over Course of Infection

	1 DPI		6 DPI		12 DPI		24 DPI	
	Lr Treatment	Vehicle	Lr Treatment	Vehicle	Lr Treatment	Vehicle	Lr Treatment	Vehicle
Unclassified Clostridiales	45.74 ± 3.62*	33.38 ± 3.91	40.58 ± 3.21	35.13 ± 3.89	31.82 ± 3.91	34.56 ± 2.57	36.39 ± 4.47	40.96 ± 3.62
Lactobacillus	21.33 ± 4.73	34.22 ± 6.25	19.59 ± 3.76	22.87± 4.68	22.91 ± 4.61	20.98 ± 4.13	14.44 ± 2.40	17.73 ± 3.29
Lachnospiraceae	7.50 ± 0.71	6.17 ± 0.68	6.92 ± 0.82	6.11 ± 0.72	6.05 ± 0.65	7.64 ± 0.84	5.58 ± 0.69	6.29 ± 0.41
S24-7	0.04 ± 0.04	1.13 ± 0.66	8.86 ± 3.25	7.51 ± 1.76	6.97 ± 1.78	6.51 ± 1.64	13.90 ± 3.78*	4.72 ± 1.92
Bacteroides	4.97 ± 0.61	3.48 ± 0.47	3.80 ± 1.00	2.73 ± 0.55	2.75 ± 0.57	3.65 ± 0.99	5.02 ± 1.30	5.16 ± 1.13
Oscillospira	3.27 ± 0.36	2.75 ± 0.45	2.70 ± 0.40	2.86 ± 0.37	2.53 ± 0.32	2.61 ± 0.27	2.44 ± 0.20	2.49 ± 0.28
Shewanella	1.19 ± 0.37	3.29 ± 1.12	1.67 ± 0.46	2.94 ± 1.29	1.93 ± 0.33	2.67 ± 0.90	2.02 ± 0.59	2.70 ± 0.43
UnclassifiedRuminococcaceae	2.82 ± 0.86	1.96 ± 0.33	2.15 ± 0.31	1.89 ± 0.15	1.99 ± 0.28	2.05 ± 0.19	1.95 ± 0.29	2.75 ± 0.43
Lachnospiraceae; Ruminococcus	2.65 ± 0.40*	1.38 ± 0.17	2.07 ± 0.23	2.04 ± 0.32	1.84 ± 0.36	1.91 ± 0.27	1.81 ± 0.17	1.80 ± 0.23
Unclassified Halomonadaceae	1.08 ± 0.30	2.69 ± 0.86	1.33 ± 0.27	1.98 ± 0.72	1.53 ± 0.22	2.27 ± 0.72	1.58 ± 0.33	2.21 ± 0.35
Halomonas	1.02 ± 0.24	2.48 ± 0.84	1.24 ± 0.24	2.35 ± 1.10	1.48 ± 0.22	2.15 ± 0.71	1.61 ± 0.42	2.24 ± 0.41
Parabacteroides	1.59 ± 0.28	1.13 ± 0.20	0.82 ± 0.14	0.96 ± 0.43	1.32 ± 0.28	1.00 ± 0.25	1.15 ± 0.23	2.42 ± 0.57
Prevotella	1.24 ± 0.18	1.07 ± 0.19	1.18 ± 0.19	1.61 ± 0.47	0.68 ± 0.15	0.76 ± 0.20	1.20 ± 0.28	1.73 ± 0.37
Enterobacteriaceae	0.01± 0.01	0.02 ± 0.01	0.99 ± 0.72	1.61 ± 1.40	3.23 ± 1.67	1.62 ± 0.98	0.13 ± 0.09	0.02 ± 0.01
Ruminococcus	1.10 ± 0.18	1.19 ± 0.23	0.95 ± 0.13	0.84 ± 0.09	0.84 ± 0.10	0.85 ± 0.09	0.86 ± 0.11	1.01 ± 0.10

Data are mean of percentage of total attained sequences ± standard error
Filtered to genera that comprise>1% total sequences
*- p<.05 vs Vehicle on same DPI

Table 14: Most Abundant Genera by Probiotic Treatment

	1 DPI		6 DPI		12 DPI		24 DPI	
	SDR	HCC	SDR	HCC	SDR	HCC		HCC
Unclassified Clostridiales	42.06 ± 3.46	38.37 ± 3.91	41.42 ± 3.92	34.34 ± 3.17	30.31 ± 3.96	35.52 ± 2.83	30.43 ± 3.86*	45.43 ± 3.23
Lactobacillus	26.33 ± 5.23	27.69 ± 6.25	13.55 ± 2.46*	28.56 ± 4.75	17.93 ± 4.44	25.56 ± 4.25	19.68 ± 3.80	13.32 ± 1.93
Lachnospiraceae	7.36 ± 0.67	6.42 ± 0.68	7.29 ± 0.79	5.76 ± 0.72	6.04 ± 0.71	7.44 ± 0.76	5.17 ± 0.53	6.57 ± 0.53
S24-7	0.19 ± 0.14	0.89 ± 0.66	12.73 ± 2.98*	3.87 ± 1.51	10.40 ± 1.90*	3.59 ± 1.23	15.71 ± 3.24*	3.88 ± 2.34
Bacteroides	4.84 ± 0.80	3.73 ± 0.47	1.88 ± 0.34*	4.54 ± 0.95	2.44 ± 0.75	3.80 ± 0.78	4.80 ± 1.38	5.33 ± 1.08
Oscillospira	3.12 ± 0.36	2.96 ± 0.45	3.06 ± 0.49	2.52 ± 0.23	2.56 ± 0.37	2.58 ± 0.24	2.15 ± 0.22	2.73 ± 0.24
Shewanella	1.54 ± 0.33	2.74 ± 1.12	2.84 ± 1.37	1.81 ± 0.45	2.92 ± 0.92	1.71 ± 0.26	2.01 ± 0.45	2.66 ± 0.54
Unclassified Ruminococcaceae	2.27 ± 0.28	2.64 ± 0.33	2.22 ± 0.27	1.82 ± 0.20	2.23 ± 0.30	1.84 ± 0.19	1.83 ± 0.35*	2.80 ± 0.37
Lachnospiraceae; Ruminococcus	2.08 ± 0.31	2.11 ± 0.17	2.18 ± 0.31	1.94 ± 0.26	2.02 ± 0.42	1.74 ± 0.22	1.73 ± 0.17	1.86 ± 0.22
Unclassified Halomonadaceae	1.33 ± 0.31	2.30 ± 0.86	1.97 ± 0.77	1.37 ± 0.26	2.40 ± 0.72	1.42 ± 0.19	1.71 ± 0.32	2.07 ± 0.36
Halomonas	1.20 ± 0.23	2.18 ± 0.84	2.32 ± 1.17	1.34 ± 0.27	2.34 ± 0.72	1.32 ± 0.17	1.73 ± 0.43	2.10 ± 0.41
Parabacteroides	1.45 ± 0.28	1.32 ± 0.20	0.39 ± 0.08*	1.37 ± 0.41	0.88 ± 0.25	1.43 ± 0.27	0.95 ± 0.12*	2.49 ± 0.54
Prevotella	1.35 ± 0.26	0.96 ± 0.19	1.12 ± 0.16	1.66 ± 0.48	0.88 ± 0.24	0.57 ± 0.10	1.49 ± 0.33	1.46 ± 0.35
Enterobacteriaceae	0.01 ± 0.01	0.02 ± 0.01	0.46 ± 0.27	2.10 ± 1.51	2.36 ± 1.33	2.58 ± 1.52	0.03 ± 0.02	0.10 ± 0.01
Ruminococcus	1.22 ± 0.20	1.05 ± 0.23	0.97 ± 0.13	0.82 ± 0.09	0.78 ± 0.10	0.90 ± 0.09	0.74 ± 0.10*	1.10 ± 0.10

Data are mean of percentage of total attained sequences ± standard error
Filtered to genera that comprise>1% total sequences
*- p<.05 vs HCC on same DPI

Table 15: Most Abundant Genera by Stress Exposure

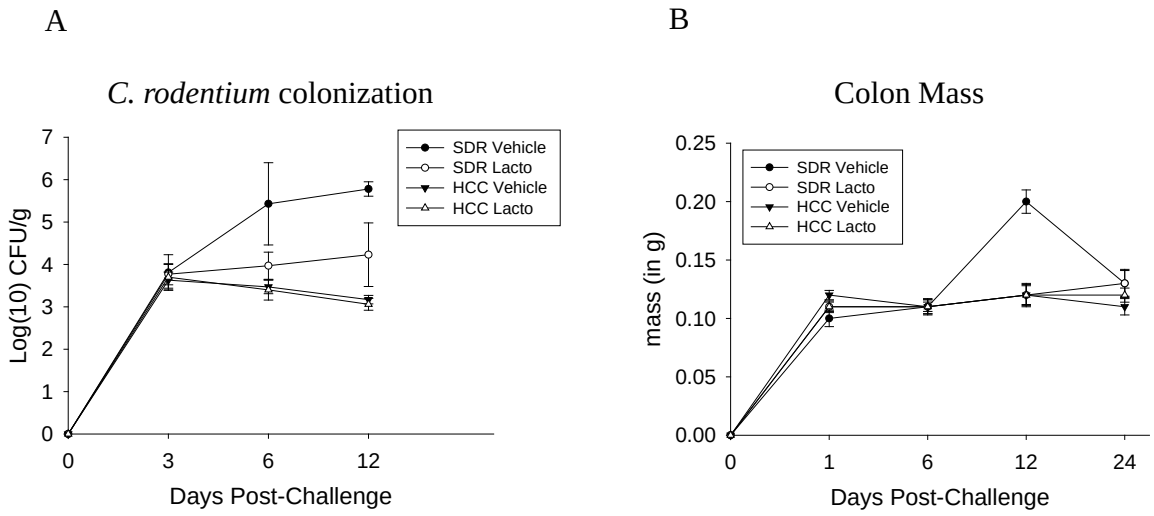


Figure 13: Probiotic *Lactobacillus reuteri* treatment abrogates stressor-induced increase in colon mass. A.) Mice that were exposed to SDR had significantly higher shed *C. rodentium* in the stool at 12 DPI than control mice. Treatment with *L. reuteri* blocked this increase in *C. rodentium* abundance in the stool. All groups n=3B.) Mice exposed to SDR had significantly higher colon mass, an indicator of colitic pathology, at 12 DPI. Mice given *L. reuteri* did not exhibit the same increase in colon mass. All groups n=9-12

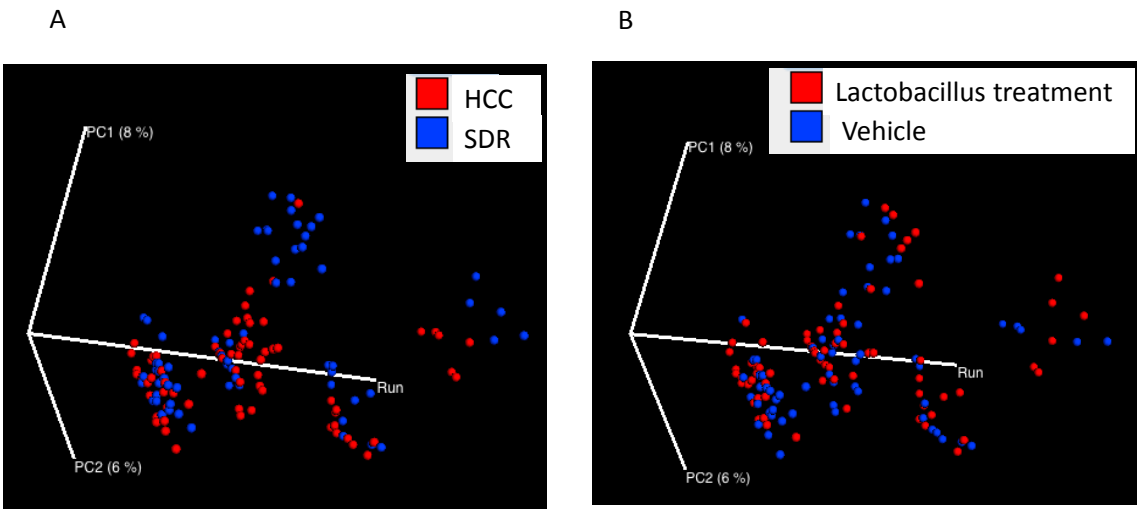


Figure 14: Stressor exposure significantly disturbs microbiota community structure in the overall sample, while probiotic treatment has no effect. A.) Mice exposed to SDR had significantly altered microbial profiles, as indicated on a principal coordinate analysis that used unweighted UniFrac distances. This clustering was found to be significant using the adonis statistic. Because the repeated experiments had a significant effect on community structure, the PCoA shows the effect of SDR in each of four repeats of the study on the third axis of the PCoA. B.) Probiotic *L. reuteri* treatment was not associated with unique clustering of microbial communities.

Figure 15: As infection progresses, microbial profiles become increasingly spread along the 3D PCoA space. A.) Uninfected mice cluster separately from all infected mice on a principal coordinate analysis (PCoA) based upon unweighted UniFrac distances, which was confirmed with the adonis statistic. B-F.) Separating each timepoint indicates dispersion of microbial communities along the first PCoA as the infection continues to 24 DPI.

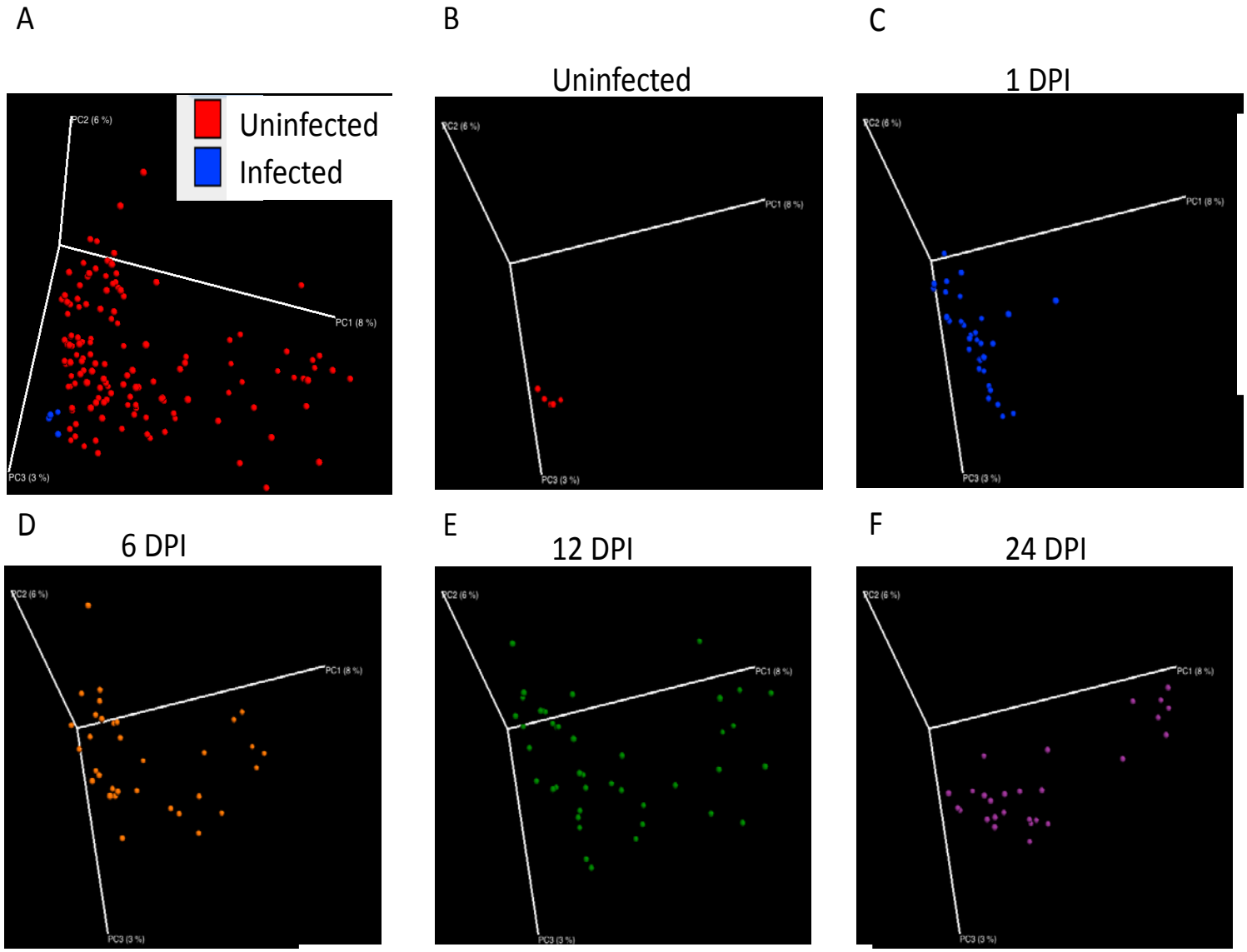

A
B
C
Uninfected
1 DPI
Uninfected
Infected
PC2 (6 %)
PC1 (8 %)
PC3 (3 %)
D
6 DPI
E
12 DPI
F
24 DPI

Figure 15.

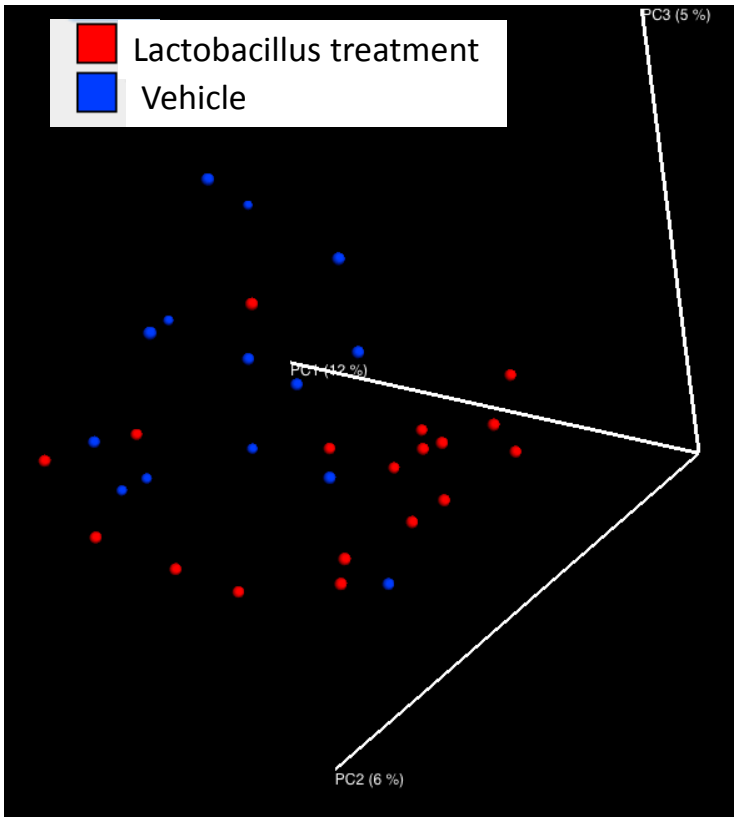


Figure 16: Probiotic treatment significantly shifts the colonic mucosal microbiota at 1 DPI. Unweighted UniFrac distances indicate significant clustering of colons treated with probiotic *L. reuteri*. No later timepoints exhibited clustering based upon probiotic treatment.

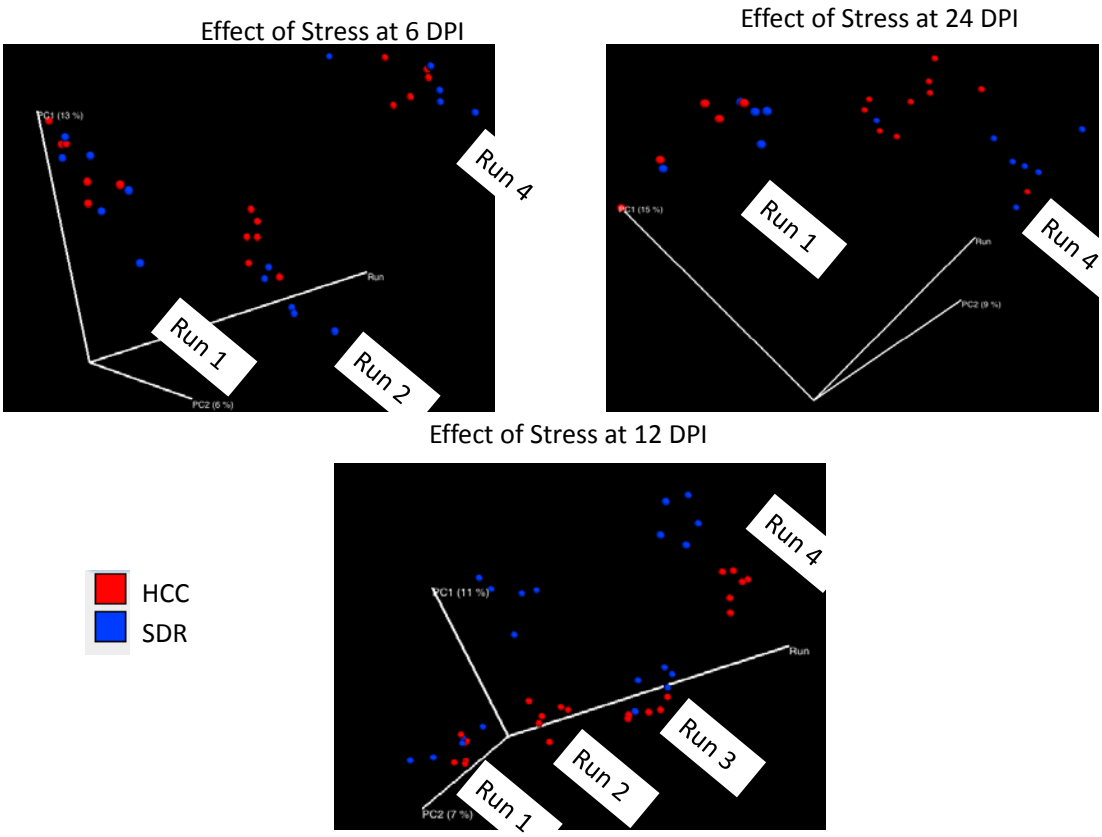


Figure 17: Exposure to SDR affects colonic mucosal microbiota structure regardless of DPI up to 19 days after cessation of exposure. A-C.) PCoAs based on unweighted UniFrac were filtered based upon experimental repeat to illustrate the continued effect of stress upon the microbiota at 6, 12, and 24 DPI.

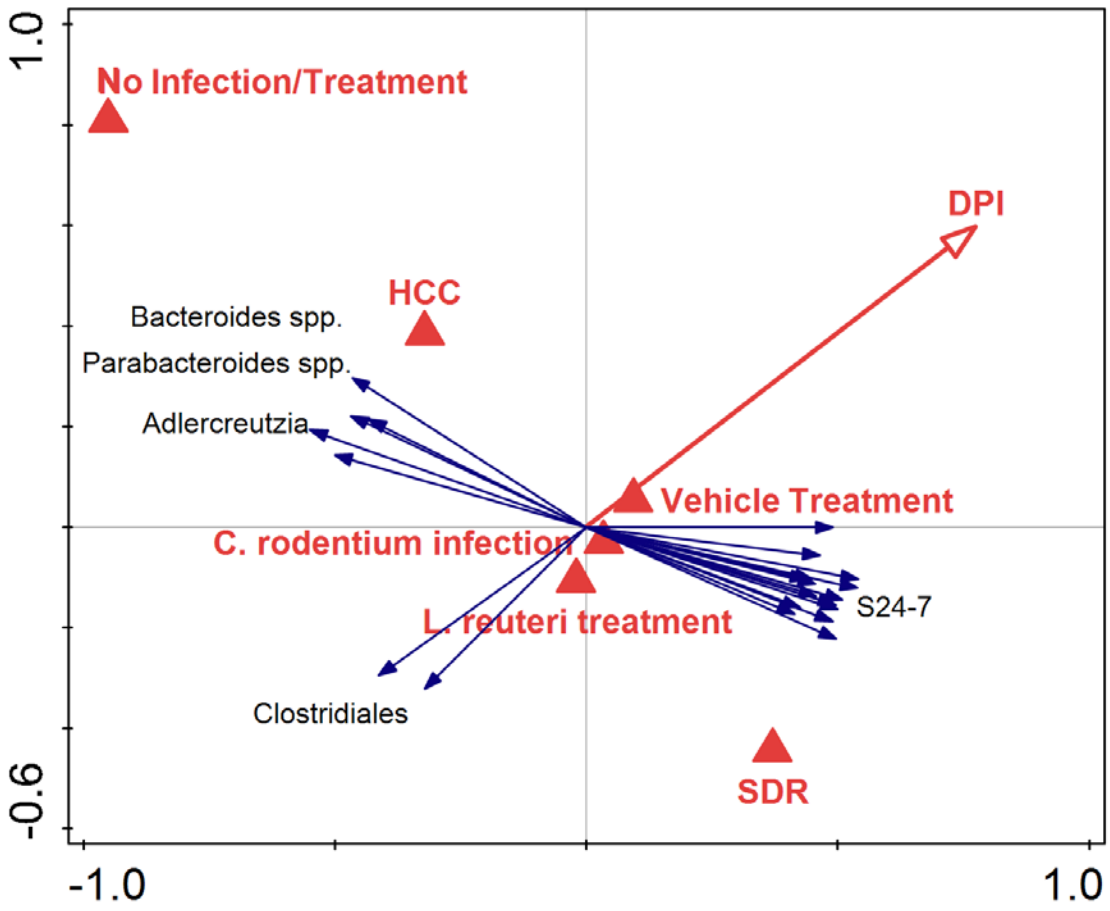


Figure 18: DPI and stressor exposure are associated with specific OTUs on an RDA biplot. OTUs that were identified in *Clostridiales* negatively associated with increasing DPI. OTUs in *Bacteroides* spp., *Parabacteroides* spp., and *Adlercreutzia* spp. associated with HCC and OTUs in *S24-7* associated with SDR. Canoco 5 software was used to construct an RDA plot. OTUs that contributed to at least 20% of the variance were included on the plot.

The previous chapters have emphasized the effect that psychological stress has in inducing dysbiosis. By altering the murine colonic microbiota, psychological stress has major downstream effects upon host health, particularly as it pertains to the mucosal immune response to enteric pathogen challenge. However, a better understanding of the translatability of dysbiosis emergence must be achieved for humans. The molecular and physiological mechanisms by which antibiotics and diet induce dysbiosis are well understood, but these are not the totality of dysbiosis induction in humans. In order to better evaluate methods by which dysbiosis begins and is sustained within human hosts, we examined the association between maternal obesity and the offspring microbiota, as parental obesity is a primary antecedent of offspring obesity in adulthood. Since the mother is also a major source of the microbiota for their child and the microbiota can be obesogenic, we hypothesized that children born to obese mothers would have a unique microbiota compared to children born to non-obese mothers. If so, maternal transmission of the microbiota could be a mechanism by which obesity is passed from mother to child.

Chapter 6: Maternal obesity is associated with alterations in the gut microbiota in toddlers[3]

Introduction

Obesity is a substantial public health problem globally. In the US, it is estimated that 16.9% of children ages 2-19 years and 33.8% of adults ≥ 20 years are obese (347, 348). However, early life antecedents of obesity are not well delineated. In children under 3 years of age, the strongest predictor of obesity in adolescence and adulthood is parental obesity (206). Compared to paternal obesity, maternal obesity has greater predictive value for body mass index (BMI) of offspring through adolescence (349, 350). However, the relative influence of genetics versus environmental pathways in the transgenerational transmission of obesity from parent to child is unknown.

A novel possible mechanistic pathway linking parental and child weight is the transmission of commensal microbiota via parental exposures, particularly maternal. The microbiota are a consortium of trillions of bacteria that are resident to a variety of human body niches (351). The vast majority of these microbes reside within the gastrointestinal (GI) tract where they form microbial communities whose structures are stable during periods of homeostasis and heavily involved in host metabolic and nutritional functions, including food digestion and vitamin synthesis (8, 352).

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Disruptions in the relative abundances of microbes that comprise these communities have been associated with obesity and high-fat diets (17, 273, 275, 353-355). For example, obese mice have abnormal levels of GI *Firmicutes* and *Bacteroidetes*, two primary phyla of the GI tract microbiota (273). Such skewed bacterial abundances may lead to alterations in energy procurement from food and related propensity toward obesity. When microbiota from obese mice are transferred into germ-free mice, recipient mice have increased body fat, providing strong evidence of a causal link between the microbiota and obesity (275).

Factors affecting the establishment of bacterial abundances in early life are not well understood. During birth, the neonate is rapidly colonized by maternal bacteria via vertical transmission from the gastrointestinal and reproductive tracts as well as environmental microbes (204, 208, 356). In very early life, mothers are likely to be primary donors of bacteria through physical contact and breast milk. Demonstrating such maternal influence, at one and six months of age, infants of obese mothers have significantly different bacterial population abundances compared to infants of non-obese mothers (357). Importantly, during the first year of life, the microbiota show great transience and volatility (203). As solid foods are introduced to the diet, the structure of the microbiota stabilizes and begins to reflect the adult profile (95). Thus, it is important to determine if maternal influences on gut microbial groups persist in children past early infancy despite competing factors.

In addition, the recent advent of next generation pyrosequencing allows for wider study of microbial communities than permitted by earlier methods, including denaturing

gradient gel electrophoresis (DGGE) and polymerase chain reaction (PCR). Utilization of this technology permits the analyses of entire bacterial communities rather than examination of smaller classification subsets selected by *a priori* hypotheses. To our knowledge, pyrosequencing has not been used in studies associating parental obesity to child microbiota communities.

Addressing these gaps in the literature, the current study examined the association between maternal obesity and the gut microbiota profiles of toddlers at approximately two years of age using pyrosequencing technology. We hypothesized that the microbiota of children born to obese mothers would have a significantly different gastrointestinal microbiota, as assessed using alpha and beta diversity measurements, when compared to children born to normal weight mothers. We also hypothesized that differences in abundances of bacterial populations previously associated with obesity would be observed in children of obese versus non-obese mothers.

Materials and Methods

Study Design- We recruited 79 women with children approximately two years of age from the general community of Columbus, Ohio. Children were excluded if their mother reported the child had a major health condition or developmental delay. Children were also excluded if they were already toilet trained. Each woman completed an online questionnaire which included assessment of her health behaviors and exposures (e.g., medications) during pregnancy as well as health and feeding behaviors in her child.

Within 7 days of completing the online questionnaire, each woman collected a stool sample from her child per the protocol detailed below. Two samples were removed

from statistical analyses due to low sequence count (<5108), resulting in final sample of 77 mother-child pairs. This study was approved by the Ohio State University Biomedical Institutional Review Board. All women completed written informed consent for themselves and provided written consent on behalf of their children. Women received modest compensation for their participation. Data collection occurred from May 2011 to December 2012.

Parental Characteristics- Women reported information about their age, race (self and child's father), marital status, education level (self and child's father), and total family income per year. Body mass index (BMI; kg/m^2) was calculated based on the provided maternal and paternal heights and weights. BMI values ≥ 30 were classified as obese.

Perinatal Health Information- Self-report data was collected regarding exposure to antibiotics during pregnancy and while breastfeeding (if applicable). With regard to birth outcomes, women reported the route of delivery (vaginal versus C-section), gestational age at the time of delivery and the child's sex.

Child Diet and Growth- Women reported the occurrence and duration of breastfeeding and the age at which formula (if applicable), cereals/grains, fruits/vegetables, and meats were introduced as part of the child's diet. The current frequency of each food type was also reported, from less than once per month to two or more times per day. Women reported the number of times their child had been exposed to antibiotic medications, with completion of a full prescription course (e.g., 10 days) considered as one exposure. Women also reported child exposure to probiotics in capsule/supplement form or in formula or food which specified it contained probiotics.

Finally, to determine the child's growth trajectory, women reported their child's height and weight percentile at the most recent well-visit to the pediatrician. A weight/height ratio was calculated and children were categorized into three groups: those whose weight percentile was greater than their height percentile (n = 11), those in the same percentile bracket (n = 31), and those whose weight percentile was lower than their height percentile (n = 33).

Stool Sample Collection and Storage- Women were provided with sterile wooden applicators and sterile 50 ml plastic conical collection tubes for collection. They were instructed to sterilely collect the stool sample from child's soiled diaper with the wooden applicator and place in the collection tube. Samples were then stored at 4° C (i.e., refrigerated) for up to 24 hours until collection by study personnel from the participant's home or delivery by the participant to OSUWMC. In the latter case, women were instructed to transport samples in a cooler with ice. Upon arrival at the Wexner Medical Center, samples were placed in long-term storage at -80°C until pyrosequencing was conducted.

bTEFAP- Bacterial tag-encoded FLX-Amplicon Pyrosequencing (bTEFAP) was performed as previously described (41, 358). The 16s rrn universal primers 27f (AGA GTT TGA TCM TGG CTC AG) and 519r (GWATTACCGCGGCKGCTG) were used for specific 16S rrn targeting. These primers were used for single-step 30 cycle PCR. The following thermoprofile was used: a single cycle of 94°C for 3 minutes, then 28 cycles of: 30 seconds at 94°C ; 40 seconds at 53°C, 1 minute at 72°C, with a single 5 minute cycle at 72°C for 5 minutes for elongation. Amplicons were pooled at equivalent

concentrations and purified (Agencourt Bioscience Corporation, MA, USA). Sequencing was performed with the Roche 454 FLX Titanium system using manufacturer's guidelines.

Sequencing Analysis- Analysis was performed using the open-source software package, Quantitative Insights Into Microbial Ecology (QIIME), v.1.7.0. (46). Sequences were provided via .fasta file and sequence quality was denoted with a .qual file. Barcodes were trimmed and low-quality reads were removed. An average quality score of 25 was used. Minimum sequence length of 200 and maximum length of 1000 were used. No mismatches were allowed in the primer sequence. An average of 14862 sequences were attained per sample, and a total of 77.06% of sequences passed quality filtering.

Sequences were clustered based upon 0.97 similarity using UClust into operational taxonomic units (OTUs)(231). A representative sequence was selected from each OTU and the RDP classifier was used to assign taxonomy to the representative sequence (44). Sequences were aligned using PyNAST (232) against a Greengenes core reference alignment database (48) and an OTU phylogenetic tree was assembled based upon this alignment (234).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, or PiCRUST, was used to identify differences in predictive metagenome function (359). In summary, OTUs were picked from a demultiplexed fasta file containing the sequences for all 77 subjects using the closed-reference procedure, against the GreenGenes 13_5 reference database(233). These OTUs were normalized by the predicted 16s copy number, and functions were predicted from these normalized OTUs

with the use of GreenGenes 13_5 database for KEGG Orthologs. From this, a BIOM table containing the predicted metagenome for each sample was attained. Each sample was rarefied at 2,000,000 before further analysis. Downstream statistical analysis was performed using STAMP (360).

Statistical Analysis- The Shannon Diversity Index (SDI), a measurement of within-sample (alpha-diversity) community diversity, as well as Chao1 (estimates richness), equitability (measures evenness), and observed_species (calculates unique OTUs) were used to ascertain differences in alpha diversity based on maternal obesity status (235). All alpha-diversity measurements were calculated with QIIME and significance was measured using a parametric t-test at a depth of 5930 sequences for comparison of all obese vs non-obese groups. Depths of 4534 sequences for comparison of maternal obesity among the high income group alone, and 5126 sequences for comparison among the low income group alone were also used. UniFrac unweighted distance matrices were calculated from the OTU phylogenetic tree for beta diversity analyses (50). A sampling depth of 5108 sequences/sample was used for beta diversity for all groups.

The adonis statistic, available through the vegan package on the open-source statistical program R, and further employed in QIIME, was used to measure differences in variance between two groups based upon their microbiota UniFrac distance matrices (47, 236). Groups were split based upon maternal and paternal BMI, as well as by income level and differences in community structure were determined using adonis. The permdisp statistic, also available through vegan, was then performed to verify equal variances between groups dichotomized by obesity.

Chi-square analyses and two-sample t-tests were used to determine the demographic and behavioral similarity between the maternal obesity groups to identify possible confounding factors. Additionally, Pearson's correlations, univariate analysis of variance (ANOVA) and regression analyses were used to examine associations between variables including maternal BMI, child's weight/height ratio and the SDI. The relative abundance of bacterial groups in samples from children of obese and non-obese mothers were compared using Mann-Whitney U-tests. All analyses were performed using SPSS v.21 (IBM, Chicago, IL). For predictive functional group analysis in STAMP, Welch's t-tests were used for two group comparisons, while Kruskal-Wallis H-tests were used for multiple group comparisons. P-values were corrected for multiple-tests using the Benjamini-Hochberg method (361), with a q-value of 0.10.

Data Availability- Sequences have been deposited in the Sequence Read Archive, under the study Accession Number SRP045568. Requests for metadata can be made to Carson Reader (Carson.Reider@osumc.edu).

Results

Participant Characteristics- This study included 77 mother-child pairs. Children were 18-27 months at the time of assessment (Mean = 23.14, SD = 2.00), with 91% falling between 21-26 months. In this sample, 87.0% (n = 67) of mothers were White, 9.1% (n = 7) were Black and 3.9% (n = 3) were Asian. The mean maternal age at the time of delivery was 31.10 ± 5.43 and 87.0% of women (n = 67) were married. In this sample, 66.2% of mothers (n = 51) were non-obese (BMI < 30) and 33.8% (n = 26) were obese (BMI ≥ 30) based self-reported height and weight prior to pregnancy. The mean BMI

among the obese women was 35.13 ± 4.48 compared to 22.65 ± 2.85 among the non-obese ($t(75) = 15.3$, $p < 0.001$).

To identify potential factors which may confound the relationship between maternal obesity and the composition of the child microbiota, we examined the demographic and behavioral similarity between obese and non-obese women (Tables 16 & 17). Obese and non-obese women did not differ significantly in race, marital status, maternal age at the time of delivery, antibiotic exposure during pregnancy or breastfeeding, or delivery route (vaginal versus C-section). Obese women had heavier male partners than did non-obese women, with BMIs of 31.20 ± 5.98 vs. 26.91 ± 4.60, respectively ($t(75) = 3.49$, $p = 0.001$). Obese women and their partners had completed less education than non-obese women and their partners ($ps \leq 0.014$). However, women did not differ in annual household income based on obesity status ($X^2(3) = 1.92$, $p = .59$), although household income was significantly correlated with both maternal ($r = .65$, $p < 0.001$) and paternal education ($r = .52$, $p < 0.001$).

Maternal obesity and beta diversity in the child gut microbiota- Unweighted UniFrac distance matrices were used to assess differences between the microbial communities, known as beta diversity, in children of obese compared to non-obese mothers. Permutational multivariate ANOVA using adonis showed that children of obese versus non-obese mothers had a different microbiota community structure ($r^2 = 0.01539$, $p = 0.044$). However, this did not result in clustering of two distinct populations using a principle coordinate analysis (PCoA) (Fig. 19). To further explain the significant adonis statistic in the absence of obvious clustering, permdisp, a statistic that measures the

extent to which variances in different populations are equivalent, was used to compare the two groups. Dispersion of the community structures of children born to obese versus non-obese mothers differed signficantly, with greater variance among children of non-obese mothers ($p = 0.035$, $F = 4.843$). In contrast, there was no difference in between-sample community structure as measured via adonis in children of obese versus non-obese fathers ($r^2 = 0.01214$, $p = 0.801$).

Next, we examined whether the strength of the association between beta diversity and maternal obesity differed among children of mothers from higher versus lower socioeconomic backgrounds. Analyses showed no main effects of socioeconomic indicators; neither maternal education ($r^2 = 0.01267$, $p = 0.615$) nor income level ($r^2 = 0.01331$, $p = 0.409$) were associated with shifts in the offspring microbial profile. Similarly, neither maternal education nor income were associated with clustering on a PCoA (Fig. 20). Next, the interaction between obesity status with both education (high school graduate or less versus college graduate or more) and income (<\$50k versus ≥\$50k) was examined. An interaction effect between income and obesity status was observed; in the high-income group, a different microbiota community structure was seen in the children of obese versus non-obese mothers ($r^2 = 0.02547$, $p = 0.041$). However, in the lower-income group, no significant effects of maternal obesity on beta diversity were observed ($r^2 = 0.03798$, $p = 0.139$). Also, in dyads from high-income households, the microbiota of children of obese mothers had greater homogeneity among the samples compared to those from non-obese mothers ($F = 11.942$, $p = 0.003$). Furthermore,

clustering based on obesity status was observed using a PCoA in the high income group only (Fig 21A-B).

Similar effects were seen when using education as an indicator of socioeconomic status. Among mothers with a high education, children born to obese mothers had a different community structure than those born to non-obese mothers (r^2 = 0.02049, p = 0.045) and this was partly explained by significantly greater homogeneity in variance (F = 6.215, p = 0.02). In contrast, among children born to women with less education, there were no significant differences in beta diversity based on maternal obesity status (r^2 = 0.05327, p = 0.61). Thus, similar results were observed in relation to income and education as indicators of socioeconomic status. Compared to education level, income was more evenly distributed in the obese and non-obese groups, providing greater statistical power. Thus, all downstream analyses focused on income.

Maternal obesity and alpha diversity in the child gut microbiota- We next examined the relationship between maternal BMI and alpha diversity of the child microbiota. First, we examined the Shannon Diversity Index (SDI), a measure of the overall diversity within a microbial community. Two samples were below the threshold for SDI, resulting in a sample of 75 for these analyses. Results showed that children of obese mothers had a significantly higher SDI than children of non-obese mothers (t(73) = 2.1, p = 0.04; Fig. 22A). Greater alpha diversity in children born to obese mothers was associated with greater equitability (t(73) = 1.96, p = 0.05; Fig. 22B) and a trend towards greater richness as estimated by Chao1 (t(73) = 1.83, p = 0.07; Fig. 22C). Furthermore, children of obese

mothers had higher number of unique OTUs as defined by QIIME variable observed_species ($t(73) = 2.25$, $p = 0.03$; Fig. 22D).

Next, we examined interactions between maternal socioeconomic status and obesity on alpha diversity of the child gut microbiota. As with beta diversity, results indicated that effects of maternal obesity on alpha diversity were driven by the high-income group. Specifically, in high income households, SDI ($t(73) = 2.30$, $p = 0.026$), Chao1 ($t(73) = 2.08$, $p = 0.043$), equitability ($t(73) = 2.20$, $p = 0.033$), and observed OTUs ($t(73) = 2.30$, $p = 0.029$) were all higher in children of obese versus non-obese mothers. However, among children in lower income households, no differences in alpha diversity were detected in relation to maternal obesity status [SDI ($t(73) = 0.537$, $p = 0.595$), Chao1 ($t(73) = -0.018$, $p = 0.992$), equitability ($t(73) = 0.498$, $p = 0.619$), observed OTUs ($t(73) = 0.674$, $p = 0.515$)] (Fig. 23A-H).

Further analyses demonstrated that the SDI was higher in children of obese versus non-obese fathers ($t(73) = 1.99$, $p = 0.05$) which corresponded to greater equitability ($t(73) = 2.10$, $p = 0.04$). However, there were no significant differences in either the Chao1 estimation or OTUs (i.e., observed_species in QIIME) between children born to obese or non-obese fathers (data not shown). When entered into a regression model together, maternal BMI remained a significant predictor of the SDI ($\beta = 0.324$, $p = 0.008$) while paternal BMI was no longer significantly associated ($\beta = 0.085$, $p = 0.48$) suggesting that maternal BMI was the critical predictor. In addition, univariate ANOVA demonstrated that the child weight/height ratio showed no association with the toddler SDI ($F(2,72) = 0.58$, $p = .565$). Moreover, maternal BMI remained a significant predictor

after including the child's WHR in the model (β = 3.178, p = 0.002), indicating an effect of maternal BMI that was independent of the child's current body composition.

Maternal obesity and phylogenetic shifts in child gut microbiota- We next examined phylogenetic shifts in the fecal microbiota of the children, to determine if differences in abundances of given genera were evident. An area graph of the phyla present in all subjects indicated that considerable variability existed across children in the abundances of the highly abundant phyla, wherein a wide range of ratios between *Firmicutes:Bacteroidetes* was observed (Fig. 24). Mann-Whitney U-tests revealed no significant differences in the two largest bacterial phyla in the gut, *Firmicutes* (p = 0.667) and *Bacteroidetes* (p = 0.914) when the relative abundances found in children from obese versus non-obese mothers were compared. When analyses were conducted separately among higher versus lower income groups, no significant effects of maternal obesity on the child gut microbiota at the phyla level were observed that withstood multiple test correction.

Next, genera-level abundances were examined. The Mann-Whitney U test was used due to the skewed distributions of the population abundances. Benjamini-Hochberg tests for multiple comparisons were used, with a q-value set at 0.10. In the overall sample, there were limited significant differences between children born to obese versus non-obese mothers after multiple test correction (Table 18). However, examination of interactions between SES and obesity status revealed multiple associations. Among children of high-income mothers, abundances of the genera *Parabacteroides* (p = 0.008,

q < 0.10), *Eubacterium* ($p = 0.021$, q < 0.10), *Blautia* ($p = 0.025$, q < 0.10), and *Oscillibacter* ($p = 0.011$, q < 0.010), as well as an undefined genus in *Bacteroidales* ($p = 0.005$, q < 0.10) differed significantly based on maternal obesity status (Table 19). In contrast, after correction for multiple tests, there were no significant differences between children born to obese versus non-obese mothers in the low-income group (Table 20).

Other behavioral and environmental influences upon the microbiota- In addition to influence by exposure to maternal bacteria, mothers could affect the toddler microbiota via control of the toddler diet, as diet is a primary factor in determining population abundances of the GI microbiota. In chi-square analyses, we found no significant differences in dietary patterns in children of obese versus non-obese women (Table 17). Specifically, children did not differ significantly in duration of breastfeeding, age at which grains/cereals or other foods were introduced, or the frequency of consuming meat or vegetables (*p's* ≥ 0.15). Children of obese versus non-obese mothers also did not differ in the extent to which they had been exposed to antibiotic medications (during pregnancy, breastfeeding, or directly during childhood) or probiotics in food or supplement form (*p's* ≥ .34).

Because significant results in this study were found predominately in high-income dyads, we further examined potential dietary differences in children born to obese versus non-obese mothers in the high income group. Results also showed no differences in breastfeeding duration, age at which grains/cereals or other foods were introduced, or the frequency of consuming meat or vegetables among children of obese versus non-obese mothers in this group (*p's* ≥ 0.13).

We also examined the potential role of three key environmental factors that may covary with maternal obesity status and SES: route of delivery (vaginal versus C-section), duration of breastfeeding, and antibiotic exposure in mothers and children. Analyses showed no significant associations between these factors and the community structure of the child gut microbiota (Table 21), and no clustering observed using PCoA (Fig. 25). Also, as described earlier, these exposures did not differ based on maternal obesity status (Table 17). Further analyses among the high-income group also showed that route of delivery, maternal antibiotic use in pregnancy/breastfeeding (combined due to low occurrence), and antibiotic exposure in the child did not differ significantly based on maternal obesity status ($ps \geq .12$).

Predictive metagenome- The predictive metagenome program, PiCrust, was used to examine if maternal obesity and other factors (duration of breastfeeding, maternal use of antibiotics during breastfeeding or pregnancy, child use of antibiotics, and birth route) were associated with altered functioning of the microbial groups. Abundances of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologies, or KOs, were highly similar across children (Fig. 26). Deeper analysis of the KOs revealed that carbohydrate metabolism was significantly lower in children born to obese mothers. However, these differences in KO abundances did not pass correction for multiple tests, due to low effect sizes (Table 22). Likewise, when high and low-income participants were examined separately, maternal obesity was not associated with any significant differences in functional group abundance after multiple test correction (Table 23), nor were differences

detected in functional groups based upon breastfeeding duration, antibiotic use by mother or child, and birth route (Tables 24-26).

Discussion

Children born to obese mothers are at greater risk for obesity in adulthood compared to children of non-obese mothers, with odds ratios ranging from 1.23 to 6.12 depending on sex and age (206, 362, 363). Factors including diet and genetics contribute to, but do not fully explain this increased risk (364). The gut microbiota may play a clinically meaningful role; bacteria that affect metabolic processes are transmitted from the mother to the infant during birth and subsequently through physical contact and, in many cases, breastfeeding (204, 208, 356). Obese adults have different microbial community profiles in the gut (17, 353, 354), and studies show that transplanting microbiota from obese mice into germ-free mice can lead to increased body fat (275), illustrating that altered profiles of microbiota can be both obesogenic and transmittable. However, the extent to which the microbiota may contribute to the intergenerational transmission of obesity in humans is not known.

This study provides novel evidence that maternal obesity is related to measurable differences in the composition of the gut microbiota in offspring, as reflected by measures of both alpha (Shannon Diversity Index, equitability, unique OTUs) and beta diversity (per adonis). Despite the lack of group clustering on a PCoA, differences in beta diversity were explained using permdisp, which indicated increased homogeneity among the microbiotas of the obese-group and increased dispersion among the non-obese group.

Our results suggest that the relationship between maternal obesity and the composition of the child gut microbiota remain after accounting for paternal BMI and indicators of child body composition, supporting an exposure rather than purely genetic pathway. This is consistent with epidemiological studies showing that maternal BMI is more strongly associated with obesity in offspring than is paternal BMI (349, 350). In addition, in metagenome function analyses using PiCRUST, lower abundances of communities related to carbohydrate metabolism were observed in children born to obese versus non-obese mothers, although this result did not remain significant after statistical correction for multiple comparisons.

Importantly, effects of maternal obesity on the composition of the gut microbiota in offspring were stronger and more consistent among those born to mothers of higher socioeconomic status (SES) as defined by income and/or education. Specifically, when higher and lower income groups were examined separately, differences in beta diversity in relation to maternal obesity (per adonis/permdisp and PCoA) were evident only in the higher income group, as were multiple measures of alpha diversity. Less dispersion of profiles among children born to obese compared to non-obese mothers, particularly among those of high SES, indicates that these children are developing microbial profiles typified by greater homogeneity of community structures. Additional studies are needed to determine if similar effects are present in older children, adolescents, and adults.

Also demonstrating effects of socioeconomic status, among the high-income group only, children born to obese versus non-obese mothers had greater abundances of *Parabacteroides* spp., *Oscillibacter* spp., and an unclassified genus of the order

Bacteroidales as well as lower *Blautia* spp., and *Eubacterium* spp. Of note, differences in *Eubacteriaceae, Oscillibacter* and *Blautia* have been found in prior studies of diet and obesity (365-367), but the clinical relevance of these bacterial types in affecting obesity risk is not fully understood. Also, when PiCRUST was used to examine metagenome function based on obesity status in the higher income group only, no significant differences were found.

The mechanisms underlying the interaction between maternal obesity and SES in predicting the composition of the child gut microbiota are not known. Obesity is a health condition with multifactorial origins, both genetic and behavioral (i.e., diet, physical activity). Research on the true interaction between social-environmental and genetic factors (i.e., moderating effects) is sparse. However, among obese adults, the relative contribution of genetic versus behavioral factors may differ in those from higher versus lower socioeconomic backgrounds (368). Relatedly, the extent to which maternal obesity confers measureable changes to the gut microbiota of offspring may differ based on the etiology of maternal obesity.

Our finding of higher SDI among children of obese versus non-obese mothers contrasts prior research linking obesity with lower alpha diversity (202, 353). However, previous studies have focused on adults or used mouse models with experimentally-induced obesity. This is one of the first studies to ascertain SDI among toddlers as a function of maternal obesity. Higher SDI in children born to obese mothers may reflect interactions between their unique beta-diversity community profile and age-related effects, possibly down-regulated immune surveillance or reduced GI motility, which

could result in greater growth and diversification of microbial groups. Due to the novelty of the study, further investigation is required.

In early life, parents largely control the diet of the child, and tend to offer solid foods that reflect their own adult diets (369). Diet can substantially affect the composition of the gut microbiota (264, 365, 370). In our sample, we found no differences in the children from obese and non-obese mothers in terms of breastfeeding behavior, age at which solid foods were introduced, or the current frequency of consumption of meat, vegetables, and cereals/grains regardless of maternal SES. This suggests that diet did not explain the observed differences in the children's gut microbiota related to maternal obesity and SES. However, this study did not include detailed food diaries that would capture the volume and quality of foods (e.g., high versus low fat meats) consumed. Thus, the possibility remains that differences in feeding behaviors contribute to the observed association with maternal obesity and/or the interaction between maternal obesity and SES.

In addition, other key factors that can affect the gut microbiota including antibiotic exposure, breastfeeding, and route of delivery were examined, but did not account for the observed effects of maternal obesity, or the interaction between maternal obesity and SES. After correction for multiple comparisons, there were not significant differences in individual KOs based upon these factors. Moreover, as described, these factors did not differ significantly based on obesity status, regardless of maternal SES. However, the role of such factors requires further attention.

If continued research supports the notion that obese mothers may pass obesogenic microbiota to their infants, interventions could target manipulation of maternal vaginal and gut microbiota. Prior research has shown that administration of antibiotics during the delivery process reduces vaginal *Lactobacillus* spp. levels in the mother and corresponds to lower levels of lactobacilli in oral samples from newborns (205). In this case, these effects are potentially detrimental, as early colonization with *Lactobacillus* spp. may have a preventative role in the development of allergic diseases. However, such studies demonstrate that interventions that affect population abundances in the mother can have downstream effects in the neonate's own microbial structure.

A strength of this study is a focus on children between 18 and 27 months of age. Prior studies have shown that infants of obese mothers have differences in the gut microbiota, specifically the numbers of *Bacteroides* spp. and *Staphylococcus* spp. in the stool (357). However, the microbiota are characterized by a lack of consistency and high volatility during the first year of life (203). These profiles generally stabilize and increase in diversity, more closely resembling adult profiles, when the range of dietary exposures for the child expands (95, 371). Thus, the current data extend prior findings and support the hypothesis that early life exposures may have lasting effects on the gut microbiota. However, considerable variability of the major phyla is still a hallmark of the 18-27 month old child microbiota. In future studies, long-term and longitudinal examination through early childhood and adolescence would be highly valuable in explicating the extent to which observed effects persist and ultimately influence weight.

This study utilized deep pyrosequencing technology which adds upon prior studies by allowing for whole bacterial community profiling of the toddler microbiota. Utilization of this technology allowed increased sensitivity in detecting differences in the gastrointestinal microbiota community structure between children born to obese and non-obese mothers. PiCRUST was used for prediction of metagenome function based upon 16s rRNA abundances. As reviewed, some effects in relation to maternal obesity were suggested, but these did not remain significant after correction for multiple tests. Unique microbial profiles would be expected to result in differences in microbiota function. True metagenomic shotgun sequencing will likely provide greater power to examine effects of factors such as maternal obesity on the function of the microbiota in children.

In this study, parental BMI as well as children's body composition indicators (height and weight percentile) were collected via maternal report rather than direct measurement. Current maternal BMI was not the focus because 1) maternal BMI may have changed considerably since the target pregnancy (e.g., due to weight retention after the target pregnancy or subsequent weight gain) and 2) women were of childbearing age, thus a meaningful proportion were pregnant with another child at the time of data collection. Prior studies suggest that among women of reproductive age, BMI classified by self-reported height and weight is generally accurate, resulting in correct categorization of 84%-87% and an underestimate in BMI of 0.8 kg/m^2 (372, 373). Because BMI by self-report tends to be slightly lower than true BMI, effects of maternal obesity on outcomes of interest may be underestimated in the current study. In addition, this study did not include collection of maternal specimens, such as vaginal or fecal

samples, which would permit profiling of maternal microbial communities. This is clearly a critical next step in establishing a direct link from maternal to child microbial profiles. In conclusion, obesity is a worldwide public health issue. Identification of modifiable early life antecedents is key to addressing this disease process. A rapidly growing body of literature indicates that the gut microbiota plays a critical role in the development of obesity. Adding to this literature, the current study provides novel evidence that maternal obesity is associated with different microbial profiles in offspring 18-27 months of age. The potential role of the gut microbiota in this intergenerational transmission of obesity risk warrants further attention. In particular, the stability of such effects into later childhood and adolescence, the clinical relevance of abundances of specific bacteria in conferring risk for obesity, and the ultimate impact of early life microbial profiles on long-term weight trajectory remains to be explicated.

	Total (n=77)	Obese (n=26)	Non-Obese (n=51)	Obese vs. Non-Obese
Maternal BMI [Mean (SD)]	26.86 (6.83)	35.13 (4.48)	22.65 (2.85)	t(75) = 15.3, *p* =.000*
Paternal BMI [Mean (SD)]	28.34(5.47)	31.2 (5.98)	26.89 (4.92)	t(75) = 3.49, *p* =.001*
Maternal Age [Mean (SD)]	31.10 (5.43)	31.96 (6.02)	30.67 (5.11)	t(75) = 0.99, *p* = .33
Child Sex [n (%)]				$X^2(1)$= 0.79, *p* = .37
Male	41 (53.2%)	12 (46.2%)	29 (56.9%)	
Female	36 (46.8%)	14 (53.8%)	22 (43.1%)	
Maternal Race				$X^2(1) = 0.47$, *p* = .47[@]
White	67 (87.0%)	21 (80.8%)	46 (90.2%)	
Black/African-American	7 (9.1%)	5 (19.2%)	2 (3.9%)	
Asian	3 (3.9%)	0 (0%)	3 (5.9%)	
Marital Status [n (%)]				$X^2(1) = 3.54$, *p* = .06
Married	67 (87.0%)	20 (76.9%)	47 (92.2%)	
Unmarried	10 (13%)	6 (23.1%)	4 (7.8%)	
Maternal Education [n (%)]				$X^2(2) = 10.67^{\ddagger}$, *p* = .005*
High school graduate or less	19 (24.7%)	12 (46.2%)	7 (13.7%)	
College graduate (2 or 4 yr)	26 (33.8%)	8 (30.8%)	18 (35.3%)	
Some graduate school or higher	32 (41.6%)	6 (23.1%)	26 (51.0%)	
Paternal Education [n (%)]				$X^2(2) = 8.51$, *p* = .014*
High school graduate or less	29 (37.6%)	12 (46.2%)	17 (33.3%)	
College graduate (2 or 4 yr)	30 (39.0%)	12 (46.2%)	18 (35.3%)	
Some graduate school or higher	18 (23.3%)	2 (7.7%)	16 (31.4%)	
Income [n (%)]				$X^2(3) = 1.92$, *p* = .59
< $ 30,000	15 (19.5%)	7 (26.9%)	8 (15.7%)	
$30,000-49,999	15 (19.5%)	5 (19.2%)	10 (19.6%)	
$50,000-99,999	30 (39.0%)	10 (38.4%)	20 (39.2%)	
≥ $100,000	17 (22.0%)	4 (15.4%)	13 (25.5%)	

Table 16: Demographic Characteristics

	Total (n=77)	Obese (n=26)	Non-Obese (n=51)	Obese vs. Non-Obese
Route of delivery [n (%)]				X2(1)= 0.17, p = .68
C-Section	33 (42.9%)	12 (46.2%)	21 (41.2%)	
Vaginal	44 (57.1%)	14 (53.8%)	30 (58.8%)	
Breastfeeding duration [n (%)]				$X^2(2) = 3.84$, $p = .147^{\#}$
Never	5 (6.5%)	4 (15.4%)	1 (2%)	
< 3 months	7 (9.1%)	3 (11.5%)	4 (7.8%)	
3 to 11 months	38 (49.4%)	11 (42.3%)	27 (52.9%)	
≥ 12 months	27 (35.1%)	8 (30.8%)	19 (37.2%)	
Grains/Cereals introduced [n (%)]				$X^2(1) = 0.06$, $p = .812$^
≤ 4 months	30 (39.0%)	12 (46.2%)	18 (35.3%)	
5 - 6 months	41 (53.2%)	11 (42.3%)	30 (58.8%)	
≥ 7 months	6 (7.8%)	3 (11.5%)	3 (5.9%)	
Vegetables, fruits, and/or meats introduced [n (%)]				$X^2(2) = 0.17$, $p = .92$
≤ 4 months	17 (22.1%)	6 (23.1%)	11 (21.6%)	
5 - 6 months	38 (49.4%)	12 (46.2%)	26 (51.0%)	
≥ 7 months	22 (28.6%)	8 (30.8%)	14 (27.5%)	
Meat frequency [n (%)]				$X^2(2) = 2.24$, $p = .33$
Less than once per day	25 (32.5%)	6 (23.1%)	19 (37.3%)	
Once per day	27 (35.1%)	9 (34.6%)	18 (35.3%)	
More than once per day	25 (32.5%)	11 (42.3%)	32 (27.5%)	
Vegetable frequency [n (%)]				$X^2(2) = 0.30$, $p = .86$
Less than once per day	17 (22.1%)	5 (19.2%)	12 (23.5%)	
Once per day	24 (31.2%)	9 (34.6%)	15 (29.4%)	
More than once per day	36 (46.8%)	12 (46.2%)	24 (47.1%)	
Antibiotic use in pregnancy [n (%)]				X2(1) = 1.07, $p = .30$
No	64 (83.1%)	20 (76.9%)	44 (86.3%)	
Yes	13 (16.9%)	6 (23.1%)	7 (13.7%)	
Antibiotic use while breastfeeding [n (%)]				X2(1) = .056, $p = .81$
No	69 (89.6%)	23 (88.5%)	46 (90.2%)	
Yes	8 (10.4%)	3 (11.5%)	5 (9.8%)	
Antibiotic exposure in child [n (%)]				X2(2) = 2.30, $p = .317$
None	23 (29.9%)	5 (19.2%)	18 (35.3%)	
One or two courses	29 (37.7%)	12 (46.2%)	17 (33.3%)	
More than two courses	25 (32.4%)	9 (34.6%)	16 (31.4%)	

Table 17: Health/Behavioral Characteristics

	Normal Weight	Obese	
Bacteroides spp.	36.24 ± 3.76	27.35 ± 4.33	
Lachnospiraceae; Other	19.31 ± 2.89	13.37 ± 2.44	
Dialister spp.	5.51 ± 1.67	7.71 ± 2.05	
Faecalibacterium spp.	5.60 ± 1.46	6.96 ± 2.24	*
Prevotella spp.	2.85 ± 1.44	9.46 ± 3.94	
Unclassified Clostridiales	5.22 ± 0.60	4.44 ± 0.73	
Roseburia spp.	2.97 ± 0.41	4.52 ± 1.21	
Veillonella spp.	3.31 ± 0.93	2.25 ± 1.62	
Ruminococcaceae; Other	2.00 ± 0.61	2.42 ± 0.91	
Parabacteroides spp.	1.78 ± 0.61	2.04 ± 0.57	
Escherichia/Shigella spp.	1.54 ± 0.79	1.09 ± 0.46	
Alistepes spp.	1.11 ± 0.34	1.88 ± 0.92	
Ruminococcus spp.	1.70 ± 0.86	0.61 ± 0.30	
Unclassified Bacteria	1.09 ± 0.09	1.44 ± 0.17	
Akkermansia spp.	0.60 ± 0.27	1.88 ± 1.54	
Klebsiella spp.	0.03 ± 0.02	2.81 ± 2.65	
Unclassified Bacteroidales	0.67 ± 0.09	0.82 ± 0.11	
Eubacterium spp.	0.80 ± 0.26	0.34 ± 0.13	**
Oscillibacter spp.	0.43 ± 0.09	1.00 ± 0.35	
Coprobacillus spp.	0.28 ± 0.13	1.00 ± 0.81	

Data are the mean relative abundance ± standard error.
** p < .05 vs. Non-Obese, passed correction for multiple comparisions
* p < .05 vs. Non-Obese;

Table 18: Top 20 Most Abundant Genera

	Normal Weight	Obese	
Bacteroides spp.	30.65 ± 4.59	31.24 ± 5.86	
Lachnospiraceae; Other	20.94 ± 3.99	11.13 ± 2.71	
Dialister spp.	6.85 ± 2.50	5.61 ± 2.66	
Faecalibacterium spp.	5.83 ± 1.86	5.92 ± 1.64	
Prevotella spp.	3.06 ± 1.90	11.79 ± 6.17	
Unclassified *Clostridiales*	5.85 ± 0.86	3.87 ± 0.66	
Roseburia spp.	3.68 ± 0.57	6.09 ± 2.12	
Veillonella spp.	4.55 ± 1.36	3.41 ± 3.00	
Parabacteroides spp.	1.66 ± 0.91	3.03 ± 0.94	**
Ruminococcaceae; Other	1.28 ± 0.25	3.10 ± 1.67	
Escherichia/Shigella spp.	2.24 ± 1.21	0.66 ± 0.34	
Alistipes spp.	0.93 ± 0.45	2.14 ± 1.52	
Ruminococcus spp.	1.40 ± 1.02	0.96 ± 0.55	
Unclassified *Bacteria*	1.07 ± 0.12	1.36 ± 0.19	
Eubacterium spp.	0.92 ± 0.38	0.41 ± 0.23	**
Unclassified *Bacteroidales*	0.53 ± 0.08	1.01 ± 0.14	**
Akkermansia spp.	0.70 ± 0.41	0.45 ± 0.26	
Oscillibacter spp.	0.28 ± 0.08	1.18 ± 0.60	**
Blautia spp.	0.53 ± 0.23	0.32 ± 0.25	**
Unclassified *Peptostreptococcaceae*	0.53 ± 0.30	0.32 ± 0.22	

Data are the mean relative abundance ± standard error.
** p < .05 vs. Non-Obese, passed correction for multiple comparisions

Table 19: Top 20 Most Abundant Genera Among High-Income Subjects

	Normal Weight	Obese
Bacteroides spp.	46.49 ± 5.97	22.81 ± 6.47 *
Lachnospiraceae; Other	16.33 ± 3.74	15.99 ± 4.25
Faecalibacterium spp.	5.19 ± 2.42	8.19 ± 4.58
Dialister spp.	3.05 ± 1.06	10.16 ± 3.14
Unclassified *Clostridiales*	4.06 ± 0.57	5.12 ± 1.39
Prevotella spp.	2.46 ± 2.19	6.76 ± 4.79
Ruminococcacae; Other	3.34 ± 1.64	1.64 ± 0.45
Klebsiella spp.	0.01 ± 0.00	5.76 ± 5.75
Roseburia spp.	1.65 ± 0.35	2.69 ± 0.63
Akkermansia spp.	0.42 ± 0.18	3.55 ± 3.33
Parabacteroides spp.	1.99 ± 0.49	0.87 ± 0.34
Alistipes spp.	1.42 ± 0.52	1.58 ± 1.00
Ruminococcus spp.	2.23 ± 1.61	0.20 ± 0.08
Unclassified *Bacteria*	1.13 ± 0.13	1.55 ± 0.30
Veillonella spp.	1.03 ± 0.50	0.89 ± 0.41
Coprobacillus spp.	0.11 ± 0.05	1.88 ± 1.75
Escherichia/Shigella spp.	0.27 ± 0.14	1.60 ± 0.92
Unclassified Bacteroidales	0.92 ± 0.19	0.59 ± 0.15
Oscillibacter spp.	0.72 ± 0.18	0.78 ± 0.34
Megasphaera spp.	1.06 ± 0.85	0.02 ± 0.02

Data are the mean relative abundance ± standard error.
** p < .05 vs. Non-Obese, passed correction for multiple comparisions
* p < .05 vs. Non-Obese; did not pass correction for multiple comparisons

Table 20: Top 20 Most Abundant Genera Among Low-Income Subjects

	P-value	R^2
Birth route (vaginal vs. caesarean)	0.661	0.0126
Breastfeeding Duration (>12 months vs. <12 months)	0.714	0.0125
Mother took antibiotics during pregnancy (yes vs. no)	0.994	0.0109
Mother took antibiotics while breastfeeding (yes vs. no)	0.363	0.0134
Child has taken antibiotics (0 courses vs. 1-2 courses vs. >2 courses)	0.926	0.0116

Data are the calculated p-value and R^2 (effect size) as measured with adonis.

Table 21: Potential Impacts Upon the Offspring Microbiota

	Non-Obese	Obese
Membrane Transport	11.33 ± 2.23	11.16 ± 2.23
Carbohydrate Metabolism	11.13 ± 0.78	10.70 ± 0.83
Amino Acid Metabolism	9.73 ± 0.30	9.71 ± 0.29
Replication and Repair	8.71 ± 0.52	8.84 ± 0.67
Energy Metabolism	5.91 ± 0.32	5.88 ± 0.35
Translation	5.42 ± 0.47	5.57 ± 0.51
Metabolism of Cofactors and Vitamins	4.51 ± 0.32	4.57 ± 0.33
Cellular Processes and Signaling	4.42 ± 0.30	4.43 ± 0.24
Nucleotide Metabolism	4.02 ± 0.26	4.11 ± 0.35
Lipid Metabolism	2.93 ± 0.18	2.93 ± 0.21
Glycan Biosynthesis and Metabolism	2.89 ± 0.82	2.93 ± 0.71
Transcription	2.79 ± 0.27	2.74 ± 0.23
Genetic Information Processing	2.55 ± 0.19	2.62 ± 0.16
Folding, Sorting and Degradation	2.48 ± 0.18	2.52 ± 0.20
Metabolism	2.53 ± 0.17	2.47 ± 0.15
Enzyme Families	2.18 ± 0.08	2.21 ± 0.12
Cell Motility	1.77 ± 0.79	1.81 ± 0.75
Metabolism of Terpenoids and Polyketides	1.63 ± 0.11	1.66 ± 0.15
Metabolism of Other Amino Acids	1.53 ± 0.14	1.56 ± 0.12
Xenobiotics Biodegradation and Metabolism	1.50 ± 0.15	1.54 ± 0.31
Signal Transduction	1.49 ± 0.21	1.50 ± 0.26
Biosynthesis of Other Secondary Metabolites	1.01 ± 0.16	1.00 ± 0.13
Cell Growth and Death	0.50 ± 0.04	0.51 ± 0.05
Transport and Catabolism	0.40 ± 0.16	0.39 ± 0.12
Signaling Molecules and Interaction	0.20 ± 0.05	0.19 ± 0.04
Environmental Adaptation	0.16 ± 0.03	0.16 ± 0.02

Data are KEGG Orthologue mean relative frequency (in %) ± standard deviation

Table 22: KEGG Orthologues, Obese vs. Non Obese

	Non-Obese	Obese
Membrane Transport	11.22 ± 2.25	11.25 ± 2.30
Carbohydrate Metabolism	11.26 ± 0.66	11.03 ± 0.69
Amino Acid Metabolism	9.73 ± 0.34	9.74 ± 0.29
Replication and Repair	8.68 ± 0.52	8.80 ± 0.52
Energy Metabolism	5.97 ± 0.27	5.96 ± 0.25
Translation	5.40 ± 0.43	5.50 ± 0.44
Metabolism of Cofactors and Vitamins	4.48 ± 0.27	4.52 ± 0.33
Cellular Processes and Signaling	4.44 ± 0.28	4.42 ± 0.22
Nucleotide Metabolism	4.01 ± 0.23	4.06 ± 0.26
Lipid Metabolism	2.94 ± 0.18	2.91 ± 0.15
Glycan Biosynthesis and Metabolism	2.87 ± 0.81	2.83 ± 0.73
Transcription	2.83 ± 0.25	2.80 ± 0.24
Genetic Information Processing	2.52 ± 0.13	2.57 ± 0.12
Metabolism	2.54 ± 0.18	2.49 ± 0.15
Folding, Sorting and Degradation	2.51 ± 0.19	2.40 ± 0.19
Enzyme Families	2.20 ± 0.08	2.21 ± 0.06
Cell Motility	1.70 ± 0.74	1.74 ± 0.70
Metabolism of Terpenoids and Polyketides	1.64 ± 0.12	1.63 ± 0.12
Metabolism of Other Amino Acids	1.54 ± 0.14	1.54 ± 0.11
Xenobiotics Biodegradation and Metabolism	1.50 ± 0.15	1.49 ± 0.11
Signal Transduction	1.49 ± 0.20	1.48 ± 0.19
Biosynthesis of Other Secondary Metabolites	1.03 ± 0.16	1.03 ± 0.12
Cell Growth and Death	0.50 ± 0.04	0.51 ± 0.04
Transport and Catabolism	0.40 ± 0.16	0.39 ± 0.14
Signaling Molecules and Interaction	0.20 ± 0.05	0.19 ± 0.05
Environmental Adaptation	0.16 ± 0.02	0.16 ± 0.02

Data are KEGG Orthologue mean relative frequency (in %) ± standard deviation

Table 23: KEGG Orthologues among High-Income Subjects

	Less than 12 Months Breastfeeding	12 Months or Greater Breastfeeding
Carbohydrate Metabolism	10.96 ± 0.86	11.22 ± 0.71
Membrane Transport	11.20 ± 2.15	10.72 ± 2.25
Amino Acid Metabolism	9.72 ± 0.30	9.79 ± 0.25
Replication and Repair	8.76 ± 0.60	8.83 ± 0.49
Energy Metabolism	5.87 ± 0.33	6.08 ± 0.26
Translation	5.47 ± 0.50	5.43 ± 0.46
Metabolism of Cofactors and Vitamins	4.53 ± 0.34	4.58 ± 0.29
Cellular Processes and Signaling	4.45 ± 0.28	4.42 ± 0.28
Nucleotide Metabolism	4.05 ± 0.30	4.09 ± 0.27
Glycan Biosynthesis and Metabolism	2.97 ± 0.75	2.90 ± 0.86
Lipid Metabolism	2.93 ± 0.20	2.92 ± 0.15
Transcription	2.76 ± 0.25	2.75 ± 0.29
Genetic Information Processing	2.58 ± 0.19	2.50 ± 0.09
Folding, Sorting and Degradation	2.50 ± 0.18	2.57 ± 0.20
Metabolism	2.52 ± 0.16	2.49 ± 0.18
Enzyme Families	2.18 ± 0.10	2.23 ± 0.07
Cell Motility	1.77 ± 0.78	1.79 ± 0.80
Metabolism of Terpenoids and Polyketides	1.64 ± 0.12	1.67 ± 0.12
Metabolism of Other Amino Acids	1.55 ± 0.14	1.54 ± 0.12
Xenobiotics Biodegradation and Metabolism	1.52 ± 0.23	1.47 ± 0.10
Signal Transduction	1.50 ± 0.23	1.46 ± 0.18
Biosynthesis of Other Secondary Metabolites	1.01 ± 0.16	1.01 ± 0.10
Cell Growth and Death	0.51 ± 0.05	0.50 ± 0.04
Transport and Catabolism	0.40 ± 0.15	0.40 ± 0.16
Signaling Molecules and Interaction	0.20 ± 0.05	0.20 ± 0.06
Environmental Adaptation	0.16 ± 0.02	0.16 ± 0.03

Data are KEGG Orthologue mean relative frequency (in %) ± standard deviation

Table 24: KEGG Orthologues, Breastfeeding Duration

	Vaginal	Caesarean
Membrane Transport	11.32 ± 2.26	11.21 ± 2.20
Carbohydrate Metabolism	10.92 ± 0.88	11.08 ± 0.73
Amino Acid Metabolism	9.75 ± 0.26	9.69 ± 0.33
Replication and Repair	8.75 ± 0.57	8.76 ± 0.59
Energy Metabolism	5.89 ± 0.35	5.92 ± 0.30
Translation	5.46 ± 0.49	5.48 ± 0.49
Metabolism of Cofactors and Vitamins	4.56 ± 0.33	4.49 ± 0.32
Cellular Processes and Signaling	4.42 ± 0.29	4.43 ± 0.27
Nucleotide Metabolism	4.04 ± 0.28	4.06 ± 0.31
Lipid Metabolism	2.92 ± 0.20	2.94 ± 0.18
Glycan Biosynthesis and Metabolism	2.91 ± 0.77	2.91 ± 0.80
Transcription	2.76 ± 0.26	2.79 ± 0.24
Genetic Information Processing	2.57 ± 0.19	2.57 ± 0.17
Metabolism	2.50 ± 0.17	2.52 ± 0.15
Folding, Sorting and Degradation	2.50 ± 0.20	2.49 ± 0.17
Enzyme Families	2.18 ± 0.09	2.19 ± 0.11
Cell Motility	1.81 ± 0.79	1.75 ± 0.76
Metabolism of Terpenoids and Polyketides	1.63 ± 0.13	1.64 ± 0.11
Metabolism of Other Amino Acids	1.54 ± 0.13	1.54 ± 0.15
Xenobiotics Biodegradation and Metabolism	1.52 ± 0.25	1.50 ± 0.17
Signal Transduction	1.51 ± 0.23	1.48 ± 0.21
Biosynthesis of Other Secondary Metabolites	1.00 ± 0.15	1.02 ± 0.15
Cell Growth and Death	0.51 ± 0.05	0.50 ± 0.04
Transport and Catabolism	0.39 ± 0.15	0.39 ± 0.14
Signaling Molecules and Interaction	0.19 ± 0.05	0.20 ± 0.04
Environmental Adaptation	0.16 ± 0.02	0.16 ± 0.02

Data are KEGG Orthologue mean relative frequency (in %) ± standard deviation

Table 25: KEGG Orthologues, Birth Route

	Zero Courses	1-2 Courses	Over 2 Courses
Membrane Transport	11.73 ± 2.32	10.30 ± 1.94	11.37 ± 2.17
Carbohydrate Metabolism	10.93 ± 0.83	11.40 ± 0.67	10.88 ± 0.82
Amino Acid Metabolism	9.73 ± 0.27	9.78 ± 0.23	9.70 ± 0.33
Replication and Repair	8.70 ± 0.58	8.81 ± 0.58	8.76 ± 0.58
Energy Metabolism	5.89 ± 0.32	5.99 ± 0.28	5.87 ± 0.35
Translation	5.48 ± 0.47	5.38 ± 0.44	5.50 ± 0.51
Metabolism of Cofactors and Vitamins	4.49 ± 0.33	4.51 ± 0.28	4.56 ± 0.33
Cellular Processes and Signaling	4.37 ± 0.30	4.52 ± 0.27	4.42 ± 0.26
Nucleotide Metabolism	3.99 ± 0.29	4.07 ± 0.28	4.07 ± 0.29
Lipid Metabolism	2.95 ± 0.19	2.98 ± 0.14	2.90 ± 0.20
Glycan Biosynthesis and Metabolism	2.68 ± 0.87	3.11 ± 0.74	2.96 ± 0.71
Transcription	2.82 ± 0.28	2.75 ± 0.21	2.75 ± 0.25
Genetic Information Processing	2.56 ± 0.18	2.53 ± 0.11	2.59 ± 0.20
Metabolism	2.47 ± 0.16	2.55 ± 0.14	2.52 ± 0.17
Folding, Sorting and Degradation	2.47 ± 0.20	2.56 ± 0.17	2.49 ± 0.18
Enzyme Families	2.17 ± 0.09	2.23 ± 0.11	2.18 ± 0.09
Cell Motility	1.93 ± 0.84	1.59 ± 0.58	1.77 ± 0.79
Metabolism of Terpenoids and Polyketides	1.62 ± 0.15	1.69 ± 0.11	1.63 ± 0.12
Metabolism of Other Amino Acids	1.52 ± 0.16	1.56 ± 0.14	1.54 ± 0.12
Xenobiotics Biodegradation and Metabolism	1.55 ± 0.34	1.51 ± 0.14	1.50 ± 0.14
Signal Transduction	1.54 ± 0.25	1.44 ± 0.15	1.49 ± 0.23
Biosynthesis of Other Secondary Metabolites	0.98 ± 0.15	1.07 ± 0.13	1.00 ± 0.15
Cell Growth and Death	0.51 ± 0.05	0.49 ± 0.04	0.51 ± 0.05
Transport and Catabolism	0.36 ± 0.15	0.45 ± 0.14	0.39 ± 0.14
Signaling Molecules and Interaction	0.18 ± 0.06	0.21 ± 0.04	0.20 ± 0.04
Environmental Adaptation	0.17 ± 0.03	0.15 ± 0.02	0.16 ± 0.02

Data are KEGG Orthologue mean relative frequency (in %) ± standard deviation

Table 26: KEGG Orthologues, Antibiotic Course

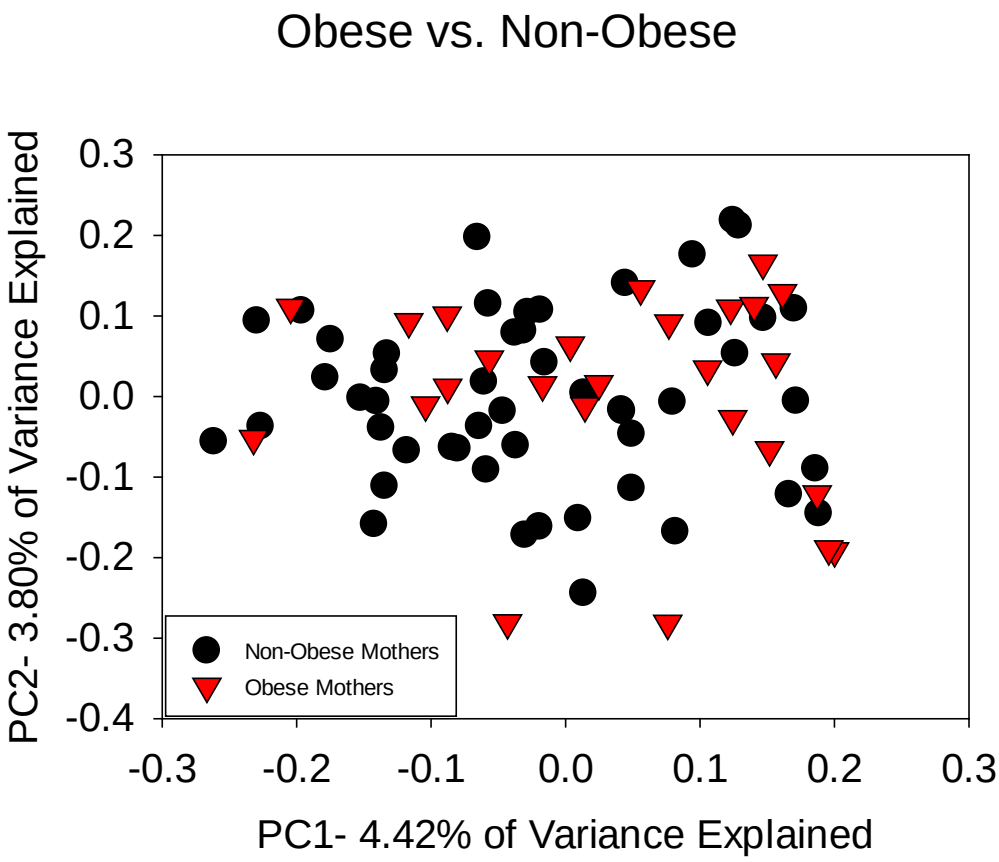


Figure 19: In the overall sample, datapoints did not cluster on a principle coordinate analysis (PCoA) scatter-plot as a function of maternal obesity. The beta-diversity non-parametric statistic adonis showed that children born to obese (n=26) versus non-obese mothers (n=51) had unique microbial profiles (p = 0.044). However, this was due to greater homogeneity among the obese group as measured with permdisp (p = 0.035).

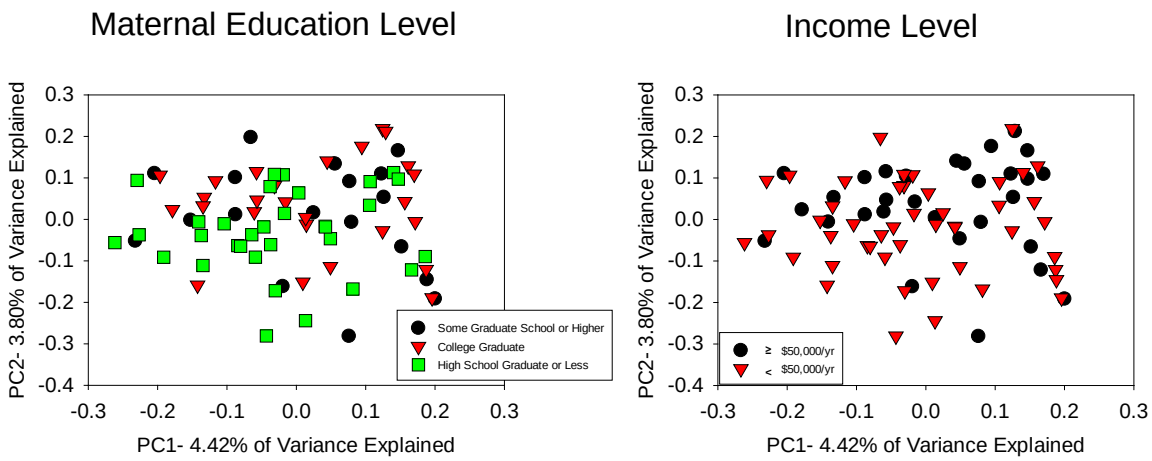


Figure 20: Indicators of socioeconomic status (SES), maternal education (A) and income (B) did not predict differences in the offspring microbiota community structure.

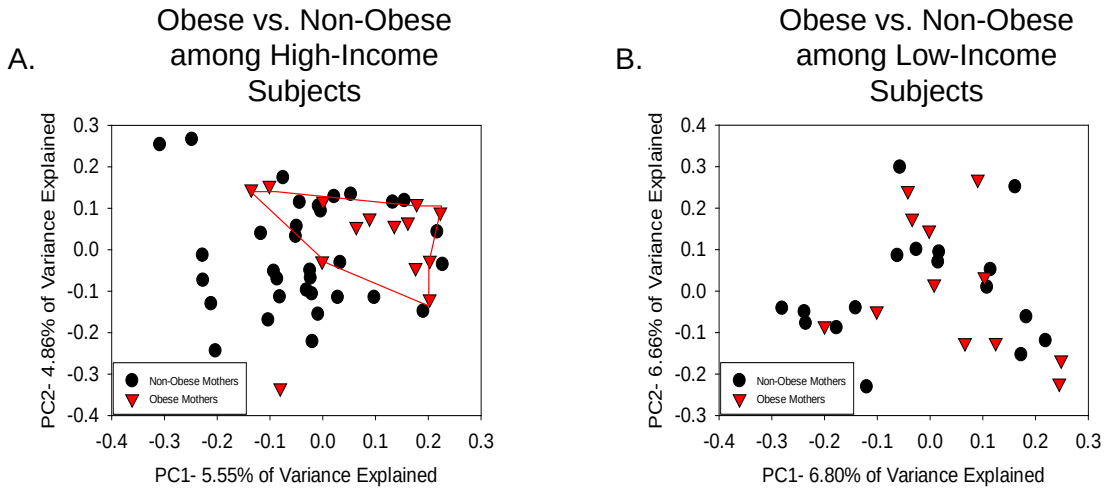


Figure 21: Interactive effects of maternal obesity and socioeconomic status were observed; effects of maternal obesity on the child microbiota were primarily seen among the higher SES group. A) In the higher income group, children born to obese versus non-obese mothers clustered (adonis, p=0.041) and had higher homogeneity (permdisp, p=0.003). B) These effects of maternal obesity were not seen in children in the lower income group.

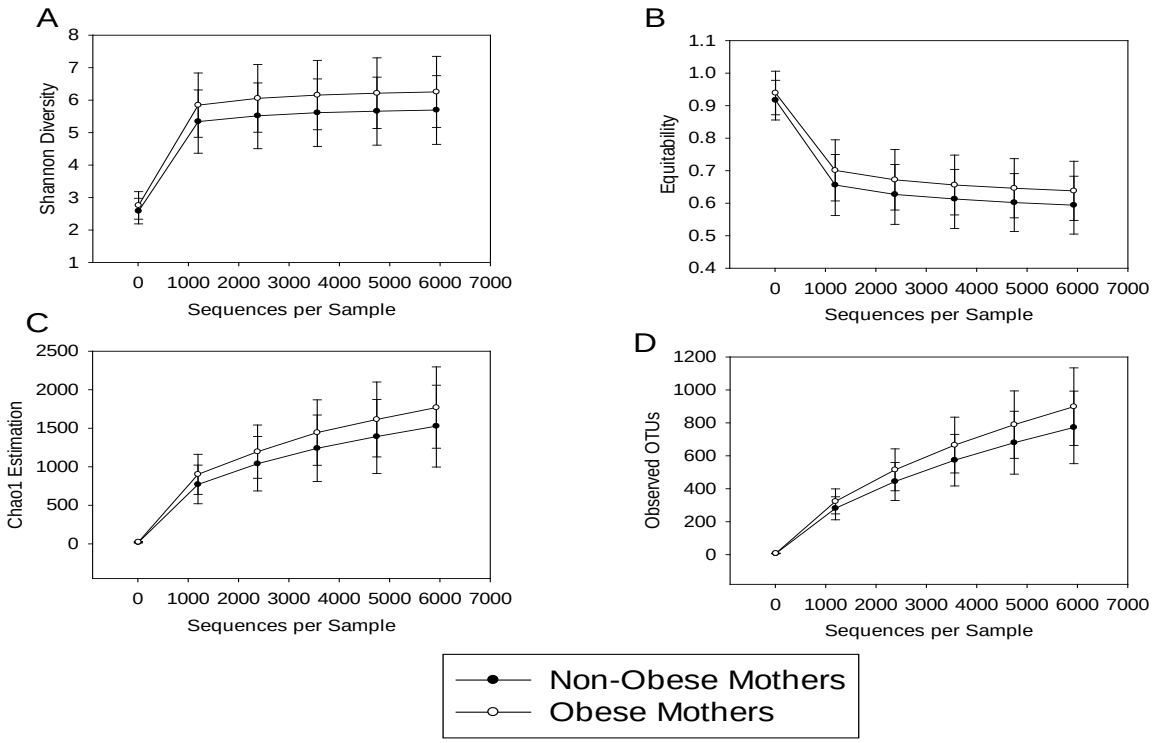


Figure 22: In the overall sample, children born to obese versus non-obese mothers had significantly greater alpha diversity as indicated by A) Shannon Diversity Index (SDI), a measure of overall alpha diversity; B) equitability, a measurement of evenness; C) Chao1, an estimation of richness; and D) the total observed operational taxonomic units (OTUs) (*p*s < .05; Means ± 1 SE).

Figure 23: As with measures of beta diversity, differences in alpha diversity in relation to maternal obesity were seen predominately in the higher SES group. In the higher-income group, children born to obese versus non-obese mothers had significantly higher A) Shannon Diversity Index, B) equitability, C) Chao1 estimation, and D) observed operational taxonomic units (OTUs) (ps ≤ 0.05). In contrast, in the lower-income group, no significant effects of maternal obesity on alpha diversity indicators were observed (E-H)

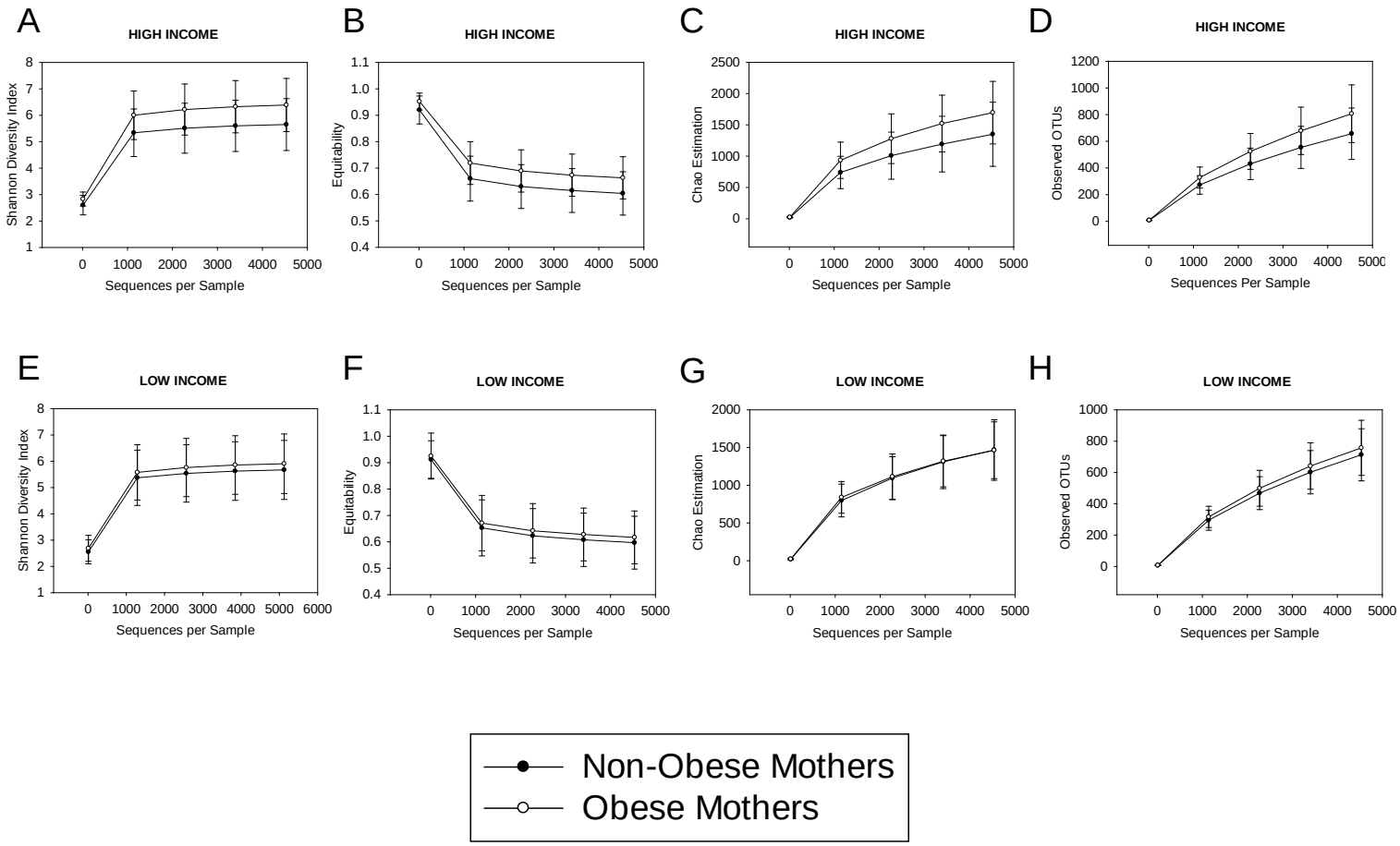

A
HIGH INCOME
Shannon Diversity Index
Sequences per Sample
B
HIGH INCOME
Equitability
Sequences per Sample
C
HIGH INCOME
Chao Estimation
Sequences per Sample
D
HIGH INCOME
Observed OTUs
Sequences Per Sample
E
LOW INCOME
Shannon Diversity Index
Sequences per Sample
F
LOW INCOME
Equitability
Sequences per Sample
G
LOW INCOME
Chao Estimation
Sequences per Sample
H
LOW INCOME
Observed OTUs
Sequences per Sample
Non-Obese Mothers
Obese Mothers

Figure 23.

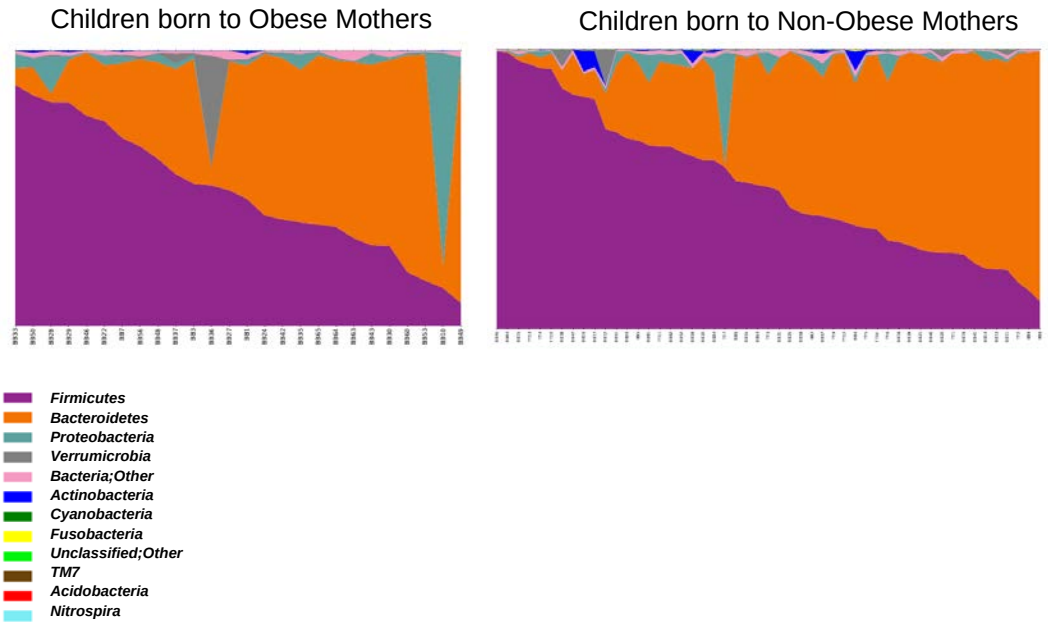
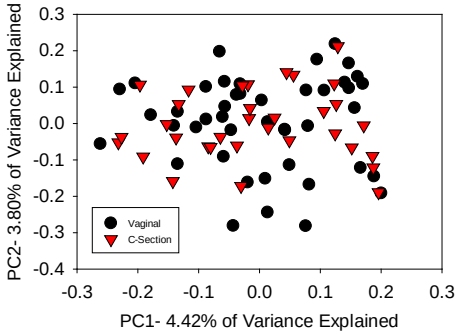


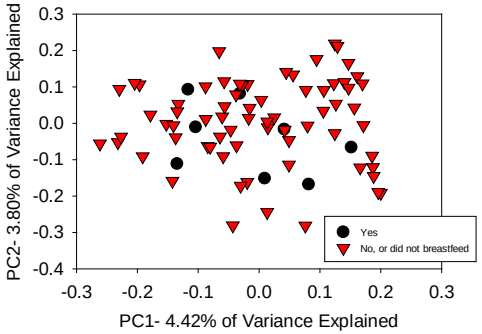
Figure 24: Across individuals, there was considerable variance in the *Firmicutes:Bacteroidetes* ratio, as shown. However, there were no differences between the children born to obese versus non-obese mothers in abundances of the major phyla, *Firmicutes* ($p = 0.667$) and *Bacteroidetes* ($p = 0.914$).

Figure 25: Other key factors which may impact the gut microbiota were not associated with differences in community structure, including (A) birth route (B) antibiotic use by the mother while breastfeeding (C) antibiotic use during pregnancy (D), child antibiotic use or (E) duration of breastfeeding.

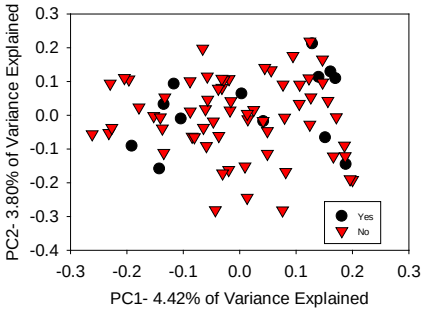
A. Birth Route



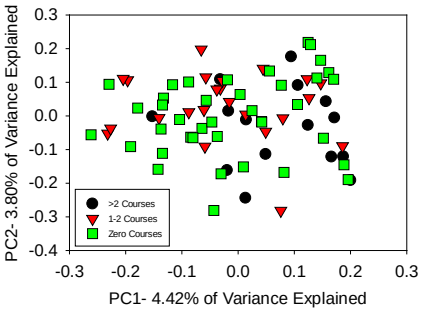
B. Antibiotic use while Breastfeeding



C. Antibiotic use during Pregnancy



D. Child Antibiotic Use



E. Duration of Breastfeeding

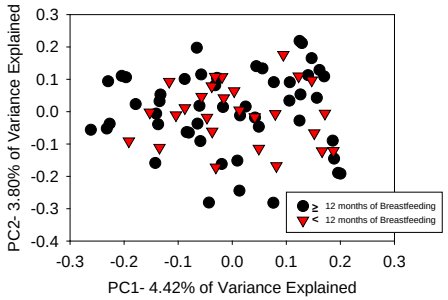


Figure 25.

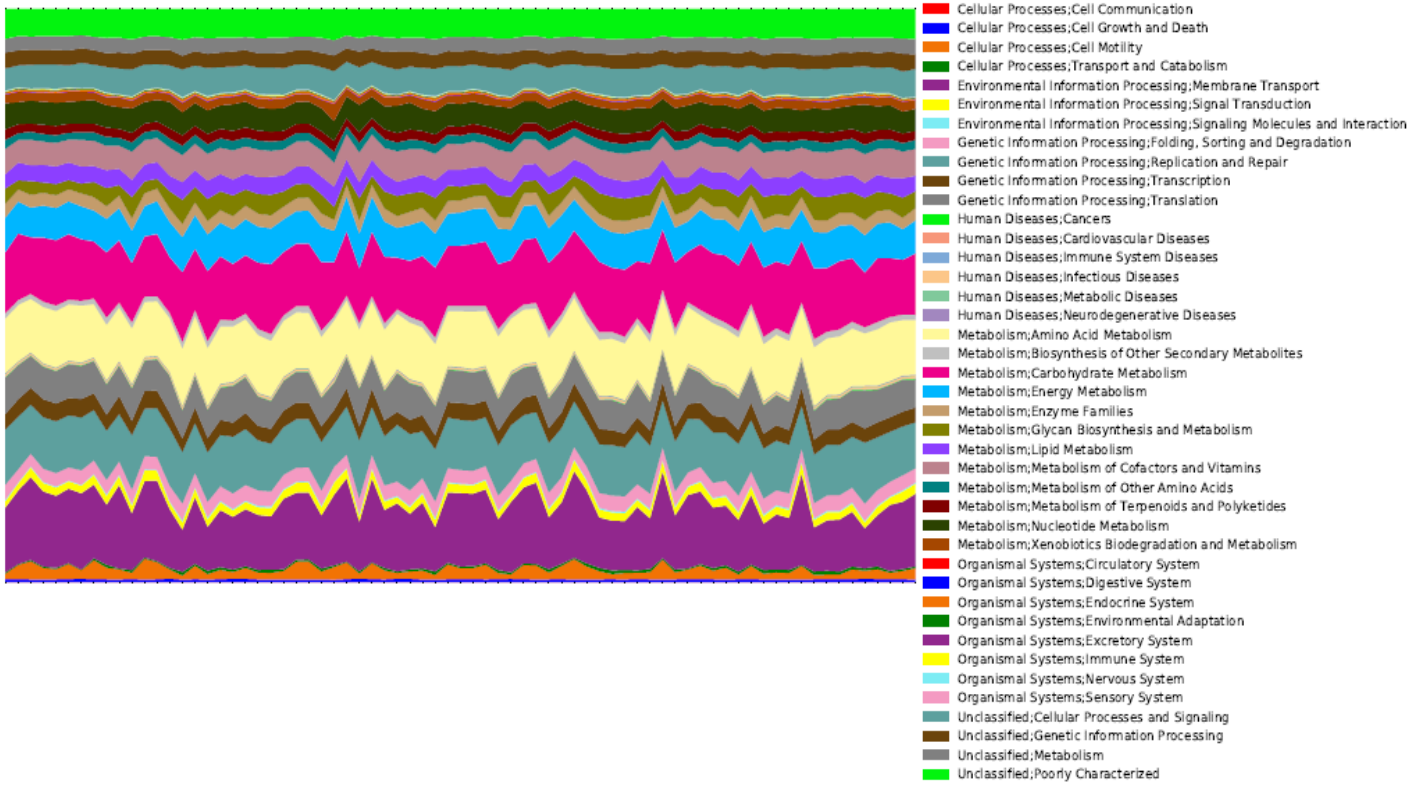


Figure 26: KEGG Orthologues (KOs) were highly similar across individuals. PiCRUST was used to predict metagenomic function of the child microbiota. An area graph produced by QIIME indicated that overall abundances of KOs were similar across samples.

Chapter 7: Discussion

The human gastrointestinal microbiota matures with the host over time, with the community structure stabilizing over the first three years of life (95, 371). Over the lifespan of the human, the microbiota plays a key role in the development and function of a variety of host physiological systems, including immunity, central nervous system activity, and metabolism (374-376). The delicate balance of the microbial populations that compose these GI communities can be disrupted by commonly studied external factors such as antibiotics and diverse dietary compositions, as well as internal factors like host genetic variances and inflammatory responses (5, 11, 216, 218). These profile alterations, termed dysbiosis, have been associated with dysfunction in normal host physiology as well as chronic and infectious diseases (172, 275, 344). Among the lesser known elements that can influence microbiota community structure is psychological stressor exposure (36, 70). Previous studies that reported stressor-induced changes to broadly defined bacterial groups as measured with fecal plating, paved the way for modern techniques that allowed for deeper analysis of overall microbial communities. Yet the effect of psychological stress on GI microbiota as it pertains to duration, severity, and type of stressor has not been elucidated to this point. Thus, in order to better characterize how psychological stress acts upon the microbiota and to what extent, two

unique stressors were applied to mice and deep pyrosequencing was performed for complete microbiota analysis. The effects these stressors have upon GI immunity and the inflammatory response to an enteric infection were also evaluated. Further, this study investigated how dysbiotic profiles come to predominate in a given niche, and sought to identify other methods by which a host might come to acquire such a profile.

Psychological stressor exposure alters the community structure of the mucosal-associated microbiota

Exposure to psychological stress has been previously shown to affect fecal lactic acid bacteria, in numerous types of hosts including humans, non-human primates, and mice (36, 38, 207). The development and use of sequencing technologies that target and quantify the hypervariable regions of the 16S rRNA has given researchers the ability to evaluate how certain variables can affect a microbial community from numerous possible sources, including living hosts and environmental habitats, and at a much greater depth. Relative taxonomic changes can be measured as deep as the species level, in addition to other efficiently performed diversity analyses. A more exact understanding of how different stressors affect the microbiota can inform hypotheses on the mechanism by which psychological stressor exposure can act upon the microbial populations. Despite unique hallmarks that define the two stressors, both restraint, a long-term chronic stressor, and SDR, an acute social stressor, both affected microbiota community structure significantly. Reductions in the relative abundance of the beneficial genus, *Lactobacillus*, were also consistent between both stressors. This effect was focused at the mucosal

niche; no reduction was observed in the luminal compartment when studies in restraint-exposed mice. A single two-hour cycle of SDR was sufficient to alter the mucosal-associated community structure and reduce *Lactobacillus*. The limited exposure to the stressor indicates that the mechanism by which stressor exposure is acting upon the microbiota is activated rapidly, and is central to the location of the microbiota. Furthermore, quantitative real-time PCR demonstrated that this effect compounded with repeated exposures to SDR; the reduction in *Lactobacillus* compared to controls increased with repeated cycles. These data demonstrate universality in the effect that psychological stressor exposure has upon the microbiota. Identifying the mechanisms that overlap in restraint and SDR would be key in understanding how stress disrupts these populations. The effect of SDR also can be observed upon the colonic mucosal microbiota up to 19 days after the cessation of the physical application of the stressor, an intriguing result that has far-reaching consequences pertaining to the long-term physiological effects stress may have on the host. It is possible that physiological and mechanical pathways, such as motility and mucus secretion within the GI tract, are practical targets given the association between stress exposure and alterations in the activity of these functions and the findings reported in this study that highlight specific changes at the mucosal niche, and at an early stage in stressor-exposure (22, 131, 132, 141).

Psychological stressor exposure amplifies the severity of the inflammatory response to challenge by an enteric pathogen

Past studies have shown that exposure to psychological stressors can affect immunological function, including major constituents of both the adaptive and innate arms of the immune response (61, 65, 71). A study by Bailey et al illustrated that restraint stress elevates the gastrointestinal inflammatory response in mice challenged with *Citrobacter rodentium*, but the reason for this was not previously known (77). This dissertation shows, through the use of sterile germ-free mice, that stressor-induced alterations to microbiota community structure within the colon is associated with the inflammatory increases in the mice exposed to restraint. Germ-free mice have been used in similar fashion in previous studies to highlight the involvement of the microbiota in obesity and immune activity (269, 275). The current study adds to those findings and demonstrate how closely microbiota structure is involved in host immune function. Further, infection with *C. rodentium* increasingly affects the colonic mucosal microbiota as infection progresses. The concurrent effects of stress and infection upon the microbiota allow for identification of microbial groups that may be implicated in dysbiotic perturbations in immunity and inflammation. Given the reported correlations between stress and IBD symptom activation in patients with IBD (145), these data can begin to explain how stressful life episodes can aggravate symptomology in IBD.

Probiotic Lactobacillus reuteri*-mediated abrogation of colitic inflammation is not due to normalization of colonic mucosa community structure in stressor-exposed mice*

The association between stressor-induced disruptions to colonic mucosa community structure and heightened inflammatory responses could be related to the

ubiquitous reduction in beneficial microbes that have been observed in multiple stressors and mammals (36, 37, 187). Commensal groups like *Bifidobacterium* and *Lactobacillus* have been implicated in pathogen defense and immunoregulation, and certain species are used in probiotic formulations (175, 176, 181). *L. reuteri* has been shown to increase the diversity of an entire microbial community (197). Probiotic *L. reuteri* gavage can reduce inflammatory marker transcripts and colitic pathology in stressor-exposed *C. rodentium*-infected mice through an as-yet-unknown mechanism. Illumina sequencing of colonic mucosal microbial communities during the course of the *C. rodentium* infection in mice that received either the probiotic or vehicle treatment demonstrated that beyond the first day post infection, probiotic treatment had no effect on the microbiota structure. These data indicate that the immunomodulatory function of *L. reuteri* is not due to a shift in community structure to one associated with health, or a pre-infection state. Thus, future studies should target such possibilities as secreted biomolecules from *L. reuteri* as the primary anti-inflammatory mediator of this particular probiotic.

Variation between sequencing technologies, Illumina and 454

Earlier research in this thesis used the 454 pyrosequencing technology to ascertain the effect of stressor exposure upon the microbiota, while the later timecourse study that evaluated the effect of probiotic *L. reuteri*, the *C. rodentium* infection, and the long-term effects of the stressor of the microbiota utilized Illumina sequencing technology. Major differences exist between the two sequencing technologies, particularly among methodology, sequence depth, and sequence length. Sequence depth is greatly increased

in Illumina sequencing, resulting in greater coverage among all samples. However, there is also a loss in length. While the 454 method can result in sequence lengths of up to 600-700 basepairs, paired-end technology in Illumina gives sequence lengths of 300 bps currently. Though this length can be slightly increased through paired-end joining, there is still a considerable reduction in length. As a result, taxonomic identification is less accurate in Illumina-derived sequences, particularly when classified using publicly-curated databases like GreenGenes, which emphasize common sequences and disregard rare sequences to greater extent. In order to properly analyze the breadth of attained sequences, privately curated databases are an ideal solution. These allow for targeted analysis with increased specificity of taxonomic classification.

Children born to high-income obese mothers are unique in comparison to children born to high-income non-obese mothers

Transmissibility of microbiota function between hosts has been previously proven in studies that use germ-free mice (275), wherein the microbiota from obese donor mice was capable of increasing adiposity and weight gain in acceptor mice. Communities can also be transmitted to conventional hosts. Mice deficient in NOD-2, which have increased levels of colitic inflammation can transmit this heighted inflammatory state by passing the microbiota to other murine hosts when co-housed (5). While a causative link cannot be established, the data demonstrating that children born to obese high-income mothers have a unique microbiota in comparison to children born to non-obese high-income mothers indicates that maternal obesity does have an association with offspring

microbiota. Though further analyses on dietary composition and maternal donor microbiota must be performed, it is evident that obesogenic profiles could be passed or cultivated by obese mothers, be it through direct transmission or environmental pressures. No changes were observed in children born to low-income mothers. Dietary intake greatly differs between SES levels, suggesting that diets common to low SES subjects might alter the microbiota to a greater extent than the effect of maternal obesity, whereas among the high SES subjects, the higher quality foods do not override the maternal effect.

The PCoAs for this human study explain a low level of variance. In many cases for human studies, sample sizes must be large, as considerable variation exists between human samples. Thus, a sample size of 77, as is the case here, results in high variance between subjects and a reduced capacity to explain effects. For example, the first PCoA axis of the overall sample comparing children born to obese and non-obese mothers explained only 4.42% of the total variance. However, this reduced effect size was partially addressed by separating high and low income subjects, which resulted in first PCoA axes of 5.55% and 6.80% respectively, and revealed an interaction between maternal obesity and SES. Future studies should make use of great sample sizes in order to overcome increased variance in microbiota samples.

Conclusions and Future Directions

The microbiota and the human host have developed mutualistically to a point that the health of one greatly impacts that of the other. The abundances of the bacterial groups

compose a microbiota structure that molds host physiology and immunity, and alterations to this structure, be it in early life or as adult, by a variety of factors, can precipitate changes to host health. Some of these factors, like antibiotics and high-fat diets, are better defined and have physical attributes and mechanisms. However, factors like psychological stress or other environmental exposures are not as easily delineated in either their effects upon the microbiota, or how these effects can influence the host downstream. This study characterized how multiple psychological stressors can affect the murine microbiota, and directly associates stressor-induced changes to the mucosal-associated microbiota with changes to the host immune and inflammatory response to an enteric pathogen. This study also establishes an association between human maternal obesity and the offspring microbiota. Understanding how a dysbiotic profile may originate in a human can promote treatments that may preclude the dominance of such profiles via therapeutic means or better target host functions that the profile may modulate negatively.

The translatability of dysbiotic profile generation within disparate mammalian hosts, as indicated by the mouse and human segments of this study, emphasizes that mammalian host microbiotas are intimately susceptible to outside impacts, beyond the well-delineated effects of antibiotics and diet, and that dysbiotic shifts can target health-associated bacterial groups. Future studies must continue to apply the concepts uncovered in murine hosts in this study to humans, by determining if specific microbiota changes that are associated with maternal obesity can also affect offspring weight gain and physiological function in later life, while also parsing out the exact means by which the

mother affects the offspring microbiota. Likewise, a deeper understanding in the mechanistic pathways by which psychological stress affects mucosal-associated microbiota must be pursued.

In conclusion, the data reported here suggest that the microbiota can be re-modeled by external factors to a negative state, and such dysbiotic disruptions may be associated with host disease. The study of dysbiosis is a burgeoning field, and a strong understanding of the multitude of means by which human-associated microbiotas can be affected is of great importance. Diseases like inflammatory bowel disease, which have reported correlations with stressor exposure, as well as obesity and the derivative health complications that stem from that physiological state, have been closely associated with dysbiotic profiles (344, 377). In order for researchers to develop preventative and targeted treatments to these costly illnesses, the full etiology of the disorders must be investigated, including how the microbiota may be implicated.

References

1. **Spor A, Koren O, Ley R.** 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Microbiol **9:**279-290.
2. **Grover M, Kashyap PC.** 2014. Germ-free mice as a model to study effect of gut microbiota on host physiology. Neurogastroenterol Motil **26:**745-748.
3. **Goldsmith JR, Sartor RB.** 2014. The role of diet on intestinal microbiota metabolism: downstream impacts on host immune function and health, and therapeutic implications. J Gastroenterol **49:**785-798.
4. **Roberts ME, Bishop JL, Fan X, Beer JL, Kum WW, Krebs DL, Huang M, Gill N, Priatel JJ, Finlay BB, Harder KW.** 2014. Lyn deficiency leads to increased microbiota-dependent intestinal inflammation and susceptibility to enteric pathogens. J Immunol **193:**5249-5263.
5. **Couturier-Maillard A, Secher T, Rehman A, Normand S, De Arcangelis A, Haesler R, Huot L, Grandjean T, Bressenot A, Delanoye-Crespin A, Gaillot O, Schreiber S, Lemoine Y, Ryffel B, Hot D, Nunez G, Chen G, Rosenstiel P, Chamaillard M.** 2013. NOD2-mediated dysbiosis predisposes mice to transmissible colitis and colorectal cancer. J Clin Invest **123:**700-711.
6. **Surana NK, Kasper DL.** 2014. Deciphering the tete-a-tete between the microbiota and the immune system. J Clin Invest **124:**4197-4203.
7. **Wu HJ, Wu E.** 2012. The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes **3:**4-14.
8. **Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI.** 2004. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A **101:**15718-15723.
9. **Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, Clemente JC, Knight R, Heath AC, Leibel RL, Rosenbaum M, Gordon JI.** 2013. The long-term stability of the human gut microbiota. Science **341:**1237439.
10. **Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, Young VB.** 2009. Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect Immun **77:**2367-2375.
11. **Daniel H, Moghaddas Gholami A, Berry D, Desmarchelier C, Hahne H, Loh G, Mondot S, Lepage P, Rothballer M, Walker A, Bohm C, Wenning M, Wagner M, Blaut M, Schmitt-Kopplin P, Kuster B, Haller D, Clavel T.** 2014. High-fat diet alters gut microbiota physiology in mice. ISME J **8:**295-308.
12. **Mason MR, Preshaw PM, Nagaraja HN, Dabdoub SM, Rahman A, Kumar PS.** 2015. The subgingival microbiome of clinically healthy current and never smokers. ISME J **9:**268-272.

13. **Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR.** 2007. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A **104:**13780-13785.
14. **Buffie CG, Jarchum I, Equinda M, Lipuma L, Gobourne A, Viale A, Ubeda C, Xavier J, Pamer EG.** 2012. Profound alterations of intestinal microbiota following a single dose of clindamycin results in sustained susceptibility to Clostridium difficile-induced colitis. Infect Immun **80:**62-73.
15. **Bassis CM, Theriot CM, Young VB.** 2014. Alteration of the Murine Gastrointestinal Microbiota by Tigecycline Leads to Increased Susceptibility to Clostridium difficile Infection. Antimicrob Agents Chemother doi:10.1128/AAC.02262-13.
16. **Turnbaugh PJ, Backhed F, Fulton L, Gordon JI.** 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe **3:**213-223.
17. **Bervoets L, Van Hoorenbeeck K, Kortleven I, Van Noten C, Hens N, Vael C, Goossens H, Desager KN, Vankerckhoven V.** 2013. Differences in gut microbiota composition between obese and lean children: a cross-sectional study. Gut Pathog **5:**10.
18. **Lupien SJ, Maheu F, Tu M, Fiocco A, Schramek TE.** 2007. The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. Brain Cogn **65:**209-237.
19. **Shibasaki T, Imaki T, Hotta M, Ling N, Demura H.** 1993. Psychological stress increases arousal through brain corticotropin-releasing hormone without significant increase in adrenocorticotropin and catecholamine secretion. Brain Res **618:**71-75.
20. **Oka T, Oka K, Hori T.** 2001. Mechanisms and mediators of psychological stress-induced rise in core temperature. Psychosom Med **63:**476-486.
21. **Schneiderman N, Ironson G, Siegel SD.** 2005. Stress and health: psychological, behavioral, and biological determinants. Annu Rev Clin Psychol **1:**607-628.
22. **Ataka K, Nagaishi K, Asakawa A, Inui A, Fujimiya M.** 2012. Alteration of antral and proximal colonic motility induced by chronic psychological stress involves central urocortin 3 and vasopressin in rats. Am J Physiol Gastrointest Liver Physiol **303:**G519-528.
23. **Abelson JL, Khan S, Giardino N.** 2010. HPA axis, respiration and the airways in stress--a review in search of intersections. Biol Psychol **84:**57-65.
24. **Allen AP, Kennedy PJ, Cryan JF, Dinan TG, Clarke G.** 2014. Biological and psychological markers of stress in humans: focus on the Trier Social Stress Test. Neurosci Biobehav Rev **38:**94-124.
25. **Smith SM, Vale WW.** 2006. The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues Clin Neurosci **8:**383-395.
26. **Buijs RM, Van Eden CG.** 2000. The integration of stress by the hypothalamus, amygdala and prefrontal cortex: balance between the autonomic nervous system and the neuroendocrine system. Prog Brain Res **126:**117-132.
27. **Gray TS.** 1993. Amygdaloid CRF pathways. Role in autonomic, neuroendocrine, and behavioral responses to stress. Ann N Y Acad Sci **697:**53-60.
28. **Matthews SG, Challis JR.** 1997. CRH and AVP-induced changes in synthesis and release of ACTH from the ovine fetal pituitary in vitro: negative influences of cortisol. Endocrine **6:**293-300.

29. **Stratakis CA, Chrousos GP.** 1995. Neuroendocrinology and pathophysiology of the stress system. Ann N Y Acad Sci **771:**1-18.
30. **Funder JW.** 1997. Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance. Annu Rev Med **48:**231-240.
31. **Sundberg M, Savola S, Hienola A, Korhonen L, Lindholm D.** 2006. Glucocorticoid hormones decrease proliferation of embryonic neural stem cells through ubiquitin-mediated degradation of cyclin D1. J Neurosci **26:**5402-5410.
32. **Sapolsky RM, Romero LM, Munck AU.** 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev **21:**55-89.
33. **Samuels ER, Szabadi E.** 2008. Functional neuroanatomy of the noradrenergic locus coeruleus: its roles in the regulation of arousal and autonomic function part II: physiological and pharmacological manipulations and pathological alterations of locus coeruleus activity in humans. Curr Neuropharmacol **6:**254-285.
34. **Crawley JN, Maas JW, Roth RH.** 1980. Evidence against specificity of electrical stimulation of the nucleus locus coeruleus in activating the sympathetic nervous system in the rat. Brain Res **183:**301-311.
35. **Sacca L, Vigorito C, Cicala M, Corso G, Sherwin RS.** 1983. Role of gluconeogenesis in epinephrine-stimulated hepatic glucose production in humans. Am J Physiol **245:**E294-302.
36. **Bailey MT, Coe CL.** 1999. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol **35:**146-155.
37. **Bailey MT, Lubach GR, Coe CL.** 2004. Prenatal stress alters bacterial colonization of the gut in infant monkeys. J Pediatr Gastroenterol Nutr **38:**414-421.
38. **Knowles SR, Nelson EA, Palombo EA.** 2008. Investigating the role of perceived stress on bacterial flora activity and salivary cortisol secretion: a possible mechanism underlying susceptibility to illness. Biol Psychol **77:**132-137.
39. **Ellekilde M, Selfjord E, Larsen CS, Jakesevic M, Rune I, Tranberg B, Vogensen FK, Nielsen DS, Bahl MI, Licht TR, Hansen AK, Hansen CH.** 2014. Transfer of gut microbiota from lean and obese mice to antibiotic-treated mice. Sci Rep **4:**5922.
40. **Sjoberg F, Nowrouzian F, Rangel I, Hannoun C, Moore E, Adlerberth I, Wold AE.** 2013. Comparison between terminal-restriction fragment length polymorphism (T-RFLP) and quantitative culture for analysis of infants' gut microbiota. J Microbiol Methods **94:**37-46.
41. **Dowd SE, Callaway TR, Wolcott RD, Sun Y, McKeehan T, Hagevoort RG, Edrington TS.** 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol **8:**125.
42. **Liu L, Li Y, Li S, Hu N, He Y, Pong R, Lin D, Lu L, Law M.** 2012. Comparison of next-generation sequencing systems. J Biomed Biotechnol **2012:**251364.
43. **Shin S, Park J.** 2014. Correction of sequence-dependent ambiguous bases (Ns) from the 454 pyrosequencing system. Nucleic Acids Res **42:**e51.
44. **Wang Q, Garrity GM, Tiedje JM, Cole JR.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol **73:**5261-5267.

45. **Kumar PS, Brooker MR, Dowd SE, Camerlengo T.** 2011. Target region selection is a critical determinant of community fingerprints generated by 16S pyrosequencing. PLoS One **6:**e20956.
46. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R.** 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods **7:**335-336.
47. **Oksanen J BF, Kindt R, Legendre R, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H. .** Vegan: community ecology package. , p R package version 2.0-3. .
48. **DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL.** 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol **72:**5069-5072.
49. **Vazquez-Baeza Y, Pirrung M, Gonzalez A, Knight R.** 2013. EMPeror: a tool for visualizing high-throughput microbial community data. Gigascience **2:**16.
50. **Lozupone C, Knight R.** 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol **71:**8228-8235.
51. **Bailey MT, Kierstein S, Sharma S, Spaits M, Kinsey SG, Tliba O, Amrani Y, Sheridan JF, Panettieri RA, Haczku A.** 2009. Social stress enhances allergen-induced airway inflammation in mice and inhibits corticosteroid responsiveness of cytokine production. J Immunol **182:**7888-7896.
52. **Engler H, Engler A, Bailey MT, Sheridan JF.** 2005. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. J Neuroimmunol **163:**110-119.
53. **Quan N, Avitsur R, Stark JL, He L, Lai W, Dhabhar F, Sheridan JF.** 2003. Molecular mechanisms of glucocorticoid resistance in splenocytes of socially stressed male mice. J Neuroimmunol **137:**51-58.
54. **Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA.** 1994. Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. Steroids **59:**436-442.
55. **Avitsur R, Stark JL, Dhabhar FS, Padgett DA, Sheridan JF.** 2002. Social disruption-induced glucocorticoid resistance: kinetics and site specificity. J Neuroimmunol **124:**54-61.
56. **Bailey MT, Avitsur R, Engler H, Padgett DA, Sheridan JF.** 2004. Physical defeat reduces the sensitivity of murine splenocytes to the suppressive effects of corticosterone. Brain Behav Immun **18:**416-424.
57. **Pace TW, Hu F, Miller AH.** 2007. Cytokine-effects on glucocorticoid receptor function: relevance to glucocorticoid resistance and the pathophysiology and treatment of major depression. Brain Behav Immun **21:**9-19.
58. **Berk M, Wadee AA, Kuschke RH, O'Neill-Kerr A.** 1997. Acute phase proteins in major depression. J Psychosom Res **43:**529-534.
59. **Wohleb ES, McKim DB, Shea DT, Powell ND, Tarr AJ, Sheridan JF, Godbout JP.** 2014. Re-establishment of anxiety in stress-sensitized mice is caused by monocyte trafficking from the spleen to the brain. Biol Psychiatry **75:**970-981.

60. **Powell ND, Sloan EK, Bailey MT, Arevalo JM, Miller GE, Chen E, Kobor MS, Reader BF, Sheridan JF, Cole SW.** 2013. Social stress up-regulates inflammatory gene expression in the leukocyte transcriptome via beta-adrenergic induction of myelopoiesis. Proc Natl Acad Sci U S A **110:**16574-16579.
61. **Bailey MT, Engler H, Powell ND, Padgett DA, Sheridan JF.** 2007. Repeated social defeat increases the bactericidal activity of splenic macrophages through a Toll-like receptor-dependent pathway. Am J Physiol Regul Integr Comp Physiol **293:**R1180-1190.
62. **Wohleb ES, Powell ND, Godbout JP, Sheridan JF.** 2013. Stress-induced recruitment of bone marrow-derived monocytes to the brain promotes anxiety-like behavior. J Neurosci **33:**13820-13833.
63. **Hanke ML, Powell ND, Stiner LM, Bailey MT, Sheridan JF.** 2012. Beta adrenergic blockade decreases the immunomodulatory effects of social disruption stress. Brain Behav Immun **26:**1150-1159.
64. **Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, Nelson RJ, Godbout JP, Sheridan JF.** 2011. beta-Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. J Neurosci **31:**6277-6288.
65. **Mays JW, Bailey MT, Hunzeker JT, Powell ND, Papenfuss T, Karlsson EA, Padgett DA, Sheridan JF.** 2010. Influenza virus-specific immunological memory is enhanced by repeated social defeat. J Immunol **184:**2014-2025.
66. **Tarr AJ, Powell ND, Reader BF, Bhave NS, Roloson AL, Carson WE, 3rd, Sheridan JF.** 2012. beta-Adrenergic receptor mediated increases in activation and function of natural killer cells following repeated social disruption. Brain Behav Immun **26:**1226-1238.
67. **Dong-Newsom P, Powell ND, Bailey MT, Padgett DA, Sheridan JF.** 2010. Repeated social stress enhances the innate immune response to a primary HSV-1 infection in the cornea and trigeminal ganglia of Balb/c mice. Brain Behav Immun **24:**273-280.
68. **Bailey MT, Kinsey SG, Padgett DA, Sheridan JF, Leblebicioglu B.** 2009. Social stress enhances IL-1beta and TNF-alpha production by Porphyromonas gingivalis lipopolysaccharide-stimulated CD11b+ cells. Physiol Behav **98:**351-358.
69. **Allen RG, Lafuse WP, Galley JD, Ali MM, Ahmer BM, Bailey MT.** 2012. The intestinal microbiota are necessary for stressor-induced enhancement of splenic macrophage microbicidal activity. Brain Behav Immun **26:**371-382.
70. **Bailey MT, Dowd SE, Galley JD, Hufnagle AR, Allen RG, Lyte M.** 2011. Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation. Brain Behav Immun **25:**397-407.
71. **Voorhees JL, Tarr AJ, Wohleb ES, Godbout JP, Mo X, Sheridan JF, Eubank TD, Marsh CB.** 2013. Prolonged restraint stress increases IL-6, reduces IL-10, and causes persistent depressive-like behavior that is reversed by recombinant IL-10. PLoS One **8:**e58488.
72. **Leem YH, Oh S.** 2015. 3,4,5-Trimethoxycinnamin acid ameliorates restraint stress-induced anxiety and depression. Neurosci Lett **585:**54-59.
73. **Hunzeker J, Padgett DA, Sheridan PA, Dhabhar FS, Sheridan JF.** 2004. Modulation of natural killer cell activity by restraint stress during an influenza A/PR8 infection in mice. Brain Behav Immun **18:**526-535.
74. **Engler A, Roy S, Sen CK, Padgett DA, Sheridan JF.** 2005. Restraint stress alters lung gene expression in an experimental influenza A viral infection. J Neuroimmunol **162:**103-111.

75. **Padgett DA, Marucha PT, Sheridan JF.** 1998. Restraint stress slows cutaneous wound healing in mice. Brain Behav Immun **12:**64-73.
76. **Mercado AM, Quan N, Padgett DA, Sheridan JF, Marucha PT.** 2002. Restraint stress alters the expression of interleukin-1 and keratinocyte growth factor at the wound site: an in situ hybridization study. J Neuroimmunol **129:**74-83.
77. **Bailey MT, Dowd SE, Parry NM, Galley JD, Schauer DB, Lyte M.** 2010. Stressor exposure disrupts commensal microbial populations in the intestines and leads to increased colonization by Citrobacter rodentium. Infect Immun **78:**1509-1519.
78. **Gershon MD, Tack J.** 2007. The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology **132:**397-414.
79. **Hansen MB, Witte AB.** 2008. The role of serotonin in intestinal luminal sensing and secretion. Acta Physiol (Oxf) **193:**311-323.
80. **Noah TK, Kazanjian A, Whitsett J, Shroyer NF.** 2010. SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells. Exp Cell Res **316:**452-465.
81. **Specian RD, Neutra MR.** 1980. Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. J Cell Biol **85:**626-640.
82. **Suzuki T.** 2013. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci **70:**631-659.
83. **Aveleira CA, Lin CM, Abcouwer SF, Ambrosio AF, Antonetti DA.** 2010. TNF-alpha signals through PKCzeta/NF-kappaB to alter the tight junction complex and increase retinal endothelial cell permeability. Diabetes **59:**2872-2882.
84. **Ma TY, Boivin MA, Ye D, Pedram A, Said HM.** 2005. Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression. Am J Physiol Gastrointest Liver Physiol **288:**G422-430.
85. **Marchiando AM, Shen L, Graham WV, Weber CR, Schwarz BT, Austin JR, 2nd, Raleigh DR, Guan Y, Watson AJ, Montrose MH, Turner JR.** 2010. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. J Cell Biol **189:**111-126.
86. **Kuhnert F, Davis CR, Wang HT, Chu P, Lee M, Yuan J, Nusse R, Kuo CJ.** 2004. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proc Natl Acad Sci U S A **101:**266-271.
87. **Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI.** 2007. The human microbiome project. Nature **449:**804-810.
88. **Sekirov I, Russell SL, Antunes LC, Finlay BB.** 2010. Gut microbiota in health and disease. Physiol Rev **90:**859-904.
89. **Yang Y, Torchinsky MB, Gobert M, Xiong H, Xu M, Linehan JL, Alonzo F, Ng C, Chen A, Lin X, Sczesnak A, Liao JJ, Torres VJ, Jenkins MK, Lafaille JJ, Littman DR.** 2014. Focused specificity of intestinal TH17 cells towards commensal bacterial antigens. Nature **510:**152-156.
90. **Van den Abbeele P, Van de Wiele T, Verstraete W, Possemiers S.** 2011. The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol Rev **35:**681-704.

91. **Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA.** 2005. Diversity of the human intestinal microbial flora. Science **308:**1635-1638.
92. **Hao WL, Lee YK.** 2004. Microflora of the gastrointestinal tract: a review. Methods Mol Biol **268:**491-502.
93. **Favier CF, de Vos WM, Akkermans AD.** 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe **9:**219-229.
94. **Hall MA, Cole CB, Smith SL, Fuller R, Rolles CJ.** 1990. Factors influencing the presence of faecal lactobacilli in early infancy. Arch Dis Child **65:**185-188.
95. **Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE.** 2011. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A **108 Suppl 1:**4578-4585.
96. **Bates JM, Mittge E, Kuhlman J, Baden KN, Cheesman SE, Guillemin K.** 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev Biol **297:**374-386.
97. **Becker S, Oelschlaeger TA, Wullaert A, Vlantis K, Pasparakis M, Wehkamp J, Stange EF, Gersemann M.** 2013. Bacteria regulate intestinal epithelial cell differentiation factors both in vitro and in vivo. PLoS One **8:**e55620.
98. **Backhed F, Manchester JK, Semenkovich CF, Gordon JI.** 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A **104:**979-984.
99. **Rabot S, Membrez M, Bruneau A, Gerard P, Harach T, Moser M, Raymond F, Mansourian R, Chou CJ.** 2010. Germ-free C57BL/6J mice are resistant to high-fat-diet-induced insulin resistance and have altered cholesterol metabolism. FASEB J **24:**4948-4959.
100. **Lamouse-Smith ES, Tzeng A, Starnbach MN.** 2011. The intestinal flora is required to support antibody responses to systemic immunization in infant and germ free mice. PLoS One **6:**e27662.
101. **Moreau MC, Ducluzeau R, Guy-Grand D, Muller MC.** 1978. Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. Infect Immun **21:**532-539.
102. **Rhee KJ, Sethupathi P, Driks A, Lanning DK, Knight KL.** 2004. Role of commensal bacteria in development of gut-associated lymphoid tissues and preimmune antibody repertoire. J Immunol **172:**1118-1124.
103. **Souza DG, Vieira AT, Soares AC, Pinho V, Nicoli JR, Vieira LQ, Teixeira MM.** 2004. The essential role of the intestinal microbiota in facilitating acute inflammatory responses. J Immunol **173:**4137-4146.
104. **Mazagova M, Wang L, Anfora AT, Wissmueller M, Lesley SA, Miyamoto Y, Eckmann L, Dhungana S, Pathmasiri W, Sumner S, Westwater C, Brenner DA, Schnabl B.** 2015. Commensal microbiota is hepatoprotective and prevents liver fibrosis in mice. FASEB J **29:**1043-1055.
105. **Van den Abbeele P, Belzer C, Goossens M, Kleerebezem M, De Vos WM, Thas O, De Weirdt R, Kerckhof FM, Van de Wiele T.** 2013. Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an in vitro gut model. ISME J **7:**949-961.

106. **Duncan SH, Barcenilla A, Stewart CS, Pryde SE, Flint HJ.** 2002. Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. Appl Environ Microbiol **68:**5186-5190.
107. **Segain JP, Raingeard de la Bletiere D, Bourreille A, Leray V, Gervois N, Rosales C, Ferrier L, Bonnet C, Blottiere HM, Galmiche JP.** 2000. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut **47:**397-403.
108. **Liu T, Li J, Liu Y, Xiao N, Suo H, Xie K, Yang C, Wu C.** 2012. Short-chain fatty acids suppress lipopolysaccharide-induced production of nitric oxide and proinflammatory cytokines through inhibition of NF-kappaB pathway in RAW264.7 cells. Inflammation **35:**1676-1684.
109. **Martinez CA, de Campos FG, de Carvalho VR, de Castro Ferreira C, Rodrigues MR, Sato DT, Pereira JA.** 2015. Claudin-3 and occludin tissue content in the glands of colonic mucosa with and without a fecal stream. J Mol Histol doi:10.1007/s10735-015-9610-y.
110. **Bordin M, D'Atri F, Guillemot L, Citi S.** 2004. Histone deacetylase inhibitors up-regulate the expression of tight junction proteins. Mol Cancer Res **2:**692-701.
111. **Hatayama H, Iwashita J, Kuwajima A, Abe T.** 2007. The short chain fatty acid, butyrate, stimulates MUC2 mucin production in the human colon cancer cell line, LS174T. Biochem Biophys Res Commun **356:**599-603.
112. **Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F, Wu GD.** 2009. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology **137:**1716-1724 e1711-1712.
113. **Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, Kim SG, Li H, Gao Z, Mahana D, Zarate Rodriguez JG, Rogers AB, Robine N, Loke P, Blaser MJ.** 2014. Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell **158:**705-721.
114. **van Klinken BJ, Einerhand AW, Duits LA, Makkink MK, Tytgat KM, Renes IB, Verburg M, Buller HA, Dekker J.** 1999. Gastrointestinal expression and partial cDNA cloning of murine Muc2. Am J Physiol **276:**G115-124.
115. **Ho SB, Roberton AM, Shekels LL, Lyftogt CT, Niehans GA, Toribara NW.** 1995. Expression cloning of gastric mucin complementary DNA and localization of mucin gene expression. Gastroenterology **109:**735-747.
116. **Hansson GC, Johansson ME.** 2010. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Gut Microbes **1:**51-54.
117. **Asker N, Axelsson MA, Olofsson SO, Hansson GC.** 1998. Dimerization of the human MUC2 mucin in the endoplasmic reticulum is followed by a N-glycosylation-dependent transfer of the mono- and dimers to the Golgi apparatus. J Biol Chem **273:**18857-18863.
118. **Atuma C, Strugala V, Allen A, Holm L.** 2001. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol **280:**G922-929.
119. **Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC.** 2008. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A **105:**15064-15069.

120. **Derrien M, Vaughan EE, Plugge CM, de Vos WM.** 2004. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol **54:**1469-1476.
121. **Sonnenburg JL, Xu J, Leip DD, Chen CH, Westover BP, Weatherford J, Buhler JD, Gordon JI.** 2005. Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science **307:**1955-1959.
122. **Sonnenburg JL, Angenent LT, Gordon JI.** 2004. Getting a grip on things: how do communities of bacterial symbionts become established in our intestine? Nat Immunol **5:**569-573.
123. **Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu XN, Kubo C, Koga Y.** 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. J Physiol **558:**263-275.
124. **Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, Collins SM.** 2011. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology **141:**599-609, 609 e591-593.
125. **Riley TP, Neal-McKinney JM, Buelow DR, Konkel ME, Simasko SM.** 2013. Capsaicin-sensitive vagal afferent neurons contribute to the detection of pathogenic bacterial colonization in the gut. J Neuroimmunol **257:**36-45.
126. **Lyte M, Varcoe JJ, Bailey MT.** 1998. Anxiogenic effect of subclinical bacterial infection in mice in the absence of overt immune activation. Physiol Behav **65:**63-68.
127. **Bercik P, Verdu EF, Foster JA, Macri J, Potter M, Huang X, Malinowski P, Jackson W, Blennerhassett P, Neufeld KA, Lu J, Khan WI, Corthesy-Theulaz I, Cherbut C, Bergonzelli GE, Collins SM.** 2010. Chronic gastrointestinal inflammation induces anxiety-like behavior and alters central nervous system biochemistry in mice. Gastroenterology **139:**2102-2112 e2101.
128. **Girard SA, Bah TM, Kaloustian S, Lada-Moldovan L, Rondeau I, Tompkins TA, Godbout R, Rousseau G.** 2009. Lactobacillus helveticus and Bifidobacterium longum taken in combination reduce the apoptosis propensity in the limbic system after myocardial infarction in a rat model. Br J Nutr **102:**1420-1425.
129. **Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, Bisson JF, Rougeot C, Pichelin M, Cazaubiel M, Cazaubiel JM.** 2011. Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. Br J Nutr **105:**755-764.
130. **Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF.** 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci U S A **108:**16050-16055.
131. **Tan W, Zhou W, Luo HS, Liang CB, Xia H.** 2013. The inhibitory effect of melatonin on colonic motility disorders induced by water avoidance stress in rats. Eur Rev Med Pharmacol Sci **17:**3060-3067.
132. **Castagliuolo I, Lamont JT, Qiu B, Fleming SM, Bhaskar KR, Nikulasson ST, Kornetsky C, Pothoulakis C.** 1996. Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells. Am J Physiol **271:**G884-892.

133. **Sun DC.** 1969. Effect of corticotropin on gastric acid, pepsin, and mucus secretion in dogs with fistulas. Am J Dig Dis **14:**107-112.
134. **Chatzaki E, Crowe PD, Wang L, Million M, Tache Y, Grigoriadis DE.** 2004. CRF receptor type 1 and 2 expression and anatomical distribution in the rat colon. J Neurochem **90:**309-316.
135. **Tsukamoto K, Nakade Y, Mantyh C, Ludwig K, Pappas TN, Takahashi T.** 2006. Peripherally administered CRF stimulates colonic motility via central CRF receptors and vagal pathways in conscious rats. Am J Physiol Regul Integr Comp Physiol **290:**R1537-1541.
136. **Overman EL, Rivier JE, Moeser AJ.** 2012. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. PLoS One **7:**e39935.
137. **Lyte M, Bailey MT.** 1997. Neuroendocrine-bacterial interactions in a neurotoxin-induced model of trauma. J Surg Res **70:**195-201.
138. **Bailey MT, Karaszewski JW, Lubach GR, Coe CL, Lyte M.** 1999. In vivo adaptation of attenuated Salmonella typhimurium results in increased growth upon exposure to norepinephrine. Physiol Behav **67:**359-364.
139. **Huerta-Franco MR, Vargas-Luna M, Montes-Frausto JB, Morales-Mata I, Ramirez-Padilla L.** 2012. Effect of psychological stress on gastric motility assessed by electrical bio-impedance. World J Gastroenterol **18:**5027-5033.
140. **Vicario M, Alonso C, Guilarte M, Serra J, Martinez C, Gonzalez-Castro AM, Lobo B, Antolin M, Andreu AL, Garcia-Arumi E, Casellas M, Saperas E, Malagelada JR, Azpiroz F, Santos J.** 2012. Chronic psychosocial stress induces reversible mitochondrial damage and corticotropin-releasing factor receptor type-1 upregulation in the rat intestine and IBS-like gut dysfunction. Psychoneuroendocrinology **37:**65-77.
141. **Shigeshiro M, Tanabe S, Suzuki T.** 2012. Repeated exposure to water immersion stress reduces the Muc2 gene level in the rat colon via two distinct mechanisms. Brain Behav Immun **26:**1061-1065.
142. **Saunders PR, Kosecka U, McKay DM, Perdue MH.** 1994. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. Am J Physiol **267:**G794-799.
143. **Stanisor OI, van Diest SA, Yu Z, Welting O, Bekkali N, Shi J, de Jonge WJ, Boeckxstaens GE, van den Wijngaard RM.** 2013. Stress-induced visceral hypersensitivity in maternally separated rats can be reversed by peripherally restricted histamine-1-receptor antagonists. PLoS One **8:**e66884.
144. **Xu D, Gao J, Gillilland M, 3rd, Wu X, Song I, Kao JY, Owyang C.** 2014. Rifaximin alters intestinal bacteria and prevents stress-induced gut inflammation and visceral hyperalgesia in rats. Gastroenterology **146:**484-496 e484.
145. **Bernstein CN, Singh S, Graff LA, Walker JR, Miller N, Cheang M.** 2010. A prospective population-based study of triggers of symptomatic flares in IBD. Am J Gastroenterol **105:**1994-2002.
146. **Murray CD, Flynn J, Ratcliffe L, Jacyna MR, Kamm MA, Emmanuel AV.** 2004. Effect of acute physical and psychological stress on gut autonomic innervation in irritable bowel syndrome. Gastroenterology **127:**1695-1703.

147. **Rigsbee L, Agans R, Shankar V, Kenche H, Khamis HJ, Michail S, Paliy O.** 2012. Quantitative profiling of gut microbiota of children with diarrhea-predominant irritable bowel syndrome. Am J Gastroenterol **107:**1740-1751.
148. **Chen L, Wang W, Zhou R, Ng SC, Li J, Huang M, Zhou F, Wang X, Shen B, M AK, Wu K, Xia B.** 2014. Characteristics of fecal and mucosa-associated microbiota in Chinese patients with inflammatory bowel disease. Medicine (Baltimore) **93:**e51.
149. **Farache J, Koren I, Milo I, Gurevich I, Kim KW, Zigmond E, Furtado GC, Lira SA, Shakhar G.** 2013. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. Immunity **38:**581-595.
150. **Ivanov, II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR.** 2009. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell **139:**485-498.
151. **Barnes MJ, Powrie F.** 2009. Regulatory T cells reinforce intestinal homeostasis. Immunity **31:**401-411.
152. **Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, Valvano MA, Foster SJ, Mak TW, Nunez G, Inohara N.** 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol **4:**702-707.
153. **Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ.** 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem **278:**8869-8872.
154. **Ma TY, Iwamoto GK, Hoa NT, Akotia V, Pedram A, Boivin MA, Said HM.** 2004. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. Am J Physiol Gastrointest Liver Physiol **286:**G367-376.
155. **Chung LP, Keshav S, Gordon S.** 1988. Cloning the human lysozyme cDNA: inverted Alu repeat in the mRNA and in situ hybridization for macrophages and Paneth cells. Proc Natl Acad Sci U S A **85:**6227-6231.
156. **Ayabe T, Satchell DP, Wilson CL, Parks WC, Selsted ME, Ouellette AJ.** 2000. Secretion of microbicidal alpha-defensins by intestinal Paneth cells in response to bacteria. Nat Immunol **1:**113-118.
157. **Cash HL, Whitham CV, Behrendt CL, Hooper LV.** 2006. Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science **313:**1126-1130.
158. **Antoni L, Nuding S, Weller D, Gersemann M, Ott G, Wehkamp J, Stange EF.** 2013. Human colonic mucus is a reservoir for antimicrobial peptides. J Crohns Colitis **7:**e652-664.
159. **Cho JH.** 2008. The genetics and immunopathogenesis of inflammatory bowel disease. Nat Rev Immunol **8:**458-466.
160. **Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, Ramos R, Britton H, Moran T, Karaliuskas R, Duerr RH, Achkar JP, Brant SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH.** 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature **411:**603-606.
161. **Higgins LM, Frankel G, Douce G, Dougan G, MacDonald TT.** 1999. Citrobacter rodentium infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease. Infect Immun **67:**3031-3039.

162. **Schauer DB, Falkow S.** 1993. Attaching and effacing locus of a Citrobacter freundii biotype that causes transmissible murine colonic hyperplasia. Infect Immun **61:**2486-2492.
163. **Deng W, Vallance BA, Li Y, Puente JL, Finlay BB.** 2003. Citrobacter rodentium translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. Mol Microbiol **48:**95-115.
164. **Hoffmann C, Hill DA, Minkah N, Kirn T, Troy A, Artis D, Bushman F.** 2009. Community-wide response of the gut microbiota to enteropathogenic Citrobacter rodentium infection revealed by deep sequencing. Infect Immun **77:**4668-4678.
165. **Mackos AR, Eubank TD, Parry NM, Bailey MT.** 2013. Probiotic Lactobacillus reuteri attenuates the stressor-enhanced severity of Citrobacter rodentium infection. Infect Immun **81:**3253-3263.
166. **Vallance BA, Deng W, De Grado M, Chan C, Jacobson K, Finlay BB.** 2002. Modulation of inducible nitric oxide synthase expression by the attaching and effacing bacterial pathogen citrobacter rodentium in infected mice. Infect Immun **70:**6424-6435.
167. **Kim YG, Kamada N, Shaw MH, Warner N, Chen GY, Franchi L, Nunez G.** 2011. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. Immunity **34:**769-780.
168. **Khan MA, Ma C, Knodler LA, Valdez Y, Rosenberger CM, Deng W, Finlay BB, Vallance BA.** 2006. Toll-like receptor 4 contributes to colitis development but not to host defense during Citrobacter rodentium infection in mice. Infect Immun **74:**2522-2536.
169. **Gibson DL, Ma C, Rosenberger CM, Bergstrom KS, Valdez Y, Huang JT, Khan MA, Vallance BA.** 2008. Toll-like receptor 2 plays a critical role in maintaining mucosal integrity during Citrobacter rodentium-induced colitis. Cell Microbiol **10:**388-403.
170. **Wang Z, Friedrich C, Hagemann SC, Korte WH, Goharani N, Cording S, Eberl G, Sparwasser T, Lochner M.** 2014. Regulatory T cells promote a protective Th17-associated immune response to intestinal bacterial infection with C. rodentium. Mucosal Immunol **7:**1290-1301.
171. **Bergstrom KS, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, Ryz N, Huang T, Velcich A, Finlay BB, Chadee K, Vallance BA.** 2010. Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. PLoS Pathog **6:**e1000902.
172. **Wlodarska M, Willing B, Keeney KM, Menendez A, Bergstrom KS, Gill N, Russell SL, Vallance BA, Finlay BB.** 2011. Antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated Citrobacter rodentium-induced colitis. Infect Immun **79:**1536-1545.
173. **Jang SO, Kim HJ, Kim YJ, Kang MJ, Kwon JW, Seo JH, Kim HY, Kim BJ, Yu J, Hong SJ.** 2012. Asthma Prevention by Lactobacillus Rhamnosus in a Mouse Model is Associated With CD4(+)CD25(+)Foxp3(+) T Cells. Allergy Asthma Immunol Res **4:**150-156.
174. **Tsai YT, Cheng PC, Liao JW, Pan TM.** 2010. Effect of the administration of Lactobacillus paracasei subsp. paracasei NTU 101 on Peyer's patch-mediated mucosal immunity. Int Immunopharmacol **10:**791-798.

175. **O'Mahony D, Murphy S, Boileau T, Park J, O'Brien F, Groeger D, Konieczna P, Ziegler M, Scully P, Shanahan F, Kiely B, O'Mahony L.** 2010. Bifidobacterium animalis AHC7 protects against pathogen-induced NF-kappaB activation in vivo. BMC Immunol **11:**63.
176. **Zakostelska Z, Kverka M, Klimesova K, Rossmann P, Mrazek J, Kopecny J, Hornova M, Srutkova D, Hudcovic T, Ridl J, Tlaskalova-Hogenova H.** 2011. Lysate of probiotic Lactobacillus casei DN-114 001 ameliorates colitis by strengthening the gut barrier function and changing the gut microenvironment. PLoS One **6:**e27961.
177. **Yun B, Oh S, Griffiths MW.** 2014. Lactobacillus acidophilus modulates the virulence of Clostridium difficile. J Dairy Sci **97:**4745-4758.
178. **Thiraworawong T, Spinler JK, Werawatganon D, Klaikeaw N, Venable SF, Versalovic J, Tumwasorn S.** 2014. Anti-inflammatory properties of gastric-derived Lactobacillus plantarum XB7 in the context of Helicobacter pylori infection. Helicobacter **19:**144-155.
179. **Watson D, O'Connell Motherway M, Schoterman MH, van Neerven RJ, Nauta A, van Sinderen D.** 2013. Selective carbohydrate utilization by lactobacilli and bifidobacteria. J Appl Microbiol **114:**1132-1146.
180. **Finamore A, Roselli M, Imbinto A, Seeboth J, Oswald IP, Mengheri E.** 2014. Lactobacillus amylovorus inhibits the TLR4 inflammatory signaling triggered by enterotoxigenic Escherichia coli via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. PLoS One **9:**e94891.
181. **Jang SE, Hyam SR, Han MJ, Kim SY, Lee BG, Kim DH.** 2013. Lactobacillus brevis G-101 ameliorates colitis in mice by inhibiting NF-kappaB, MAPK and AKT pathways and by polarizing M1 macrophages to M2-like macrophages. J Appl Microbiol **115:**888-896.
182. **Tien MT, Girardin SE, Regnault B, Le Bourhis L, Dillies MA, Coppee JY, Bourdet-Sicard R, Sansonetti PJ, Pedron T.** 2006. Anti-inflammatory effect of Lactobacillus casei on Shigella-infected human intestinal epithelial cells. J Immunol **176:**1228-1237.
183. **Oh PL, Benson AK, Peterson DA, Patil PB, Moriyama EN, Roos S, Walter J.** 2010. Diversification of the gut symbiont Lactobacillus reuteri as a result of host-driven evolution. ISME J **4:**377-387.
184. **Frese SA, Benson AK, Tannock GW, Loach DM, Kim J, Zhang M, Oh PL, Heng NC, Patil PB, Juge N, Mackenzie DA, Pearson BM, Lapidus A, Dalin E, Tice H, Goltsman E, Land M, Hauser L, Ivanova N, Kyrpides NC, Walter J.** 2011. The evolution of host specialization in the vertebrate gut symbiont Lactobacillus reuteri. PLoS Genet **7:**e1001314.
185. **Walter J, Britton RA, Roos S.** 2011. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the Lactobacillus reuteri paradigm. Proc Natl Acad Sci U S A **108 Suppl 1:**4645-4652.
186. **Rosander A, Connolly E, Roos S.** 2008. Removal of antibiotic resistance gene-carrying plasmids from Lactobacillus reuteri ATCC 55730 and characterization of the resulting daughter strain, L. reuteri DSM 17938. Appl Environ Microbiol **74:**6032-6040.
187. **Galley JD, Nelson MC, Yu Z, Dowd SE, Walter J, Kumar PS, Lyte M, Bailey MT.** 2014. Exposure to a social stressor disrupts the community structure of the colonic mucosa-associated microbiota. BMC Microbiol **14:**189.
188. **Roos S, Jonsson H.** 2002. A high-molecular-mass cell-surface protein from Lactobacillus reuteri 1063 adheres to mucus components. Microbiology **148:**433-442.

189. **Mackenzie DA, Jeffers F, Parker ML, Vibert-Vallet A, Bongaerts RJ, Roos S, Walter J, Juge N.** 2010. Strain-specific diversity of mucus-binding proteins in the adhesion and aggregation properties of Lactobacillus reuteri. Microbiology **156:**3368-3378.
190. **Madsen KL, Doyle JS, Jewell LD, Tavernini MM, Fedorak RN.** 1999. Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology **116:**1107-1114.
191. **Smits HH, Engering A, van der Kleij D, de Jong EC, Schipper K, van Capel TM, Zaat BA, Yazdanbakhsh M, Wierenga EA, van Kooyk Y, Kapsenberg ML.** 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. J Allergy Clin Immunol **115:**1260-1267.
192. **Livingston M, Loach D, Wilson M, Tannock GW, Baird M.** 2010. Gut commensal Lactobacillus reuteri 100-23 stimulates an immunoregulatory response. Immunol Cell Biol **88:**99-102.
193. **Talarico TL, Casas IA, Chung TC, Dobrogosz WJ.** 1988. Production and isolation of reuterin, a growth inhibitor produced by Lactobacillus reuteri. Antimicrob Agents Chemother **32:**1854-1858.
194. **Spinler JK, Taweechotipatr M, Rognerud CL, Ou CN, Tumwasorn S, Versalovic J.** 2008. Human-derived probiotic Lactobacillus reuteri demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. Anaerobe **14:**166-171.
195. **Francavilla R, Lionetti E, Castellaneta SP, Magista AM, Maurogiovanni G, Bucci N, De Canio A, Indrio F, Cavallo L, Ierardi E, Miniello VL.** 2008. Inhibition of Helicobacter pylori infection in humans by Lactobacillus reuteri ATCC 55730 and effect on eradication therapy: a pilot study. Helicobacter **13:**127-134.
196. **Kohler GA, Assefa S, Reid G.** 2012. Probiotic interference of Lactobacillus rhamnosus GR-1 and Lactobacillus reuteri RC-14 with the opportunistic fungal pathogen Candida albicans. Infect Dis Obstet Gynecol **2012:**636474.
197. **Preidis GA, Saulnier DM, Blutt SE, Mistretta TA, Riehle KP, Major AM, Venable SF, Finegold MJ, Petrosino JF, Conner ME, Versalovic J.** 2012. Probiotics stimulate enterocyte migration and microbial diversity in the neonatal mouse intestine. FASEB J **26:**1960-1969.
198. **Macfarlane GT, Macfarlane S.** 2011. Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. J Clin Gastroenterol **45 Suppl:**S120-127.
199. **Kashyap PC, Marcobal A, Ursell LK, Larauche M, Duboc H, Earle KA, Sonnenburg ED, Ferreyra JA, Higginbottom SK, Million M, Tache Y, Pasricha PJ, Knight R, Farrugia G, Sonnenburg JL.** 2013. Complex interactions among diet, gastrointestinal transit, and gut microbiota in humanized mice. Gastroenterology **144:**967-977.
200. **Payne AN, Chassard C, Zimmermann M, Muller P, Stinca S, Lacroix C.** 2011. The metabolic activity of gut microbiota in obese children is increased compared with normal-weight children and exhibits more exhaustive substrate utilization. Nutr Diabetes **1:**e12.
201. **Ferrer M, Ruiz A, Lanza F, Haange SB, Oberbach A, Till H, Bargiela R, Campoy C, Segura MT, Richter M, von Bergen M, Seifert J, Suarez A.** 2013. Microbiota from the distal guts of lean and obese adolescents exhibit partial functional redundancy besides clear differences in community structure. Environ Microbiol **15:**211-226.

202. **Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI.** 2009. A core gut microbiome in obese and lean twins. Nature **457:**480-484.
203. **Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO.** 2007. Development of the human infant intestinal microbiota. PLoS Biol **5:**e177.
204. **Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R.** 2010. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A **107:**11971-11975.
205. **Keski-Nisula L, Kyynarainen HR, Karkkainen U, Karhukorpi J, Heinonen S, Pekkanen J.** 2013. Maternal intrapartum antibiotics and decreased vertical transmission of Lactobacillus to neonates during birth. Acta Paediatr **102:**480-485.
206. **Whitaker RC, Wright JA, Pepe MS, Seidel KD, Dietz WH.** 1997. Predicting obesity in young adulthood from childhood and parental obesity. N Engl J Med **337:**869-873.
207. **Tannock GW, Savage DC.** 1974. Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infect Immun **9:**591-598.
208. **Makino H, Kushiro A, Ishikawa E, Kubota H, Gawad A, Sakai T, Oishi K, Martin R, Ben-Amor K, Knol J, Tanaka R.** 2013. Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. PLoS One **8:**e78331.
209. **Furness JB.** 2012. The enteric nervous system and neurogastroenterology. Nat Rev Gastroenterol Hepatol **9:**286-294.
210. **Holzer P.** 2006. Efferent-like roles of afferent neurons in the gut: Blood flow regulation and tissue protection. Auton Neurosci **125:**70-75.
211. **de Jonge WJ.** 2013. The Gut's Little Brain in Control of Intestinal Immunity. ISRN Gastroenterol **2013:**630159.
212. **Whitehead WE, Crowell MD, Robinson JC, Heller BR, Schuster MM.** 1992. Effects of stressful life events on bowel symptoms: subjects with irritable bowel syndrome compared with subjects without bowel dysfunction. Gut **33:**825-830.
213. **Lee KJ, Tack J.** 2010. Altered intestinal microbiota in irritable bowel syndrome. Neurogastroenterol Motil **22:**493-498.
214. **Huffnagle GB.** 2010. The microbiota and allergies/asthma. PLoS Pathog **6:**e1000549.
215. **Allison SD, Martiny JB.** 2008. Colloquium paper: resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A **105 Suppl 1:**11512-11519.
216. **Dethlefsen L, Huse S, Sogin ML, Relman DA.** 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol **6:**e280.
217. **Hakansson A, Tormo-Badia N, Baridi A, Xu J, Molin G, Hagslatt ML, Karlsson C, Jeppsson B, Cilio CM, Ahrne S.** 2014. Immunological alteration and changes of gut microbiota after dextran sulfate sodium (DSS) administration in mice. Clin Exp Med doi:10.1007/s10238-013-0270-5.
218. **Schwab C, Berry D, Rauch I, Rennisch I, Ramesmayer J, Hainzl E, Heider S, Decker T, Kenner L, Muller M, Strobl B, Wagner M, Schleper C, Loy A, Urich T.** 2014. Longitudinal study of murine microbiota activity and interactions with the host during acute inflammation and recovery. ISME J doi:10.1038/ismej.2013.223.

219. **Shilov VM, Lizko NN, Borisova OK, Prokhorov VY.** 1971. Changes in the microflora of man during long-term confinement. Life Sci Space Res **9:**43-49.
220. **Lizko NN, Silov VM, Syrych GD.** 1984. [Events in he development of dysbacteriosis of the intestines in man under extreme conditions]. Nahrung **28:**599-605.
221. **Nocker A, Burr M, Camper AK.** 2007. Genotypic microbial community profiling: a critical technical review. Microb Ecol **54:**276-289.
222. **Hong PY, Croix JA, Greenberg E, Gaskins HR, Mackie RI.** 2011. Pyrosequencing-based analysis of the mucosal microbiota in healthy individuals reveals ubiquitous bacterial groups and micro-heterogeneity. PLoS One **6:**e25042.
223. **Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, de Vos WM.** 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol **68:**3401-3407.
224. **O'Malley D, Julio-Pieper M, Gibney SM, Dinan TG, Cryan JF.** 2010. Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour. Stress **13:**114-122.
225. **Buynitsky T, Mostofsky DI.** 2009. Restraint stress in biobehavioral research: Recent developments. Neurosci Biobehav Rev **33:**1089-1098.
226. **Cavigelli SA, McClintock MK.** 2003. Fear of novelty in infant rats predicts adult corticosterone dynamics and an early death. Proc Natl Acad Sci U S A **100:**16131-16136.
227. **Dobbs CM, Vasquez M, Glaser R, Sheridan JF.** 1993. Mechanisms of stress-induced modulation of viral pathogenesis and immunity. J Neuroimmunol **48:**151-160.
228. **Dobbs CM, Feng N, Beck FM, Sheridan JF.** 1996. Neuroendocrine regulation of cytokine production during experimental influenza viral infection: effects of restraint stress-induced elevation in endogenous corticosterone. J Immunol **157:**1870-1877.
229. **Bonneau RH, Sheridan JF, Feng N, Glaser R.** 1993. Stress-induced modulation of the primary cellular immune response to herpes simplex virus infection is mediated by both adrenal-dependent and independent mechanisms. J Neuroimmunol **42:**167-176.
230. **Stark JL, Avitsur R, Hunzeker J, Padgett DA, Sheridan JF.** 2002. Interleukin-6 and the development of social disruption-induced glucocorticoid resistance. J Neuroimmunol **124:**9-15.
231. **Edgar RC.** 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics **26:**2460-2461.
232. **Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R.** 2010. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics **26:**266-267.
233. **McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P.** 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J **6:**610-618.
234. **Price MN, Dehal PS, Arkin AP.** 2010. FastTree 2--approximately maximum-likelihood trees for large alignments. PLoS One **5:**e9490.
235. **Shannon CE, Weaver W.** 1949. The mathematical theory of communication. University of Illinois Press, Urbana,.
236. **Development RCT.** R: a language and environment for statistical computing. R Foundation for Statistical Computing, Coventry, United Kingdom

237. **Greene BR, Blanchard EB, Wan CK.** 1994. Long-term monitoring of psychosocial stress and symptomatology in inflammatory bowel disease. Behav Res Ther **32:**217-226.
238. **Sun Y, Zhang M, Chen CC, Gillilland M, 3rd, Sun X, El-Zaatari M, Huffnagle GB, Young VB, Zhang J, Hong SC, Chang YM, Gumucio DL, Owyang C, Kao JY.** 2013. Stress-induced corticotropin-releasing hormone-mediated NLRP6 inflammasome inhibition and transmissible enteritis in mice. Gastroenterology **144:**1478-1487, 1487 e1471-1478.
239. **Garcia-Rodenas CL, Bergonzelli GE, Nutten S, Schumann A, Cherbut C, Turini M, Ornstein K, Rochat F, Corthesy-Theulaz I.** 2006. Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. J Pediatr Gastroenterol Nutr **43:**16-24.
240. **Thomas CM, Hong T, van Pijkeren JP, Hemarajata P, Trinh DV, Hu W, Britton RA, Kalkum M, Versalovic J.** 2012. Histamine derived from probiotic Lactobacillus reuteri suppresses TNF via modulation of PKA and ERK signaling. PLoS One **7:**e31951.
241. **Dicksved J, Schreiber O, Willing B, Petersson J, Rang S, Phillipson M, Holm L, Roos S.** 2012. Lactobacillus reuteri maintains a functional mucosal barrier during DSS treatment despite mucus layer dysfunction. PLoS One **7:**e46399.
242. **Geuking MB, Cahenzli J, Lawson MA, Ng DC, Slack E, Hapfelmeier S, McCoy KD, Macpherson AJ.** 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. Immunity **34:**794-806.
243. **Khosravi A, Yanez A, Price JG, Chow A, Merad M, Goodridge HS, Mazmanian SK.** 2014. Gut microbiota promote hematopoiesis to control bacterial infection. Cell Host Microbe **15:**374-381.
244. **Baba N, Samson S, Bourdet-Sicard R, Rubio M, Sarfati M.** 2009. Selected commensal-related bacteria and Toll-like receptor 3 agonist combinatorial codes synergistically induce interleukin-12 production by dendritic cells to trigger a T helper type 1 polarizing programme. Immunology **128:**e523-531.
245. **Ouwehand AC, Salminen S, Arvola T, Ruuska T, Isolauri E.** 2004. Microbiota composition of the intestinal mucosa: association with fecal microbiota? Microbiol Immunol **48:**497-500.
246. **Bekassy ZD, Calderon Toledo C, Leoj G, Kristoffersson A, Leopold SR, Perez MT, Karpman D.** 2011. Intestinal damage in enterohemorrhagic Escherichia coli infection. Pediatr Nephrol **26:**2059-2071.
247. **Moxley RA.** 2004. Escherichia coli 0157:H7: an update on intestinal colonization and virulence mechanisms. Anim Health Res Rev **5:**15-33.
248. **Theriot CM, Koenigsknecht MJ, Carlson PE, Jr., Hatton GE, Nelson AM, Li B, Huffnagle GB, J ZL, Young VB.** 2014. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. Nat Commun **5:**3114.
249. **Sham HP, Yu EY, Gulen MF, Bhinder G, Stahl M, Chan JM, Brewster L, Morampudi V, Gibson DL, Hughes MR, McNagny KM, Li X, Vallance BA.** 2013. SIGIRR, a negative regulator of TLR/IL-1R signalling promotes Microbiota dependent resistance to colonization by enteric bacterial pathogens. PLoS Pathog **9:**e1003539.
250. **Lone AG, Selinger LB, Uwiera RR, Xu Y, Inglis GD.** 2013. Campylobacter jejuni colonization is associated with a dysbiosis in the cecal microbiota of mice in the absence of prominent inflammation. PLoS One **8:**e75325.

251. **Ghosh S, Dai C, Brown K, Rajendiran E, Makarenko S, Baker J, Ma C, Halder S, Montero M, Ionescu VA, Klegeris A, Vallance BA, Gibson DL.** 2011. Colonic microbiota alters host susceptibility to infectious colitis by modulating inflammation, redox status, and ion transporter gene expression. Am J Physiol Gastrointest Liver Physiol **301:**G39-49.
252. **Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J.** 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut **55:**205-211.
253. **Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, Ferrante M, Verhaegen J, Rutgeerts P, Vermeire S.** 2013. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut doi:10.1136/gutjnl-2013-304833.
254. **Sokol H, Seksik P, Furet JP, Firmesse O, Nion-Larmurier I, Beaugerie L, Cosnes J, Corthier G, Marteau P, Dore J.** 2009. Low counts of Faecalibacterium prausnitzii in colitis microbiota. Inflamm Bowel Dis **15:**1183-1189.
255. **Carroll IM, Ringel-Kulka T, Siddle JP, Ringel Y.** 2012. Alterations in composition and diversity of the intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterol Motil **24:**521-530, e248.
256. **Allegretti JR, Hamilton MJ.** 2014. Restoring the gut microbiome for the treatment of inflammatory bowel diseases. World J Gastroenterol **20:**3468-3474.
257. **Kao D, Hotte N, Gillevet P, Madsen K.** 2014. Fecal Microbiota Transplantation Inducing Remission in Crohn's Colitis and the Associated Changes in Fecal Microbial Profile. J Clin Gastroenterol doi:10.1097/MCG.0000000000000131.
258. **Hermann G, Tovar CA, Beck FM, Sheridan JF.** 1994. Kinetics of glucocorticoid response to restraint stress and/or experimental influenza viral infection in two inbred strains of mice. J Neuroimmunol **49:**25-33.
259. **Crumeyrolle-Arias M, Jaglin M, Bruneau A, Vancassel S, Cardona A, Dauge V, Naudon L, Rabot S.** 2014. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. Psychoneuroendocrinology **42:**207-217.
260. **Zhang D, Kishihara K, Wang B, Mizobe K, Kubo C, Nomoto K.** 1998. Restraint stress-induced immunosuppression by inhibiting leukocyte migration and Th1 cytokine expression during the intraperitoneal infection of Listeria monocytogenes. J Neuroimmunol **92:**139-151.
261. **Park AJ, Collins J, Blennerhassett PA, Ghia JE, Verdu EF, Bercik P, Collins SM.** 2013. Altered colonic function and microbiota profile in a mouse model of chronic depression. Neurogastroenterol Motil **25:**733-e575.
262. **Goehler LE, Gaykema RP, Opitz N, Reddaway R, Badr N, Lyte M.** 2005. Activation in vagal afferents and central autonomic pathways: early responses to intestinal infection with Campylobacter jejuni. Brain Behav Immun **19:**334-344.
263. **Foster JA, McVey Neufeld KA.** 2013. Gut-brain axis: how the microbiome influences anxiety and depression. Trends Neurosci **36:**305-312.
264. **Kim KA, Gu W, Lee IA, Joh EH, Kim DH.** 2012. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. PLoS One **7:**e47713.

265. **Lozupone CA, Stombaugh J, Gonzalez A, Ackermann G, Wendel D, Vazquez-Baeza Y, Jansson JK, Gordon JI, Knight R.** 2013. Meta-analyses of studies of the human microbiota. Genome Res **23:**1704-1714.
266. **Reinhardt C, Bergentall M, Greiner TU, Schaffner F, Ostergren-Lunden G, Petersen LC, Ruf W, Backhed F.** 2012. Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. Nature **483:**627-631.
267. **Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:**881-884.
268. **Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K, Honda K.** 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. Science **331:**337-341.
269. **El Aidy S, van Baarlen P, Derrien M, Lindenbergh-Kortleve DJ, Hooiveld G, Levenez F, Dore J, Dekker J, Samsom JN, Nieuwenhuis EE, Kleerebezem M.** 2012. Temporal and spatial interplay of microbiota and intestinal mucosa drive establishment of immune homeostasis in conventionalized mice. Mucosal Immunol **5:**567-579.
270. **Williams AM, Probert CS, Stepankova R, Tlaskalova-Hogenova H, Phillips A, Bland PW.** 2006. Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse. Immunology **119:**470-478.
271. **Rehman A, Sina C, Gavrilova O, Hasler R, Ott S, Baines JF, Schreiber S, Rosenstiel P.** 2011. Nod2 is essential for temporal development of intestinal microbial communities. Gut **60:**1354-1362.
272. **Xu J, Gordon JI.** 2003. Honor thy symbionts. Proc Natl Acad Sci U S A **100:**10452-10459.
273. **Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI.** 2005. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A **102:**11070-11075.
274. **Nava GM, Friedrichsen HJ, Stappenbeck TS.** 2011. Spatial organization of intestinal microbiota in the mouse ascending colon. ISME J **5:**627-638.
275. **Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI.** 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature **444:**1027-1031.
276. **Chang JY, Antonopoulos DA, Kalra A, Tonelli A, Khalife WT, Schmidt TM, Young VB.** 2008. Decreased diversity of the fecal Microbiome in recurrent Clostridium difficile-associated diarrhea. J Infect Dis **197:**435-438.
277. **Sekirov I, Tam NM, Jogova M, Robertson ML, Li Y, Lupp C, Finlay BB.** 2008. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. Infect Immun **76:**4726-4736.
278. **Schreiber O, Petersson J, Phillipson M, Perry M, Roos S, Holm L.** 2009. Lactobacillus reuteri prevents colitis by reducing P-selectin-associated leukocyte- and platelet-endothelial cell interactions. Am J Physiol Gastrointest Liver Physiol **296:**G534-542.
279. **Barouei J, Moussavi M, Hodgson DM.** 2012. Effect of maternal probiotic intervention on HPA axis, immunity and gut microbiota in a rat model of irritable bowel syndrome. PLoS One **7:**e46051.

280. **Dickhaus B, Mayer EA, Firooz N, Stains J, Conde F, Olivas TI, Fass R, Chang L, Mayer M, Naliboff BD.** 2003. Irritable bowel syndrome patients show enhanced modulation of visceral perception by auditory stress. Am J Gastroenterol **98:**135-143.
281. **Sartor RB.** 2008. Microbial influences in inflammatory bowel diseases. Gastroenterology **134:**577-594.
282. **Garrett WS, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, Glickman JN, Glimcher LH.** 2007. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. Cell **131:**33-45.
283. **Carroll IM, Chang YH, Park J, Sartor RB, Ringel Y.** 2010. Luminal and mucosal-associated intestinal microbiota in patients with diarrhea-predominant irritable bowel syndrome. Gut Pathog **2:**19.
284. **Bajaj JS, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, Monteith P, Noble NA, Sikaroodi M, Gillevet PM.** 2012. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. Am J Physiol Gastrointest Liver Physiol **303:**G675-685.
285. **Allen RG, Lafuse WP, Powell ND, Webster Marketon JI, Stiner-Jones LM, Sheridan JF, Bailey MT.** 2012. Stressor-induced increase in microbicidal activity of splenic macrophages is dependent upon peroxynitrite production. Infect Immun **80:**3429-3437.
286. **Callaway TR, Dowd SE, Edrington TS, Anderson RC, Krueger N, Bauer N, Kononoff PJ, Nisbet DJ.** 2010. Evaluation of bacterial diversity in the rumen and feces of cattle fed different levels of dried distillers grains plus solubles using bacterial tag-encoded FLX amplicon pyrosequencing. J Anim Sci **88:**3977-3983.
287. **Williams WL, Tedeschi LO, Kononoff PJ, Callaway TR, Dowd SE, Karges K, Gibson ML.** 2010. Evaluation of in vitro gas production and rumen bacterial populations fermenting corn milling (co)products. J Dairy Sci **93:**4735-4743.
288. **Chen J, Yu Z, Michel FC, Jr., Wittum T, Morrison M.** 2007. Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. Appl Environ Microbiol **73:**4407-4416.
289. **Anderson K, Yu Z, Chen J, Jenkins J, Courtney P, Morrison M.** 2008. Analyses of Bifidobacterium, Lactobacillus, and total bacterial populations in healthy volunteers consuming calcium gluconate by denaturing gradient gel electrophoresis and real-time PCR. . Int J Probiotics Prebiotics **3:**31-36.
290. **Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP.** 2001. Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol **67:**2578-2585.
291. **Heilig HG, Zoetendal EG, Vaughan EE, Marteau P, Akkermans AD, de Vos WM.** 2002. Molecular diversity of Lactobacillus spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. Appl Environ Microbiol **68:**114-123.
292. **Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A.** 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol **97:**1166-1177.

293. **Brisbin JT, Gong J, Orouji S, Esufali J, Mallick AI, Parvizi P, Shewen PE, Sharif S.** 2011. Oral treatment of chickens with lactobacilli influences elicitation of immune responses. Clin Vaccine Immunol **18:**1447-1455.
294. **Tong J, Liu C, Summanen P, Xu H, Finegold SM.** 2011. Application of quantitative real-time PCR for rapid identification of Bacteroides fragilis group and related organisms in human wound samples. Anaerobe **17:**64-68.
295. **Head CC, Farrow MJ, Sheridan JF, Padgett DA.** 2006. Androstenediol reduces the anti-inflammatory effects of restraint stress during wound healing. Brain Behav Immun **20:**590-596.
296. **Friswell MK, Gika H, Stratford IJ, Theodoridis G, Telfer B, Wilson ID, McBain AJ.** 2010. Site and strain-specific variation in gut microbiota profiles and metabolism in experimental mice. PLoS One **5:**e8584.
297. **Hildebrand F, Nguyen TL, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, Liston A, Raes J.** 2013. Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol **14:**R4.
298. **Bouwknecht JA, Paylor R.** 2002. Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains. Behav Brain Res **136:**489-501.
299. **Julio-Pieper M, O'Mahony CM, Clarke G, Bravo JA, Dinan TG, Cryan JF.** 2012. Chronic stress-induced alterations in mouse colonic 5-HT and defecation responses are strain dependent. Stress **15:**218-226.
300. **Cuevas M, Flores I, Thompson KJ, Ramos-Ortolaza DL, Torres-Reveron A, Appleyard CB.** 2012. Stress exacerbates endometriosis manifestations and inflammatory parameters in an animal model. Reprod Sci **19:**851-862.
301. **Craven M, Egan CE, Dowd SE, McDonough SP, Dogan B, Denkers EY, Bowman D, Scherl EJ, Simpson KW.** 2012. Inflammation drives dysbiosis and bacterial invasion in murine models of ileal Crohn's disease. PLoS One **7:**e41594.
302. **Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB.** 2007. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe **2:**119-129.
303. **Collins SM, McHugh K, Jacobson K, Khan I, Riddell R, Murase K, Weingarten HP.** 1996. Previous inflammation alters the response of the rat colon to stress. Gastroenterology **111:**1509-1515.
304. **Kinsey SG, Bailey MT, Sheridan JF, Padgett DA, Avitsur R.** 2007. Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. Brain Behav Immun **21:**458-466.
305. **Bangsgaard Bendtsen KM, Krych L, Sorensen DB, Pang W, Nielsen DS, Josefsen K, Hansen LH, Sorensen SJ, Hansen AK.** 2012. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. PLoS One **7:**e46231.
306. **Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ, Danska JS.** 2013. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science **339:**1084-1088.
307. **Bennett EJ, Tennant CC, Piesse C, Badcock CA, Kellow JE.** 1998. Level of chronic life stress predicts clinical outcome in irritable bowel syndrome. Gut **43:**256-261.

308. **Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, Wekerle H, Krishnamoorthy G.** 2011. Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination. Nature **479:**538-541.
309. **Buljevac D, Hop WC, Reedeker W, Janssens AC, van der Meche FG, van Doorn PA, Hintzen RQ.** 2003. Self reported stressful life events and exacerbations in multiple sclerosis: prospective study. BMJ **327:**646.
310. **Kappelman MD, Moore KR, Allen JK, Cook SF.** 2013. Recent trends in the prevalence of Crohn's disease and ulcerative colitis in a commercially insured US population. Dig Dis Sci **58:**519-525.
311. **Bernstein MT, Graff LA, Targownik LE, Downing K, Shafer LA, Rawsthorne P, Bernstein CN, Avery L.** 2012. Gastrointestinal symptoms before and during menses in women with IBD. Aliment Pharmacol Ther **36:**135-144.
312. **Subasinghe D, Nawarathna NM, Samarasekera DN.** 2011. Disease characteristics of inflammatory bowel disease (IBD): findings from a tertiary care centre in South Asia. J Gastrointest Surg **15:**1562-1567.
313. **Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, Liang S, Zhang W, Guan Y, Shen D, Peng Y, Zhang D, Jie Z, Wu W, Qin Y, Xue W, Li J, Han L, Lu D, Wu P, Dai Y, Sun X, Li Z, Tang A, Zhong S, Li X, Chen W, Xu R, Wang M, Feng Q, Gong M, Yu J, Zhang Y, Zhang M, Hansen T, Sanchez G, Raes J, Falony G, Okuda S, Almeida M, LeChatelier E, Renault P, Pons N, Batto JM, Zhang Z, Chen H, Yang R, Zheng W, Li S, Yang H, et al.** 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature **490:**55-60.
314. **Chen P, Starkel P, Turner JR, Ho SB, Schnabl B.** 2015. Dysbiosis-induced intestinal inflammation activates tumor necrosis factor receptor I and mediates alcoholic liver disease in mice. Hepatology **61:**883-894.
315. **De Minicis S, Rychlicki C, Agostinelli L, Saccomanno S, Candelaresi C, Trozzi L, Mingarelli E, Facinelli B, Magi G, Palmieri C, Marzioni M, Benedetti A, Svegliati-Baroni G.** 2014. Dysbiosis contributes to fibrogenesis in the course of chronic liver injury in mice. Hepatology **59:**1738-1749.
316. **Duboc H, Rajca S, Rainteau D, Benarous D, Maubert MA, Quervain E, Thomas G, Barbu V, Humbert L, Despras G, Bridonneau C, Dumetz F, Grill JP, Masliah J, Beaugerie L, Cosnes J, Chazouilleres O, Poupon R, Wolf C, Mallet JM, Langella P, Trugnan G, Sokol H, Seksik P.** 2013. Connecting dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel diseases. Gut **62:**531-539.
317. **Kim YG, Udayanga KG, Totsuka N, Weinberg JB, Nunez G, Shibuya A.** 2014. Gut dysbiosis promotes M2 macrophage polarization and allergic airway inflammation via fungi-induced PGE(2). Cell Host Microbe **15:**95-102.
318. **Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, Darfeuille-Michaud A, Barnich N.** 2014. Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. Gut **63:**116-124.
319. **Galley JD, Yu Z, Kumar P, Dowd SE, Lyte M, Bailey MT.** 2014. The structures of the colonic mucosa-associated and luminal microbial communities are distinct and differentially affected by a prolonged murine stressor. Gut Microbes **5:**748-760.

320. **Ivanov, II, Frutos Rde L, Manel N, Yoshinaga K, Rifkin DB, Sartor RB, Finlay BB, Littman DR.** 2008. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe **4:**337-349.
321. **Niess JH, Adler G.** 2010. Enteric flora expands gut lamina propria CX3CR1+ dendritic cells supporting inflammatory immune responses under normal and inflammatory conditions. J Immunol **184:**2026-2037.
322. **Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI.** 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science **341:**1241214.
323. **Aronesty E.** 2011. ea-utils. http://code.google.com/p/ea-utils. Accessed
324. **Engler H, Bailey MT, Engler A, Sheridan JF.** 2004. Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. J Neuroimmunol **148:**106-115.
325. **Chiba S, Numakawa T, Ninomiya M, Richards MC, Wakabayashi C, Kunugi H.** 2012. Chronic restraint stress causes anxiety- and depression-like behaviors, downregulates glucocorticoid receptor expression, and attenuates glutamate release induced by brain-derived neurotrophic factor in the prefrontal cortex. Prog Neuropsychopharmacol Biol Psychiatry **39:**112-119.
326. **Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB, Reading NC, Villablanca EJ, Wang S, Mora JR, Umesaki Y, Mathis D, Benoist C, Relman DA, Kasper DL.** 2012. Gut immune maturation depends on colonization with a host-specific microbiota. Cell **149:**1578-1593.
327. **Philippe D, Favre L, Foata F, Adolfsson O, Perruisseau-Carrier G, Vidal K, Reuteler G, Dayer-Schneider J, Mueller C, Blum S.** 2011. Bifidobacterium lactis attenuates onset of inflammation in a murine model of colitis. World J Gastroenterol **17:**459-469.
328. **Cano PG, Santacruz A, Trejo FM, Sanz Y.** 2013. Bifidobacterium CECT 7765 improves metabolic and immunological alterations associated with obesity in high-fat diet-fed mice. Obesity (Silver Spring) **21:**2310-2321.
329. **Le TK, Hosaka T, Le TT, Nguyen TG, Tran QB, Le TH, Pham XD.** 2014. Oral administration of Bifidobacterium spp. improves insulin resistance, induces adiponectin, and prevents inflammatory adipokine expressions. Biomed Res **35:**303-310.
330. **Moya-Perez A, Romo-Vaquero M, Tomas-Barberan F, Sanz Y, Garcia-Conesa MT.** 2014. Hepatic molecular responses to Bifidobacterium pseudocatenulatum CECT 7765 in a mouse model of diet-induced obesity. Nutr Metab Cardiovasc Dis **24:**57-64.
331. **Jang MO, An JH, Jung SI, Park KH.** 2015. Refractory Clostridium difficile Infection Cured With Fecal Microbiota Transplantation in Vancomycin-Resistant Enterococcus Colonized Patient. Intest Res **13:**80-84.
332. **Youngster I, Russell GH, Pindar C, Ziv-Baran T, Sauk J, Hohmann EL.** 2014. Oral, capsulized, frozen fecal microbiota transplantation for relapsing Clostridium difficile infection. JAMA **312:**1772-1778.
333. **Larsen N, Vogensen FK, van den Berg FW, Nielsen DS, Andreasen AS, Pedersen BK, Al-Soud WA, Sorensen SJ, Hansen LH, Jakobsen M.** 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS One **5:**e9085.

334. **Zhang YZ, Li YY.** 2014. Inflammatory bowel disease: pathogenesis. World J Gastroenterol **20:**91-99.
335. **Kamada N, Nunez G.** 2014. Regulation of the immune system by the resident intestinal bacteria. Gastroenterology **146:**1477-1488.
336. **Liu Y, Fatheree NY, Mangalat N, Rhoads JM.** 2012. Lactobacillus reuteri strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-kappaB signaling in the intestine. Am J Physiol Gastrointest Liver Physiol **302:**G608-617.
337. **Jan RL, Yeh KC, Hsieh MH, Lin YL, Kao HF, Li PH, Chang YS, Wang JY.** 2012. Lactobacillus gasseri suppresses Th17 pro-inflammatory response and attenuates allergen-induced airway inflammation in a mouse model of allergic asthma. Br J Nutr **108:**130-139.
338. **Yoda K, He F, Kawase M, Miyazawa K, Hiramatsu M.** 2014. Oral administration of Lactobacillus gasseri TMC0356 stimulates peritoneal macrophages and attenuates general symptoms caused by enteropathogenic Escherichia coli infection. J Microbiol Immunol Infect **47:**81-86.
339. **Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, Angenent LT, Knight R, Ley RE.** 2012. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. ISME J **6:**94-103.
340. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-410.
341. **Kverka M, Zakostelska Z, Klimesova K, Sokol D, Hudcovic T, Hrncir T, Rossmann P, Mrazek J, Kopecny J, Verdu EF, Tlaskalova-Hogenova H.** 2011. Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. Clin Exp Immunol **163:**250-259.
342. **Belzer C, Gerber GK, Roeselers G, Delaney M, DuBois A, Liu Q, Belavusava V, Yeliseyev V, Houseman A, Onderdonk A, Cavanaugh C, Bry L.** 2014. Dynamics of the microbiota in response to host infection. PLoS One **9:**e95534.
343. **Collins JW, Keeney KM, Crepin VF, Rathinam VA, Fitzgerald KA, Finlay BB, Frankel G.** 2014. Citrobacter rodentium: infection, inflammation and the microbiota. Nat Rev Microbiol **12:**612-623.
344. **Walker AW, Sanderson JD, Churcher C, Parkes GC, Hudspith BN, Rayment N, Brostoff J, Parkhill J, Dougan G, Petrovska L.** 2011. High-throughput clone library analysis of the mucosa-associated microbiota reveals dysbiosis and differences between inflamed and non-inflamed regions of the intestine in inflammatory bowel disease. BMC Microbiol **11:**7.
345. **De Fazio L, Cavazza E, Spisni E, Strillacci A, Centanni M, Candela M, Pratico C, Campieri M, Ricci C, Valerii MC.** 2014. Longitudinal analysis of inflammation and microbiota dynamics in a model of mild chronic dextran sulfate sodium-induced colitis in mice. World J Gastroenterol **20:**2051-2061.
346. **Mar JS, Nagalingam NA, Song Y, Onizawa M, Lee JW, Lynch SV.** 2014. Amelioration of DSS-induced murine colitis by VSL#3 supplementation is primarily associated with changes in ileal microbiota composition. Gut Microbes **5:**494-503.
347. **Ogden CL, Carroll MD, Kit BK, Flegal KM.** 2012. Prevalence of obesity and trends in body mass index among US children and adolescents, 1999-2010. JAMA **307:**483-490.

348. **Flegal KM, Carroll MD, Ogden CL, Curtin LR.** 2010. Prevalence and trends in obesity among US adults, 1999-2008. JAMA **303:**235-241.
349. **Linabery AM, Nahhas RW, Johnson W, Choh AC, Towne B, Odegaard AO, Czerwinski SA, Demerath EW.** 2013. Stronger influence of maternal than paternal obesity on infant and early childhood body mass index: the Fels Longitudinal Study. Pediatr Obes **8:**159-169.
350. **Whitaker KL, Jarvis MJ, Beeken RJ, Boniface D, Wardle J.** 2010. Comparing maternal and paternal intergenerational transmission of obesity risk in a large population-based sample. Am J Clin Nutr **91:**1560-1567.
351. **Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI.** 2005. Host-bacterial mutualism in the human intestine. Science **307:**1915-1920.
352. **Coates ME, Ford JE, Harrison GF.** 1968. Intestinal synthesis of vitamins of the B complex in chicks. Br J Nutr **22:**493-500.
353. **Verdam FJ, Fuentes S, de Jonge C, Zoetendal EG, Erbil R, Greve JW, Buurman WA, de Vos WM, Rensen SS.** 2013. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obesity (Silver Spring) **21:**E607-615.
354. **Ley RE, Turnbaugh PJ, Klein S, Gordon JI.** 2006. Microbial ecology: human gut microbes associated with obesity. Nature **444:**1022-1023.
355. **Park DY, Ahn YT, Park SH, Huh CS, Yoo SR, Yu R, Sung MK, McGregor RA, Choi MS.** 2013. Supplementation of Lactobacillus curvatus HY7601 and Lactobacillus plantarum KY1032 in diet-induced obese mice is associated with gut microbial changes and reduction in obesity. PLoS One **8:**e59470.
356. **Favier CF, de Vos WM, Akkermans ADL.** 2003. Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe **9:**219-229.
357. **Collado MC, Isolauri E, Laitinen K, Salminen S.** 2010. Effect of mother's weight on infant's microbiota acquisition, composition, and activity during early infancy: a prospective follow-up study initiated in early pregnancy. Am J Clin Nutr **92:**1023-1030.
358. **Dowd SE, Wolcott RD, Sun Y, McKeehan T, Smith E, Rhoads D.** 2008. Polymicrobial nature of chronic diabetic foot ulcer biofilm infections determined using bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP). PLoS One **3:**e3326.
359. **Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C.** 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol **31:**814-821.
360. **Parks DH, Tyson GW, Hugenholtz P, Beiko RG.** 2014. STAMP: Statistical analysis of taxonomic and functional profiles. Bioinformatics doi:10.1093/bioinformatics/btu494.
361. **Benjamini YH, Y. .** 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological) **57:**289-300.
362. **Koupil I, Toivanen P.** 2008. Social and early-life determinants of overweight and obesity in 18-year-old Swedish men. Int J Obes (Lond) **32:**73-81.
363. **Stuebe AM, Forman MR, Michels KB.** 2009. Maternal-recalled gestational weight gain, pre-pregnancy body mass index, and obesity in the daughter. Int J Obes (Lond) **33:**743-752.

364. **Thompson AL.** 2013. Intergenerational impact of maternal obesity and postnatal feeding practices on pediatric obesity. Nutr Rev **71 Suppl 1:**S55-61.
365. **Lam YY, Ha CW, Campbell CR, Mitchell AJ, Dinudom A, Oscarsson J, Cook DI, Hunt NH, Caterson ID, Holmes AJ, Storlien LH.** 2012. Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. PLoS One **7:**e34233.
366. **Clarke SF, Murphy EF, O'Sullivan O, Ross RP, O'Toole PW, Shanahan F, Cotter PD.** 2013. Targeting the microbiota to address diet-induced obesity: a time dependent challenge. PLoS One **8:**e65790.
367. **Martinez I, Lattimer JM, Hubach KL, Case JA, Yang J, Weber CG, Louk JA, Rose DJ, Kyureghian G, Peterson DA, Haub MD, Walter J.** 2013. Gut microbiome composition is linked to whole grain-induced immunological improvements. ISME J **7:**269-280.
368. **Faith MSaKTVE.** 2006. Social, Environmental, and Genetic Influences on Obesity and Obesity-Promoting Behaviors: Fostering Research Integration, p 232-235. *In* Hernandez LMaBDG (ed), Genes, Behavior, and the Social Environment: Moving Beyond the Nature/Nurture Debate. National Academies Press.
369. **Anzman SL, Rollins BY, Birch LL.** 2010. Parental influence on children's early eating environments and obesity risk: implications for prevention. Int J Obes (Lond) **34:**1116-1124.
370. **Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, Brown D, Stares MD, Scott P, Bergerat A, Louis P, McIntosh F, Johnstone AM, Lobley GE, Parkhill J, Flint HJ.** 2011. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J **5:**220-230.
371. **Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI.** 2012. Human gut microbiome viewed across age and geography. Nature **486:**222-227.
372. **Brunner Huber LR.** 2007. Validity of self-reported height and weight in women of reproductive age. Matern Child Health J **11:**137-144.
373. **Holland E, Moore Simas TA, Doyle Curiale DK, Liao X, Waring ME.** 2013. Self-reported pre-pregnancy weight versus weight measured at first prenatal visit: effects on categorization of pre-pregnancy body mass index. Matern Child Health J **17:**1872-1878.
374. **Cryan JF, Dinan TG.** 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci **13:**701-712.
375. **Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, Pettersson S.** 2012. Host-gut microbiota metabolic interactions. Science **336:**1262-1267.
376. **Borre YE, Moloney RD, Clarke G, Dinan TG, Cryan JF.** 2014. The impact of microbiota on brain and behavior: mechanisms & therapeutic potential. Adv Exp Med Biol **817:**373-403.
377. **Machiels K, Joossens M, Sabino J, De Preter V, Arijs I, Eeckhaut V, Ballet V, Claes K, Van Immerseel F, Verbeke K, Ferrante M, Verhaegen J, Rutgeerts P, Vermeire S.** 2014. A decrease of the butyrate-producing species Roseburia hominis and Faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut **63:**1275-1283.