EXPLORING HOST GENETIC DIFFERENCES IN GASTROINTESTINAL MICROBIOTA AND HOMEOSTASIS, THROUGH THE PRODUCTION OF FECAL MIRNA

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Abstract

Research has shown that our gut microbiota confers many beneficial functions, including aiding the development of the immune system, metabolism, modulating stress reactivity and behaviour. The diverse population of the gut microbiota has been shown to be heterogeneous between individuals, with host genetic factors emerging as a contributor to gut microbiota composition. Recent work suggests that microRNA may act as a mediator of communication between the host and resident gut microbiota. Here we explore host genetic differences in gut microbiota composition and fecal miRNA profiles in two inbred mouse strains BALB/C and C57BL/6, in relation to gastrointestinal homeostasis. Furthermore, we evaluate the role of host genetics in response to perturbation of the gut microbiota using broad-spectrum antibiotic treatment. Distinct differences in the gut microbiota composition evaluated by fecal 16s rRNA gene sequencing between BALB/c and C57BL/6 mice were found with notable significant differences in genera Prevotella, Alistipes, Akkermansia and Ruminococcus. Significant host genetic differences were also observed in fecal miRNA profiles evaluated using the nCounter Nanostring platform. A BLASTn analysis was used to identify conserved fecal miRNA target regions in bacterial metagenomes, which identified numerous bacterial gene targets. Of those miRNA targets that were conserved in our dataset, 14 significant correlations were found between fecal miRNA and predicted taxa relative abundance. Treatment with broad-spectrum antibiotics for a period of 2 weeks resulted in BALB/c mice exhibiting a decrease in barrier permeability while C56BL/6 barrier permeability remained unchanged, demonstrating a host-specific physiological response to antibiotics at the gastrointestinal barrier. Differential response to antibiotics was also observed in the expression of barrier

regulating genes in both host strains. Individual taxa were found to respond differentially by host strain, with *Parabacteroides* and *Bacteroides* associating with changes in barrier function. Together these findings suggest that host genetics play a role in determining the host-microbe relationship in both healthy homeostatic conditions and altered microbial conditions.

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List of Abbreviations

Germ-Free	(GF)
Specific pathogen-free mice	(SPF)
Brain-derived neurotrophic factor	(BDNF)
Short chain fatty acid	(SCFA)
Mucin-2	(muc-2)
Claudin	(Cldn)
Occuldin	(Ocln)
Tight junction	(TJ)
Zonula Occludens	(ZO)
Quantitative trait loci	(QTL)
Lipopolysaccharide	(LPS)
Genome-wide association studies	(GWAS)
Monozygotic	(MZ)
Dizygotic	(DZ)
MicroRNA	(MiRNA)
Immunoglobulin	(Ig)
Toll-like receptor 4	(TLR-4)
Intestinal epithelial	(IEC)
Human Microbiome Project	(HMP)
Unconjugated fluorescein isothiocyanate	(FITC)
Principal Coordinate Analysis	(PCoA)

Erythromycin	(ERY)
Operational taxonomic units	(OTUs)
False discovery rate	(FDR)
Principal Component Analysis	(PCA)
3’ untranslated regions	(UTR)
Extracellular matrix	(ECM)

Declaration of academic achievement:

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1.0 Introduction

1.1 The Gut Microbiome

The trillions of microorganisms that colonize the human body have existed long before the human species, and thus our co-evolution and development has been intrinsically linked. The largest population of microorganisms that reside within the human body are found in the gastrointestinal tract and are often referred to as the gut microbiota (Gill et al., 2006). The term microbiota is used to describe the organisms themselves, which harbour diverse genomes collectively known as the microbiome (Turnbaugh et al., 2007). Until recently, the gut microbiota was thought of by the majority of scientists as pathogenic or commensal with the host, providing the benefit of nutrients and an optimal environment to grow but, received no benefit in return. Within the last two decades, research focused on exploring the gut microbiota's role in human health and development has challenged this perception and revealed a more complex mutualistic and symbiotic relationship. Research has uncovered several functional contributions made by the gut microbiota to the host. A major contribution is the regulation and augmentation to energy metabolism of the host (Backhed et al., 2004) through the harvesting of otherwise inaccessible nutrients and carbohydrates, as well as regulation of fat storage and lipid metabolism (Murphy et al., 2010; Velagapudi et al., 2010). Additionally, it has been demonstrated that the gut microbiota is intrinsically linked to the development of the immune system. Germ-free (GF) mice, which are devoid of microbiota, exhibit extensive deficits in the gut-associated lymphoid tissues (Macpherson and Uhr, 2004), antibody production (Lamouse-Smith et al., 2011), T-cell development (Ostman et al., 2006) and differentiation as compared to healthy specific pathogen-free mice (SPF). Deficits in immune system development due to the

absence of microbiota persist even after conventionalization with normal gut microbiota, indicating a role for the microbiota in programing immune response early in development (Hansen et al., 2012). Interestingly, it has been identified that particular populations of the gut microbiota, such as spore-forming genus *Clostridium*, promote the induction of colonic regulatory T-cells, which play a critical role in mediating immune homeostasis (Atarashi et al., 2011). Lastly, the gut microbiota population play a protective role towards the host, preventing the invasion of pathogens by limiting available resources and modulating the host immune system. Due to the complex role the gut microbiome plays in human development, changes to the composition of gut microbiota are often linked with health outcomes, with recent work identifying the role of gut microbiota in intestinal and metabolic disorders such as ulcerative colitis (Macfarlane et al., 2005), irritable bowel syndrome (Collins et al., 2009), type 2 diabetes (Zhang et al., 2013) and obesity (Ley et al., 2006).

1.2 Gut-Brain Axis

The exploration into the influences of gut microbiota on human health has revealed numerous complex connections, one of the more intriguing associations comes from the emerging evidence of a gut-brain axis. A seminal study by Sudo and colleagues in 2004 showed that germ-free mice have alterations to the hypothalamic-pituitary axis, exhibiting an exaggerated response to stress, which was restored with colonization of *Bifidobacterium infantis* after 5 weeks of life, but not 9 (Sudo et al., 2004). The work that followed this influential study explored the role of gut microbiota in affecting behaviour. For example, Lyte and colleagues provided evidence of gut microbiota's influence on behaviour, mediated by vagal sensory neurons (Lyte et al., 2006). Additionally, work using the germ-

free mouse model found changes in anxiety-like behaviour and alterations to brain-derived neurotropic factor BDNF, as well as alterations to serotonin receptor expression (Neufeld et al., 2011). Treatment with broad-spectrum antibiotics was also found to induce behavioural changes, which were accompanied by changes to BDNF protein levels in the hippocampus and amygdala (Bercik et al., 2011). Heijtz and colleagues confirmed the reduced anxiety-like behavioural phenotype in the GF model and provided additional evidence of the effect of early life colonization of GF mice with normalization of the behavioural phenotype and alteration of synaptic plasticity genes (Diaz Heijtz et al., 2011). Sex-dependent changes in the hippocampus of germ-free mice were exhibited by Clarke and colleagues in 2013, and their work reinforced the existence of a critical window for conventionalization of germ-free mice to ameliorate behavioural changes (Clarke et al., 2013). More recent studies have explored the role of the gut microbiota in interacting with brain microstructures and resident microglia cells (Erny et al., 2015; Fernandez-Real et al., 2015). Work by Erny et al in 2015, showed that loss of microbiota results in immature microglia cells with impaired immune responses, and that these changes were mediated through the signalling of bacterial metabolites short chain fatty acids (SCFA). Indicating the role of metabolites and immune signalling in gut-brain communication (Erny et al., 2015). Novel work has begun to explore the use of psychobiotics, a term used to describe probiotic strains and prebiotic products that may produce positive psychological effects when given in adequate amounts (Dinan et al., 2013; Sarkar et al., 2016). Both human and animal work has begun to test the effect of probiotic strains, focusing on genera *Bifidobacterium* and *Lactobacillus*, evaluating their effectiveness in treating mood disorders (Foster et al., 2017; Wallace and Milev, 2017). The numerous studies focused on the gut-brain axis have slowly

begun to elucidate the mechanistic routes of the bi-directional communication from the gut to the brain. There is now evidence to support the involvement of the vagus nerve, enteric nervous system, immune and endocrine signalling, as well as metabolite signalling in mediating gut-brain communication (Cryan and Dinan, 2012). While evidence suggests the involvement of these numerous systems, the complete mechanism has yet to be elucidated.

1.3 Gastrointestinal Barrier Microbe Interaction

The gastrointestinal tract contains the highest density of microbial cells in the human body. Therefore, the interface at the gastrointestinal barrier between the gut microbiota and the rest of the body is a critical site for understanding the relationship between the host and its microbiota. The distribution of microbial cells is uneven throughout the gastrointestinal tract, with the highest numbers found in the distal colon region, with a gradient of decreasing density moving upward along the tract. This disparity in density is also accompanied by differences in microbial populations and diversity, with the small intestine, large intestine, colon and stomach consisting of regionally specific populations of microbiota (Eckburg et al., 2005; Lu et al., 2014). Despite these differences in populations, they all share a common interaction with the gastrointestinal barrier.

The intestinal barrier is comprised of intestinal epithelial cells (IECs), secretory goblet cells, Paneth cells, and Enteroendocrine cells. Together they express a wide range of genes that aid in maintaining homeostasis (Wells et al., 2017). The barrier is also maintained and protected by the production of a mucus layer, which is formed by the secretion of highly glycosylated mucin proteins predominantly consisting of mucin-2 (muc-2)(Johansson et al., 2008). The distribution of mucus thickness and functionality is regionally specific. The small intestine has a diffuse layer of mucus, with a high

concentration of antimicrobial peptides close to the epithelium (Johansson et al., 2008). Contrastingly, the large intestine employs a densely striated mucus layer, which forms a physical barrier between the microbiota and epithelial cells (Johansson et al., 2008). The intestinal epithelial barrier regulates the absorptions and diffusion of ions, water and small molecules by controlling paracellular permeability through the expression of apical tight junction complexes (Van Itallie and Anderson, 2006). Tight junction (TJ) complexes consist of several transmembrane proteins such as claudins (cldn), occludens (ocln), and junction adhesion molecules (Arumugam et al., 2011), as well as scaffold proteins such as zonula occludens (ZO) (Lu et al., 2013). Disruption of TJ proteins have been shown to largely affect paracellular permeability, with increases in paracellular permeability linked disruption of the intestinal barrier and increased inflammation (Nusrat et al., 2000). Alterations in permeability are thought to facilitate interaction between bacteria and the host through enhanced signalling and physical translocation, which may contribute to the etiology of diseases in which gut microbiota have been implicated (Knoop et al., 2016).

Bacterial products have been found to have significant effects on barrier permeability. A study by Guo et al 2013 exhibited the effect of lipopolysaccharide (LPS), a common bacterial endotoxin, on barrier permeability, with exposure to LPS resulting in increased barrier permeability (Guo et al., 2013). Guo also showed that this process was immune mediated, with increases in TLR-4 and immune protein CD14 critical for the effect of LPS on barrier permeability. Interestingly, certain bacteria have been found to have positive effects on barrier permeability and function. The genus *Bifidobacterium* has been found to stabilize claudins and tight junction complexes in the mouse model of necrotizing enterocolitis (Bergmann et al., 2013). Recent work has also shown that treatment with

Bifidobacterium causes an up-regulation of ZO-1, cldn3 and ocln, which attenuates LPS treatment-related changes to barrier permeability and decreases inflammation in both cultured enterocyte monolayer and a rat model of necrotizing enterocolitis (Ling et al., 2016). Additional genera such as *Lactobacillus* (Yu et al., 2015) and *Bacteroides* (Hsiao et al., 2013) have also been positively associated with barrier function, although the complete mechanism for this positive relationship remains poorly understood. One mechanism by which bacteria influence barrier permeability is through the production of short chain fatty acids (SCFA), which are bacterial fermentation end products consisting mainly of acetate, butyrate and propionate (Peng et al., 2009). Research has shown the butyrate has a positive effect on barrier permeability and causes an upregulation of tight junction proteins through the activation of the AMP-activated protein kinase (AMPK) pathway (Peng et al., 2009). Additionally, it has been shown that butyrate can prevent the negative effect of LPS on barrier permeability through down-regulation of TLR-4 and inhibition of LPS downregulation of Akt/mTOR pathway (Yan and Ajuwon, 2017). It is important to note that not all bacteria can produce SCFAs. Furthermore, only a subset of SCFA producers are known to produce butyrate, therefore it is critical to understand the impact of the compositional changes to the gut microbiota, as well as explore individual taxa differences and how they relate to host specific changes.

1.4 Host Genetics

The advent of next generation sequencing technology has allowed investigators to retrieve compositional data for individual microbiomes by 16S rRNA sequencing, greatly improving our overall understanding of the microbiome's impact on host health and development. These advances in techniques and their subsequent use have revealed the

vast heterogeneity of the microbiome (Huttenhower et al., 2012), as well as allowed researchers to explore numerous factors that can influence microbiome composition. Two dominant factors have emerged as the strongest contributors to determining the gut microbiota composition: host genetic factors (Benson et al., 2010; Goodrich et al., 2016; Goodrich et al., 2014) and environmental factors such as diet (Cotillard et al., 2013). The impact of host genetics on the human gut microbiome was first addressed using twin studies (Goodrich et al., 2014; Turnbaugh et al., 2009). In 2014, Goodrich et al. demonstrated significant differences between monozygotic (MZ) and dizygotic (DZ) twins' gut microbiomes, showing that MZ twins had more similar gut microbiomes than DZ (Goodrich et al., 2014). In addition to reaching significance, Goodrich and colleagues were also able to estimate the heritability of individual taxa and demonstrated that within the UK twin data set, the family Christensenellaceae was the most heritable taxon. This study assessed not only the host genetic impact but also, the effect of an early shared environment on the microbiome. By evaluating the heritability of individual taxa, Goodrich and colleagues were able to demonstrate host genetic effects across various taxa. Some individual taxa were strongly impacted by host genetics, while others identified were driven by environmental factors (Goodrich et al., 2016; Goodrich et al., 2014).

Work by Davenport et al 2015 and Bonder et al 2016, both utilizing genome-wide association studies (GWAS), confirmed the relationship between human genetics and microbial population. Specifically, they identified a relationship between lactase encoding gene LCT single nucleotide polymorphism and the presence of the genus *Bifidobacterium* (Bonder et al., 2016; Davenport et al., 2015). More recent work has utilized data collected as part of the Human Microbiome Project (HMP), which has further established the

relationship between host genetics and microbial composition, as well as providing evidence of site-specific host genetic control (Kolde et al., 2018).

Many animal studies have also been performed to evaluate the role of host genetics on the microbiome, as they can strongly control erroneous environmental factors. Benson et al in 2010 were the first to show that the composition of the gut microbiota behaves as a polygenic trait in the murine model (Benson et al., 2010). They identified 18 host quantitative trait loci (QTL) that correlated to relative abundances of microbial groups(Benson et al., 2010). Many of the QTL identified by Benson et al. in 2010 have been replicated (Leamy et al., 2014; Org et al., 2015). While other studies have focused on host genetic production of secretory IgA and its relationship to microbiota composition. A study by Fransen and colleagues revealed a distinct difference in IgA production between inbred strains of mice BALB/c and C57Bl/6, which related directly to differences observed in gut microbiota composition (Fransen et al., 2015). Despite the growing evidence indicating the role of host genetics in microbial composition, the underlying mechanisms of how host genetics influences the gut microbiome remains unclear.

1.5 MiRNA relationship with microbiota

One proposed mechanism for genetic regulation of the microbiota comes from exploring the role of small non-coding microRNA (miRNA). MicroRNA are non-coding 18-22 nucleotide long RNA molecules that regulate genes through selective binding to mRNA targets in the 3'-untranslated region, resulting in repression of translation or degradation of the transcript (Djuranovic et al., 2012). The degree to which miRNA regulates a target depends on the level of complementarity between the miRNA sequence and the target sequence (Bartel, 2004). Due to the ability of miRNA to bind to only partially

complementary sequences, a single miRNA can have numerous targets. MicroRNAs have been determined to play numerous roles in physiological processes such as development, cell proliferation, apoptosis and cancer, however, their role in the regulation of microbial and intestinal cell communication is just starting to be explored.

1.5.1 Fecal MIRNA

A study by Liu and colleagues in 2016 demonstrated the role of miRNA in regulating the gut microbiota and exhibited the potential of host-derived miRNA to directly impact bacterial gene expression (Liu et al., 2016). They provided evidence of miRNAs presence in the fecal sample of both mice and humans, with 17 of the 50 most abundant fecal miRNA from each species overlapping, providing promising translational targets. This work showed that miRNA from intestinal epithelial cells, Paneth cells and Goblet cells were exported in to the lumen by means of exosomes, a process known as exosomal miRNA transfer (Liu et al., 2016; Valadi et al., 2007). They also identified and quantified the present miRNA in the fecal samples by utilizing a miRNA profile hybridization assay technique referred to as nCounter Nanostring. To demonstrate miRNA's role in gut homeostasis, Liu and colleagues removed Dicer, a key miRNA- processing enzyme, which led to the loss of mature miRNA and produced significant alterations to gut microbiota composition and barrier regulating tight junction genes. Additionally, they evaluated fecal miRNA's ability to target bacterial genes through sequence similarity, showing that human miRNA miR-515-5p and miR-1226-5p are capable of entering bacterial cells and regulating bacterial gene expression in vitro (Liu et al., 2016). While Liu and colleagues observed an upregulation of the targeted bacterial genes, upregulation of eukaryotic genes by miRNA is less common but has also been shown to occur (Vasudevan, 2012), therefore it is plausible

for eukaryotic miRNA to have an up regulatory effect on bacterial genes in a similar manner. They provided additional evidence of this relationship between miRNA and gut microbiota by feeding miR-1226-5p mimics through drinking water to wild-type mice, which led to an increase in relative abundance of the predicted target *E.coli*. This demonstrates a plausible mechanism for direct genetic influence on microbial populations via host-derived miRNA, but the details have yet to be explored.

1.5.2 Intestinal miRNA relationship to microbiota

The relationship between the gut microbiota and intestinal miRNA expression has revealed a specific mechanism of control and communication between the microbiota and the host. Using a germ-free mouse model Dalmasso and colleagues found that the microbiota controls host miRNA expression, with nine differentially expressed miRNA between GF mice and colonized mice (Dalmasso et al., 2011). A similar study using GF and SPF mice found 16 differentially expressed miRNA, and found that the predicted targets of the altered miRNAs are responsible for maintaining the gastrointestinal barrier (Singh et al., 2012). Prior work has identified intestinal miRNAs as master regulators of gastrointestinal homeostasis, revealing miRNAs role in intestinal epithelial cell differentiation, architecture and barrier function (McKenna et al., 2010). As previously mentioned, the gastrointestinal barrier is a critical site for communication between the host and gut microbiota, and current work now suggests that the communication between the microbiota and the gastrointestinal barrier may be mediated through miRNA production. Individual IEC subtypes have been found to have differing miRNA profiles, as well as specific levels of sensitivity to microbial exposure (Peck et al., 2017). Recent work has revealed that IEC expression of mir-21-5p is responsive to microbiota exposure, with

expression induced through microbial activation of Toll-like receptors 2 and 4 (Nakata et al., 2017). Mir-21-5p was also found to regulate intestinal permeability indirectly through downregulation of ARF4, a small GTPase (Nakata et al., 2017). Often alterations at the gastrointestinal barrier due to microbiota exposure has been found to be immune-mediated, with mounting evidence providing a role of miRNA is facilitating this communication.

1.5.3 MiRNA responsive role in immune system modulation of gut microbiota

The gastrointestinal tract is exposed to trillions of foreign microbial cells and yet surprisingly, under normal physiological conditions, the host immune system forms a homeostatic relationship with the resident microbiota (Hooper and Macpherson, 2010). Recent evidence has found miRNAs to act as critical regulators of the immune system both in an immune tolerance capacity and regulatory response to pathogenic exposure. A specific example of this, is the negative regulation of mir-10a by the commensal microbiota (Xue et al., 2011). Mir-10a is downregulated through TLR signalling, which leads to a regulation of critical cytokines IL-12 and IL-23 which have known effects on the development of Th1 and Th17 helper T- cells, and subsequently, gut immune homeostasis (Xue et al., 2011). Several other intestinal and macrophage miRNAs have been found to be under the control of TLR signalling and cytokine production such as let-7 (Schulte et al., 2011), miR-155 (Tili et al., 2007), miR-146a/b (Miyata et al., 2015; Taganov et al., 2006), miR-9 (Bazzoni et al., 2009) all of which have been found to play critical roles in host-microbe immune response. A study by Schulte et al 2011 found that let-7a acts as a post-transcriptional brake for the production of IL-6 and IL-10 (Schulte et al., 2011). However, activation of TLR-4 by Salmonella results in a decrease of let-7a and subsequent increase in

production of cytokines, thus enabling an appropriate host immune response to the pathogen (Schulte et al., 2011). A recent study in 2018 also found evidence for the role of mir-146a in controlling pathogenic infection of *Listeria monocytogenes*, as well as controlling gut microbiota composition (Du et al., 2018). Mice deficient in miR-146a were found to be resistant to infection and had significant differences in gut microbiota composition, which were beneficial to the host, resulting in reduced infection (Du et al., 2018). Work by Taibi and colleagues found a specific role of mir-148 in mediating the anti-inflammatory response to *Bifidobacterium.bifidum* MIMBb75, by the downregulation of an immune system mediated transcription factor EPAS1, reinforcing the positive modulatory effect of the strain (Guglielmetti et al., 2011; Taibi et al., 2017).

1.5.4 Gut microbiota and Brain connection through regulation of miRNA

The aforementioned gut-brain axis has multiple routes of bidirectional communication, and the use of the germ-free mouse model has confirmed the role of the microbiota in determining anxiety-like behaviours along with the identification of a critical window for conventionalization (Neufeld et al., 2011;Diaz Heijtz et al., 2011; Bercik et al., 2011). Additional work using a combination of animal and clinical evidence has now revealed that the gut microbiota may be associated with other psychiatric conditions, with numerous links made to Major Depressive Disorder (Akkasheh et al., 2016; Jiang et al., 2015; Kelly et al., 2016; Naseribafrouei et al., 2014; Zheng et al., 2016). The development of anxiety-like behaviour, as well as depressive-like behaviours in the mouse model, is accompanied by a number of changes to gene expression in the hippocampus, prefrontal cortex (PFC) and amygdala (Hoban et al., 2016; Neufeld et al., 2011; Stilling et al., 2015). Work within the last year using germ-free mice has now revealed a relationship between

the microbiome and the expression of miRNA in these brain regions. A study by Chen and colleagues found that GF mice had a total of 50 altered hippocampal miRNAs, with 17 miRNAs increased and 33 miRNAs down-regulated as compared to SPF mice (Chen et al., 2017). Colonization of adolescent GF mice resulted in the normalization of 2 upregulated and 5 downregulated miRNA in GF mice, and altered the expression of 139 mRNA, but failed to normalize the decrease anxiety phenotype of the GF mouse (Chen et al., 2017). Interestingly a pathway analysis of miRNA restored by colonization revealed that metabolic processes are the most significantly affected pathways; previous work has linked alterations to both metabolism and the microbiota with depression (Chen et al., 2017; Zheng et al., 2016). Additional evidence of the gut microbiota-brain-miRNA connection was provided by Hoban and colleagues, who showed alterations in miRNA expression in the PFC and amygdala in both GF mice and antibiotically treated rats (Hoban et al., 2017). Intriguingly they found that the differentially expressed miRNA had a high degree of regionality, with only a few miRNAs normalizing with conventionalization. Mir-183-5p and Mir-182-5p were found to be downregulated in the amygdala of GF mice and normalized with conventionalization (Hoban et al., 2017). Both these miRNAs have been linked to amygdala dependant fear and stress output (Griggs et al., 2013; Meerson et al., 2010), indicating a role for microbiota in controlling these behaviours (Hoban et al., 2017). Mir-206-3p a known regulator of BDNF (Miura et al., 2012) was also found to be responsive to microbiota, demonstrated by alterations in both GF mice and in antibiotically treated rats, indicating a potential mechanistic role for miRNA in mediating the gut-brain behaviour effect observed by Neufeld et al. 2011 using GF-mice and Bercik et al. 2011 in antibiotic-treated mice.

1.5.5 Fecal miRNA as biomarkers

As outlined above miRNA play a critical role in almost all aspects of communication and cellular processes, from cellular structure to immune response, intestinal permeability and neurotrophic expression. Mounting evidence supports miRNA role in regulating alterations to gene expression in disease states. Therefore, due to their complex role in homeostasis, peripheral miRNAs are now being explored as novel biomarkers in numerous diseases. The identification of novel biomarkers for both disease states and normal biological differences between healthy states serves to provide valuable information for diagnosis, monitoring treatment response, as well as identifying biological differences underlying variability in symptomology. Currently the use of fecal miRNA as a biomarker has been identified for uses in colorectal cancer (Link et al., 2010), ulcerative colitis and Crohn's disease (Schonauen et al., 2018), all of which have tentative links to alteration in gut microbiota compositions. Previous work performed by the Foster lab demonstrated that inbred mouse strains BALB/c, C57BL/6 and FvB have distinct anxiety-like behavioural phenotypes, which are accompanied by unique gut microbiota composition. It is proposed here that differences in gut microbiota composition along with behavioural differences between inbred mouse strains BALB/c and C57Bl/6 may be reflective of differing fecal miRNA levels.

1.6 Antibiotic studies

The use of broad-spectrum antibiotics to deplete or alter the gut microbiota composition is a common alternative method to the germ-free mouse model used to study the causal relationship between the gut microbiota and host outcomes. As mentioned previously, the germ-free model has numerous alterations in host physiology, with gross alterations to the gastrointestinal tract architecture, immune system, and brain chemistry.

It additionally represents a paradigm not found in nature, therefore the translational value of the germ-free model is limited. Work using a combination of antibiotics has replicated the behavioural and neurochemical changes observed in the germ-free model (Bercik et al., 2011). Antibiotic use not only affects the microbiota composition and diversity, but also has an impact on host physiology and processes (Perez-Cobas et al., 2013). Work in rodent models has found that differing classes of antibiotics have varying effects on the gastrointestinal barrier, with metronidazole exhibiting a direct effect on the host mucus layer (Wlodarska et al., 2011), and bacitracin and neomycin resulting in increased expression of tight junctions (Nevado et al., 2015). Short-term use has been linked to a decrease in paracellular permeability, which may be indicative of the occasional beneficial effects observed with short-term use of antibiotics (Nevado et al., 2015; Tulstrup et al., 2015). While extensive use of antibiotics has been linked to more detrimental effects, such as prolonged changes to microbiota communities resulting in shifts to the microbiome. Metagenomic analysis revealed prolonged antibiotic use induced changes to metabolite profiles and SCFA production, as well as induces host changes in inflammatory tone and metabolism (Choo et al., 2017). There is also increasing evidence of antibiotic's impact on behaviour (Bercik et al., 2011; Desbonnet et al., 2015; Frohlich et al., 2016; Leclercq et al., 2017). A study by Frohlich et al in 2016 exhibited the effects of short-term antibiotic use in mice and found that in addition to alterations to gut microbiota composition and metabolites, there was also an effect on blood-brain barrier tight junction expression and cognition(Frohlich et al., 2016). These differences were accompanied by changes in BNDF, N-methyl-D-aspartate receptor subunit 2B, serotonin transporter, and corticosterone levels. A similar study looked at the effect of low dose penicillin in the perinatal period and

found alterations to anxiety-like and social behaviours that persisted after cessation of antibiotic treatment (Leclercq et al., 2017). Treatment with probiotic Lactobacillus rhamnosus JB-1 circumvented some of these alterations, indicating a protective role of a singular species, further demonstrating the need for understanding how specific taxa effect the host, under altered microbial conditions (Leclercq et al., 2017).

1.7 Objective

The first primary objective of this study was to explore host genetic differences in gut microbiota composition in two inbred mouse strains BALB/c and C57Bl/6. Followed by evaluating the host genetic mechanism of fecal miRNA in controlling gut microbiota composition under healthy conditions. Additional objectives included exploring differences in host physiology at the gastrointestinal barrier in response to perturbation of the gut microbiota composition, determining if host genetics plays a role in the host's response to antibiotic treatment, and identifying if individual taxa are responsible or associated with changes in host physiology. The final objective was to explore the use of peripheral fecal miRNA as biomarkers for behaviour.

2.0 METHODS

2.1 Animals and Experimental design

Female BALB/c and C57BL/6 mice were obtained from Charles River (St. Constant, Canada; Kingston, US, respectively) at 8 weeks of age. The mice were maintained in specific-pathogen-free housing in sanitized cages with filter bonnets, 2 mice per cage at St. Joseph's Healthcare animal facility, and under a 12 h light–12 h dark cycle with lights on at 5 AM. At 10 weeks of age, the mice were identified by ear punch. Mice were divided into 3 treatment groups (n=6 per treatment per strain); treatment A consists of Ampicillin 1mg/ml, Neomycin 2mg/ml, Primaricin 1.2µg/ml (AMP+NEO); treatment B consisting of

Erythromycin 1mg/ml, Primaricin 1.2µg/ml (ERY) and control (CON) distilled water. Across the two treatment groups, the antibiotics were administered in a volume of 200 ml of sterile water, which was changed twice weekly for a period of 2 weeks. Weight, food and water consumption data collection occurred twice weekly during cage maintenance. Two-minute handling took place at the same time. Fecal pellets were collected directly from individual mice 1 day prior to administering antibiotics and on day 14, and pellets were immediately frozen and stored at -80°C until DNA/RNA extraction. Mice were assessed for anxiety-like behaviour in the elevated plus maze during the light cycle two weeks after the start of antibiotic treatment as described by (Sidor et al., 2010 K., Foster, J.A., 2010). All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board, McMaster University, Hamilton, Ontario, Canada.

2.2 Small Intestinal Permeability

The permeability of the small intestine was measured after 14 days of antibiotic treatment via gavage of a fluorescent probe, unconjugated fluorescein isothiocyanate (FITC) (F1906; Invitrogen, Eugene, OR) and quantification of its recovery in serum. Mice were fasted, but allowed water for 3 hours and weighed prior to gavage. Mice were administered 200 µl of 1.25 mg/ ml FITC solution by gavage technique and decapitated 3 hours post-gavage, blood and small intestinal tissue were collected. Blood was collected at room temperature, allowed to clot and then centrifuged (10,000 G, 10 min, ambient temperature) to collect the serum. Fluorescence was measured for each serum sample in 96 well microtiter plates using SpectraMax Gemini EM microplate reader with excitation 485nm and emission 528nm (Molecular Devices, San Jose, CA). A standard curve for calculating serum recovery

of FITC was obtained by diluting unconjugated FITC 1mg/ml in water. FITC serum recovery was normalized to initial mouse weight, and measures of intestinal permeability are presented as the concentration of recovered FITC in serum normalized to mouse body weight to the power of 10^4(mg/ml/g x 10^4).

2.3 16S rRNA sequencing and OTU processing

DNA was extracted from fecal pellets as previously described (Bartram et al., 2011; Whelan et al., 2014). Sequences of the 16S rRNA gene variable 3 (v3) region were amplified with a previously described procedure(Bartram et al., 2011) using the modifications by Whelan and colleagues (Whelan et al., 2014), and sequenced using the Illumina MiSeq platform. The MiSeq sequence reads were then processed using an in-house pipeline (Whelan et al., 2014). In summary the pipeline uses Cutadapt (Martin, 2011) to trim reads outside of the v3 region, aligns remaining paired-end sequences with PANDAseq (Masella et al., 2012), and picks operational taxonomic units (OTUs) with AbundantOTU+ (Ye, 2011) with a clustering threshold of 97% sequence similarity. Finally, the Ribosomal Database Project (RDP) classifier (Wang et al., 2007) assigns taxonomy down to the genus level using the Greengenes 2011 reference database (February 4, 2011 release;(DeSantis et al., 2006)). Using QIIME version 1.9.1 (Caporaso et al., 2010), singleton OTU's were removed and the final OTU table generated for further analysis.

2.4 16S data analysis

Alpha diversity and beta diversity analyses were completed using the vegan package in R version 3.3.1 (Team, 2016). Analyses were done at the OTU level unless specified. An initial comparison of bacterial diversity between untreated BALB/c and C57BL/6 mice (n=24) was completed to identify strain-related differences in bacterial taxa. A single

sample from C57BL/6 control group was removed due to sampling error, which resulted in a n=5 for control group for C57BL/6.

2.4.1 Alpha Diversity

Individual-based rarefaction curves were produced to account for differences in sequencing depth (sample size) between 16S samples. Individual samples were subsampled without replacement (rarefied) 10 times at multiple depths up to the minimum sequencing depth of all samples in the analysis. The mean diversity of all 10 rarefactions was taken as the diversity measure for each sample at each depth. T-tests were used to examine strain differences at various depths. Diversity metrics used in the analysis included the Chao1 index and the Shannon index.

2.4.2 Beta Diversity

Beta diversity between samples was explored using Principal Coordinate Analysis (PCoA) with Bray-Curtis, and binary (presence-absence) Bray-Curtis distance metrics applied to rarefied OTU count data. Multiple rarefaction PCoA was used for all metrics, as differences in sequencing depth between samples has been shown to effect distance metrics that are sensitive to rare taxa (Weiss et al., 2017). PCoA was done separately on 100 random rarefactions at a depth of 11353. Median coordinates, interquartile range of coordinates, and median variance explained across all 100 analyses were used to create PCoA plots. Significant baseline strain differences were assessed with PERMANOVA, using 100000 permutations and the distance metrics above.

2.4.3 Differential abundance and association analysis of bacterial taxa

Two analyses were conducted, a baseline comparison of bacterial composition between C57BL/6 and BALB/c mice (n=24) and a comparison of baseline and post-treatment bacterial composition (n=6 per strain per treatment). All analyses were done on relative

taxa abundances of the unrarefied OTU table collapsed to the genus (L6) level. Permutation t-tests were used to assess differential abundance between groups. Median taxon abundance differences between strains, median change pre to post were used as effect size estimates to add interpretability to microbiome analysis results. The Benjamini-Hochberg (BH) procedure was used to correct for false discovery rate (FDR) across all taxa.

A permutation-based repeated-measures factorial ANOVA was used to assess the effects of antibiotic treatment over time and how these effects differed between mouse strains. Analyses were run separately on the abundances of each taxon. The BH procedure was used to correct for FDR within main effect and interaction terms across all taxa and FDR < 0.05 was taken as significant. The statistical method was implemented using the multi-stratum analysis function aovp in the lmPerm package (setting Ca = 0.000001 and maxIter = 100000). All treatment groups, including controls, were included in the analysis to isolate effects that are specifically caused by antibiotics and not due to random shifts in microbiota composition over time. Individual mice were taken as the strata or blocks, allowing only permutations between pre and post-treatment samples within individuals. Interactions of time with treatment and the three-way interaction of treatment by strain by time were assessed to find main effects of antibiotic treatment and differential effects of treatment between strains, respectively. For each mouse strain, separate post-hoc repeated measures t-tests within treatment groups, including the control group, were done to explore significant treatment effects and significant treatment by strain interactions. FDR correction was used across taxa included in these analyses.

2.5 Fecal RNA isolation

RNA was extracted from fecal samples (BALB/c n=6, C57BL/6 n=6) using Stool total RNA purification kit (#49400 Norgen Biotech Corporation, Thorold, ON) with the following modifications; fecal pellets were removed from -80^{o}C storage and thawed on ice in lysis buffer for 10 minutes, followed by manual homogenization of fecal pellet. Samples were then vortexed for an additional 10 minute beyond the manufacturer's recommended 5 minutes. An additional wash step was added prior to eluting purified RNA total volume of 40ul. Isolated RNA samples were treated using DNA-free™ DNase kit (Ambion) as per manufacturer guidelines. RNA yield was assessed by A260/A280 and A260/A230 ratios, analyzed with a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was accessed using Agilent 2100 Bioanalyzer with a eukaryote total RNA Nano assay and Agilent small RNA Kit for small RNA. The electropherograms were analyzed using the Agilent 2100 Expert Bioanalyzer Software.

2.6 Quantitative NanoString nCounter Fecal miRNA Analysis

Nanostring nCounter technology allows expression analysis of multiple genes from a single sample. nCounter® mouse miRNA v1.5 Assay Kit (NanoString Technologies) was used to detect miRNA in fecal RNA samples. Approximately 200 ng of total RNA was loaded in nCounter analysis following the manufacturer's protocol. Data was processed and analyzed with nSolver™ Analysis Software 4.0 with background subtraction set to the average of the negative control reads + 2× SD of the negative control read, and normalization to positive and ligation controls.

2.7 miRNA Statistical Data analysis

Differential miRNA expression between BALB/c and C57BL/6 mice were determined by fold change, evaluated by t-tests, and results were visualized by volcano plot generated

ggplot2 package in R version 3.3.4 (Team, 2016). To compare miRNA expression profile between groups, Principal Component Analysis (PCA) performed using R package DESEQ2, miRNA counts were transformed using regularized log (Love et al., 2014) to account for variation in counts across samples, top 50 variable miRNA were selected for PCA. The PCA results were plotted used ggplot2. Highly abundant miRNA were determined by examining miRNA count data. MiRNA probes with average counts >24 above the background subtraction across all samples were considered abundant. A pathway analysis of differentially expressed miRNAs and highly abundant miRNAs was carried out with DIANA-miRPath v3.021 (Vlachos et al., 2015), using predicted microRNA targets from the DIANA-microT-CDS v5.0 algorithm and Gene Ontology genesets derived from KEGG. The p-value threshold was set to 0.05 and MicroT threshold to 0.8, statistical correction for multiple comparisons was used.

2.8 Computation analysis of bacterial gene targets of abundant fecal miRNA

The most abundant fecal miRNA sequences were analyzed for potential targeting of bacterial genes by BLASTn alignment(Altschul et al., 1997) of local database consisting of the mouse gut microbial gene catalog database (Xiao et al., 2015) composed of 2 572 074 genes, obtained from the GigaScience Database (http://gigadb.org). Genes that were selected by the BLASTn analysis were matched to taxonomic classification using CARMA3 integrated US National Center for Biotechnology Information (NCBI-NR) database. The relationship between the top expressed miRNA and the predicted taxa was explored further by assessing the Spearman's rank correlation between fecal miRNA count data and the relative abundance of the selected taxa in compositional 16S RNA data from the same mice.

2.9 Ileum RNA extraction, cDNA generation and gene expression analysis

Small intestinal tissue previously collected post-FITC gavage and stored in -80°C, was analyzed by qRT-PCR for expression of tight junction protein mRNAs including Occludin (Ocln), Claudin 7 (Cldn7), Zonula occludens-1 (ZO-1) and mucus-related mucin-2 (Muc-2) mRNA. Total RNA was extracted using Norgen Biotek Animal Tissue RNA Purification kit according to the manufacturer's protocol (Norgen Biotek Corp, Thorold Ontario, Canada). Isolated RNA samples were treated using DNA-freeTM DNase kit (Ambion) as per manufacturer guidelines. RNA quality and yield were assessed by A260/A280 and A260/A230 ratios analysed with a Nano-Drop® ND-1000 spectrophotometer (NanoDrop Technologies). A total of 1µg of RNA was transcribed to cDNA in a 20 ul reaction using Superscript III reverse transcriptase kit as per manufactures protocol (Invitrogen), no reverse transcriptase reactions were utilized to assess DNA contamination. PCR amplification efficiency was determined for each primer pair target and reference gene used by generating calibration curves with 10-fold dilution of cDNA. Amplification efficiency was determined from the slope of the log-linear portion of the calibration curve for each gene tested. The relative gene expression of Ocln, Cldn7, ZO-1 and Muc-2 mRNAs in the small intestinal tissue was determined by RT-PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), the amplification reactions were carried out in a CXF96 Real-Time Detection system (Bio-Rad). The comparative ddCt – method was used to determine the amount of target gene normalized to endogenous references (β-Actin and GAPDH) and relative to a calibrator in the untreated control group. The purity of the PCR products was verified by melting curves and gel electrophoresis.

2.10 Statistical analysis of FITC recovery and correlations with bacterial abundance and tight junction expression

FITC recovery treatment and strain effects were assessed with permutation ANOVA and post-hoc permutation t-tests (perm.t.test in the Deducer package with 100000 permutations). $p < 0.05$ was taken as the significance level. Correlations of bacterial abundance after treatment with FITC recovery were analyzed using Spearman's rank-based correlation tests. A targeted analysis was done using only taxa with significant treatment effects or significant treatment by strain interactions in the above repeated-measures analysis. To account for baseline strain differences in permeability, FITC recovery values for BALB/c and C57BL/6 mice in treatment AMP+NEO and ERY were transformed to z-score distances away from respective control group means for each strain. These FITC z-scores are representative of the change in FITC recovery compared to control levels for each mouse strain. An FDR of 0.05 was taken as significant among results from the targeted taxon list. Differences between treatment groups and the control groups gene expression were assessed by the unpaired t-test or the non-parametric Mann-Whitney test, which was used to assess Cldn7 due to non-normality. Correlation between gene expression and intestinal permeability were measured using Pearson r, and the correlation between gene expression and bacterial relative abundance were assessed using Spearman's r.

3.0 RESULTS

3.1 Differences in microbiota composition and diversity between BALB/c and C57BL/6 mice

Microbial compositions of fecal samples collected from healthy BALB/c and C57BL/6 mice were analyzed by 16S rRNA sequencing. Sequencing data resulted in 5060

different OTUs belonging to 207 different assigned taxonomies, with a minimum and maximum number of reads per sample of 11353 and 139193, and a median of 71380 reads. Differences in alpha and beta diversity were observed between BALB/c and C57BL/6 (Fig. 1). BALB/c mice were found to have significantly lower alpha diversity than C57BL/6 mice (Chao1: $p < 0.05$, Shannon: $p < 0.05$ at all rarefaction depths larger than 1000; Fig. 1). Samples also clustered significantly by mouse strain in PCoA plots (PERMANOVA: median $p < 0.05$ for all metrics; Fig. 1C-E). Bray-Curtis distances were calculated using both untransformed and presence-absence (binary) abundance tables at the OTU and genus level. This approach provides a visualization of diversity based on abundant and rare taxa. Bray-Curtis distance metric is more sensitive to changes in highly abundant taxa, and less sensitive to rare taxa, while binary Bray-Curtis distances are equally sensitive to all taxa. The observed strain-specific clustering across all analyses suggests the presence of host mouse strain-related differences in both abundant and rare OTUs.

The relative abundance of bacterial taxa in BALB/c and C57BL/6 mice at the family level is shown in Fig. 1C. Inter-individual variability in taxa was evident in the abundance plots of individual mice. Average relative abundance plots are also provided (Fig. 1C). Permutation median t-tests revealed several taxa that had significant differences in abundance between C57BL/6 and BALB/c mice (Table 1). A total of 22 taxa showed increased abundance in C57BL/6 mice with the largest increase in abundance in *Prevotella*, as well as increases in *Akkermansia*, and *Ruminococcus* in comparison to BALB/c. Five taxa were uniquely found in C57BL/6 mice including *Allobaculum*. A total of 26 taxa showed increased abundance in BALB/c mice compared to C57BL/6 mice (Table 1) and 3 taxa were detected only in BALB/C mice. Interestingly, 17 of the 26 taxa increased in BALB/c mice

belonged to the phyla Firmicutes, with families Lachnospiraceae, Catabacteriaceae and Ruminococcaceae accounting for the largest differences in abundance. Additionally, at the genus level, there was a significant increase in abundance of *Alistipes* and *Oscillospira* in BALB/c mice.

BALB/c mice were found to have increased inter-individual variability in compositions at the genus level compared to C57BL/6 mice. This was demonstrated by the increasing spread and interquartile range (IQR) of BALB/c samples in PCoA plots (Fig. 1B) and in the increased variability of BALB/c compositions at higher phylogenetic levels (Fig. 1C). At the OTU level, BALB/c mice had increased inter-individual variability when analyzed using the Bray-Curtis distance, but not using binary Bray-Curtis distance. This suggests that within C57BL/6 and BALB/c mice, consistent OTU's are present, while the abundances of these OTU's shift more between BALB/c mice than between C57BL/6 mice.

Table 1. Significant differences in microbiota composition between C57Bl/6 and BALB/c mice

Consensus.Lineage	Difference
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	↑↑↑ C57BL/6
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales	↑ C57BL/6
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_	↑ C57BL/6
p_Cyanobacteria;c_4C0d-2;o_YS2;f_;g_	↑ C57BL/6
p_Firmicutes;c_Bacilli;o_Turicibacterales;f_Turicibacteraceae;g_	↑ C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_	↑ C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Ruminococcus	↑ C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptococcaceae;g_	↑ C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae	↑ C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Subdoligranulum	↑ C57BL/6
p_Proteobacteria;c_Alphaproteobacteria;o_;f_;g_	↑ C57BL/6
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_	↑ C57BL/6
p_Tenericutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae	↑ C57BL/6
p_TM7;c_TM7-3;o_CW040;f_F16;g_	↑ C57BL/6
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Akkermansia	↑ C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Roseburia	(↑) C57BL/6
p_TM7;c_TM7-3;o_CW040	(↑) C57BL/6
p_Actinobacteria;c_Actinobacteria;o_Coriobacteriales;f_;g_	Only C57BL/6
p_Cyanobacteria;c_Oscillatoriophycideae;o_Chroococcales;f_Prochloraceae;g_	Only C57BL/6
p_Firmicutes;c_Clostridia;o_Clostridiales;f_ClostridialesFamilyXIII.IncertaeSedis;g_	Only C57BL/6
p_Proteobacteria;c_Alphaproteobacteria	Only C57BL/6
p_Tenericutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Allobaculum	Only C57BL/6
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Alistipes	↑↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Catabacteriaceae;g_	↑↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae	↑↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	↑↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Oscillospira	↑↑ BALB/c
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales	↑ BALB/c
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Corynebacteriaceae;g_Corynebacterium	↑ BALB/c
p_Actinobacteria;c_Actinobacteria;o_Coriobacteriales;f_Coriobacteriaceae	↑ BALB/c
p_Actinobacteria;c_Actinobacteria;o_Coriobacteriales;f_Coriobacteriaceae;g_Slackia	↑ BALB/c
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Carnobacteriaceae;g_	↑ BALB/c
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Lactococcus	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_ClostridialesFamilyXIII.IncertaeSedis;g_Eubacterium	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Anaerostipes	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Oribacterium	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Anaerotruncus	↑ BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ethanoligenens	↑ BALB/c
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Escherichia	↑ BALB/c
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Moraxella	↑ BALB/c
p_Tenericutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_	↑ BALB/c
p_Tenericutes;c_Mollicutes;o_Anaeroplasmatales;f_Anaeroplasmataceae;g_Anaeroplasma	↑ BALB/c
p_Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_	Only BALB/c
p_Firmicutes;c_Bacilli;o_Bacillales;f_Bacillaceae;g_Bacillus	Only BALB/c
p_Firmicutes;c_Clostridia;o_Clostridiales;f_ClostridialesFamilyXI.IncertaeSedis;g_	Only BALB/c

↑↑↑, difference between medians is > 10%; ↑↑, > 1%; ↑, < 1%; (↑), increased presence

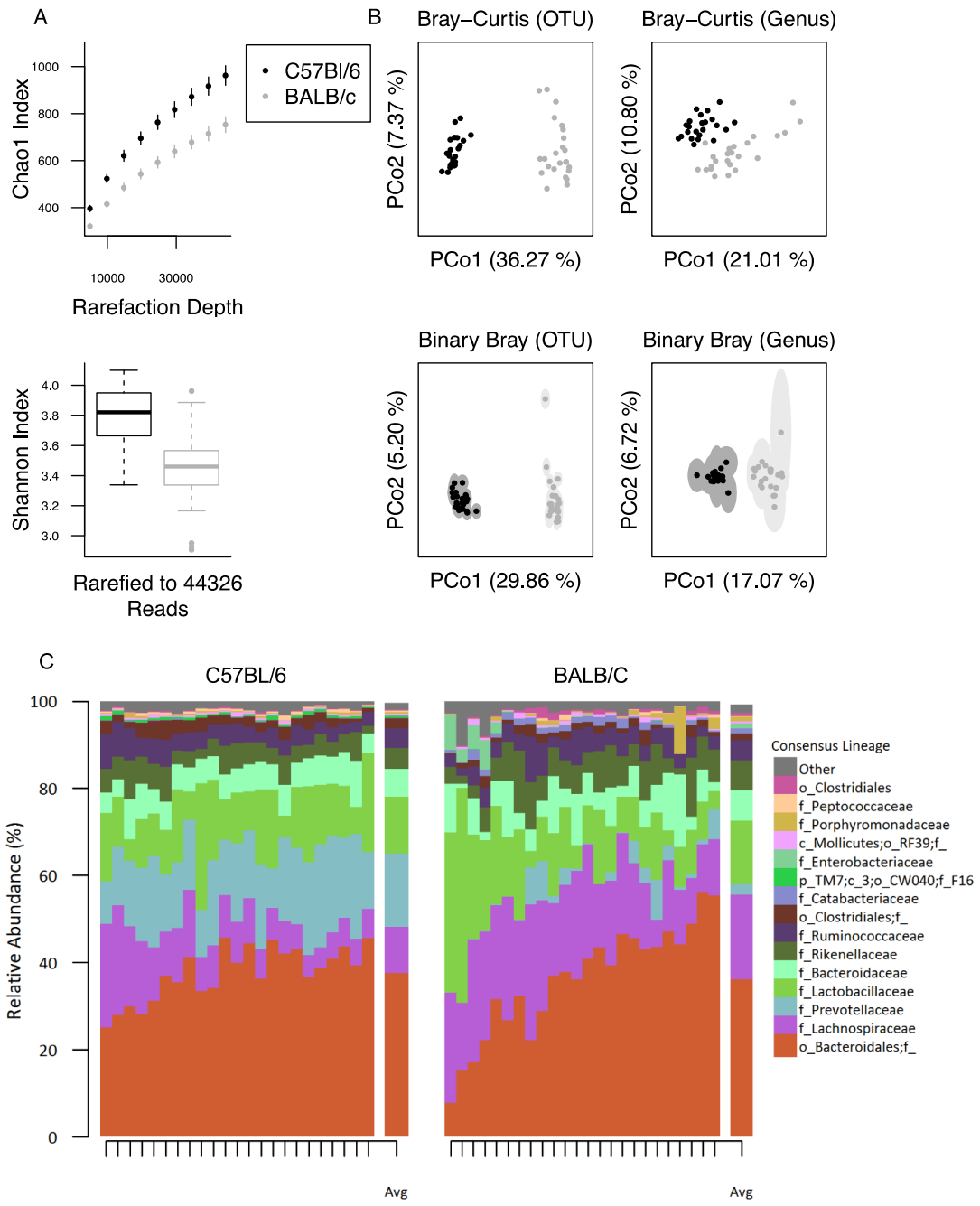


Figure 1. 16S rRNA sequencing of BALB/c and C57BL/6 mice revealed host genetic differences in bacteria composition at the OTU and genus level. A) Alpha diversity using Chao1 and Shannon indexes. B) Beta diversity analysis, a Principle Coordinate Analysis (PCoA) was done using the Bray-Curtis distance metric at the genus and OTU levels. Presence-absence (binary) Bray-Curtis distances were examined to display differences in rare taxa. PCoA was performed on 100 rarefactions with points representing median coordinates and ellipse representing interquartile ranges across 100 analyses. C) Relative abundance of bacterial taxa > 1% classified to the family level taxonomy. Plots shown for individual mice (n=24 BALB/c, n=24 C57BL/6, and average relative abundance for respective groups.

3.2 Identification of murine fecal miRNA profile

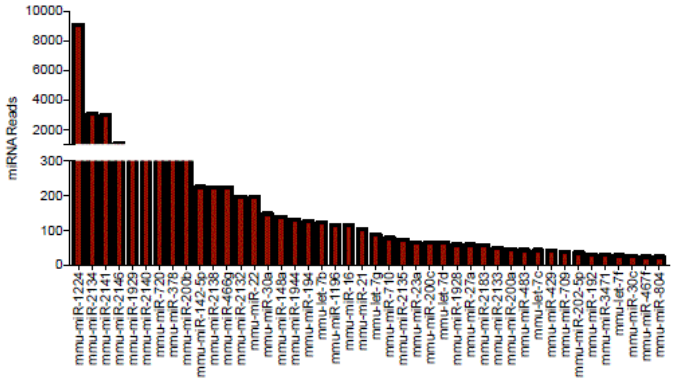
Of the 599 miRNAs tested 75 were found to be detectable and 44 of those 75 considered abundant with mmu-miR-1224, mmu-miR-2134, mmu-miR-2141, mmu-miR-2146, mmu-miR-1929, mmu-miR-2140 and mmu-miR-720 being the most abundant miRNA (Fig 2A). To investigate if fecal miRNA profiles differed by host genotype from BALB/c and C57BL/6 mice, we performed a principle component analysis and found separation and clustering by host strain (Fig 2E). Comparing fecal miRNA expression between BALB/c and C57BL/6 mice revealed differentially expressed miRNA (Fig 2B.), with miR-2141, miR-2140, miR-1224-5p and miR-30a identified as the most significantly changed ($p<0.005$).

3.3 Functionality of fecal miRNA

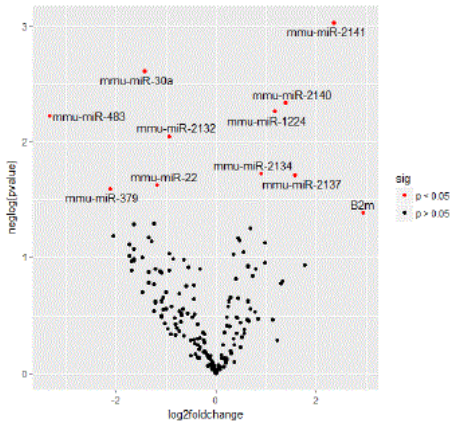
Eukaryotic miRNA negatively regulates mRNA translation via complementary binding to 3' untranslated regions (UTRs) resulting in either repression or degradation of the mRNA transcript (Fabian et al., 2010). To understand the functional impact of the detected fecal miRNAs on the host, we assessed their predicted gene targets and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways using an *in silico* analysis DIANA-miRPath v3 (Vlachos et al., 2015). Using DIANA-miRPath, all the significantly targeted pathways by the differentially expressed and abundant fecal microRNAs were evaluated(Fig. 2C, D). Of the 10 differentially expressed miRNAs; mir-2141, miR-2140, miR-2134, and miR-2132 were excluded from the pathway analysis as they are not currently annotated in miRbase (Griffiths-Jones et al., 2008). Of the pathways significantly targeted by the differentially expressed miRNA, the most significantly targeted pathway was found to be mucin-type O-glycan biosynthesis. This pathway is responsible for the glycosylation

of mucin, a ubiquitous intestinal glycoprotein that makes up the mucus layer, which adheres to the intestinal epithelial cells on the lumen side of the gastrointestinal barrier (Bergstrom and Xia, 2013). Significantly targeted pathways for the abundant miRNA across both strains involved complex glycoprotein synthesis, with targets specifically in the extracellular matrix (ECM): collagen, integrins, lamins, acetyl-galactoaminyltransferases, chondroitin sulfates synthases and fucosyltransferase 9 in addition to mucin glycan biosynthesis. This result is interesting in the context of host-microbe relationship as the ECM is highly dynamic and plays a large role in maintaining intestinal homeostasis with its constant remodelling, which is critical to the intestinal epithelium responsiveness to host-derived and microbial signals (Vllasaliu et al., 2014).

A



B



C

KEGG pathway	p-value	#genes	#miRNAs
ECM-receptor interaction	<1e-325	22	9
Mucin type O-Glycan biosynthesis	<1e-325	10	10
Proteoglycans in cancer	3.58E-10	59	10
Axon guidance	8.22E-06	35	7
Glycosphingolipid biosynthesis - lacto and neolacto series	1.76E-05	3	7
Fatty acid biosynthesis	0.000836	1	1
MAPK signaling pathway	0.00087	47	10
Signaling pathways regulating pluripotency of stem cells	0.001577	44	10
Lysine degradation	0.007422	17	6
PI3K-Akt signaling pathway	0.00798	70	6
Fatty acid metabolism	0.027836	6	3
FoxO signaling pathway	0.03073	23	4
TGF-beta signaling pathway	0.034112	20	3
Amoebiasis	0.036955	11	5
Thyroid hormone signaling pathway	0.037033	30	5
Regulation of actin cytoskeleton	0.044687	44	4

D

KEGG pathway	p-value	#genes	#miRNAs
Mucin type O-Glycan biosynthesis	2.07E-12	5	3
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	1.55E-07	1	1
Thyroid hormone synthesis	0.001131	2	1
Glycosphingolipid biosynthesis - lacto and neolacto series	0.023198	1	1
Axon guidance	0.03826	23	2

E

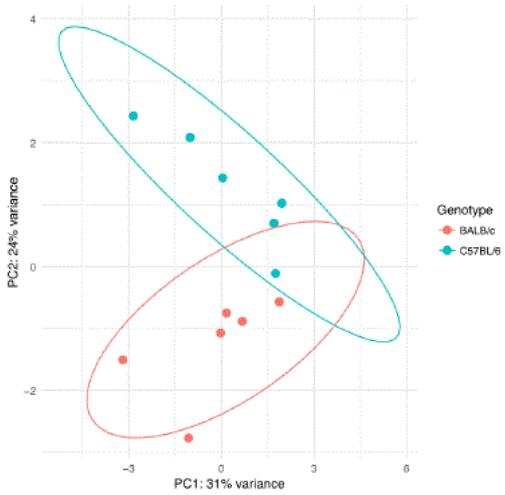


Figure 2. Host genetic related difference in expression of murine fecal miRNA. (A) Mean values for the 24 most abundant miRNAs in mouse fecal samples collected from both BALB/c (n=6) and C57BL/6 (n=6). (B) Volcano plot of fecal miRNA detected by Nanostring, BALB/c versus C57BL/6, x-axis log2 Fold change of expression level between fecal miRNA from BALB/c and C57BL/6 mice, y-axis log10 p-value from unequal variances t-test between the compared groups. (C-D) Pathway analysis murine fecal miRNA performed using DIANA-miRPath v3.021, with predicted miRNA targets derived from MicroT-CDS v5.0, and Kyoto Encyclopedia of genes and genomes (KEGG) biological pathways. (C) Pathways of top 24 expressed murine miRNA excluded mmu-miR-2134, mmu-miR-2141, mmu-miR-2146, mmu-miR-2140, mmu-miR-720, mmu-miR-2138, mmu-miR-2132, mmu-miR-1944, mmu-miR-1196, mmu-miR-2137, mmu-miR-2135, mmu-miR-2133, (D) Pathways analysis of differential expressed fecal miRNA BALB/c v C57BL/6. (E) Principle component analysis (PCA) plot of fecal miRNA counts of top 50 variable miRNA.

3.4 Host fecal miRNA target bacterial genes

Using a publicly available mouse gut metagenome (Xiao et al., 2015), we evaluated if any bacterial genes within the mouse gut metagenome had nucleic acid sequences that could be targeted by fecal miRNA based on sequence homology. Bacterial genes contain no introns, therefore alignment of miRNA sequence to a bacterial gene is predictive of miRNA-mRNA complementary to the bacterial mRNA transcript. The BLASTn alignment of the 44 top expressed miRNA sequences against the 2,572,074 genes in the gut microbial gene catalogue resulted in 991 significant alignments (Supplemental XLS1). Of these 991 alignments, 11 were mapped to genes classified at the species level, 91 at the genus level and 107 at the family level. 335 of the total 991 alignments did not have a taxonomic classification associated with the targeted gene as only 67.8% of the total genes in the metagenome database were assigned taxonomy. Following up on the predicted relationship of fecal miRNA and bacterial gene expression, associations between fecal miRNA expression and relative abundance of predicted taxa were explored in 16S rRNA compositional data obtained from the same mice. A total of 14 significant correlations between 13 miRNA abundances and their metagenomic-alignment-predicted taxa were found, including significant positive correlations with *Escherichia* (Spearman's $\rho = 0.79$, $p < 0.01$), *Akkermansia* (Spearman's $\rho = 0.65$, $p < 0.05$), and *Staphylococcus* (Spearman's $\rho = 0.6$, $p < 0.05$), and significant negative correlations with *Parabacteroides* (Spearman's $\rho = -0.77, -0.82$, $p < 0.001$), *Prevotella* (Spearman's $\rho = -0.66$, $p < 0.05$), and *Clostridium* (Spearman's $\rho = -0.63$ to -0.77, $p < 0.05$ to 0.01; Table 2).

Table 2.Conserved predicted fecal miRNA bacterial gene significantly correlate with taxa abundance

	miRNA-16S data correlation analysis		miRNA-metagenome database BLASTn alignment			
miRNA	Taxa	Spearman ρ (p-value)	Potential bacterial gene target	eggNOG3 annotation	Score (bit)	E value
mmu-miR-2141	Escherichia	0.79 (0.006)	11_GL0122160	NOG242382	30.2	4.6
mmu-miR-21	Akkermansia	0.65 (0.031)	10_GL0030843	NOG77418	30.2	7.6
mmu-miR-2140	Staphylococcus[1]	0.60 (0.049)	2A-dyr14-07_GL0001188	COG1109	30.2	4.6
mmu-let-7b	Parabacteroides[2]	-0.82 (0.004)	2A-dyr13-06_GL0013439	NA	32.2	1.9
mmu-let-7c	Parabacteroides[2]	-0.77 (0.008)	2A-dyr13-06_GL0013439	NA	30.2	7.6
mmu-miR-2134	Prevotella	-0.66 (0.031)	16_GL0035169	NA	30.2	4.6
mmu-miR-16	C. Clostridium	-0.77 (0.005)	G1-5A_GL0185204	NA	30.2	7.6
mmu-miR-16	C. Clostridium	-0.77 (0.005)	S-Fe12_GL0134416	NA	30.2	7.6
mmu-miR-16	C. Clostridium	-0.77 (0.005)	S-Fe20_GL0097563	NA	30.2	7.6
mmu-miR-200c	C. Clostridium	-0.77 (0.005)	Group2-8A_GL0215737	COG0726	30.2	9.1
mmu-miR-200c	C. Clostridium	-0.77 (0.005)	MH-0-5_GL0117732	COG3209	36.2	0.15
mmu-miR-1196	C. Clostridium	-0.73 (0.010)	2A-dyr13-06_GL0004680	COG0673	32.2	1.5
mmu-miR-1196	C. Clostridium	-0.73 (0.010)	35_GL0040992	COG0577	30.2	6.1
mmu-miR-1196	C. Clostridium	-0.73 (0.010)	S-Fe9_GL0022151	NA	30.2	6.1
mmu-let-7d	C. Clostridium	-0.68 (0.021)	6-2_GL0008179	NOG26044	30.2	7.6
mmu-let-7d	C. Clostridium	-0.68 (0.021)	6-7_GL0078312	NOG26044	30.2	7.6
mmu-miR-21	C. Clostridium	-0.68 (0.022)	S-Fe3_GL0200235	NA	30.2	7.6
mmu-miR-1944	C. Clostridium	-0.67 (0.024)	1-3_GL0087789	NA	32.2	3.9
mmu-miR-1944	C. Clostridium	-0.67 (0.024)	7-3_GL0015756	NA	32.2	3.9
mmu-miR-1944	C. Clostridium	-0.67 (0.024)	S-Fe16_GL0006390	NA	32.2	3.9
mmu-let-7g	C. Clostridium	-0.66 (0.027)	S-Fe11_GL0093182	NA	30.2	7.6
mmu-miR-23a	C. Clostridium	-0.63 (0.038)	1A-dyr2-07_GL0021636	COG0714	30.2	6.1
mmu-miR-23a	C. Clostridium	-0.63 (0.038)	Group2-5A_GL0024298	NOG39127	30.2	6.1
mmu-miR-23a	C. Clostridium	-0.63 (0.038)	S-Fe9_GL0008839	NA	30.2	6.1

C. Clostridium: Clostridiaceae Clostridium

1. Alignment was to a metagenomics read classified to Staphylococcaceae. As Staphylococcus was the only genus found from the Staphylococcaceae family in the 16S data set, correlations with the aligned miRNA were done at the genus level.
2. Alignment was to a metagenomics read classified to Porphyromonadaceae. As Parabacteroides was virtually the only genus found from the Porphyromonadaceae family in the 16S data set (mean +- std. Porphyromonadaceae proportion: 1.00 +- 0.0005), correlations with the aligned miRNA were done at the genus level.

3.5 Fecal miRNA as biomarker for locomotor behaviour

No significant differences in anxiety-like behaviour were found between mouse genotypes, as well as no treatments effects were observed. Significant differences in closed arm metrics were observed between BALB/c CON and C57BL/6 CON, with C57Bl/6 exhibiting significant increases in closed arm time, entries and distance travelled within the closed arm (Fig 3.). Increased distance within the closed arm indicates an increase in locomotion in the C57Bl/6 mice within the closed arm. A correlation analysis was

performed to evaluate if any of the abundant fecal miRNA showed any association with these locomotion differences. A total of nine fecal miRNA correlated with closed arm distance across BALB/c CON and C57BL/6 CON (Table 3). Of those 9 miRNAs, six were previously found to be differentially expressed between BALB/c and C57BL/6 (miR-2132, miR-22, miR-30a, miR-483, mir-2141, miR-2137).

Table 3. Fecal miRNA correlation to locomotion in the closed arm of BALB/c and C57BL/6 mice

	Pearson's r	Spearman rho	p-value
Closed Arm Distance (cm) vs. mmu-miR-2132	0.7183		0.0085
Closed Arm Distance (cm) vs. mmu-miR-22	0.6673		0.0178
Closed Arm Distance (cm) vs. mmu-miR-30a	0.75		0.005
Closed Arm Distance (cm) vs. mmu-let-7b	0.598		0.04
Closed Arm Distance (cm) vs. mmu-miR-1196	0.5959		0.0409
Closed Arm Distance (cm) vs. mmu-miR-483	n/a	0.8741	0.0004
Closed Arm Distance (cm) vs. mmu-miR-2141	n/a	-0.7622	0.005
Closed Arm Distance (cm) vs.mmu-miR-21	n/a	0.6294	0.0323
Closed Arm Distance (cm) vs. mmu-miR-2137	n/a	-0.6853	0.0170

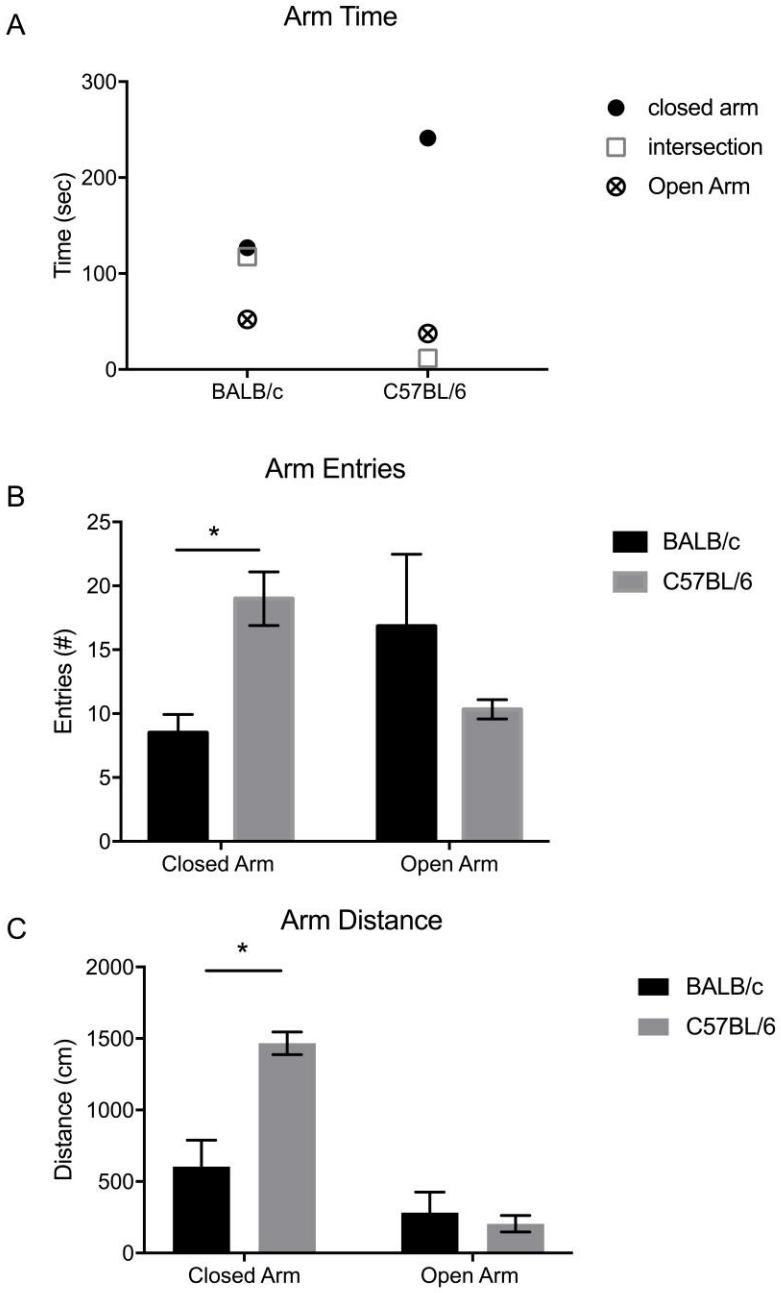


Figure 3. Activity-related behaviour of inbred mouse strains BALB/C and C57BL/6 control groups (n=6 per strain). A) Elevated Plus Maze time spent in open and closed arms, no significant difference in open arm time, significant difference in closed arm time $p<0.05$. B) Elevated plus maze number of entries into closed and open arms significant difference only in closed arm entries $p<0.05$. C) Elevated plus maze distance traveled in open and closed arms no significant difference in open arm time, significant increase in locomotion in closed arm to C57Bl/6 mice $p<0.05$. Values are mean +/- SEM.

3.6 Antibiotic treatment shifts in microbiota composition

To explore the host genetic influence on gut microbiota composition and gastrointestinal homeostasis under altered microbial conditions, two different treatments of broad-spectrum antibiotics were administered to inbred mouse strains BALB/c and C57BL/6. The first treatment was a combination of ampicillin and neomycin (AMP+NEO) and the second treatment consisted of erythromycin (ERY). An anti-fungal was also administered with both treatments to prevent up growth of fungi post-treatment. Antibiotic treatment significantly reduced the number of taxa and their overall distribution in both C57BL/6 and BALB/c mice as shown by the reduction in alpha diversity for both groups post-treatment (Appendix. Fig. 1). To assess shifts in gut microbiome composition after antibiotic treatment, repeated-measures Adonis analysis was done on sample distances matrices. A significant interaction was found between treatment and time in both Bray-Curtis and binary Bray-Curtis analysis. Antibiotic treatments shifted microbiota compositions greatly in both mouse strains, as seen in family level bar plots (Fig. 4A). An interaction between strain, treatment, and time was significant at the genus and OTU level using binary Bray-Curtis distance and at the OTU level using untransformed Bray-Curtis. This three-way interaction between strain, treatment, and time point indicates that antibiotic treatment differentially affected BALB/c and C57BL/6 microbiota compositions. No strain by treatment by time point interaction was found with untransformed Bray-Curtis at the genus level. These results suggest that both antibiotic treatments affected dominant genus-level taxa similarly in C57BL/6 and BALB/c mice, while rare taxa were more often differentially affected between mouse strains. The results of the permutation-based repeated measures factorial ANOVA analysis done at the genus level confirm the

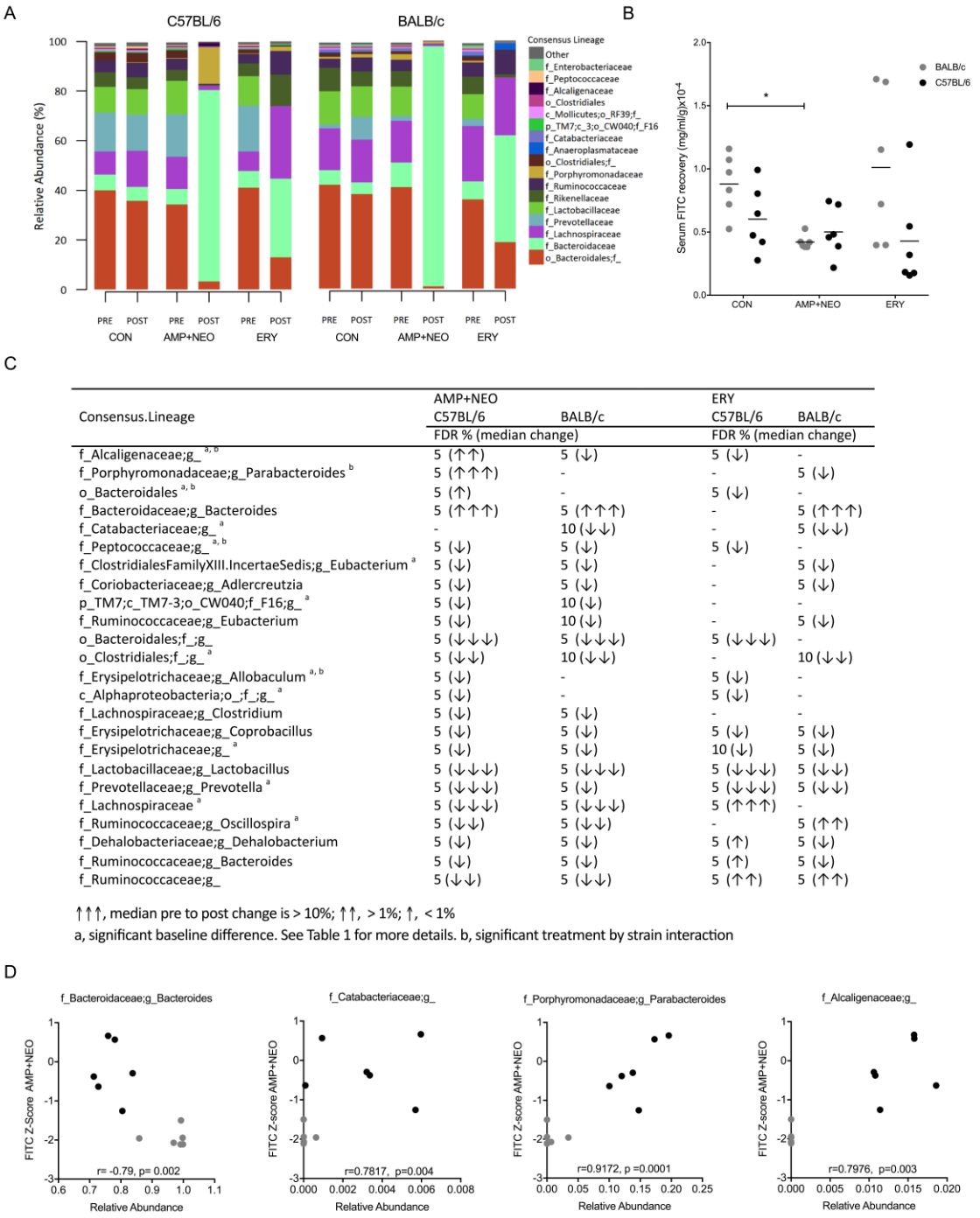
composition-wide analysis, finding mainly large shifts in the same direction in taxa affected by antibiotics in both C57BL/6 and BALB/c mice and small shifts in taxa affected in only C57BL/6 or BALB/c mice (Fig. 4C).

We identified treatment effects on the microbial composition at the genus level specific to each host mouse strain. *Prevotella, Lactobacillus, Coprobacillus*, and an unknown genus of the Erysipelotrichaceae family were the only taxa identified to significantly decrease in response to both antibiotic treatments independent of host mouse strain. While, an unidentified genus member of the Ruminococcacea family exhibited a consistent decrease in response to AMP+NEO treatment for both strains but an increase in response to ERY. There were 5 taxa identified with a significant strain interaction in response to treatment including *Parabacteroides*, an unidentified genus of *Peptococcaceae* family and an unclassified member of the *Bacteroidales* order. For all three of these taxa, the largest response occurred in C57BL/6 mice. The other two detected interactions found within abundances of *Allobaculum* and an unknown *Alcaligenaceae* genus were primarily driven by the difference in abundance of these bacteria in BALB/c mice prior to treatment. Five identified taxa, *Eubacterium* (Incertae Sedis), *Dehalobacterium*, *Bacteroides* (Ruminococcaceae), *Adlercreutzia*, and *Eubacterium* (Ruminococcaceae) also responded differentially by strain to treatment though non-significant interactions after correcting for FDR.

3.6 Disruption of barrier in AMP+NEO treated BALB/c mice small intestine permeability

Permeability was assessed by the recovery of FITC in serum. A permutation ANOVA of FITC recovery found a significant main effect of strain ($p < 0.05$) and an interaction of

strain by treatment that tended toward significance ($p < 0.1$; Fig. 4B). In separate follow-up analyses done within each strain, treatment significantly altered gut permeability in the BALB/c mice (permutation ANOVA: $p < 0.05$), but not C57BL/6 mice (permutation ANOVA: $p > 0.1$). Specifically, BALB/c mice treated with AMP+NEO had decreased permeability compared to BALB/c controls (permutation t-test: $p < 0.05$). When comparing FITC recovery between strains within treatment groups, BALB/c mice tended to have increased permeability in both the control group (permutation t-test: $p < 0.1$) and after ERY (permutation t-test: $p < 0.1$).



Consensus.Lineage	AMP+NEO C57BL/6	AMP+NEO BALB/c	ERY C57BL/6	ERY BALB/c
	FDR % (median change)		FDR % (median change)	
f_Alcaligenaceae;g_ [a, b]	5 (↑↑)	5 (↓)	5 (↓)	-
f_Porphyromonadaceae;g_Parabacteroides [b]	5 (↑↑↑)	-	-	5 (↓)
o_Bacteroidales [a, b]	5 (↑)	-	5 (↓)	-
f_Bacteroidaceae;g_Bacteroides	5 (↑↑↑)	5 (↑↑↑)	-	5 (↑↑↑)
f_Catabacteriaceae;g_ [a]	-	10 (↓↓)	-	5 (↓↓)
f_Peptococcaceae;g_ [a, b]	5 (↓)	5 (↓)	5 (↓)	-
f_ClostridialesFamilyXIII.IncertaeSedis;g_Eubacterium [a]	5 (↓)	5 (↓)	-	5 (↓)
f_Coriobacteriaceae;g_Adlercreutzia	5 (↓)	5 (↓)	-	5 (↓)
p_TM7;c_TM7-3;o_CW040;f_F16;g_ [a]	5 (↓)	10 (↓)	-	-
f_Ruminococcaceae;g_Eubacterium	5 (↓)	10 (↓)	-	5 (↓)
o_Bacteroidales;f_;g_	5 (↓↓↓)	5 (↓↓↓)	5 (↓↓↓)	-
o_Clostridiales;f_;g_ [a]	5 (↓↓)	10 (↓↓)	-	10 (↓↓)
f_Erysipelotrichaceae;g_Allobaculum [a, b]	5 (↓)	-	5 (↓)	-
c_Alphaproteobacteria;o_;f_;g_ [a]	5 (↓)	-	5 (↓)	-
f_Lachnospiraceae;g_Clostridium	5 (↓)	5 (↓)	-	-
f_Erysipelotrichaceae;g_Coprobacillus	5 (↓)	5 (↓)	5 (↓)	5 (↓)
f_Erysipelotrichaceae;g_ [a]	5 (↓)	5 (↓)	10 (↓)	5 (↓)
f_Lactobacillaceae;g_Lactobacillus	5 (↓↓↓)	5 (↓↓↓)	5 (↓↓↓)	5 (↓↓)
f_Prevotellaceae;g_Prevotella [a]	5 (↓↓↓)	5 (↓)	5 (↓↓↓)	5 (↓↓)
f_Lachnospiraceae [a]	5 (↓↓↓)	5 (↓↓↓)	5 (↑↑↑)	-
f_Ruminococcaceae;g_Oscillospira [a]	5 (↓↓)	5 (↓↓)	-	5 (↑↑)
f_Dehalobacteriaceae;g_Dehalobacterium	5 (↓)	5 (↓)	5 (↑)	5 (↓)
f_Ruminococcaceae;g_Bacteroides	5 (↓)	5 (↓)	5 (↑)	5 (↓)
f_Ruminococcaceae;g_	5 (↓↓)	5 (↓↓)	5 (↑↑)	5 (↑↑)

↑↑↑, median pre to post change is > 10%; ↑↑, > 1%; ↑, < 1%

a, significant baseline difference. See Table 1 for more details. b, significant treatment by strain interaction

Figure 4. Impact of antibiotic treatment on gut microbiota composition and gastrointestinal barrier integrity. (A) Relative abundance of bacterial taxa classified to the family-level taxonomy pre and post treatment with antibiotics: ampicillin + neomycin (AMP+NEO) and erythromycin (ERY) for in BALB/c and C57BL/6 mice. (B) Small Intestinal epithelial permeability to fluorescein isothiocyanate (FITC) of control group (CON) C57BL/6 and BALB/c, and permeability post antibiotic treatment AMP+NEO and ERY, $p < 0.05$. (C) Significant treatment effects on relative abundance at 16S genus level. (D) Changes in small intestinal permeability post antibiotic treatment with AMP+NEO correlate with bacterial taxa relative abundance post treatment. Spearman correlation of FITC Z-score to relative abundance of genera *Bacteroides,* unclassified *Catabacteriaceae, Parabacteroides, and* unclassified *Alcaligenaceae* genus.

3.7 Taxa correlations with small intestine permeability

Overall AMP+NEO affected BALB/c and C57BL/6 microbiota compositions similarly, consistently eliminating many of the same taxa, with only a few taxa significantly responding to treatment in a mouse strain-dependent manner. Significant differences in the host physiological response to the antibiotic treatment were found for small intestine permeability. BALB/c mice small intestine permeability was significantly reduced in response to AMP+ NEO however, C57BL/6 mice permeability remained relative unchanged after the same treatment regime (Fig. 4B). < 15%), along with a negative correlation with Bacteroidaceae *Bacteroides* (Spearman's $\rho = -0.57$ FDR < 15%), though none of these effects remained significant after correcting for multiple comparisons (Appendix. Fig. 2). No significant correlations were found in C57BL/6 mice.

Interestingly, *Parabacteroides* and unclassified *Alcaligenaceae*, both increased in response to AMP+NEO in C57BL/6 mice only. To determine which bacteria may be contributing to shifts in gut permeability found in BALB/c mice and which bacteria may be resisting shifts in C57BL/6 mice, an analysis comparing values of both strains together with FITC within each treatment was completed. Analyzing the correlation of FITC z-scores with taxon relative abundances revealed that taxa that responded differentially to AMP+NEO treatment between mouse strains may account for differential shifts in FITC recovery observed between these two mouse strains in response to AMP+NEO. Positive correlations with FITC z-score values were found with AMP+NEO treatment and *Parabacteroides* (Spearman's $\rho = 0.90$, FDR < 1%), an unknown *Alcaligenaceae* genus, (Spearman's $\rho = 0.80$, FDR < 5%), an unknown *Catabacteriaceae* genus (Spearman's $\rho = 0.78$, FDR < 5%), and an unclassified Bacteroidales taxon (Spearman's $\rho = 0.77$, FRD < 5%). A negative correlation

was also found with *Bacteroidaceae Bacteroides* (Spearman's ρ = -0.75, FDR < 5%). No significant correlations were found in ERY-treated mice (Fig. 4D).

3.8 Antibiotics treatment AMP+NEO differentially effect tight junction expression based on host physiology

To determine if alterations in small intestine permeability were associated with changes in expression of tight junctions and mucin, RT-qPCR on RNA isolated from sections of ileum tissue collected post-FITC gavage was performed (Fig. 5 A-C). Within the control groups, the only significant difference found was in the expression of mucin-producing gene Muc-2 mRNA, with C57BL/6 mice exhibiting an increase in relative expression as compared to BALB/c mice (Fig. 5A). Significant increases in relative expression of the tight junction protein Claudin 7 mRNA (Cldn7) was observed in C57BL/6 mice but not in BALB/c mice after AMP+NEO compared to control group, as well as a decrease in Muc-2 mRNA expression (Fig. 5B). Despite significant alterations to Cldn7 mRNA and Muc-2 mRNA expression, there was no significant correlation to FITC recovery in C57BL/6 AMP+NEO treated mice, indicating these genes may have additional roles outside of barrier tightening. A significant increase in tight junction scaffolding protein ZO-1 was found in response to AMP+NEO treatment only in BALB/c mice. A negative correlation (Spearman's ρ =-0.71, p >0.05) was found with the expression of ZO-1 mRNA and FITC z-score for AMP+NEO (Fig 5D), indicating that increased expression of ZO-1 mRNA correlated with a reduction of small intestine permeability. An analysis of the taxa that were previously shown to correlate with FITC Z-scores confirms the relationship between the taxa and barrier function, as *Parabacteroides* and unknown *Alcaligenaceae* genus relative abundance's negatively correlated with ZO-1 mRNA expression in AMP+NEO treated BALB/c and C57BL/6 mice (Spearman's ρ =-0.9, p<0.01, ρ=0.8 p<0.01 respectively). A

positive correlation with relative abundances of *Bacteroidaceae Bacteroides* and ZO-1 mRNA expression was found in response to AMP+ NEO treatment (Spearman's $\rho = 0.75$, $p < 0.01$ (Fig. 5F). No significant differences in tight junction expression or Muc-2 mRNAs were found for ERY treatment in either mouse strains.

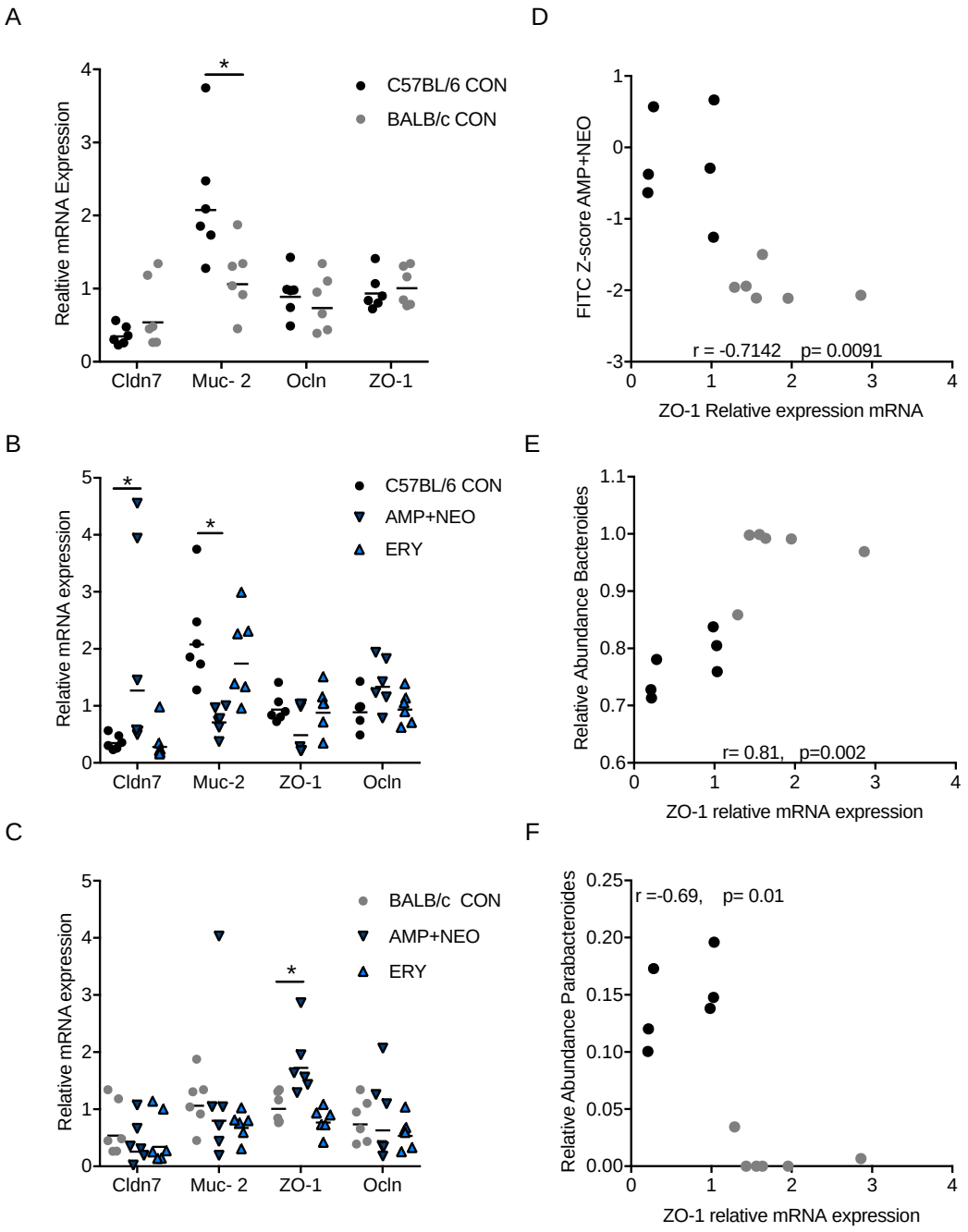


Figure 5. mRNA expression of intestinal barrier regulating genes A) Small intestine claudin 7 (Cldn7), mucin 2 (Muc-2), occludin (OCLN), and zonuline-1 (ZO-1) mRNA expression normalized to GAPDH and β-Actin gene relative expression in C57BL/6 and BALB/c control groups (muc-2: p< 0.05). B) C57BL/6 mRNA expression post treatment AMP+NEO and ERY (Cldn7: p<0.01, Muc-2: p<0.01) C) BALB/c mRNA expression post AMP+NEO and ERY (ZO-1: p< 0.05). (D) Correlation analysis of small intestinal barrier permeability post AMP+NEO treatment with expression of ZO-1 mRNA (r=-0.71). (E-F) Spearman's correlation between relative abundance of the genera Bacteroides (r=0.75) and Parabacteroides (r=-0.65) and the relative expression of ZO-1, post treatment AMP+NEO in both BALB/c and C57BL/6 mice.

4.0 DISCUSSION

Differences in gut microbiota composition in inbred mouse strains BALB/c and C57Bl/6

Exploring the gut microbiota composition of two inbred mouse strains, BALB/c and C57BL/6, revealed distinct differences in composition. At the genus level, differences were observed in relative abundance of notable genera *Prevotella*, *Alistipes* and *Akkermansia*, with significantly increased relative abundance of *Prevotella and Alistipes* in BALB/c and increased relative abundance of *Akkermansia* in C57BL/6 respectively. These results are in accordance with previous studies focused on host genetic influence on murine gut microbial compositions (Hildebrand et al., 2013; Krych et al., 2013; Xiao et al., 2015). The increase in relative abundance of *Prevotella* in BALB/c mice is of interest, as *Prevotella* is one of the defining taxa associated with human enterotypes (Arumugam et al., 2011; de Moraes et al., 2017; Wu et al., 2011). Previous work has also identified the relative abundance of *Alistipes* to be under the control of a quantitative trait loci (QTL) in mice, confirming the role of host genetics in its relative abundance (Leamy et al., 2014). Interestingly, the genus *Akkermansia* has previously been shown to be increased in the genus for mice of C57BL/6 background (Fransen et al., 2015). This may be due to biological differences at the gastrointestinal barrier, as *Akkermansia* is known to attach to and degrade the mucus layer of the gastrointestinal tract, with studies indicating the ability of *Akkermansia muciniphila* to degrade Muc-2 O-glycans in vitro (Png et al., 2010). Glycosylated Mucin 2 (Muc-2) makes up the largest component of the mucus layer of the small intestine, providing a physical barrier separating the host from its microbiota, thus aiding in maintaining gut homeostasis (Bergstrom and Xia, 2013). Utilization of Muc-2 by *Akkermansia* in C57BL/6 may be a contributing factor to the observed increase in

expression of Muc-2 mRNA in the small intestine of C57BL/6 mice. Furthermore, an increased expression of Muc-2 in C57BL/6 mice may provide a niche environment for the genus to thrive. As factors known to affect gut microbiota composition such as diet and environment are strictly controlled, here we attribute the observed differences to biological related genetic differences between the two inbred mouse strains. Understanding these baseline differences is a key consideration when using mouse models to study diseases influenced by gut microbiota, as well as being a necessary step in defining the limitations of comparability between gut microbiota studies that utilize differing strains of mice.

The results show that perturbation of bacterial composition with antibiotics alters barrier integrity and function in both a taxa-dependent and taxa-independent manner. Previous studies have evaluated host genetic differences between BALB/c and C57BL/6 mice at the gastrointestinal barrier (Volynets et al., 2016a), specifically finding differences in IgA production (Fransen et al., 2015) and the expression of antimicrobial peptides (Volynets et al., 2016b). The intestinal barrier is a critical site for host-microbe interaction (Wells et al., 2011) and the IECs that make up this barrier have a pivotal role in maintaining gut homeostasis. BALB/c mice exhibited a decrease in barrier permeability post-treatment with AMP+NEO, indicating a tightening of the gastrointestinal barrier while no significant effects were observed in C57BL/6 mice of the same treatment regime. Previous work in rats has shown that changes in gastrointestinal permeability may be linked to different antibiotic classes, which may account for the lack of significant change with erythromycin treatment observed here (Tulstrup et al., 2015). The analysis of the alterations of microbial composition at the genus level in response to antibiotic treatment revealed significant

treatment by strain interactions for *Parabacteroides* and an unclassified genera of *Peptococcaceae*. The genus *Parabacteroides* has previously been shown to carry ampicillin resistance genes (Nakano et al., 2011), which may account for its up growth in C57BL/6 mice, but does not account for the lack of response in BALB/c mice of the same treatment regime. A significant association of barrier permeability and the relative abundance of *Parabacteroides* was found, with an increase in *Parabacteroides* correlating to increased barrier permeability. The increase in *Parabacteroides* in C57BL/6 in response to antibiotics may be counteracting antibiotic-mediated tightening of the gastrointestinal barrier. A role for *Parabacteroides*-host signalling influencing intestinal barrier integrity was further supported by a significant negative correlation between *Parabacteroides* and expression of barrier regulating ZO-1 mRNA. BALB/C mice exhibited a host-specific change in expression of ZO-1 mRNA, a universally expressed tight junction adaptor protein (Volynets et al., 2016a). We additionally, confirmed previous work demonstrating ZO-1 expression correlates to intestinal permeability, with the expression of ZO-1 in both C57BL/6 and BALB/c mice post-treatment significantly correlating to changes in small intestinal permeability (Volynets et al., 2016a). Similarly to *Parabacteroides*, an association between the relative abundance of genus *Bacteroides* and barrier permeability was observed, with *Bacteroides* abundance exhibiting a positive impact on barrier function. This result supports previous reports of *Bacteroides* sp. increasing barrier function (Hooper et al., 2001; Hsiao et al., 2013). Notably, Hsiao and colleagues demonstrated the clinical relevance of the probiotic *Bacteroides fragilis*, which was found to restore alterations in barrier function, microbiota composition, and reduced behavioural defects in a mouse model of autism spectrum disorder. The results provide evidence of a host-microbe mediated

process underlying antibiotic-related alterations to the gut microbiota composition and intestinal permeability.

Differential responses to antibiotic treatment were found in the mRNA expression of barrier regulating tight junction protein Cldn7 in response to AMP+NEO only in C57BL/6 mice. These subtle changes in gene expression are not surprising as the exposure period in this study was 2 weeks. It is likely that longer exposure to antibiotics would lead to greater changes in host gene expression and barrier function. The increased expression of Cldn7 mRNA in C57BL/6 mice is of interest, since claudins are known to play critical structural and functional roles in tight junction complexes that moderate epithelial permeability (Van Itallie and Anderson, 2006). In the current work, Cldn7 mRNA increased independent of changes in intestinal permeability and was not associated with specific bacterial taxa. Recent evidence has shown that Cldn7 has additional roles maintaining intestinal homeostasis (Ding et al., 2012). Cldn7 is expressed in the basolateral membrane and interacts critically with the extracellular matrix (ECM) components, with loss of function resulting in inflammation and uncontrolled remodelling of ECM (Ding et al., 2012). The increase in Cldn7 mRNA expression may be indicative of alterations in ECM composition or immune signalling cascades, as well part of the host-specific response to maintaining intestinal homeostasis under altered microbial conditions.

Here we provide additional evidence that host-miRNA signalling influences bacterial composition in a strain-dependent manner. A bidirectional relationship between the host miRNA expression and gut microbiota composition has been reported (Dalmasso et al., 2011; Liu et al., 2016; Singh et al., 2012), demonstrating a role for microbiota in regulating

host miRNA expression in IECs and on bacterial diversity (Liu et al., 2016). Fecal miRNA profiles from BALB/c and C57Bl/6 mice clustered by host mouse strain, providing evidence of host genetic-based differences in the local environment of the gastrointestinal tract. Exploring the functional eukaryotic gene targets of the differentially expressed fecal miRNA revealed targets genes localized to the mucin O-type biosynthesis pathway, the end products of which makes up the mucus layer of the gastrointestinal tract. Differences in mucus layer organization and composition have been previously shown to impact gastrointestinal microbiota, as the glycosylated mucins provide a unique energy source to mucolytic bacteria (Staubach et al., 2012). Differences in mucus layer thickness effect secretory immunoglobulin (sIgA) distribution (Johansson et al., 2009), a factor known to play a critical role in maintaining intestinal homeostasis (Corthesy, 2013). The functional pathway analysis of the top expressed fecal miRNA yielded similar significant pathways involving complex glycoprotein synthesis, with targets specifically in the extracellular matrix, mucus O-glycan biosynthesis and proteoglycans pathways. Interestingly, many of the eukaryotic glycan processing genes targeted by the fecal miRNA have prokaryotic homologues (El Kaoutari et al., 2013).

Evaluating the potential for eukaryotic host miRNA to target bacterial genes is a recent consideration, and published work provides evidence of potential regulation of bacterial genes by human miRNA (Liu et al., 2016; Moloney et al., 2018). Here the analysis demonstrates that highly expressed fecal miRNAs have the potential to target murine gut bacterial genes. Previous work looking at bacterial sRNA gene regulation have found multiple possible binding sites for bacterial sRNA to bacterial genes (De Lay et al., 2013), thus understanding the role of miRNA in host-microbe interactions is needed. Exploring

the possibility of eukaryotic miRNA targeting of bacterial genes isn't limited to complementary binding regions in the 3' UTR as it is in eukaryotic miRNA-mRNA interactions (De Lay et al., 2013). Of those taxa at the genus and family level that were conserved within our dataset, 14 significant correlations between fecal miRNA counts and the relative abundance of the predicted taxa were found. Most notably there was a strong negative correlation with let-7b-5p and the relative abundance of *Parabacteroides*. Let-7 family miRNA expression has previously been shown to be involved in the host anti-bacterial defense through modulation of immune response (Schulte et al., 2011). Intriguingly, *Parabacteroides* has also been associated with inflammatory markers (Conley et al., 2016), and here has been found to respond in a host genetics mediated way to antibiotic treatment. The predicted relationship here provides a potential host genetics-based mechanism for the regulation of *Parabacteroides* abundance in the gastrointestinal tract.

The positive association between mir-21a-5p and the relative abundance of predicted genus *Akkermansia* observed here may be reflective of a mechanistic link between *Akkermansia* abundance and miR-21 expression in energy metabolism of the host. Previous work has indicated that *A. muciniphila* plays a role in energy metabolism, specifically in regulating adiposity by regulating mRNA expression of markers of adipocyte differentiation and lipid oxidation (Everard et al., 2013). Mir-21a-5p has also been shown to play a critical role in regulating the proliferation of human adipose tissue (Kim et al., 2012). The relationship observed here provides a plausible mechanism of host genetic control in the relationship between *Akkermansia* and host energy homeostasis.

Prevotella was found to be one of the most divergent taxa between BALB/c and C57Bl/6. Interestingly, miR-2134 was predicted to regulate a *Prevotella* classified gene, based on BLASTn analysis, and is negatively correlated with *Prevotella* abundance. As previously mentioned, *Prevotella* abundance contributes to known human enterotypes (Wu et al., 2011). While, much of the current work has focused on the diet aspect of enterotypes, results here now suggests that host genetics may also contribute. Several negative correlations were found with miRNA predicted to affect Clostridiacea *Clostridum* and the abundance of Clostridiacea *Clostridum*, indicating this genus may be under strict host genetic control. The demand for host derived regulation of *Clostridium* is apparent as numerous *Clostridum.*spp can be pathogenic. Additionally, there is evidence of *Clostridum's* role in modulating host physiology and homeostasis by controlling regulatory T-cell development and producing barrier-modifying SCFAs (Smith et al., 2013). It is likely that the targeting of these bacterial genes could affect bacterial growth. This suggestion is supported by the current analysis, as there was a negative correlation between miRNA expression and relative abundance of these taxa. However, this relationship requires further *in vivo* investigation.

Of the 9 miRNAs significantly correlated to distance traveled in the closed arm, only miR-30a, miR-22 and miR-21 have been previously assessed in the context of behavior or psychological disordered behaviour. Mir-30a-5p exhibited a positive correlation with distance travelled, and has been found to be decreased in PFC of schizophrenic patients, targets BDNF, and is under the control of early growth response protein transcription factor (ERG1) (Liu et al., 2017). Loss of EGR1 in mouse models has a dramatic effect on locomotion, with general decrease in overall movement measured by open field

(MacDonald et al., 2017). A negative correlation has also been observed between increase in mir-30a-5p and the reduction of negative symptoms of schizophrenia (PANSS score) post treatment, indicating a potential use as a treatment response biomarker (Liu et al., 2017). MiR-21 has been shown to be responsive to stress, but studies have exhibited mixed results with either increases or decreases of miR-21 levels found in blood or saliva post stress (Beech et al., 2014; Gidron et al., 2010; Wiegand et al., 2018). Interestingly, here we see a positive relationship between fecal miR-21 counts, locomotion and *Akkermansia* relative abundance. A recent study also found a positive correlation between women with increased activity and *Akkermansia* relative abundance (Bressa et al., 2017), furthering the association between *Akkermansia* and energy homeostasis. A miR-22 SNP is associated with panic disorder, and also regulates BDNF , which has been shown to play a role in regulating locomotion in mice(Kernie et al., 2000). While these correlations observed here do represent an interesting concept, it should be noted that, 6 of the 9 miRNAs were found to be differentially expressed between BALB/c and C57Bl/6 prior to correlation analysis with locomotion, therefore the significant correlations here may represent a coincidental or spurious result. To confirm the association between fecal miRNA, microbial composition and specific behavioural phenotypes, a much larger sample size would be required with additional behavioural tests.

The results here provide useful insights into the mechanisms involved in host-microbe interactions. The collective work on understanding the roles of intestinal miRNAs has revealed their potential to act as a master regulator of gut homeostasis, with work indicating specific miRNome for distinct cell types of the gastrointestinal tract (Peck et al., 2017). Understanding the complex relationships between the host and its gut microbiota,

and the mechanisms that mediate its homeostasis is of critical importance. Since the discovery of the gut brain axis, the field has rapidly progressed, with differences in gut microbiota composition in clinical populations becoming evident. Much of the current work in clinical populations has focused on differences in observed taxa, with efforts currently shifting focus to the use of probiotic therapies as a way to modulate the gut microbiota (Sarkar et al., 2016). Little effort has been made in the field of the gut brain axis to understand the mechanisms responsible for the differences observed. Here, we advance our understanding of host genetic influence on the gut microbiota composition, how manipulating microbiota composition impacts barrier integrity and function, as well as provide preliminary evidence of a potential mechanism of host miRNA-microbe interactions. The information gained here could be used to further identify both environmental and biological factors that contribute to the emerging gut brain axis in psychiatric disorders.

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Appendix

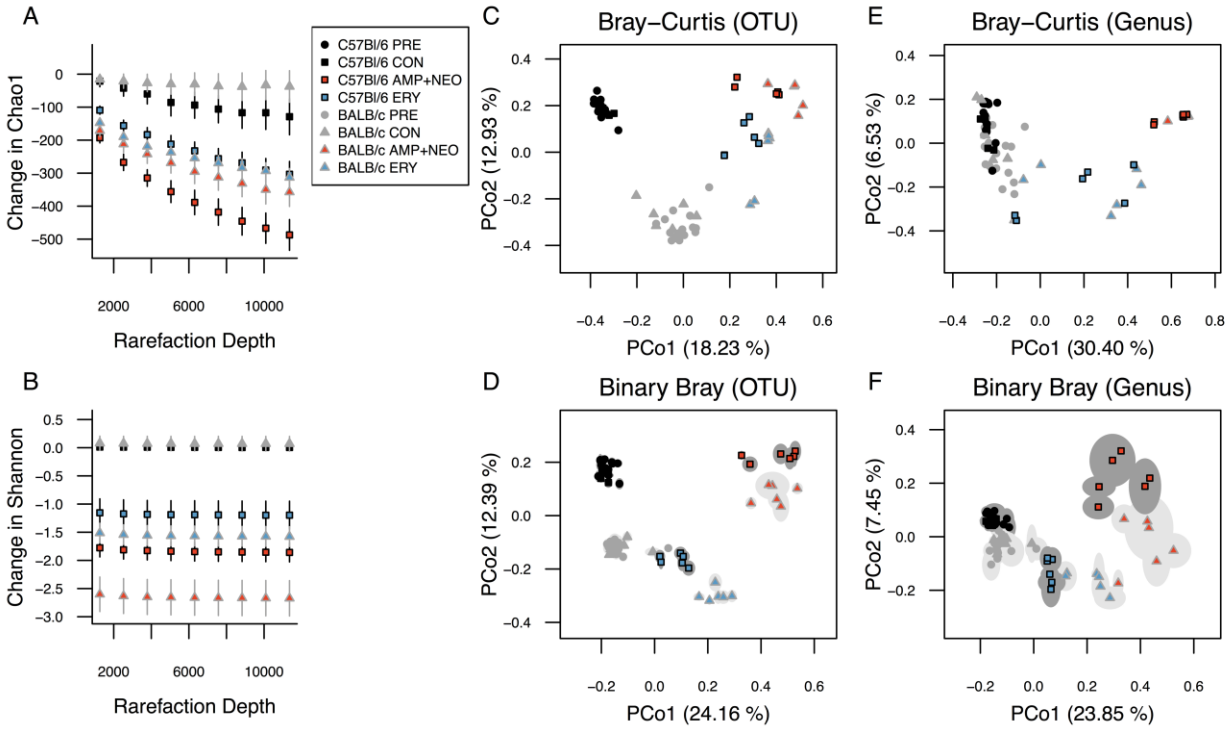

Figure 1. Effect of antibiotic treatment on Alpha and Beta diversity. (A) Change in alpha diversity post treatment with AMP+NEO and ERY in BALB/c and C57Bl/6 as measure by Chao1, and (B) Shannon index. (C-E) Effect of antibiotic treatment on beta diversity, as measured by Bray-Curtis for both OTU and relative abundance genus level. (D-F) Effect of antibiotic treatment on Beta diversity as measured by the binary Bray-Curtis at the OTU and genus level.

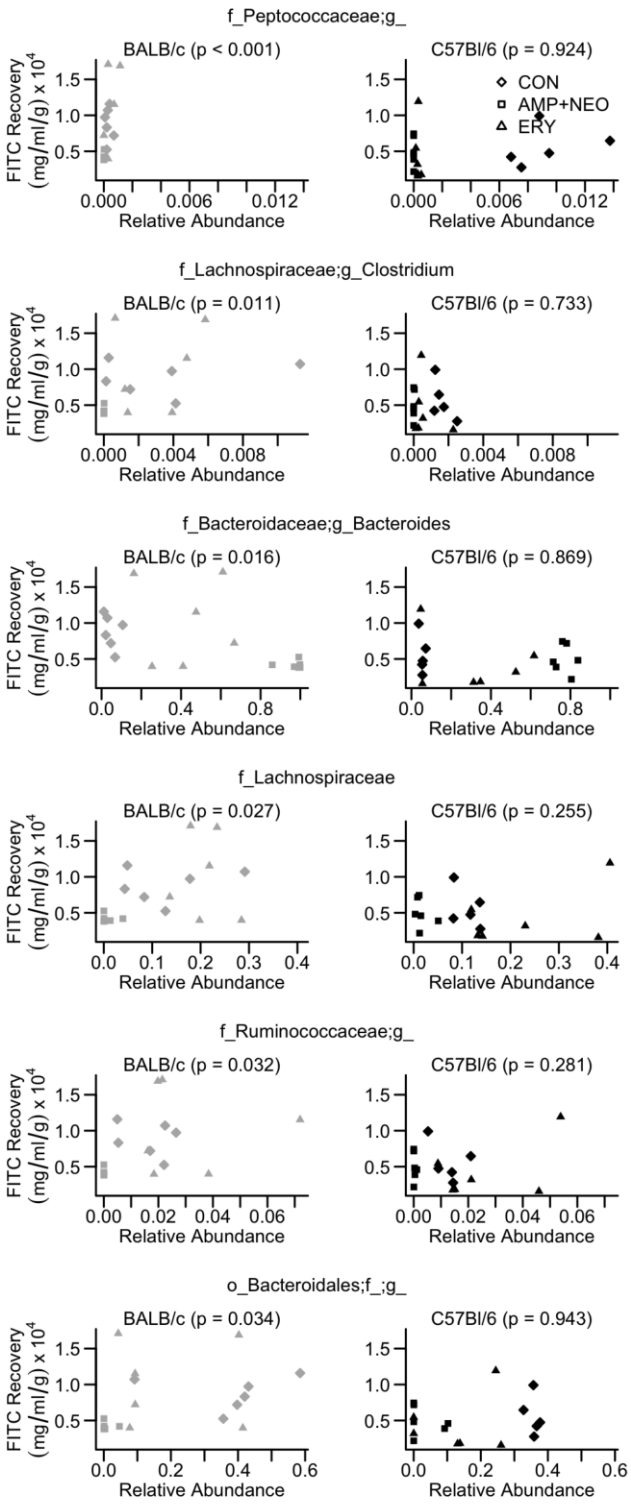

Figure 2. Spearmen's rank correlation analysis between differentially antibiotic responding taxa as identified in Figure 4C and small intestinal barrier permeability as measured by FITC recovery.