Modulation of Neurodevelopmental Outcomes using *Lactobacillus* in a Model of Maternal Microbiome Dysbiosis

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Translational Biology, Medicine, and Health

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August 22, 2019
Blacksburg, Virginia

Keywords: Cx3cr1; gut-brain axis; Lactobacillus; maternal microbiome dysbiosis; microbiota; microglia; neurobehavior; neurodevelopment

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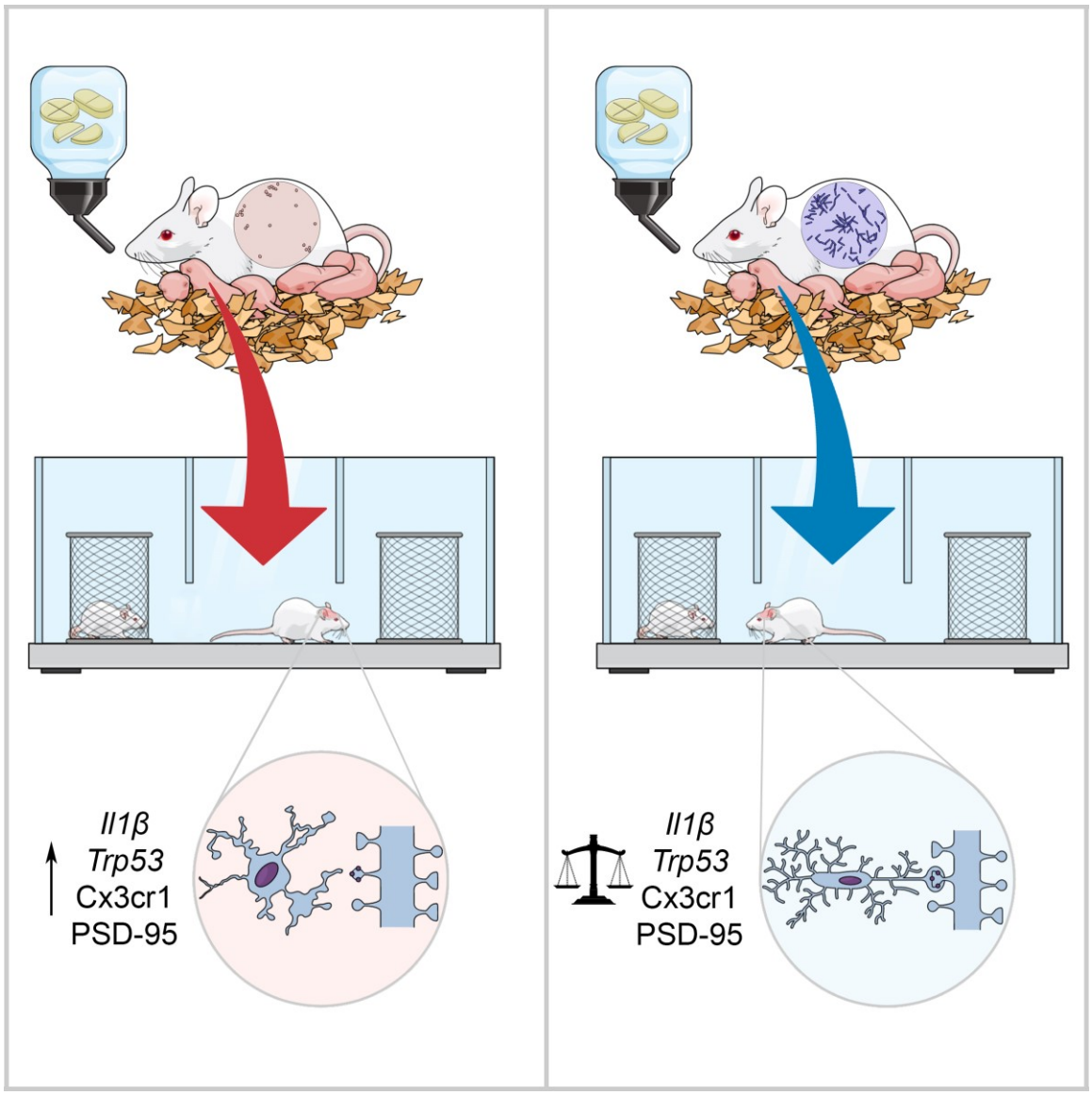
GRAPHICAL ABSTRACT



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ACADEMIC ABSTRACT

Neurodevelopmental disorders, such as autism spectrum disorders, schizophrenia, and attention deficit hyperactivity disorder, are a heterogeneous set of developmental disorders affecting the central nervous system. Studies into their etiology remain challenging, as neurodevelopmental disorders frequently present with a wide range of biological, behavioral, and comorbid symptomologies. Increasing epidemiological reports of antibiotic use during pregnancy as a significant correlate of subsequent mental disorder diagnosis in children suggest a mechanism of influence via the maternal gut-fetal brain axis. Importantly, antibiotics cause dysbiosis of the gut microbiome and disrupt the delicate composition of the microbial inoculum transferred from mother to child, which is critical for development of the immune system and holds implications for long-term health outcomes. The research objective of this dissertation is to reveal a causal mechanism of maternal microbial influence on neurodevelopment by examining the brain's resident immune cells, microglia, and corresponding behavioral outcomes in a mouse model of antibiotics-driven maternal microbiome dysbiosis (MMD). We identify early gross motor deficits and social behavior impairments in offspring born to MMD dams, which paralleled hyperactivated microglia in brain regions specific to cognition and social reward. The MMD microglia also exhibited altered transcriptomic signatures reflective of premature cellular senescence that support evidence of impaired synaptic modeling found in MMD brains. We report that these deficits are rescued in the absence of *Cx3cr1*, a chemokine receptor expressed ubiquitously on microglia, to highlight a pathway in which maternal microbiota may signal to neonatal microglia to undergo appropriate neurodevelopmental actions. Finally, we characterize *Lactobacillus murinus* HU-1, a novel strain of an important gut bacterium found in native rodent microbiota, and demonstrate its use as a probiotic to restore microglial and behavioral dysfunction in MMD offspring.

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GENERAL AUDIENCE ABSTRACT

Population studies on neurodevelopmental disorders, such as autism spectrum disorders, schizophrenia, and attention deficit hyperactivity disorder, highlight antibiotic use during pregnancy as a major correlate of subsequent diagnoses in children. These findings support a growing body of evidence from animal and human studies that the microbial ecosystems ("microbiome") found in and on our bodies play significant roles in mental health, including mood, cognition, and brain function. Importantly, antibiotics during pregnancy create an imbalance of the gut microbiome ("dysbiosis") and disrupt the microbial inoculum transferred from mother to child, which is critical for maturation of the infant immune system and holds implications for long-term health outcomes. Thus, the research objective of this dissertation is to identify a mechanism of influence from the mother's gut to the neonate's brain by examining the brain's resident immune cells ("microglia") in a mouse model of antibiotics-driven maternal microbiome dysbiosis (MMD). We uncover autism-like behavioral deficits and dysfunctional microglia in MMD offspring, and characterize signaling cues specific to microglia by which improper neurodevelopment may be taking place. We also reveal that the detrimental effects of MMD are reversed in mice born to mothers pretreated with a probiotic candidate, *Lactobacillus murinus* HU-1, to suggest maternally-derived *Lactobacillus* may help to mediate proper neurodevelopment.

To Hugh

Acknowledgements

My deepest gratitude goes to Dr. Michelle Theus, whose nurture and expertise were critical for my development as a doctoral trainee. This body of work would not be possible without her foresight and fortitude. I am also utterly indebted to my lab mates, Xia, Ben, Amanda, Kisha, Liz, Kristobal, and Alison, and friends, Meghna and Christina, for sharing their skills, humor, and sympathy with me over the years.

I am grateful for the members of my dissertation committee, Dr. Coy Allen, Dr. Angela Scarpa, and Dr. Harald Sontheimer, who are extraordinary researchers in their respective fields and whose salient advice and incisive questions helped to focus this cross-disciplinary work. I am also thankful to Dr. Paul Morton, Dr. Alicia Pickrell, Dr. Terry Hrubec, and Dr. Nanda Nanthakumar for their mentorship and scientific input.

I would like to acknowledge the organizations that helped to fund this research: VCOM-VMCVM Center for One Health Research, Dannon, VT Center for Autism Research, and VT Graduate Student Assembly. And I would like to acknowledge the PhD Program in Translational Biology, Medicine, and Health and the Regenerative Medicine Interdisciplinary Graduate Education Program for their support of my graduate studies.

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YL wrote the manuscript. YL and VR generated figures. IA and MT conducted final editing and review of the manuscript.

Chapter 3: Research Manuscript

Lebovitz, Y., Kowalski, E.A., Wang, X., Kelly, C., Lee, M., McDonald, V., Ward, R., Creasey, M., Mills, W., Gudenschwager-Basso, E.K., Hazy, A., Hrubec, T., and Theus, M.H. (2019). *Lactobacillus* rescues postnatal neurobehavioral and microglial dysfunction in a model of maternal microbiome dysbiosis. *Brain Behav Immun*, doi: 10.1016/j.bbi.2019.07.025.

YL and MHT conducted research, wrote the manuscript, and generated figures. EAK, XW, and EKG performed RNA isolation and qPCR. XW performed microglial isolation. WM contributed confocal images in Figure 2. CK, ML, VM, RW, and MC contributed to animal husbandry, reagent preparation, behavior testing, immunohistochemistry, and stereology. AH contributed RNAseq data analysis. VM and TH produced embryonic mouse data.

Chapter 4: Research Manuscript

Lebovitz, Y., and Theus, M.H. (2019). Molecular phenotyping and genomic characterization of a novel neuroactive bacterium strain, *Lactobacillus murinus* HU-1. *Front Pharmacol,* Under review.

YL conducted research, wrote the manuscript, and generated figures. MHT conducted final editing and review of the manuscript.

Chapter 1: Introduction

The neuroimmune system, composed primarily of glial cells, is distinct from the peripheral immune system and plays a significant role in brain development and function (Bilimoria and Stevens, 2015). Emerging evidence suggests microglial disturbances may interrupt typical developmental processes, such as neuronal function and synaptic pruning, to result in physiological and behavioral traits of neurodevelopmental disorders (Meltzer and Van de Water, 2017). Interestingly, the gut microbiome is an important regulator of both peripheral and neuroimmune function (Blacher et al., 2017; Dinan and Cryan, 2017). Disruption of this milieu via antibiotic treatment in animal models can induce adult microglial dysfunction and aberrant behaviors (Mohle et al., 2016; Tochitani et al., 2016). These preclinical results support clinical findings that antibiotics can cause defective inflammatory signaling in immune cells (Lankelma et al., 2016), and that dietary probiotic supplementation resulted in measurable psychotropic responses and enhanced brain functional connectivity (Messaoudi et al., 2011; Tillisch et al., 2013). Moreover, recent epidemiological studies highlight antibiotic use during pregnancy as a major event associated with subsequent mental disorder diagnoses in children (Atladottir et al., 2012; Hisle-Gorman et al., 2018). As such, the maternal microbiome may be inextricably linked to proper neurodevelopment. To date, however, the maternal gut-fetal brain axis remains understudied in clinical research, and preclinical studies have mainly focused on neurobehavioral outcomes of pregnancy events in adult animals (De Palma et al., 2015; Buffington et al., 2016). And although gut microbiota has observable control over various subsets of immune cells and can modulate systemic immunity in the adult

(Wu and Wu, 2012; Benakis et al., 2016), its role in regulating immature microglia and the impact on neurodevelopmental processes are relatively unknown.

The doctoral research described in this dissertation is based on the hypothesis that *Lactobacillus* bacteria, which is a key member of both the healthy gut and vaginal microbiomes, enables homeostatic conditions in maternal microbiota that are necessary for proper neurodevelopment. In order to test this hypothesis, we assume a clinically-relevant experimental design using antibiotics to induce maternal microbiome dysbiosis in a murine model of pregnancy. As measures of appropriate neurodevelopment, we conduct longitudinal assessments of the offspring's behavior until time of weaning, and then examine the state of microglia in brain regions pertinent to neurodevelopmental disorders—all in the presence and absence of a lab-generated novel strain of a rodent-specific commensal gut bacterium, *Lactobacillus murinus* HU-1. We also utilize transgenic animals that do not possess functional *Cx3cr1*, a chemokine receptor expressed almost exclusively by microglia in the brain and is critical for microglia-neuron communication, in order to delineate the contribution of *Lactobacillus* from microglia-specific activity on offspring behavioral outcomes.

To present the findings of this work in an integrative manner, this dissertation has been organized as a series of published manuscripts. Chapter 2 introduces the basis for studying the maternal gut-fetal brain axis by drawing on links between microbiome, microglia, and neurodevelopment and synthesizes emerging scientific literature across neuroscience, microbiology, immunology, and developmental biology fields. This content was published in *Frontiers in Immunology* in 2018. Chapter 3 is the culmination of the majority of this doctoral research and describes in full the development of our

model of maternal microbiome dysbiosis, experiments to validate the model, and its application. This content was published in *Brain, Behavior, and Immunity* in 2019. Chapter 4 explores the probiotic potential of *Lactobacillus murinus* HU-1 through computational analysis of its genome structure. This content was submitted to *Frontiers in Pharmacology.*

Given the complex etiology of disordered neurodevelopment and the common medical need for antibiotics during pregnancy, the goal of this body of work is to identify protective factor(s) that reduce the severity of neurobehavioral symptoms through controlled manipulation of the maternal gut microbiome. The results of this study contribute information on temporal susceptibility of the neonatal brain to probiotic therapy, developmental onset of gross motor delays and their potential as predictors of subsequent social behavior impairments, Cx3cr1-signaling as a previously unrecognized communication pathway of the gut-brain axis, and the role of maternally-derived *Lactobacillus murinus* in preventing a premature senescent-like phenotype in offspring microglia.

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Chapter 2: Review of Literature

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This work was published in *Frontiers in Immunology* as "Emerging Developments in Microbiome and Microglia Research: Implications for Neurodevelopmental Disorders." https://doi.org/10.3389/fimmu.2018.01993. Open access.

2.1 Abstract

From immunology to neuroscience, interactions between the microbiome and host are increasingly appreciated as potent drivers of health and disease. Epidemiological studies previously identified compelling correlations between perinatal microbiome insults and neurobehavioral outcomes, the mechanistic details of which are just beginning to take shape thanks to germ-free and antibiotics-based animal models. This review summarizes parallel developments from clinical and preclinical research that suggest neuroactive roles for gut bacteria and their metabolites. We also examine the nascent field of microbiome-microglia crosstalk research, which includes pharmacological and genetic strategies to inform functional capabilities of microglia in response to microbial programming. Finally, we address an emerging hypothesis behind neurodevelopmental disorders, which implicates microbiome dysbiosis in the atypical programming of neuroimmune cells, namely microglia.

2.2 Introduction

The various microbial ecosystems ("microbiota") and their component genes ("microbiomes") existing on and within the host body are increasingly recognized as significant contributors to a functional host immune system. Commensal microbiota consist of bacteria, fungi, viruses, and other microorganisms that make up distinct microbial ecologies of various host systems, such as the gastrointestinal tract, skin, mouth, and genitourinary tract (Human Microbiome Project, 2012). Within each physiological niche, microbial compositions can vary widely according to the environment and mutualistic functions that they may serve in conjunction with the host (Segata et al., 2012). In the gut, where microbial density is the highest, some of these functions include forming a physical barrier against infection by pathogenic microbes, acting as a bioreactor for digestion and nutrient absorption, and sensitizing the host immune system (Brestoff & Artis, 2013). The latter interaction is especially critical in early development, as the colonization of the infant gut with an initial inoculum of maternal microbiota primes the neonatal peripheral immune system (Backhed et al., 2015; Gomez de Aguero et al., 2016).

Given the wealth of evidence depicting microbiota as a driver of early peripheral immunity, an important follow-up question remains as to whether the microbiome may also drive immune development in the brain. The neuroimmune system, primarily comprised of glial cells, is distinct from the peripheral immune system in part due to anatomical barriers and developmental sequence. Microglia, the resident macrophage-like cells in the brain, play an especially critical role in neurodevelopment through their numerous functions in patterning and wiring of the maturing brain. Accordingly, recent

studies on neurodevelopmental disorders, such as autism spectrum disorders (ASD), Rett syndrome, and schizophrenia, whose complex pathologies include neuronal and synaptic dysfunction, suggest improper microglial activity as a contributor to these disorders' neurobiological and behavioral outcomes (Hui et al., 2018; A. S. Lee, Azmitia, & Whitaker-Azmitia, 2017; Maezawa & Jin, 2010). Interestingly, additional studies suggest that microglia, not unlike peripheral macrophages, may be susceptible to microbiome changes (Erny et al., 2015; Thion et al., 2018). Altogether, the demonstration of microbial influence on brain function via microglial mediators raises the possibility that manipulation of microbe-immune crosstalk represents a promising strategy for treating neurological diseases.

2.3 Microbiome and Neurodevelopment

A growing number of studies pointing to distinct gut microbiome profiles among psychiatric patient populations allude to microbiota as an important corollary of disease pathology (De Angelis et al., 2013; Dickerson, Severance, & Yolken, 2017; Jiang et al., 2018). The purported mechanisms for microbial linkages to aberrant neurobehavioral outcomes are broadly considered to be due to impaired gut-brain communication, including but not limited to those cause by cytokine imbalance, vagal nerve signaling, and hypothalamic-pituitary-adrenal (HPA) axis responses (Bravo et al., 2011; N. Sudo et al., 2004; M. L. Wong et al., 2016).

Disruptions to gut microbiota are also implicated in aberrant neurodevelopmental outcomes (Diaz Heijtz et al., 2011; Hsiao et al., 2013; S. Kim et al., 2017). Here, the impaired gut-brain pathways are extrapolated to include both mother and fetus, i.e., the

maternal gut-fetal brain axis. Permeability of maternal gut epithelium, the placental barrier, and fetal blood-brain barrier are potential factors of maternal gut-fetal brain communication, as are neuroactive microbial metabolites that are small enough to bypass these barriers (Braniste et al., 2014; Gomez de Aguero et al., 2016). The conceptual basis for how the maternal microbiome may drive offspring neurodevelopment is illustrated in **Figure 1**. Maternal skin and vaginal microbiota play a critical role in seeding the infant microbiota and were shown to contribute equally to various body site taxa in infants up to 6 weeks of age in vaginal deliveries and cesarean deliveries accompanied by active labor (Chu et al., 2017). In addition, amniotic fluid, placenta, and umbilical cord blood possess their own niche microbiomes, although the manner and extent to which these microbial communities communicate with the mother or fetus are not yet clear (Jordan et al., 2017).

While the gut microbiome is constantly evolving in response to dietary and environmental changes, longitudinal sampling of infant stool during the first three years of life demonstrated resiliency in its ability to return to original homeostatic conditions following short periods of antibiotic usage (Yassour et al., 2016). Additional studies suggest that the overall composition of the gut microbiome may remain stable across multiple decades of life (Faith et al., 2013). Interestingly, developmental shifts in gut microbial composition align with milestones in brain development, such as neuronal migration and proliferation, myelination, and synaptic pruning (**Figure 2**). Although these developmental correlations do not necessarily indicate a causal relationship, strong evidence from experimental models using germ-free (GF) and antibiotic-treated rodents showed that the complete absence or severe reduction of gut microbiota, respectively, resulted in altered brain chemistry, transcriptional changes, and atypical behaviors

compared to controls (Diaz Heijtz et al., 2011; Leclercq et al., 2017; Tochitani et al., 2016).

Nonetheless, large-scale epidemiological studies highlight compelling correlations between certain microbiome-modifying pregnancy events and subsequent diagnoses of neurodevelopmental disorders in children. Maternal infection and antibiotic use during pregnancy are often highlighted as potential risk factors for ASD (Atladottir et al., 2012; Hisle-Gorman et al., 2018; Zerbo et al., 2013). Of the former, meta-analysis of studies reporting ASD risk of maternal infections resulted in significant associations with bacterial infections during second and third trimesters. The odds of subsequent ASD diagnosis were slightly greater than those reported for viral infections, but not as high as any maternal infection combined with a hospital visit (Jiang et al., 2016). Such results also comport with numerous animal studies that used viral or bacterial components to elicit maternal immune activation and resulted in broad-based ASD- and schizophrenia-like phenotypes in the offspring, including neuroinflammation, dysregulated neural circuitry, behavioral deficits, and gene expression changes (Fatemi et al., 2008; Garay, Hsiao, Patterson, & McAllister, 2013; Y. Li et al., 2018; Onore, Schwartzer, Careaga, Berman, & Ashwood, 2014; Sherman, Zaghouani, & Niklas, 2015). Within the context of the mother-child dyad, these findings suggest that acute, immune insults as a result of microbial dysbiosis during pregnancy may be more influential on neurodevelopmental outcomes than chronic conditions that mainly affect the mother alone.

2.3.1 Gut bacteria

Gastrointestinal issues are a common comorbidity in ASD, which remains a factor even when considering non-autistic sibling controls (Isaksson, Pettersson, Kostrzewa, Diaz Heijtz, & Bolte, 2017). Comparative gut microbial profiling studies in ASD suggest that, in the absence of a pathogenic infection, the lack of commensal microbes and/or microbial communities found in neurotypical counterparts may contribute to adverse health outcomes. Pyrosequencing of fecal bacteria DNA in children diagnosed with ASD determined lower abundance of gut bacteria species known to ferment complex carbohydrates, such as *Prevotella*, *Coprococcus*, and *Veillonellaceae* compared to non-autistic children (Kang et al., 2013). Meanwhile, other studies reported increased abundance of *Bacteroides*, *Ruminococcus*, and *Sutterella* in autistic children compared to controls (Finegold et al., 2010; L. Wang et al., 2013). A recent open-labeled clinical study showed that fecal microbiota transplantation resulted in mitigation of both gastrointestinal and behavioral symptoms in autistic children that corresponded with increased diversity of gut microbiota and increased abundance of previously low populations, such as *Prevotella* (Kang et al., 2017). Accordingly, targeted communities of commensal gut microbiota are currently under investigation as possible catalysts for gut-brain signaling. At present, research on these microbes are bacteria-specific and frequently coincide with research on probiotics. Of clinical interest are bacterial species found in maternal microflora during pregnancy and in the neonatal gut, such as *Lactobacillus*, *Bifidobacterium*, and *Bacteroides*, whose presence represent homeostatic conditions during healthy development (Dominguez-Bello et al., 2010).

Lactobacillus is a key component of a complete and diverse gut microbiome and represents a genus of bacteria naturally found in the gut of healthy mammals. The human vaginal tract microbiome is also primarily dominated by *Lactobacillus* spp. followed by anaerobic species from *Prevotella* and *Sneathia* spp. (Dominguez-Bello et al., 2010). During pregnancy, the vaginal microbiome undergoes remodeling that results in reduced diversity, increased stability, and enrichment of Lactobacilli. This is thought to protect against pathogenic infection through increased lactic acid production and decreased pH levels (Aagaard et al., 2012; McLean & Rosenstein, 2000). Parallel sampling of maternal and neonatal microbiota showed that the gut microbiota of vaginally-delivered infants reflects bacterial species found in the maternal gut microbiome, whereas cesarean-delivered infants were more likely to harbor bacteria from maternal skin microbiome, e.g., *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. (Dominguez-Bello et al., 2010). Meanwhile, the use of intrapartum antibiotics resulted in infant gut dysbiosis at 3 months and 12 months of age regardless of the mode of delivery (Azad et al., 2016).

As a commensal microbe, *Lactobacilli* in the gastrointestinal tract confer beneficial effects to digestion through lactic acid fermentation of foods (G. T. Macfarlane & Macfarlane, 2012) and prime immune cells via interactions with leukocytes in mesenteric lymph nodes and/or via dendritic cell sampling of gut lumen contents (Aktas et al., 2015; Christensen, Frokiaer, & Pestka, 2002; Fink et al., 2012). The influence on immune system priming has been shown to have significant effects in biological and behavioral outcomes. For example, changes to HPA axis signaling and reduced corticosterone levels were observed in mice following oral administration with various

Lactobacilli strains, such as *L. rhamnosus*, (Bravo et al., 2011). Similarly, *L. reuteri* represents another well-studied strain with reports of restoration to ventral tegmental area synaptic plasticity and oxytocin production in mice born to dams on a high fat diet (Buffington et al., 2016). Meanwhile, administration of *L. helveticus* resulted in decreased levels of inflammatory cytokines and improved performance in spatial memory and anxiety-related behavior tasks in IL-$10^{-/-}$ mice (Ohland et al., 2013).

Given the broad evidence base supporting the molecular and physiological impact of *Lactobacillus* spp., the continued focus on this genus of bacteria is not surprising. Nonetheless, other bacteria, such as *Bifidobacteria* and *Bacteroides*, have also demonstrated ability to regulate immune response and behavior. For example, oral feeding of *Bacteroides fragilis* to weaned mice in a maternal immune activation model of autism resulted in the recovery of gut barrier proteins, Claudin-5 and -8, and rescue of anxiety-like and stereotypic behaviors (Hsiao et al., 2013). These findings suggest that bacterial functionality, such as the ability to sensitize immune cells or produce bioactive metabolites, may be a better indicator of gut-brain interaction than mere taxonomy.

2.3.2 Gut bacterial metabolites

The discovery of penicillin by Alexander Fleming popularized the notion that byproducts of microbial metabolism could serve as potent chemicals (Fleming, 1929). These metabolic byproducts, or metabolites, range broadly in terms of their molecular assembly and function. They can act as quorum sensing molecules, energy substrates, or even competitive antimicrobials against other microbes (Biggs et al., 2017; Chow & Jewesson, 1985; G. Li & Young, 2013). Microbial metabolism is also one of the critical

functions of the gut microbiome in maintaining host health; the mammalian digestive system is incapable of extracting many key nutrients, such as vitamins, amino acids, and energy, from diet and relies on commensal gut microbes for these tasks (Velagapudi et al., 2010).

The most abundant products of gut bacterial metabolism are short-chain fatty acids (SCFAs), which result from bacterial fermentation of complex carbohydrates and proteins in the colon. SCFAs refer to fatty acids consisting of one to six carbon atoms, but predominantly consist of acetic acid, butyric acid, and propionic acid in the mammalian gut (S. Macfarlane & Macfarlane, 2003). Of the three, acetic acid (anion: acetate) makes up the largest portion of SCFA distribution in the colon, where it readily enters the circulatory system to act as a vasodilator or energy substrate for peripheral tissues (Bergman, 1990). Radiolabeled colonic acetate has been shown to pass the blood-brain barrier to serve as an energy substrate for astrocytes, but also to preferentially accumulate in the hypothalamus where it is converted to acetyl-CoA leading to downstream suppression of appetite-related hormones, Neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Frost et al., 2014; Wyss, Magistretti, Buck, & Weber, 2011). Butyric acid (anion: butyrate) is an important energy substrate for colonocytes and a well-documented histone deacetylase (HDAC) inhibitor with pharmaceutical potential for neurodegenerative diseases (Ferrante et al., 2003; Govindarajan, Agis-Balboa, Walter, Sananbenesi, & Fischer, 2011). Interestingly, exposing microglia to sodium butyrate in vitro resulted in differential inflammatory responses wherein rat primary cells, hippocampal slice cultures, and neural co-cultures (consisting of microglia, astrocytes, and neurons) resulted in an anti-inflammatory effect against LPS, but cultured murine N9

microglial cells elicited a pro-inflammatory response (Huuskonen, Suuronen, Nuutinen, Kyrylenko, & Salminen, 2004). Propionic acid (anion: propionate) appears to be the only SCFA to demonstrate adverse effects in the brain, as direct intracerebroventricular injection of propionic acid in rats yielded a wide range of autism-like neurobehavioral changes, including repetitive motion and increased markers for astrocyte and microglia immunoreactivity (GFAP and CD68, respectively) (MacFabe et al., 2007).

While SCFAs' best known functions are to serve as fuel for colonocytes and regulators of host metabolism, recent investigations revealed that SCFAs directly interact with the nervous system via G protein-coupled receptors, GPR41 and GPR43 (or free fatty acid receptor 3 [FFAR3] and FFAR2, respectively) (Kimura et al., 2011; Nohr et al., 2013). Previously deemed "orphan" receptors, GPR41 and GPR43 are now understood to be expressed broadly on host tissues and immune cells and are involved in the resolution of inflammatory responses (Brown et al., 2003; Haghikia et al., 2015; Maslowski et al., 2009). Within the brain, GPR41 is expressed at low levels in the cerebral cortex, hippocampus, caudate, and cerebellum and preferentially binds to butyrate and propionate, whereas GPR43 is expressed at moderate levels in the caudate and preferentially binds to acetate and propionate (Brown et al., 2003; Uhlen et al., 2015). Much remains to be discovered about the signaling mechanisms of these receptors, but emerging studies point to downstream activation of immune responses, such as IgA promotion and inhibition of NF-κB pathway, that are specifically triggered according to the type of metabolite ligand and location of the receptor (S. U. Lee et al., 2013; W. Wu et al., 2017).

2.4 Microbiome and Microglia

Microglia are primarily recognized as the resident macrophage-like cells in the brain that serve as first-responders to pathogens, apoptotic cells, and debris with the ability to secrete soluble factors that modulate inflammatory responses. Recent studies paint a more complex portrayal of these highly-motile glial cells, which have since been found ubiquitously throughout the central nervous system, including the spinal cord, with region-specific phenotypes (Caldero, Brunet, Ciutat, Hereu, & Esquerda, 2009; De Biase et al., 2017; Zhu et al., 2008). Microglia originate as yolk sac progenitors (**Figure 2**) and are purported to migrate to the brain during early prenatal development, as demonstrated through single-cell RNAseq studies (Matcovitch-Natan et al., 2016; Thion et al., 2018). Perhaps appropriate given their presence in the embryo, microglia serve numerous critical functions in wiring and patterning of the developing brain. Through the production of neurotrophic factors, microglia contribute to neurogenesis and guidance of sprouting vessels, as well as phagocytosing synapses and shaping neuronal circuitry (Shigemoto-Mogami, Hoshikawa, Goldman, Sekino, & Sato, 2014; Weinhard et al., 2018). The stepwise processes in which these actions occur are not yet fully clear, although recent studies form a widening picture of microglial contribution to the proper maturation of the brain.

Current hypotheses surrounding the underlying etiology of neurodevelopmental disorders focus on microglia's dual immune and trophic capacities. Neuroimaging studies of ASD patients showed hypermyelination in both left and right medial frontal cortex, hypomyelination of the left temporo-parietal junction, and decreased local and long-range functional connectivity (Carmody & Lewis, 2010; Khan et al., 2013). These findings are

supported by murine models of ASD, whereby impediments to microglial functions resulted in under-pruning of synapses, hypermyelination of the prefrontal cortex, and reduced long-range functional brain connectivity (Hoban et al., 2016; H. J. Kim et al., 2017; Zhan et al., 2014). In contrast, rodent models of schizophrenia allude to an over-pruning effect of dysfunctional microglia wherein pharmaceutical intervention using minocycline resulted in stalled engulfment activity and rescued behavioral deficits (Giovanoli et al., 2016; Hui et al., 2018; Mallya, Wang, Lee, & Deutch, 2019). Genomic analysis of psychiatric disorders indicated upregulation of astrocyte-related genes and downregulation of neuronal/microglia-related genes across ASD, schizophrenia, and bipolar disorder to further propose a shared susceptibility in neuroimmune-specific gene networks (Gandal et al., 2018).

In the gastrointestinal tract, microbiota and associated metabolites have been shown to elicit both pro- and anti-inflammatory peripheral immune cell responses and to control cellular proliferation and epithelial barrier integrity (Christensen et al., 2002; Furusawa et al., 2013; Hayashi et al., 2013; Pull, Doherty, Mills, Gordon, & Stappenbeck, 2005). Evidence of increased blood-brain barrier disruption, altered microglia morphology, and increased microglia density in GF mice suggests that the microbiome may have a similar influence on the brain (Braniste et al., 2014; Erny et al., 2015; Thion et al., 2018). However, contrary to expectations, gene expression analysis of postmortem cerebral cortex and cerebellum tissues from ASD patients showed upregulation of genes associated with barrier proteins (e.g., Claudin-5 and TRiC) compared to controls; the same analysis of small intestine duodenal tissue revealed decreased expression of barrier protein-associated genes (Fiorentino et al., 2016).

Together, these data suggest that the epithelial cell barrier integrity in the gut is compromised, but barrier protein expression in the brain is increased in ASD.

The microbiome is also implicated in the neuroinflammation hypothesis underlying the ontogeny of neurodevelopmental disorders. With respect to ASD, anatomical evidence of unusual and sustained brain overgrowth in autistic children first alluded to improper cellular responses wherein the hyperproliferative stage of early neurodevelopment continues unchecked and unresolved (Courchesne, Carper, & Akshoomoff, 2003; Courchesne et al., 2001). Thus, the neuroinflammation hypothesis stipulates neonatal microbial dysbiosis leads to improper priming of the immune system, which leads to reduced synaptic pruning and brain overgrowth in ASD. Paradoxically, immunohistochemical and cytokine profiling of brain tissue and cerebrospinal fluid samples from ASD patients showed abundance of activated microglia and increased expression of macrophage chemoattractant factor-1 (MCP-1) and tumor growth factor-β1 (TGF- β1) in cerebral cortex, white matter, and cerebellum (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). Additional studies of ASD postmortem brain tissue also depicted decreased numbers of ramified, or resting state, microglia in grey and white matter and increased primed, or activated, microglia in grey matter of ASD brains compared to control samples (A. S. Lee et al., 2017; P. M. Smith et al., 2013). The exact mechanism by which activated microglia neglect to phagocytose excess myelin, synapses and/or neurons, and whether this is mediated by a microbiome-immune crosstalk, is yet unknown.

Increasing evidence suggests the microbiome plays a contributing role in the function of microglia in neurodevelopment. A number of models were used to evaluate

cause and effect through a reductive process that includes the use of GF models. Erny, et al., found that compared to specific-pathogen free (SPF) mice, GF microglia had marked differences in mRNA expression profiles, including a reduction in genes associated with cell activation and immune signal transduction (Erny et al., 2015). While no gross or histologic abnormalities were observed in the central nervous system, GF microglia displayed an immature morphology, reduced capacity to respond to a viral infection challenge, and significant reduction in expression of regulators of microglia cell proliferation, differentiation, activation, and transformation (Erny et al., 2015). Additionally, microglia of antibiotic-treated and Ffar2-/- mice that do not express the receptor gene for SCFA-binding also resembled a GF phenotype, which could be rescued by oral feeding of SCFAs in the former group but not the latter (Erny et al., 2015). Thus, bacteria-derived metabolites likely play a necessary and sufficient role in proper microglia development, although the mechanism(s) regarding how microbiota influence microglia-neuron interactions during development and the behavioral consequences that ensue following dysbiosis remain elusive.

2.5 Conclusion

The numerous microbiome-related studies across disparate scientific disciplines agree on a prevailing hypothesis that the microbiome is capable of communicating via immune, metabolic, and endocrine signals to modulate brain health and disease. The current evidence base from both human and animal studies to support this hypothesis, however, are largely correlational without definitive understanding of cause. The mechanisms by which the microbiome asserts microglial changes during

neurodevelopment are also not yet known, although transgenic mice and single cell sequencing methods continue to inform molecular processes that are likely to be involved. At present, the majority of microbiome-related studies are conducted in adult mice and assume a linear direction of influence from microbiota to the host. In recognition of the microbiome's role in neurodevelopmental processes, future studies should include appropriate experimental models that address the maternal gut-fetal gut axis as well as the possibility of multidirectional signaling pathways.

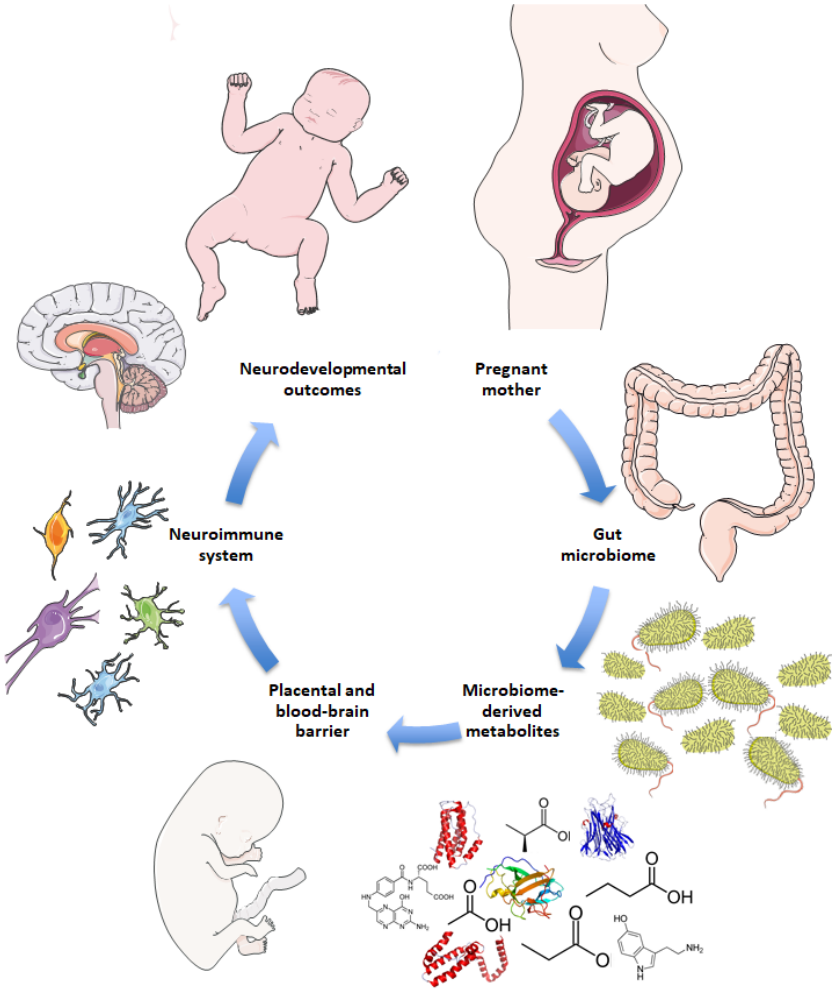

Figure 1. Schematic for maternal microbiome influence on neurodevelopment. Current hypotheses propose disruptions to the maternal gut microbiome during pregnancy, such as antibiotic use, lead to altered gut microbial communities and subsequently altered levels of microbe-derived metabolites and impaired immune signaling. Microbial metabolites include neurotransmitters, neuropeptides, and short-chain fatty acids that are small enough to bypass the placental and fetal blood-brain barriers. Microbial metabolites may serve neuroactive roles through immune priming interactions with microglia in the fetal brain to potentially drive neurodevelopmental changes and behavioral outcomes later in life.

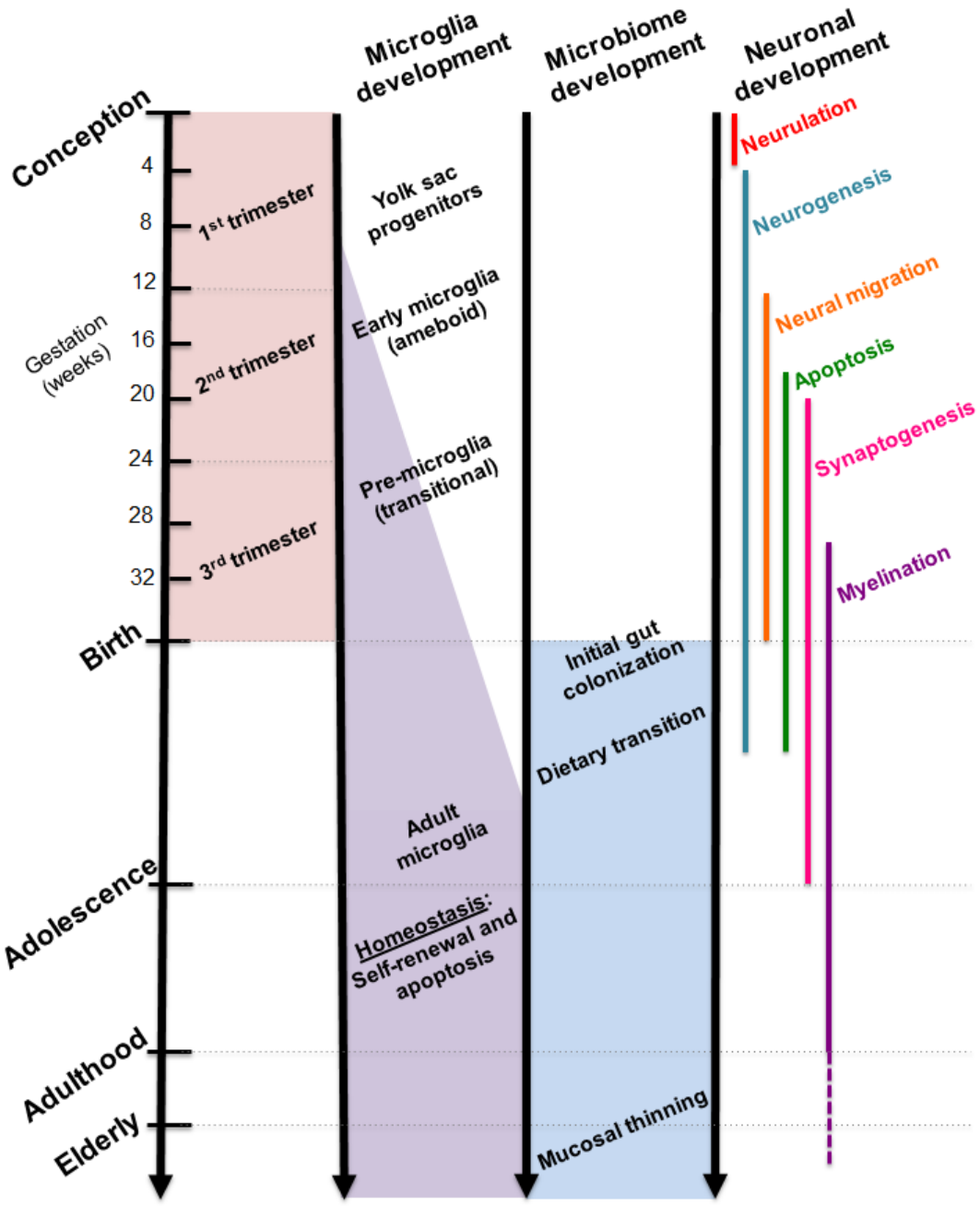

Figure 2. Comparative timelines for human microglia, gut microbiome, and neuronal development. Critical stages in brain development coincide with infant gut colonization to suggest maternal microbiome may serve as an important inoculum in priming the neuroimmune system.

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Chapter 3: *Lactobacillus* Rescues Postnatal Neurobehavioral and Microglial Dysfunction in A Model of Maternal Microbiome Dysbiosis

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This work was published in *Brain, Behavior, and Immunity*.

https://doi.org/10.1016/j.bbi.2019.07.025.

3.1 Abstract

Increasing reports of pregnancy events leading to maternal microbiome dysbiosis (MMD) show strong correlates with atypical neurodevelopmental outcomes. However, the mechanism(s) driving microbiome-mediated behavioral dysfunction in offspring remain understudied. Here, we demonstrate the presence of a novel gut commensal bacterium strain, Lactobacillus murinus HU-1, was sufficient to rescue behavioral deficits and brain region-specific microglial activation observed in MMD-reared murine offspring. We further identified a postnatal window of susceptibility that could prevent social impairments with timed maternal administration of the symbiotic bacterium. Moreover, MMD increased expression of microglial senescence genes, Trp53 and Il1β, and Cx3cr1 protein in the prefrontal cortex, which correlated with dysfunctional modeling of synapses and accompanied dysbiosis-induced microglial activation. MMD male offspring harboring Lactobacillus murinus HU-1 or lacking Cx3cr1 showed amelioration of these effects. The current study describes a new avenue of influence by which maternally transferred Lactobacillus drives proper development of social behavior in the offspring through microglia-specific regulation of Cx3cr1 signaling.

3.2 Introduction

An emerging evidence base of large-scale, retrospective epidemiological studies on pregnancy risk factors for neurodevelopmental disorders have converged on acute fever, severe infections, and broad spectrum antibiotic use as significant correlates of mental disorders, such as autism and schizophrenia (Atladottir et al., 2012; Lee et al., 2015; Jiang et al., 2016; Al-Haddad et al., 2019; Lydholm et al., 2019). Acute fever and severe infections during pregnancy are directly attributed to maternal immune activation and subsequent disruptions to fetal development, including that of the central nervous system (Mortensen et al., 2007; Smith et al., 2007; Choi et al., 2016). Meanwhile, antibiotics represent the most prescribed medication during pregnancy and early postpartum periods (Andrade et al., 2004). These factors are hypothesized to disrupt maternal microbiota and influence the critical microbial inoculum that is transferred from mother to child. However, the exact nature of the relationship between bacteria-related microbiome dysbiosis and atypical neurodevelopment remains unclear.

Under typical conditions, symbiotic bacteria in the gut, vagina, skin, and breast are uniquely consolidated around specific groups (e.g., *Lactobacillus*) and these specialized microbial environments are thought to be necessary for maintaining immune balance and for priming the naïve immune system (Backhed et al., 2015; Nuriel-Ohayon et al., 2016; Sharon et al., 2016; Vuong et al., 2017). Accordingly, germ-free conditions result in the aberrant development of resident microglia in the brain, which can be rescued by restoring a complex microbiota (Erny et al., 2017; Hammond et al., 2018). These effects have been postulated to have a major impact on microglial functions, including maintenance of synapses and control of neuroinflammation. Moreover, these

findings suggest the gut-brain axis is central in supporting normal neuron-microglia communication. Recent studies also demonstrate that *Lactobacillus* species can correct social impairments in several mouse models of autism due to afferent vagal nerve regulation of brain regions involved in social reward (Sgritta et al., 2019). The exact mechanism(s) of action underlying the role of gut microbiota on social development remains unclear.

Animal models of gut dysbiosis, whether due to reduced bacterial diversity or complete germ-free status, indicate that the microglia in the adult offspring show aberrations with respect to morphology, functionality, and expression of inflammatory genes (Erny et al., 2015; Matcovitch-Natan et al., 2016; Thion et al., 2018). Adult offspring reared under altered gut microbial conditions exhibit behavioral anomalies likened to autism-like symptoms, such as a lack of social preference (Tochitani et al., 2016; Leclercq et al., 2017). Importantly, microglial and behavioral anomalies could be rescued in adult mice with microbiome dysbiosis following re-introduction of healthy gut microbiota and/or mono-colonization with a symbiotic bacterium, such as *Lactobacillus* (Diaz Heijtz et al., 2011; Erny et al., 2015; Jang et al., 2018). While these studies highlight microbiota effects in adult brain and behavior, little is known about the cellular and molecular mechanism(s) regulating these changes during perinatal development and whether they can be similarly rescued through probiotic interventions.

A cross-disciplinary examination of studies from gut immunology and neurodevelopment suggests a potential avenue of microbiota-gut-brain signaling via the chemokine receptor, Cx3cr1, which is expressed on macrophages, natural killer cells, and certain subsets of T cells and dendritic cells (Panek et al., 2015). In the brain, Cx3cr1 is

primarily expressed on microglia where it serves as the specific receptor for neuronally expressed Cx3cl1 (fractalkine), a transmembrane glycoprotein that can be released by neurons after proteolytic cleavage under cytotoxic or other stimuli (Paolicelli et al., 2014). A major form of cross-talk between microglia and neurons in the brain is the Cx3cr1-Cx3cl1 pathway, which is instrumental in regulating synaptic pruning and limiting microglial activation. Interestingly, neuronal fractalkine expression is regionally restricted to areas of the brain known to regulate social behavior, such as the cortex, hippocampus, and amygdala. Due to the emerging role that Cx3cr1 plays in both the gut and brain, it represents an important pathway to evaluate microglia-neuron interactions in response to dysbiosis in the context of neurodevelopment (Verheijden et al., 2015; Dorfman et al., 2017).

In the present study, we developed an antibiotics-based bacterial depletion model of maternal microbiota dysbiosis (MMD) with which to study neurobehavioral development and microglial responses in the offspring. Notably, we identified a novel postnatal window of susceptibility where social deficits and microglial activation observed in MMD offspring can be prevented with timed administration of a symbiotic, antibiotic-resistant gut bacterium, *Lactobacillus murinus* HU-1 (MMD^{Lacto}). We further show that MMD-induced microglial activation is accompanied by increased expression of senescence genes, *Trp53* and *Il1β*, and microglia-specific Cx3cr1 protein in the prefrontal cortex, the loss of which ($Cx3cr1^{GFP/GFP}$) also rescued MMD-induced social and microglial dysfunction. Our novel findings demonstrate that maternal gut dysbiosis mediates dysregulation of key cellular and molecular pathways that regulate social behavior in murine offspring.

3.3 Materials and Methods

Animals. All mice were housed in an AAALAC accredited, virus/specific antigen-free facility with a 12 h light-dark cycle; food (Teklad 2918) and water provided *ad libitum.* Outbred CD-1 IGS mice were purchased from Charles River (Strain code 022), and inbred C57BL/6J (Stock no. 000664) and B6.129P-*Cx3cr1*^{tm1Litt}/J mice (Stock no. 005582) were purchased from Jackson Laboratory. Heterozygous *Cx3cr1*$^{GFP/+}$ mice were bred using C57BL/6J males and B6.129P-*Cx3cr1*^{tm1Litt}/J females. Homozygous *Cx3cr1*$^{GFP/GFP}$ mice were bred using B6.129P-*Cx3cr1*^{tm1Litt}/J males and females, as described (Jung et al., 2000). Breeding pairs (n=4-6) for all strains were 6-8 weeks old and females were used to generate one litter only to prevent potential confounding generational effects in the offspring. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #17-043).

Maternal Microbiome Dysbiosis (MMD) Protocol. Mice were either administered an antibiotic cocktail (MMD), given a single oral dose of *Lactobacillus murinus* strain HU-1 (10^9 CFU) followed by an antibiotic cocktail (MMD^{Lacto}), or water (CONV) to establish conditions of MMD, MMD plus probiotic, and conventional housing conditions, respectively. Antibiotic cocktail of 0.4 mg/ml kanamycin, 850 U/ml colistin, 0.215 mg/ml metronidazole (Bio-World, Dublin, OH), 0.035 mg/ml gentamicin (Vet One, Boise, ID), and 0.045 mg/ml vancomycin (Hospira Inc., Lake Forest, IL) was based on a previous

protocol using clinically-relevant drug dosages (Chen et al., 2008), with the addition of 0.5 mg/ml amoxicillin/clavulanic acid (Zoetis, Parsippany, NJ). Antibiotics were administered via drinking water to treatment groups one week prior to breeding and maintained throughout the duration of the experiment until sacrifice at pup weaning (P22). MMD^{Lacto} mice received a single, oral gavage of a novel, lab-generated strain of *L. murinus* HU-1 three days prior to beginning the antibiotic cocktail. Re-conventionalization experiments were conducted by moving MMD dams to dirtied conventional housing near parturition (approx. ~E19-21) to replenish their gut microbiota via coprophagic behavior (MMD^{CONV}). Additional MMD dams were also re-conventionalized in this fashion and given an oral dose of *L. murinus* HU-1 to ensure colonization with this specific bacterium ($MMD^{CONV+Lacto}$). MMD conditions were confirmed via PCR testing of fecal DNA for 16s rRNA (8F forward 5'-AGAGTTTGATCCTGGCTCAG-3' and U1429 reverse 5'-ACGGTTACCTTGTTACGACTT-3') and *L. murinus* (forward 5'-GCAATGATGCGTAGCCGAAC-3' and reverse 5'-GCACTTTCTTCTCTAACAACAGGG-3') prior to breeding, during gestation, and in the offspring near weaning (P18-21).

Microbiome Profiling. Fresh fecal pellets from dams were cultured on BHI, MacConkey, and MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ). Fecal cultures from experimental breeders using rich media agar plates revealed numerous and diverse colony formations from CONV dams, monocolonization by *L. murinus* HU-1 only from MMD^{Lacto} dams (confirmed by MALDI-TOF), and no growth

from MMD dams (data not shown). Additional culture plates were used to confirm antibiotic resistance in *L. murinus* HU-1 by Kirby-Bauer testing. Fecal DNA was extracted via kit (SKU D6010, Zymo Research, Irvine, CA) and submitted to Argonne National Laboratory for 16s rRNA sequencing using Illumina MiSeq.

Behavioral Assays. Individual offspring were tested at P1, P11, and P21 against milestones of typical behavioral development in motor-reflexive tasks: righting—ability to re-orient body on all four paws after being placed on back; cliff aversion—ability to stop from falling off a surface edge; and negative geotaxis—ability to orient body upwards when placed head-down at a steep angle, as described (Wu et al., 1997). Each test was repeated 4 times and scored as a percentage of successful attempts with 100% indicating proper motor-reflexive development and 0% indicating poor motor-reflexive development. Weanling pups (P21) were tested additionally for social preference via the three-chamber social test, as described (Silverman et al., 2010); behavior was recorded and quantified by EthoVision XT software (Noldus, Leesburg, VA). Briefly, individual pups from a litter were allowed to habituate inside a three-chambered box for 10 minutes before the 10-minute testing period in which the pup was observed for interactions with a social and non-social stimulus that were placed in the outer chambers. Age, sex, and strain-matched mice were used as the social stimulus and a green plastic block was used as the non-social stimulus. Social preference was scored as time spent with the social stimulus divided by total time spent with both social and non-social stimuli such that a score of 1.0 indicates highest social preference and 0.0 indicates lowest social preference. Significant interaction between sex and treatment were not found, therefore, both male

and female pups were tested. All individual behavior scores were averaged for each litter and represented as n=1.

Fecal Metabolite Analysis. Fresh fecal pellets from pregnant dams were collected directly into individual 1.5 ml centrifuge tubes and weighed, and homogenized with 1 ml of deionized water. The tubes were allowed to settle at room temperature for 2 hours and then submitted to Virginia Tech Environmental and Water Resources Research Facility for quantification of various volatile fatty acids via Hewlett-Packard 5890 gas chromatograph fitted with a flame ionization detector (GC-FID) coupled with a Nukol GC column and a Hewlett-Packard 7673 GC/SFC injector.

Tissue Processing and Staining. Brains from male offspring were collected at P22, perfused with 10% formalin, cryopreserved overnight in 20% sucrose and then frozen in OCT compound (Fisher Scientific, Hampton, NH). Whole brains were processed on a cryostat (CryoStar NX50 Cryostat) into 30 μM coronal serial sections spaced 10 sections apart with 5 sections per slide and each section 300 μM apart. Tissue sections were stored in -80°C until use. Sections were fixed with 10% formalin for 10 minutes followed by 3x washed with 1X PBS then blocked in 2% fish gelatin and 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. Primary antibodies (CD68 Cat. #MA5-16674, Invitrogen, Carlsbad, CA; Neurofilament Cat. # MAB1621, Chemicon International, Temecula, CA; Cx3cr1 Cat. #Ab8021 Abcam, Cambridge, MA) were diluted in block and was applied to each individual slide at 1:200 dilution and incubated overnight in 4°C. The following day, sections were washed in 1X PBS before application

of secondary antibody for 1.5-2 hours at room temperature (1X PBS solution with secondary antibody 1:500 donkey anti-rat Alexa-Fluor 594, Invitrogen Cat. #A-21209, Carlsbad, CA). The sections were washed with 1X PBS before prior to mounting in DAPI Fluoromount-G (Southern Biotech, Birmingham, AL). Cortical mantle thickness was determined using Nissl-stained slides using cresyl violet acetate 2.5% solution (Electron Microscopy Science, Hatfield, PA), as described previously (Brickler et al., 2018). Thickness was averaged from three measurements of the cortical layer spanning from the dorsal corpus callosum to the meninges and measured across five serial coronal sections from approx. -1.38 to -2.58 mm from bregma (n=4).

Stereological and Confocal Image Analyses. Cell counts for the hippocampus (HPC), cortex (CTX), and prefrontal cortex (PFC) of *Cx3cr1*$^{GFP/+}$ and *Cx3cr1*$^{GFP/GFP}$ male offspring were assessed by a blinded investigator using Optical Fractionator from StereoInvestigator (MicroBrightField, Williston, VT) and an upright Olympus BX51TRF motorized microscope (Olympus America, Center Valley, PA). Grid size was set at 300 x 300mm with a 75 x 75mm counting frame for HPC and PFC, and 700 x 700mm with a 300 x 300mm counting frame for CTX. HPC and CTX regions were estimated to be located at approx. -1.38 to -2.58 mm A/P bregma and PFC regions at approx. 1.82 to 0.62 mm A/P bregma per Allen Mouse Brain Atlas (Lein et al., 2007). Subregions of the hippocampus were analyzed as a single unit. The right hemisphere consisted of 5 coronal serial sections. Confocal images were obtained using an inverted Zeiss 880 (Carl Zeiss AG, Oberkochen, Germany).

Proteome Profiling and Western Blotting. To identify cytokine levels in the gut, protein was isolated from 100 mg of cecal content from male offspring at P22, as described (Dillenburg-Pilla et al., 2016), for measurement via Proteome Profiler Mouse XL Cytokine Array (R&D Systems, Minneapolis, MN) per manufacturer's instructions. To identify protein levels in the cortex, dissected cortices from P22 male offspring were lysed in RIPA buffer (Tris-base 50 mM, NaCl 150 mM, EDTA 1mM, NP-40 1%, Sodium deoxycholate 0.25%, NaF 20 mM, 1 mM Na3VO4 1 mM, β-glycerophosphate 10 mM, Azide 0.02%) with Roche Proteinase Inhibitor Cocktail (Cat. # 25178600, Indianapolis, IN) and Thermo Fisher Scientific Pierce™ Phosphatase Inhibitors (Catalog # 88667, Waltham, MA), aliquoted and kept in -80°C until use. The total amount of protein was quantified by Lowry method (Bio-Rad, Hercules, CA). 50 ug total protein of each sample was separated by 8% SDS-PAGE, then blotted on to Immin-Blot™ PVDF membrane (Bio-Rad, Hercules, CA). Membranes were incubated with primary antibodies (PSD-95 Cat. #3450, Cell Signaling, Danvers, MA; beta-actin Cat. #3700, Cell Signaling, Danvers, MA) in blocking solution: TBS/0.1% Tween20 (TBST)/5% bovine serum albumin (BSA) for overnight at 4°C, washed 4x with TBST, and incubated with secondary antibodies (anti-rabbit IgG Dylight^{TM} conjugate 680 or anti-mouse IgG Dylight^{TM} conjugate 800; Cell Signaling Technology, Danvers, MA) for 2 hrs in blocking solution at room temperature. Following 4x wash with TBST, images were acquired by using LI-COR Odyssey Imaging Systems (LI-COR, Inc), and band intensities were quantified by using NIH ImageJ software.

RNA Sequencing and Analysis. Whole cortex was dissected from brains of *Cx3cr1*$^{GFP/+}$ male offspring at P22, placed in Leibovitz's L-15 dissecting media on ice (ThermoFisher, Waltham, MA), subjected to neural dissociation (Kit from Miltenyi Biotec, Auburn, CA), and placed in FACS buffer (PBS, 5% FBS, 0.1% Sodium azide). Two brains were pooled for each individual isolation per experimental group. Each group consisted of three biological triplicates. GFP^{+} cells were then collected via flow cytometry (BD FACS Aria, San Jose, CA). Total RNA was isolated from sorted cells using TRIzol® reagent (Life Technologies, Carlsbad, CA) per manufacturer's instructions. RNA quantification was carried out by measuring absorbance with spectrophotometer ND-1000 (NanoDrop). Isolated RNA was submitted to BGI Americas Corp. (Cambridge, MA) for RNA quantification sequencing using Illumina platform. Fragments per kilobase of transcript per million reads (FPKM) values were calculated for each gene useGalaxy.org and Cufflinks software (version 2.2.1). Significant changes were calculated using Cuffdiff (version 2.2.1) and normalized using geometric method. Files were then passed to the Cummerbund, an R Core Team package https://www.R-project.org/ version 3.1.2 to determine the significantly differentially expressed genes (FDR< 0.05). Gene ontology (GO) biological processes of significantly upregulated and downregulated genes were conducted via GeneCodis version 3.0 (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009; Tabas-Madrid et al., 2012) and PANTHER version 14 enrichment analysis tools (Mi et al., 2019).

qPCR. RNA was reverse transcribed into cDNA with iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) per manufacturer's specifications. For qRT-PCR analysis, 50 ng

cDNA per reaction was amplified using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA). Expression changes were calculated using ΔCq values with reference to *β-actin* internal control gene. Relative expression was calculated then normalized and compared to appropriate treated or control samples. All primers were designed to span exon junctions and were tested for primer efficiency which ranged from 87-110% prior to use.

Gene	**Primer sequence (5'-3')**
β-actin	Fw: TCGTACCACAGGCATTGTGATGGA
	Rv: TGATGTCACGCACGATTTCCCTCT
C1qc	Fw: TAGGGCCAGAAGAAACAGCA
	Rv: AGGCCTGAAGTCCCTTACAC
Ccr3	Fw: ACTGGACTCATAAAGGACTTAGCA
	Rv: GTGCCCACTCATATTCATAGGG
Cd68	Fw: TCACCTTGACCTGCTCTCTCT
	Rv: GGACCAGGCCAATGATGAGA
Hdac5	Fw: AACAGAGCACGCTCATAGCA
	Rv: GGTGCCTCGGGAGCTTAC
Il1β	Fw: TGTGTAATGAAAGACGGCACAC
	Rv: CCATCTTCTTCTTTGGGTATTGCT
Trp53	Fw: CAGTCTGGGACAGCCAAGTC
	Rv: CCAGCTGGCAGAATAGCTTA

Statistics. Data were graphed using Prism software (Version 7, GraphPad, San Diego, CA). Student's unpaired two-tailed t test was used for comparison of two experimental groups. For three or more groups, multiple comparisons were done using one-way and two-way ANOVA where appropriate, followed by Tukey's multiple comparisons test or Bonferroni where appropriate. Changes were identified as significant at $p < 0.05$. Mean values were reported together with the SEM.

3.4 Results

Model of maternal microbiome dysbiosis (MMD) and treatment with *L. murinus* HU-1

To determine whether disrupting the maternal gut microbiome influences neurobehavioral outcomes in the offspring, we utilized an antibiotics-based model of maternal microbiome dysbiosis (MMD) by administering an antibiotic cocktail via drinking water to both female and male mice one week prior to breeding and producing offspring for behavioral and histological assessments (Fig. 1A). MMD mice were maintained on antibiotic drinking water throughout gestation and parturition until time of offspring weaning at postnatal day 21 (P21). Due to previous evidence of neuroprotective effects of probiotics, such as *Lactobacillus rhamnosus* JB-1 (Leclercq et al., 2017) and *L. reuteri* (Sgritta et al., 2019) in adult mice, we added a second group of MMD mice that were pretreated with an oral dose of our lab-generated strain of commensal gut bacterium, *L. murinus* HU-1. *L. murinus* HU-1 is an antibiotic-resistant strain of *L. murinus*, a common gut bacterium found in many mammal species with potential

probiotic applications (Greetham et al., 2002; Vasconcelos et al., 2003; Gardiner et al., 2004). It is a key component of the Altered Schaedler Flora that is genetically similar to other food fermenter bacteria, such as *L. animalis* (Fig. 1B) (Schaedler et al., 1965; Sarma-Rupavtarm et al., 2004; Wymore Brand et al., 2015). To examine the effects of *L. murinus* HU-1 on the neurodevelopmental outcomes following MMD exposure, mice were treated for three days with *L. murinus* HU-1 via oral gavage prior to the onset of MMD (MMD^{Lacto}) while the control untreated groups were maintained under conventional specific pathogen-free housing (CONV) (Fig. 1A).

MMD status was evident in the enlarged cecum size of MMD dams compared to CONV, which indicated the inability to properly break down food fibers due to gut dysbiosis. MMD^{Lacto} dams also exhibited enlarged cecum sizes which demonstrated the presence of *L. murinus* HU-1 was insufficient to fully restore the gut microbiota to a conventional state (Fig. 1C). MMD and MMD^{Lacto} status was regularly tested in breeding mice for the presence of 16s rRNA and *L. murinus*, respectively (Supplemental Fig. 1A). Next, 16s rRNA sequencing of maternal fecal bacteria was performed to assess the impact of MMD on gut microbiota composition. At the phylum level, CONV and MMD^{Lacto} dams shared similar gut microbial composition primarily consisting of *Firmicutes* and *Bacteroidetes* bacteria, whereas MMD dams were characterized predominantly by *Proteobacteria* (Fig. 1D). At the genus level, however, MMD^{Lacto} dams exhibited a distinct microbial profile from CONV dams with comparatively low diversity of bacteria genera and primarily consisted of *Lactobacillus* and *Parabacteroides*, which was replicated in microbial profiles of male offspring at P21 (Supplemental Fig. 1B). Although RT-PCR of MMD dams' fecal bacteria showed near complete depletion of gut

bacteria (Supplemental Fig. 1A), deep sequencing identified numerous genera of bacteria in MMD dams' feces that can be characterized as environmental bacteria with pathogenic potential (e.g., *Xanthomonadaceae* and *Acinetobacter*) (LaSala et al., 2007; Wong et al., 2017). These treatment-specific differences in maternal microbiota were consistent in dams and offspring across different mouse strains (outbred CD-1 versus inbred *Cx3cr1*$^{GFP/GFP}$) (Fig. 1D and Supplemental Fig. 1B).

Recent studies of the gut-brain axis revealed that gut bacteria-derived metabolites likely serve neuroactive roles and are capable of passing the placental and blood-brain barriers to modulate neural function and behavior (Braniste et al., 2014; Erny et al., 2015; Sampson and Mazmanian, 2015; Vuong et al., 2017). To test whether MMD status during pregnancy may influence expression of gut bacteria-related metabolites, we performed chemical analysis of CD-1 maternal feces via gas chromatography. We found a significant reduction in three prominent gut bacteria-derived metabolites (acetic acid, propionic acid, and butyric acid) in both MMD (7.818±1.471 mmol/L, 0.0±0.0 mmol/L, 0.0±0.0 mmol/L, respectively) and MMD^{Lacto} (9.761±1.058 mmol/L, 0.0±0.0 mmol/L, 0.0±0.0 mmol/L, respectively) compared to CONV (25.63±4.315 mmol/L, 4.558±1.291 mmol/L, 4.592±1.580 mmol/L, respectively) (Fig. 1E-G). Interestingly, MMD dams exhibited a significant increase in isovaleric acid (0.02±0.01 mmol/L) compared to MMD^{Lacto} (0.0±0.0 mmol/L) and CONV (0.0±0.0 mmol/L) (Fig. 1H). Whereas both MMD and MMD^{Lacto} dams displayed increased levels of isocaproic acid (0.391±0.125 mmol/L and 0.598±0.219 mmol/L) and heptanoic acid (20.69±3.602 mmol/L and 17.78±1.09 mmol/L) compared to CONV dams (0.0±0.0 mmol/L and 6.888±1.432 mmol/L, respectively) (Fig. 1I-J). These findings demonstrate MMD induced significant

remodeling of the maternal gut microbiota and resulted in altered production of key gut bacteria-derived metabolites. The presence of *L. murinus* HU-1 during MMD treatment partially prevented maternal gut dysbiosis, but could not recover altered production of most bacterial metabolites.

L. murinus HU-1 attenuates MMD-induced social deficits in offspring

To evaluate whether MMD status influences postnatal (P) offspring behavior, a battery of developmental tests on CD-1 neonatal pups were performed at P1, P11, and at weaning on P21. These dates were selected to provide longitudinal measurements across three distinct time points in murine postnatal development (Theiler, 1972; Chen et al., 2017). Both male and female pups from the first litter of each experimental dam were tested for physical, reflexive, gross motor, and social milestones. Four to six individual dams were used per experimental group with an average of 9 pups per litter, totaling approximately 45 pups tested per group. At P1, neonatal pups weighed and performed similarly in cliff aversion and negative geotaxis tests regardless of the treatment group (Fig. 2A-C). However, offspring born to MMD dams showed reduced righting ability compared to CONV and MMD^{Lacto} offspring (Fig. 2D). While no significant weight differences or righting ability were found at P11 across treatment groups (Fig. 2E, H), MMD offspring showed deficits in cliff aversion and negative geotaxis tests compared to CONV and MMD^{Lacto} (Fig. 2F-G). These atypical findings were corrected by P21, as pups performed similarly in all three behavioral tests regardless of treatment (Fig. 2I-L). However, differences arose with respect to the three-chamber social test wherein MMD

offspring exhibited a lack of social preference compared to CONV and MMD^{Lacto} offspring (Fig. 2M).

Given that the prenatal presence of *L. murinus* HU-1 (MMD^{Lacto}) can reverse the postnatal behavioral deficits observed in MMD offspring, we sought to identify whether this was temporally restricted. To ascertain this, MMD dams were re-conventionalized at parturition by placing them inside conventional housing (MMD^{CONV}) while a separate group of MMD dams were placed in conventional housing and provided a single oral dose of *L. murinus* HU-1 bacterium ($MMD^{CONV+Lacto}$) (Supplemental Fig. 2A). Gut microbiota and *L. murinus* HU-1 recolonization were confirmed using RT-PCR (Supplemental Fig. 2B). Although MMD^{CONV} fecal DNA analysis showed recolonization with bacteria by 16s rRNA as early as 2 days following re-conventionalization, *L. murinus* could not be detected until 60 days later. In contrast, $MMD^{CONV+Lacto}$ fecal DNA analysis showed immediate recolonization with 16s rRNA and *L. murinus* at 3 days following re-conventionalization (Supplemental Fig. 2B). Offspring from recolonized MMD dams were then evaluated against the same battery of behavior tests as described above. Neither recolonization strategies fully corrected the atypical behaviors induced by MMD at P1 or P11 (Supplemental Fig. 2C-E). Although the MMD^{CONV} pups displayed the same social deficits as MMD pups at P21, the $MMD^{CONV+Lacto}$ pups showed amelioration of social dysfunction (Fig. 2N). Finally, we show no gross difference in cortical thickness between the treatment groups at P21 (Fig. 2O-R). These findings suggest postnatal gut-brain axis dysregulation is a major driver of the development of social impairments in mice and that postnatal microbiota recolonization required the presence of *L. murinus* HU-1 to restore typical social behavior.

L. murinus HU-1 rescues MMD-induced microglial morphological changes and Cx3cr1 alterations in the prefrontal cortex

Next, we asked whether the social deficits found in MMD offspring correlated with impaired microglia morphology and Cx3cr1 expression. To visualize the morphology of microglia in the brain, we stained serial coronal sections and performed confocal image analysis of the prefrontal cortex of male *Cx3cr1*$^{GFP/+}$ offspring at P21 from CONV, MMD, and MMD^{Lacto} breeders (Fig. 3A, 3B, and 3C, respectively), which express green fluorescent protein in microglia of the brain. Compared to CONV and MMD^{Lacto}, MMD microglia exhibited highly dystrophic, fragmented processes and spheroid soma indicative of cytoplasmic deterioration and atrophy (Fig. 3B) (Streit et al., 2014). This dystrophic state did not correlate with greater overlap or co-labeling of GFP and neurite process marker neurofilament (Fig. 3B1-B3) indicating these cells are not actively engulfing neuronal elements. Moreover, staining for Cx3cr1 revealed increased expression in the soma and processes of microglia of MMD offspring brains (Fig. 3E, I) compared to CONV (Fig. 3D, I) and MMD^{Lacto} (Fig. 3F, I). Together, these data show MMD treatment induced dystrophic morphology in MMD microglia, which may be the direct result of Cx3cr1 overexpression and impaired functional state.

MMD induces significant gene expression changes in cortical microglial

In an effort to elucidate how the presence of *L. murinus* HU-1 mitigated behavioral and microglial changes in MMD offspring, GFP^{+} microglia were purified by FACS from the cortices of P22 *Cx3cr1*$^{GFP/+}$ heterozygous male offspring reared from MMD, MMD^{Lacto}, and CONV dams followed by RNAseq analysis. Comparative analysis

found a small number of differentially-expressed genes from MMD and MMD^{Lacto} offspring with an even smaller set of heterogeneous genes shared between the two treatment groups (Fig. 4A-B) (Hulsen et al., 2008). Gene ontology enrichment analysis of MMD cortical microglia genes again showed a heterogeneous assortment of upregulated and downregulated genes with the largest grouping of genes related to metabolic processes (Fig. 4C-D) (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009; Tabas-Madrid et al., 2012). Of these, MMD upregulated genes, such as *Acot1* in acyl-CoA degradation, represented opposing biological processes of downregulated genes, such as *Acsl6* in acyl-CoA synthesis. MMD^{Lacto} microglia also predominantly expressed genes related to metabolic processes, but frequently in the opposite manner of MMD. For example, while *Hdhd3*, *Rnf19a*, *Fn3k*, *Tst*, and *Car4* were downregulated in MMD microglia, the same genes were significantly upregulated in MMD^{Lacto} microglia (Supplemental Fig. 3) (Mi et al., 2019).

Prior studies using the maternal immune activation model suggested microglial inflammation as the key driver of dysfunction (Hammond et al., 2018; Lenz and Nelson, 2018). However, our model of MMD appeared to induce microglial effects beyond immune signaling, including expression of genes relevant to cell cycle, senescence, and cell aging. Closer examination of target genes by qPCR provided further validation and emphasized the increased differential expression of *Il1β* (Fig. 4E), *Ccr*3 (Fig. 4F) and *Trp53* (Fig. 4G), while reduced expression was seen for *Hdac5* (Fig. 4H), *C1qc* (Fig. 4I) and *Cd68* (Fig. 4J) in MMD microglia compared to CONV and MMD^{Lacto}. These results support our RNAseq findings and suggest that MMD conditions may induce an immune-mediated senescent phenotype in microglia.

MMD-induced expression of CD68 is attenuated by *L. murinus* HU-1 and loss of *Cx3cr1*

We further examined the functional state of microglia following MMD in the presence and absence of *L. murinus* HU-1 treatment by staining for CD68, a lysosomal protein implicated in antigen processing by macrophages and a marker for microglial activation (Korzhevskii and Kirik, 2016; Chistiakov et al., 2017). Importantly, given the overexpression of Cx3cr1 found on MMD microglia, which is restored in MMD^{Lacto}, we sought to evaluate whether loss of Cx3cr1 may affect these changes. Therefore, we utilized $Cx3cr1^{GFP/GFP}$ knockout and $Cx3cr1^{GFP/+}$ heterozygous P22 male offspring reared from CONV, MMD, and MMD^{Lacto} breeders. Serial coronal sections were evaluated for $GFP^+/CD68^+$ immunoreactivity and quantified in the cortex and hippocampal regions due to their associations with complex behaviors that frequently underlie neurodevelopmental disorders (Morgan et al., 2010; Edmonson et al., 2014; Gacias et al., 2016). Non-biased stereology showed no quantifiable differences in the total number of GFP^+ microglia across treatments and genotypes. However, the number of $GFP^+/CD68^+$ cells was significantly increased in the parietal (Fig. 5A) and prefrontal cortex (Fig. 5C) of $Cx3cr1^{GFP/+}$ MMD compared to $Cx3cr1^{GFP/+}$ CONV mice at multiple bregma levels (Supplemental Fig. 4). Hippocampal regions largely did not exhibit differences across treatment groups, except at bregma -2.6 in which MMD mice showed increased $GFP^+/CD68^+$ cells compared to CONV (Fig. 5B and Supplemental Fig. 4). Importantly, this effect was abrogated in $Cx3cr1^{GFP/GFP}$ knockout mice and in mice treated with *L. murinus* HU-1. Similar findings were found when analyzing the proportion of GFP^+ cells that were $CD68^+$ versus $CD68^-$ (Fig. 5D-I). While similar assessment of the total numbers

of prenatal GFP^+ and $CD68^+/GFP^+$ microglia at E17 did not show any significant changes following MMD (Supplemental Fig. 5A), both MMD and MMD^{Lacto} treated *Cx3cr1*$^{GFP/+}$ pups showed increased proportions of $CD68^+/GFP^+$ versus $CD68^-/GFP^+$ cells compared to CONV or *Cx3cr1*$^{GFP/GFP}$ knockout treated mice (Supplemental Fig. 5B), and suggest the rescue effects by *L. murinus* HU-1 are due to mechanism(s) present after the gut becomes colonized by bacteria following birth. Collectively, our data indicate MMD drives microglial activation in specific brain regions, which are highly regulated by Cx3cr1 signaling.

Cx3cr1 signaling mediates MMD-induced social deficits and PSD-95 cortical expression

Lastly, we evaluated whether the effects of Cx3cr1 loss-of-function on preventing MMD deficits in microglia correlated with changes in social behavior. We found *Cx3cr1*$^{GFP/+}$ MMD mice replicated the social deficits previously observed in CD-1 MMD offspring and also showed restored social preference in the presence of *L. murinus* HU-1. Both male and female offspring were tested from 4-5 individual dams per experimental group with an average of 7 pups per litter and approximately 29.5 pups per group. Interestingly, loss of *Cx3cr1* attenuated social deficits induced by MMD (Fig. 6A). Furthermore, sex-specific analysis of social preference behavior in *Cx3cr1*$^{GFP/+}$ mice indicated similar pattern between male and female offspring with no significant interaction between sex and treatment (Fig. 6B).

Given the role of Cx3cr1 in mediating MMD-induced microglial activation and behavioral changes, we quantified excitatory postsynaptic protein, PSD-95, in the

cortices of *Cx3cr1*$^{GFP/+}$ and *Cx3cr1*$^{GFP/GFP}$ P22 male mice as a measure of synaptic status. Over- or underpruning of synapses by dysregulated microglia have been suggested as a possible contributing factor for the altered behavior and brain sizes found in neurodevelopmental disorders (Courchesne et al., 2003; Morgan et al., 2010; Inta et al., 2017; Lee et al., 2017; Whitelaw, 2018). Here, we observed increased expression of PSD-95 in the cortices of *Cx3cr1*$^{GFP/+}$ MMD offspring and a clear absence of this effect in *Cx3cr1*$^{GFP/GFP}$ MMD knockout offspring (Fig. 6C-E). Thus, our results demonstrate offspring reared under MMD conditions developed atypical behavioral outcomes due to dysfunction of the Cx3cr1 signaling pathway and impaired synaptic modeling.

3.5 Discussion

Neurodevelopmental disorders represent rapidly increasing maladies worldwide (Boyle et al., 2011). While their etiology remains ill-defined, the gut microbiome is gaining attention as having a pervasive influence with far reaching consequences (Kinross et al., 2011). The current study demonstrates that antibiotics-driven, maternal microbiome dysbiosis (MMD) impairs proper neurobehavioral development, enhances PSD-95 expression, and induces a dysregulated state in microglia of the prefrontal and parietal cortices. Moreover, MMD resulted in the overexpression of Cx3cr1 on microglia, which correlated with increased expression of senescence-associated genes *Il1β* and *Trp53*. In contrast, these outcomes were significantly attenuated in pups born to dams colonized with a commensal bacterium (*L. murinus* HU-1) either during the MMD duration or at parturition suggesting these deficits may be rescued postnatally via oral gavage. We further demonstrate that MMD-induced impairments can also be rescued

using *Cx3cr1*$^{GFP/GFP}$ knockout mice. These findings provide novel insight into the role that gut microbiota play in social development and suggest that microglia may be pivotal in the gut-brain axis during development through yet unexplored bacteria-derived signals.

The social preference index at P21 utilized in this study is a well-characterized behavioral assay for autism-like social deficit (Silverman et al., 2010) and served to emphasize aberrant behavioral outcomes in offspring as a result of experimental MMD. Albeit arguably less utilized in studies of neurodevelopmental disorders due to challenges posed by requisite testing of neonatal mice, the righting reflex test at P1 and cliff aversion and negative geotaxis tests at P11 parallel oligodendrocyte maturation at P1-3 and peak gliogenesis at P7-10, respectively, and are reflective of appropriate murine sensorimotor and reflexive developmental milestones (Wu et al., 1997; Semple et al., 2013; Feather-Schussler and Ferguson, 2016). Indeed, we observed transient inability to perform these tasks in MMD pups, as portrayed by recovery of P1 righting reflex deficit (Fig. 2D) at P11 (Fig. 2H) and recovery of cliff aversion/negative geotaxis deficits at P11 (Fig. 2F-G) by weaning at P21 (Fig. 2J-K). It is unclear why MMD conditions prompted these developmental delays, although previous studies of germ-free mice suggested altered myelination compared to conventional controls (Hoban et al., 2016). Notably, the delayed motor skills discovered in our study reflect a growing body of clinical evidence that argues gross motor delays in infancy may serve as an early predictor of autism spectrum disorders (ASD) (Bhat et al., 2012; Bedford et al., 2016; Harris, 2017; LeBarton and Landa, 2019). Furthermore, the restoration of typical behavior in offspring reared by MMD dams supplemented with antibiotic-resistant *L. murinus* HU-1 (MMD^{Lacto}) served to prove that certain gut bacterial communities are sufficient to enable typical

neurobehavioral development despite overall dysbiotic conditions during pregnancy. Indeed, numerous adult animal studies have demonstrated improvements in sociability, stress, anxiety, and depression-related behaviors following probiotic treatment (Desbonnet et al., 2015; Wang et al., 2015; Buffington et al., 2016). Here, we also demonstrate that such rescue occurs under a developmental setting.

Interestingly, we did not find the same rescue effect when dams were passively placed into conventional housing at parturition (MMD^{CONV}). This is not surprising, as fecal analysis in this group did not show the re-emergence of *Lactobacillus* until 60 days following re-conventionalization compared to 3 days following oral gavage in $MMD^{CONV+Lacto}$. *L. murinus*, in particular, is a member of the Altered Schaedler Flora, which is a collection of eight commensal gut bacteria deemed to be necessary for laboratory rodent health and further serves to underscore the likely developmental importance of *Lactobacilli* presence in maternal microbiota (Schaedler et al., 1965; Dewhirst et al., 1999; Wymore Brand et al., 2015). Accordingly, the lack of an additional control group, $CONV^{Lacto}$, in this study is due to the endemic nature of *L. murinus* in conventionally-housed mice, as well as evidence from previous studies suggesting difficulties in discerning purported benefits of probiotics under non-pathological conditions (Bravo et al., 2011; Kelly et al., 2015).

The notion of a "window of susceptibility" in the early postnatal period was demonstrated using germ-free mice, which showed reduced brain-derived neurotrophic factor, norepinephrine and serotonin levels in the cortex that could be reversed with probiotic treatment or partially restored with re-conventionalization if conducted within six weeks of birth (Sudo et al., 2004; Sudo, 2006). Although we captured longitudinal

changes in behavior from birth through weaning, future studies should consider introducing treatments at various time points to better define critical periods in postnatal neurodevelopment that may be susceptible to MMD conditions and whether *L. murinus* HU-1 can prevent atypical outcomes. Nevertheless, our behavioral findings comport with these previous studies and further highlight the potential benefit of concurrent probiotic use during episodes of gut microbiome disruption in pregnancy or in early childhood development.

While the mechanism(s) by which microbiota dysbiosis impairs the development of typical social behavior remains unclear, our findings uncover a dysfunctional microglia phenotype under MMD conditions that correlates with increased PSD-95 expression and social deficits. MMD microglia show increased Cx3cr1 and CD68 protein expression, which is attenuated in the presence of *L. murinus* HU-1. Postmortem tissue analysis of autistic brains previously identified increased CD68 expression, as well as other indicators of activated microglia and neuroinflammation, in the cortex and prefrontal cortex that were paradoxically accompanied by overgrowth of grey and white matter (Courchesne et al., 2003; Vargas et al., 2005; Morgan et al., 2010; Lee et al., 2017). This correlation has lent support for the hypothesis that aberrantly activated microglia fail to regulate proper growth and pruning of neural circuitry in ASD (Matta et al., 2019). Indeed, the concept of hyperactivated microglia that fail to phagocytose has been described in neurodegenerative disorders wherein Cx3cr1 signaling mediates this phenotype in which microglia ineffectively surround amyloid-beta plaques in lieu of phagocytosis; in contrast, deletion of the *Cx3cr1* permitted more efficient phagocytosis and function (Liu et al., 2010). Our data suggest that MMD may lead to an aberrant

microglial phenotype in specific brain regions of the offspring that are directly regulated by gut signals to prevent appropriate modeling of synapses, and that this effect is mediated through Cx3cr1 signaling pathway.

Besides increased CD68 expression, we did not find additional evidence of overzealous neuroinflammation under MMD conditions. Unlike studies on maternal immune activation and age-related neuroinflammation, our cytokine profiling of maternal serum and offspring gut (cecal contents) in MMD-treated animals did not reveal elevated levels of pro-inflammatory markers, such as *Il6* and *Il17a*, compared to controls (data not shown). Nor did RNAseq analysis of cortical microglia from MMD offspring reveal a particularly pro- or anti-inflammatory phenotype with the exception of *Il12b* upregulation and *C1qc* downregulation. Our qPCR findings show *Il1β* was also increased, which is a known inflammatory cytokine as well as a key mediator cell senescence. Combined with the observed increase in *Trp53*, this suggests MMD microglia display a senescent phenotype. MMD microglia also showed greater expression of genes related to stress-induced premature senescence, such as *Sirt1*, *Wnt16*, and *Mapkapk5*, while MMD^{Lacto} microglia showed greater expression of *Sumf1, Steap3, Prkag2,* and *Spi1* that relate to processes which mitigate cellular stress, such as oxidoreductase activity, DNA repair, and apoptosis. Microglial senescence has been linked to lysosomal inclusions and immune dysfunction because of age-related myelin degradation (Safaiyan et al., 2016). Age-based microglial senescence is often cause by radical-mediated oxidative damage, whereas premature microglial senescence is said to be associated with cellular stress (Streit et al., 2014). Both types of senescent microglia are characterized by dystrophic morphology consisting of de-ramified and fragmented appearance, similar to our MMD microglia

(Streit, 2006). Our findings suggest that MMD may permit a premature senescence-like state in microglia, however, additional studies are needed to identify the underlying mechanisms.

It remains unclear how the presence of *L. murinus* HU-1 in the gut of MMD^{Lacto} dams rescued neurobehavioral and microglial dysfunction in offspring. Recent studies on the gut-brain axis suggest several potential modes of influence, including microbial signaling via the vagus nerve and the production of microbe-derived neuroactive molecules. While we observed a significant loss of fecal SCFAs metabolites following MMD, these levels did not restore in the presence of *L. murinus* HU-1 suggesting the effects of MMD is unlikely to be metabolite-driven. We did identify, using a multiplex cytokine profiler, an upregulation of Ccl21, CRP, Cystatin C, Endostatin, and Reg3G in the cecum of the P21 MMD offspring, which was attenuated in MMD^{Lacto} pups (Supplemental Fig. 6). Moreover, several recent findings implicate the interactions between gut *Lactobacillus* and the vagus nerve in the regulation of brain regions that may affect social behavior. Bravo, et al., described ameliorative effects of *L. rhamnosus* JB-1 in a mouse model of anxiety and depression that corresponded with alterations in GABA receptor gene expression in the prefrontal cortex and amygdala (Bravo et al., 2011). They found this anxiolytic and antidepressant effect was attenuated in vagotomized mice (Bravo et al., 2011). Sgritta, et al., also recently demonstrated reversal of social deficits through *L. reuteri* treatment across multiple adult mouse models of ASD, which was attenuated in oxytocin receptor-deficient and vagotomized mice (Sgritta et al., 2019). Combined with our findings, these data suggest the presence of *Lactobacillus* sp. in the gut could play a critical role in modulating social behavior as well as microglial function

by influencing the communication between afferent vagal nerve inputs and social reward circuits. However, additional studies are needed to evaluate Cx3cr1 expression and the microglial response to vagotomy following MMD in the presence and absence of *L. murinus* HU-1.

In conclusion, our findings describe a new model of maternal gut dysbiosis that results in atypical social development in offspring, which can be rescued by *L. murinus* HU-1 or loss of *Cx3cr1*. The current study suggests MMD-induced behavioral outcomes may result from premature senescence of microglia that could influence synaptic pruning in regions of the brain that regulate social preference. Future studies aimed at identifying the mechanisms by which gut *Lactobacillus* communicates with the social reward center of the brain will advance our understanding and treatment of neurodevelopmental disorders.

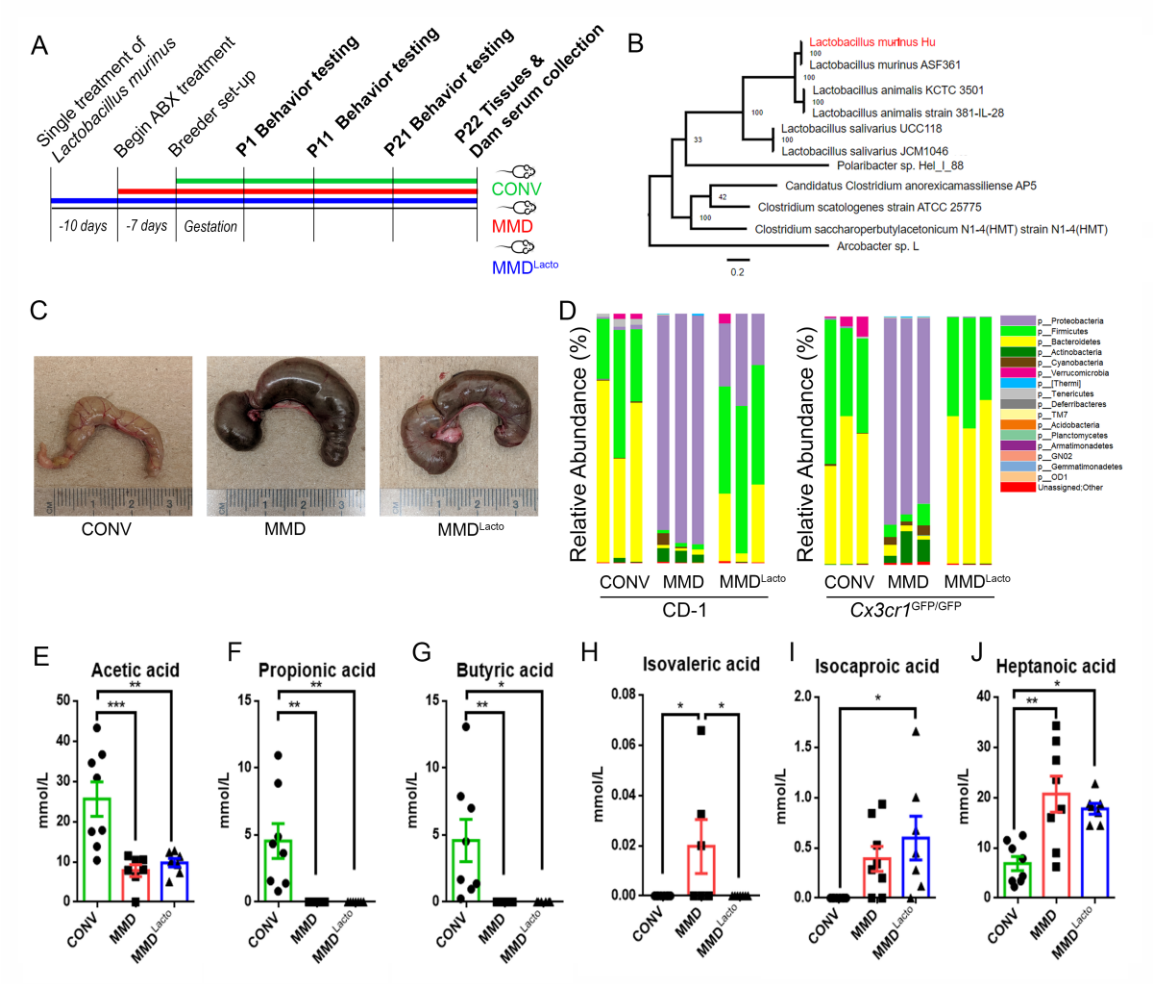

Figure 1. Maternal microbiome dysbiosis (MMD) results in distinct maternal gut microbiome profiles. (A) Study design schematic for treatment times, breeding, and offspring assessment for conventionally-housed dams (CONV), antibiotics-treated dams (MMD), and MMD dams pre-treated with *Lactobacillus murinus* HU-1 (MMD^{Lacto}) in two strains, CD-1 and *Cx3cr1*-GFP breeders. (B) Phylogenetic tree of lab-generated *L. murinus* HU-1 with nearest-neighbor analysis indicate closest similarity to food fermenting species, *L. animalis*. (C) MMD dams at time of offspring weaning exhibit enlarged cecum size compared to CONV and MMD^{Lacto} dams. (D) Relative abundance of maternal fecal bacteria shows similarity between CONV and MMD^{Lacto}-treated dams at

the phylum level regardless of mouse strain with predominantly *Firmicutes* and *Bacteroidetes* bacteria, whereas MMD dams show *Proteobacteria* abundance (n=3 dams per group). (E-J) Maternal fecal levels of short-chain fatty acids, acetic acid, propionic acid, butyric acid, isovaleric acid, isocaproic acid, and heptanoic acid, respectively. (n=7-8 dams per group). *P<0.05, **P<0.05, and ***P<0.001.

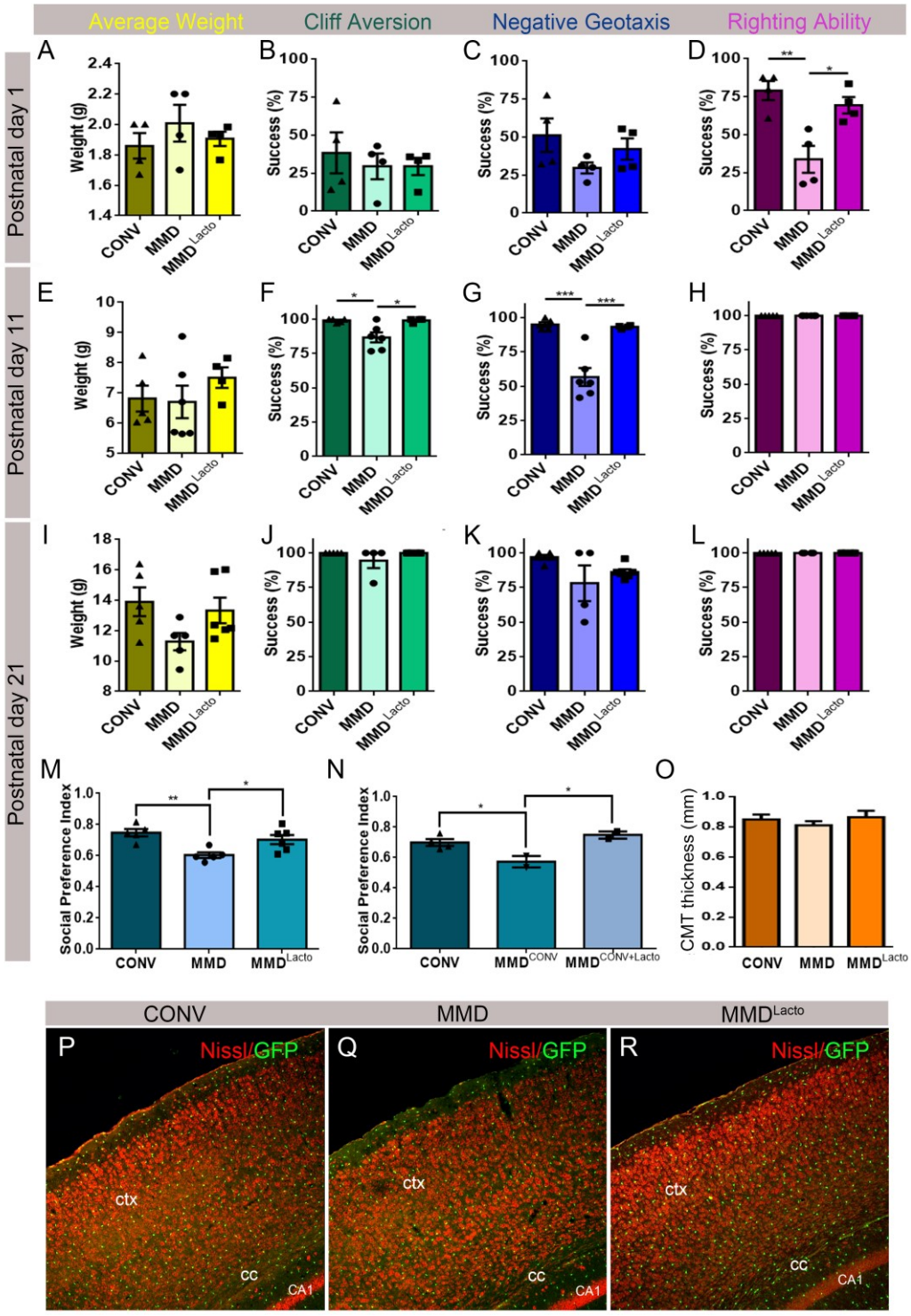

Figure 2. MMD results in offspring with gross motor developmental delays and impaired social behavior that are attenuated in the presence of *L. murinus* HU-1. (A-D) At P1, MMD offspring show significant deficit in righting reflex test, whereas

MMD^{Lacto} perform similarly to CONV. (E-H) At P11, MMD offspring exhibit new deficits in cliff aversion and negative geotaxis tests, whereas MMD^{Lacto} remain comparable to CONV. (I-L) At P21, previous gross motor deficits are recovered in MMD offspring. (M) MMD offspring exhibit lack of social preference in three-chamber test compared to CONV and MMD^{Lacto}. (N) Re-conventionalization of MMD dams (MMD^{CONV}) near parturition does not rescue social deficits. Re-conventionalization of MMD dams plus oral dosing with *L. murinus* HU-1 ($MMD^{CONV+Lacto}$) results in rescue of these behaviors. (O) No gross difference was observed in cortical mantle thickness between treatment groups. (P-R) Representative fluorescent confocal images of the parietal cortex stained with Nissl (red) and GFP for microglia. (n=4-6 litters per group, average 38-49 pups per group). *P<0.05, **P<0.05, and ***P<0.001.

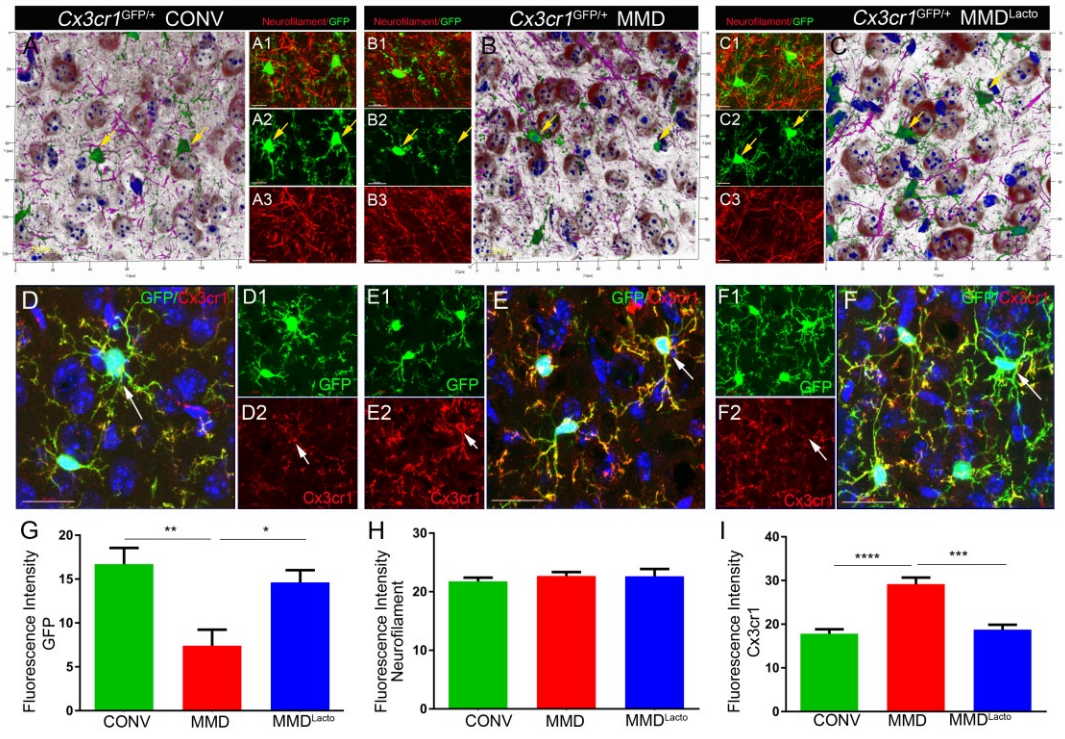

Figure 3. MMD male offspring exhibit microglial dystrophy and increased Cx3cr1 expression in the prefrontal cortex. Representative confocal images analysis of GFP^{+} microglia using *Cx3cr1*$^{GFP/+}$ male offspring under CONV, MMD, and MMD^{Lacto} conditions. (A-C) 3D rendering of z-stack images from serial sections tripled labeled with GFP, Nissl (red), and neurofilament (pink). Insets represent single channel images of GFP+ microglia and co-labeling for neurite processes showing microglia morphology. (D-F) Z-stack maximum projected confocal images showing Cx3cr1 (red) in each condition shows increased expression in microglial soma and processes under MMD, compared to CONV and MMD^{Lacto}. Scale=10µm in A1-A3, B1-B3 and C1-C3. Scale=20µm in D-F. (G-I) Quantification of fluorescence intensity of GFP (G), neurofilament (H), and Cx3cr1 expression (I). (n=5 mice per group). *P<0.05, **P<0.05, ***P<0.001, and ****P<0.0001.

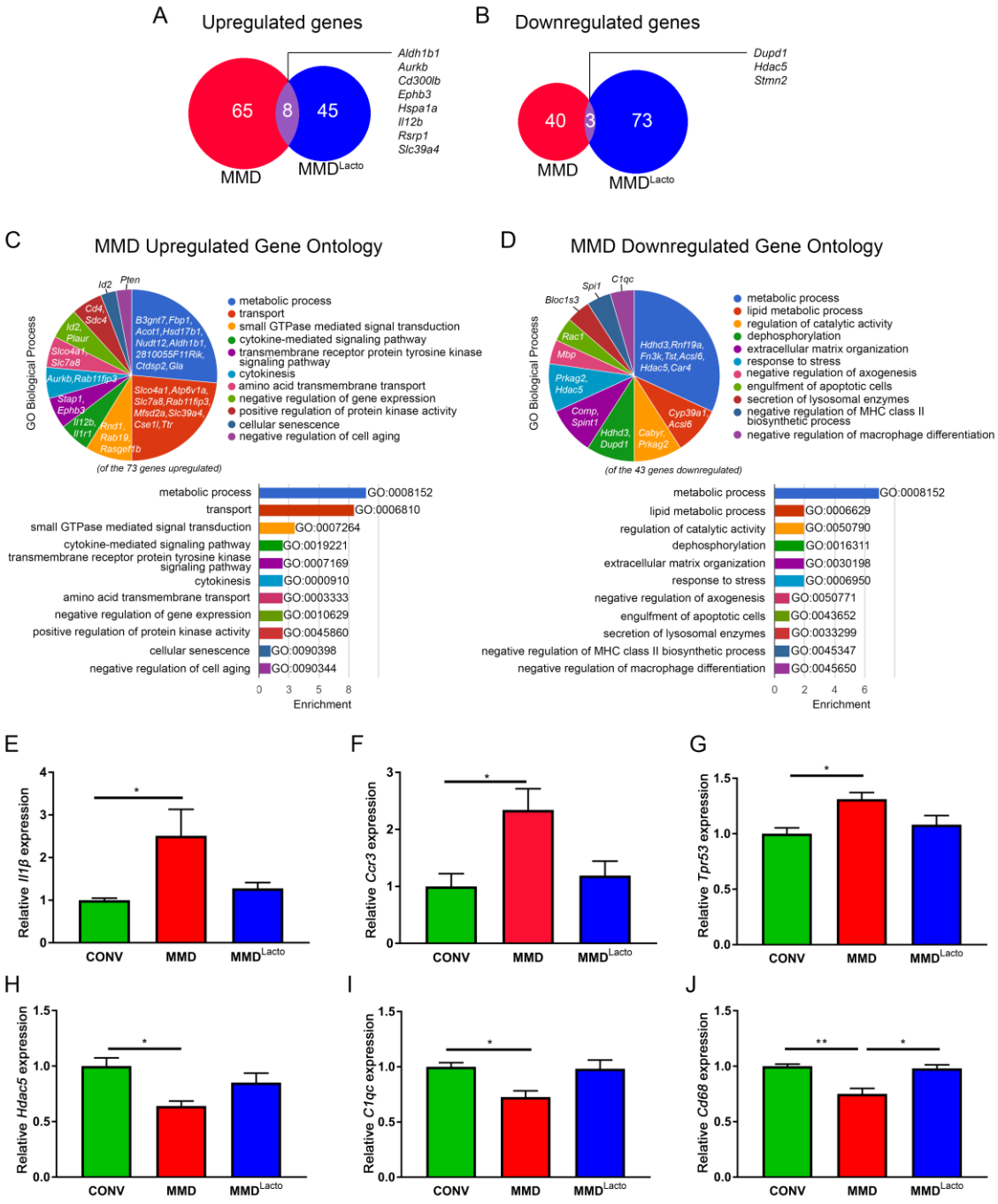

Figure 4. Gene expression changes in cortical microglia of MMD male offspring. (A) Venn diagram of upregulated genes in MMD and MMD^{Lacto} microglia compared to CONV. (B) Venn diagram of downregulated genes in MMD and MMD^{Lacto} microglia compared to CONV. (C-D) Gene ontology enrichment analysis indicates a variety of

biological processes represented by upregulated genes (C) and downregulated genes (D) with the majority of observed to be related to metabolic and cell cycle processes. (E-J) Validation of targeted genes relevant to microglia inflammation, reactivity and cellular survival, senescence by qPCR. *P<0.05 and **P<0.05.

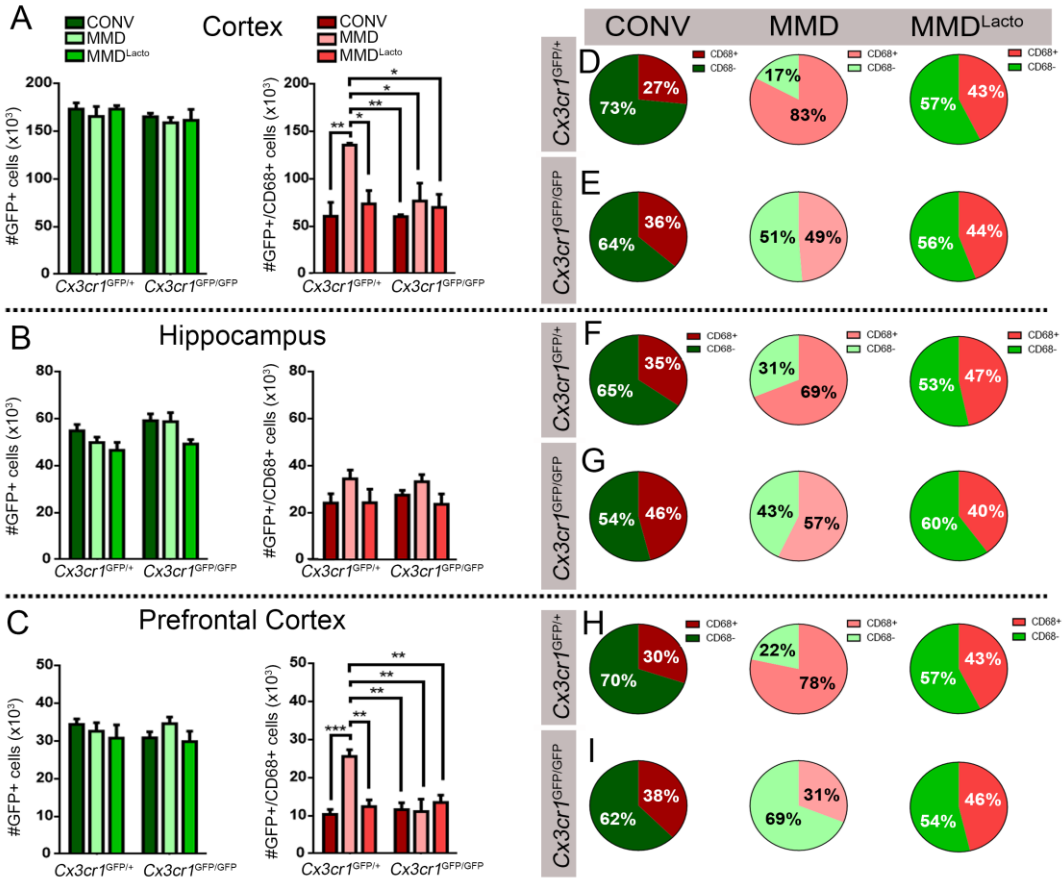

Figure 5. MMD-induced microglial CD68-immunoreactivity is attenuated in the absence of *Cx3cr1* and in MMD^{Lacto}. (A-C) Non-biased stereological analysis of the estimated total number of GFP^{+} and double-labeled CD68^{+}/GFP^{+} cells in the parietal cortex, hippocampus, and prefrontal cortex of P22 male offspring. While no difference in the total microglia numbers regardless of genotype or treatment was found, the co-labeled CD68^{+}/GFP^{+} cells were increased in the cortex and prefrontal cortex but not hippocampus of heterozygous MMD offspring only. Subregions of the hippocampus were analyzed as a single unit. (D-I) Pie charts organized by brain region and genotype representing the average proportion of CD68^{+}/GFP^{+} versus CD68^{-}/GFP^{+} cells (n=4 litters). *P<0.05, **P<0.05, and ***P<0.001.

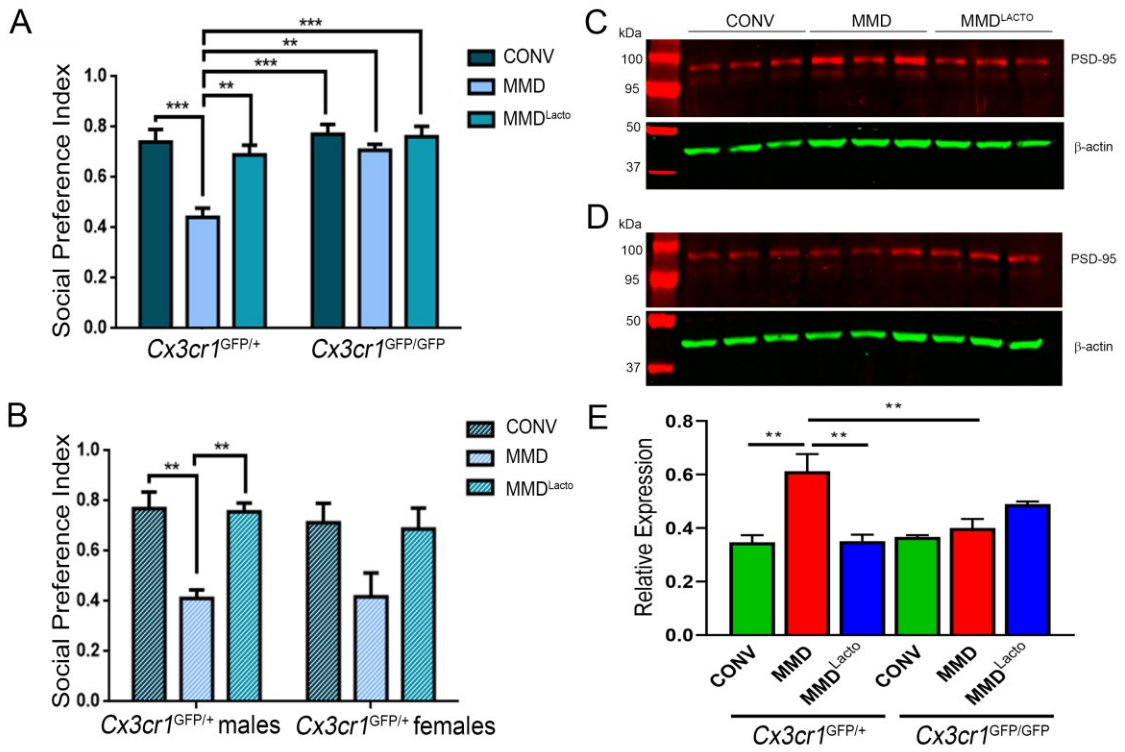

Figure 6. Loss of *Cx3cr1* attenuates MMD-induced social deficits and PSD-95 expression. (A) Heterozygous *Cx3cr1*$^{GFP/+}$ MMD offspring exhibit social deficits at P21 compared to heterozygous CONV and MMD^{Lacto} offspring. These deficits reach significance in males, however, lack of social preference was reflected in both MMD males and females and no significant interaction was found between sex and treatment. Importantly, social deficits were attenuated in *Cx3cr1*$^{GFP/GFP}$ knockout MMD offspring. MMD^{Lacto} mice showed no social deficits regardless of genotype. (C) PSD-95 protein expression in *Cx3cr1*$^{GFP/+}$ or (D) *Cx3cr1*$^{GFP/GFP}$ cortices of male offspring (n=3 per group). (E) Quantification of PSD-95 expression shows increased levels in MMD offspring in the presence of functional *Cx3cr1* only. (n=4-5 litters, average 29-30 pups per group). **P<0.05 and ***P<0.001.

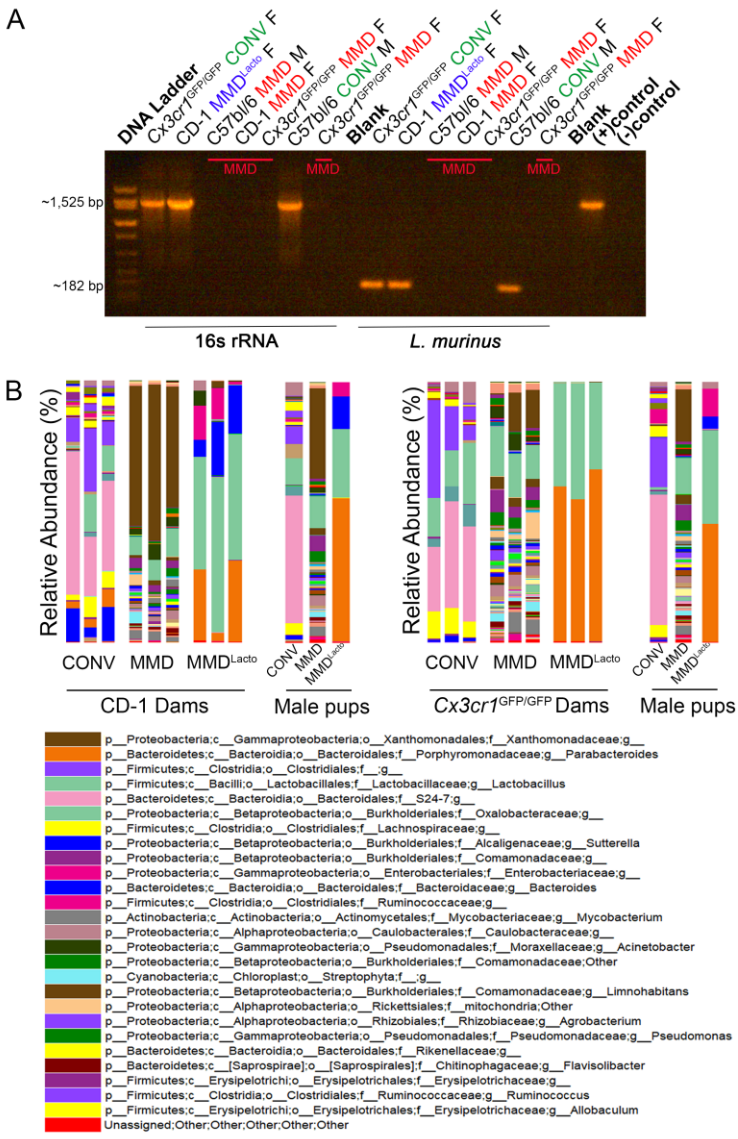

Figure S1. Reverse transcriptase PCR validation of MMD status and gut bacteria composition. (A) Representative gel electrophoresis image showing amplified bacterial DNA from breeding animals' feces. Lanes indicate individual animals. (B) Relative abundance of maternal fecal bacteria at the genus level showed reduced diversity in MMD^{Lacto} dams compared to CONV and denote less gut bacterial depletion compared to MMD dams. Fecal bacteria from male pups at time of weaning (P21) also resembled dams' microbiota. Each bar graph denotes individual animal.

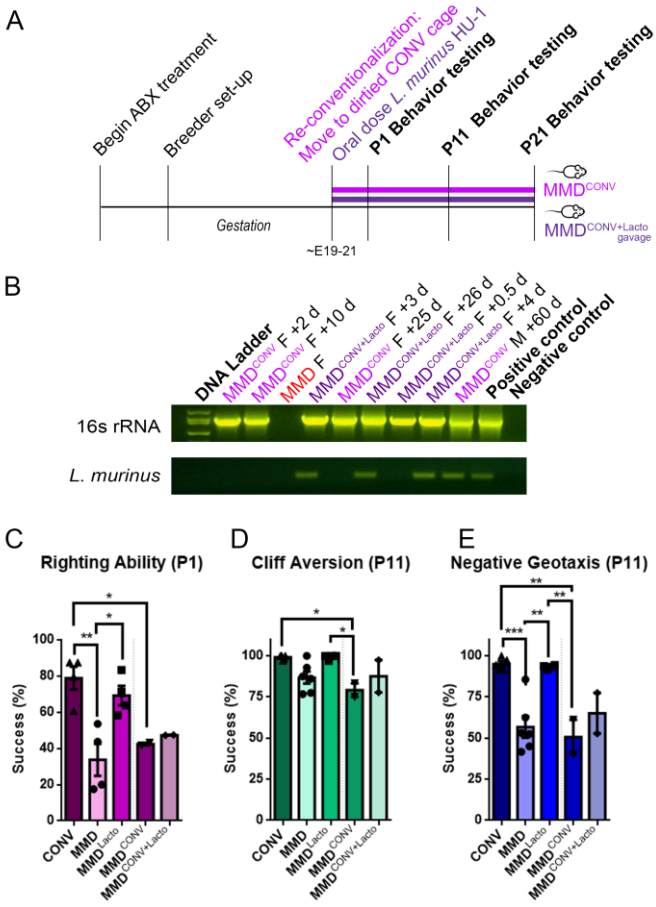

Figure S2. Re-conventionalization of MMD dams rescues behavioral deficits in offspring following oral gavage with *L. murinus* HU-1. (A) Study design schematic for introducing conventional microbiota to CD-1 MMD breeders at the end of pregnancy with and without oral gavage with *L. murinus* HU-1. (B) Representative gel image showing amplified fecal bacterial DNA from ex-MMD breeders. Lanes indicate individual animals with number of days since re-conventionalization. (C) At P1, both MMD^{CONV} and $MMD^{CONV+Lacto}$ offspring perform poorly in righting ability test. (D-E) At P11, MMD^{CONV} and $MMD^{CONV+Lacto}$ offspring show deficits in Cliff Aversion and Negative Geotaxis tests compared to CONV and MMD^{Lacto} offspring. (n=2 litters, 19 pups per group). M=male, F=female. *P<0.05, **P<0.05, and ***P<0.001.

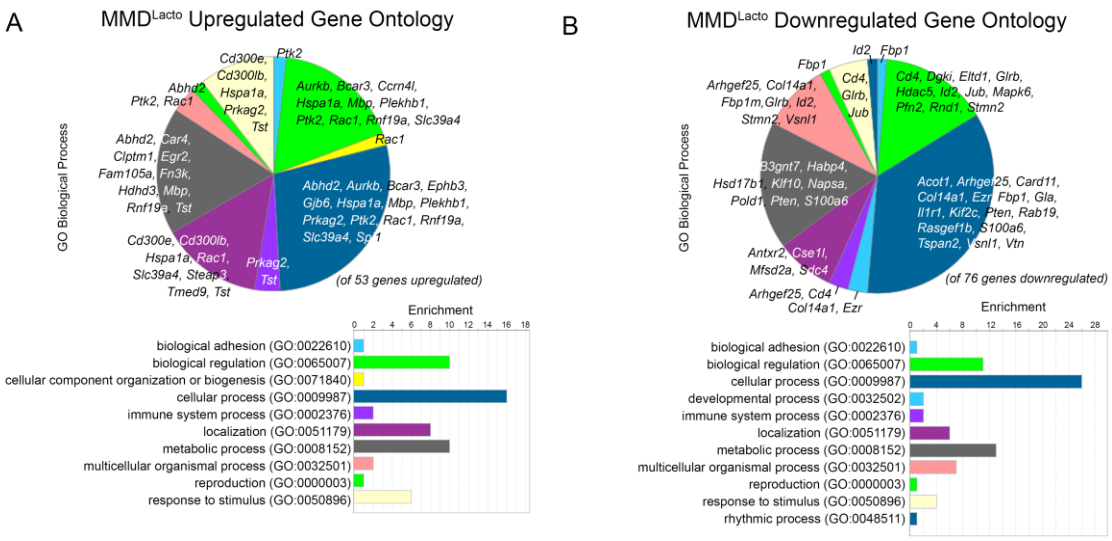

Figure S3. Gene expression changes in cortical microglia of MMD^{Lacto} male offspring. (A-B) Gene ontology enrichment analysis indicates a variety of biological processes represented by upregulated genes (A) and downregulated genes (B) with the majority of observed to be related to cellular processes and metabolic processes, followed by biological regulation and localization.

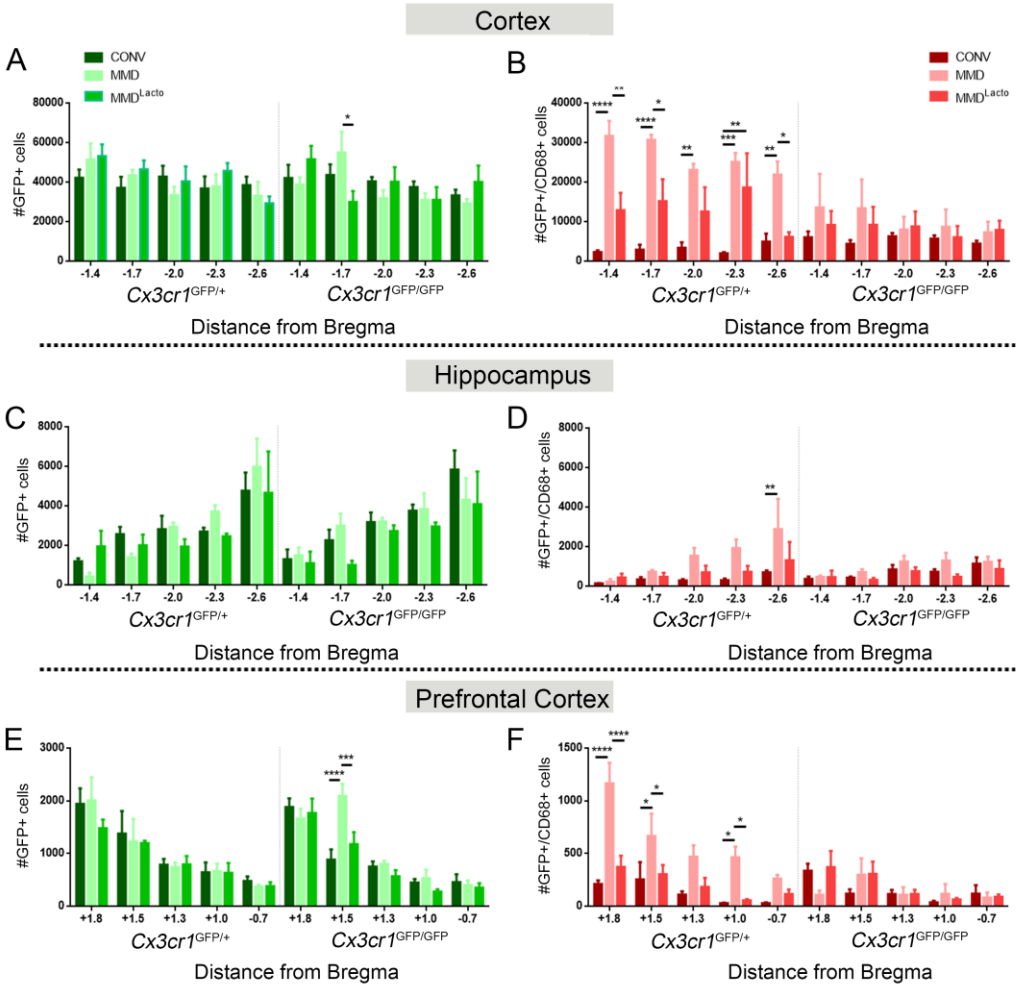

Figure S4. Stereological counts of estimated total GFP$^+$ microglia and CD68$^+$/GFP$^+$ activated microglia in brain regions of *Cx3cr1*$^{GFP/+}$ and *Cx3cr1*$^{GFP/GFP}$ male offspring by bregma level. Estimated cell counts are consistent with stereological counts for entire brain regions in *Cx3cr1*$^{GFP/+}$ and *Cx3cr1*$^{GFP/GFP}$ male offspring at P22. Minimal difference was seen in the total GFP$^+$ cell population in the parietal cortex, hippocampus and prefrontal cortex (A, C, E; respectively). Significant differences were seen in CD68$^+$/GFP$^+$ cell numbers in the parietal cortex (B) and prefrontal cortex (F). No difference was seen in the hippocampus except at bregma level -2.6 in *Cx3cr1*$^{GFP/+}$ mice (D). (n=4 litters). *P<0.05, **P<0.05, and ***P<0.001.

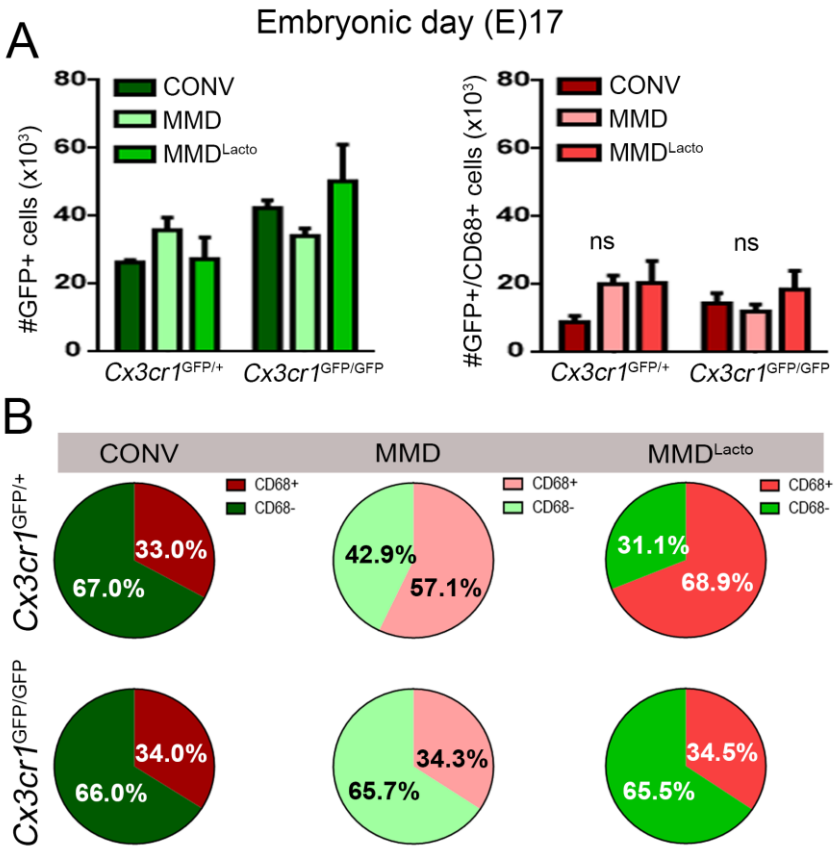

Figure S5. Hemispheric stereological counts of microglia in embryonic day 17 CONV, MMD, and MMD^{Lacto} brains of *Cx3cr1*$^{GFP/+}$ and *Cx3cr1*$^{GFP/GFP}$ mice. (A) No significant difference was found in the estimated total number of GFP^{+} or CD68^{+}/GFP^{+} cells at E17 following non-biased stereology on six serial coronal sections. (B) Pie charts depicting the proportion of CD68^{-}/GFP^{+} versus CD68^{+}/GFP^{+} cells in the three groups in the presence and absence of *Cx3cr1*. Data shows increased CD68^{+}/GFP^{+} cells in brains of heterozygous MMD and MMD^{Lacto} offspring which was attenuated in *Cx3cr1*$^{GFP/GFP}$ knockout mice. (n=4 litters).

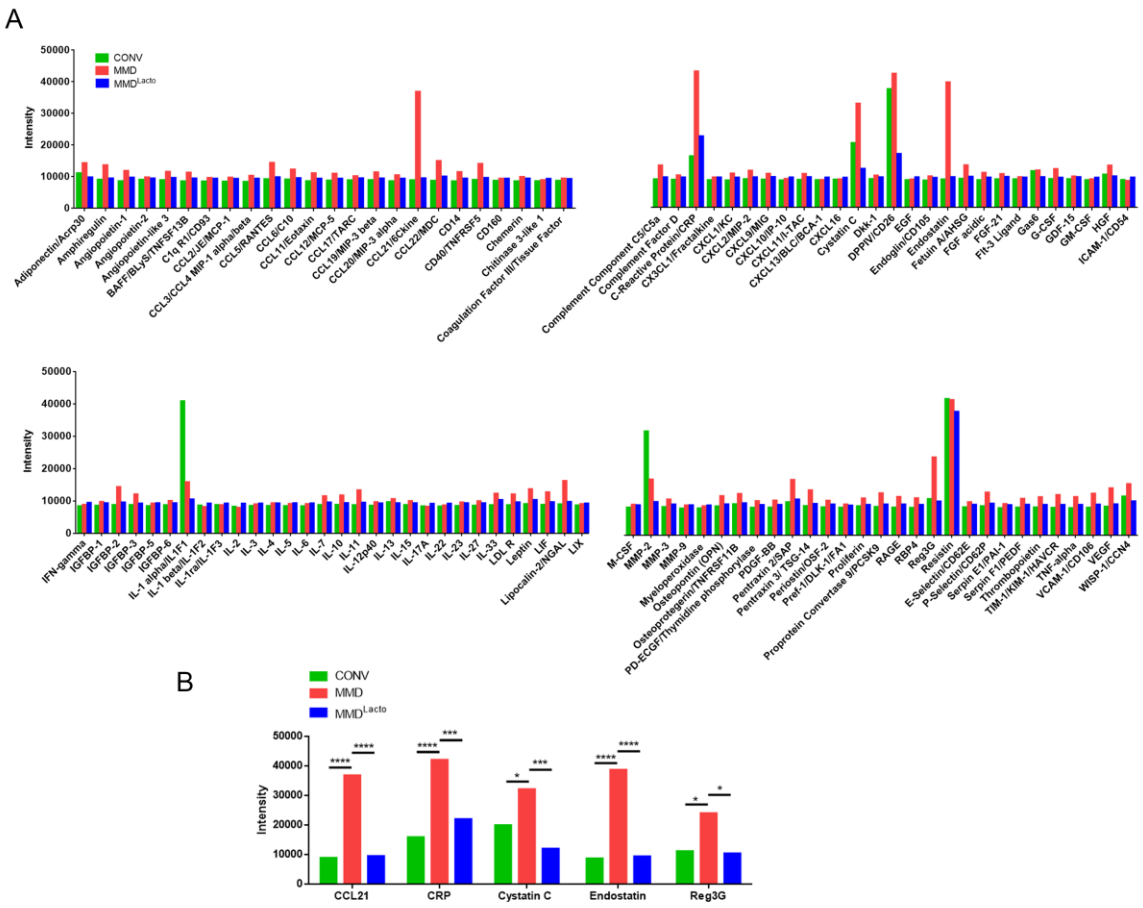

Figure S6. Multiplex proteome profile mouse cytokine array analysis of intestinal cecum from CONV, MMD, and MMD^{Lacto} male offspring. (A) Full panel of comparative inflammatory cytokines expressed in cecal contents of male offspring at P22 in CONV, MMD, and MMD^{Lacto} conditions. Measurements based on dot blot analysis and intensity outcomes using ImageLab. (B) Selected cytokines from the multiplex cytokine panel demonstrating significant changes. MMD induces expression of CCL21, CRP, Cystatin C, Endostatin and Reg3G in the cecum, which was attenuated in MMD^{Lacto}. (n=3 per group). *P<0.05, ***P<0.001, and ****P<0.0001.

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Chapter 4: Molecular Phenotyping and Genomic Characterization of a Novel Neuroactive Bacterium Strain, *Lactobacillus murinus* HU-1

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This work was submitted to *Frontiers in Pharmacology.*

4.1 Abstract

Modulation of brain development and behavior by probiotics represents exciting new therapeutic targets for a wide range of psychological disorders, including autism, depression, memory loss, and anxiety. Previously, we demonstrated that oral supplementation with a novel strain of gut bacteria, *Lactobacillus murinus* HU-1, protected brain development and social behavior in mice offspring despite antibiotics-driven maternal gut dysbiosis during pregnancy. *Lactobacillus murinus* is a commensal gut bacterium found in the gastrointestinal tracts of healthy mammals with demonstrable impact on preventing infection by pathogens, strengthening gut barrier function, and maintaining host immune balance. Despite genetic similarity to known probiotic species, only few studies have set out to address the genomic characteristics of *L. murinus* and no known studies of this bacterium within the context of the gut-brain axis exist. Here, we describe the genetic and molecular traits of *L. murinus* HU-1 in an effort to highlight features that are unique to this species and this particular strain, which may be of pharmacological interest for the probiotics field.

4.2 Introduction

Over a century ago, Elie Metchnikoff observed unusual longevity among Bulgarian populations that consumed soured milk containing lactic acid bacteria (Metchnikoff and Mitchell, 1908). He theorized that the production of lactic acid by such bacteria prevented "intestinal putrefaction" and popularized the deliberate consumption of *Lactobacilli*-cultured milk for health purposes (Cavaillon and Legout, 2016). Since Metchnikoff's time, the contributions of *Lactobacilli* to host health have been greatly expanded to include roles in immune homeostasis, production of key nutrients and vitamins, and even as a physical barrier against infection by pathogenic microorganisms (Macfarlane and Macfarlane, 2012). Recent investigations into the gut-brain axis revealed possible additional functions of lactic acid bacteria in regulating mood and cognition when ingested orally as a probiotic supplement. Indeed, many *Lactobacillus* sp. have been correlated with improved psychological outcomes, especially for neurodevelopmental, mood, stress, and anxiety disorders (Bravo et al., 2011; Buffington et al., 2016; Liu et al., 2019; Marotta et al., 2019; Sgritta et al., 2019).

While the neurological mechanisms behind probiotic consumption have yet to be fully understood, current evidence suggests *Lactobacilli* likely confer mental health benefits through both direct and indirect pathways, such as vagal nerve signaling and T_{reg} regulation (Bravo et al., 2011; Wells, 2011). Gut bacterial production of known neurotransmitters, such as gamma-aminobutyric acid (GABA), serotonin, and glutamate (Lyte, 2011; Dienel, 2012; Steenbergen et al., 2015), as well as a newfound appreciation for neuroactive potential of common bacterial metabolites, such as lactate and short-chain

fatty acids, further suggest additional pathways in which *Lactobacilli* may contribute to neurological health (Proia et al., 2016; Oleskin et al., 2017).

Previously, we reported neuroprotective effects of *Lactobacillus murinus* HU-1, a mutant strain isolated from mouse, in preventing development of premature senescence in cortical microglia and social behavior deficits in murine offspring reared under antibiotics-driven maternal microbiome dysbiosis (Lebovitz et al., 2019). A key component of a complete and diverse gut microbiome, *L. murinus* represents a commensal gut bacterium naturally found in the gut of healthy mammals, including rodents, dogs, pigs, and poultry (Kurzak et al., 1998; Greetham et al., 2002; Gardiner et al., 2004). Compared to other *Lactobacilli*, *L. murinus* is a relatively understudied species that only recently gained attention as a probiotic candidate, including potential applications regarding neonatal necrotizing enterocolitis (Isani et al., 2018), antimicrobial production (Nardi et al., 2005), pathogen antagonism (Vasconcelos et al., 2003), intestinal barrier function (Delucchi et al., 2017), food allergy (Huang et al., 2016), type 1 diabetes (Sane et al., 2018), hypertension (Wilck et al., 2017), age-associated inflammation (Pan et al., 2018), and bacterial translocation (Ma et al., 1990). Here, we characterize the genome of a novel strain, *L. murinus* HU-1, and profile its molecular features in an effort to better understand its influence on host physiology and neurobehavior.

4.3 Materials and Methods

Bacterial isolation and growth. *Lactobacillus murinus* HU-1 originally isolated from murine gut was maintained as frozen stock in 20% glycerol at -80°C until needed. Frozen stock was directly cultured overnight in MRS broth or streaked onto MRS agar (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C, as described (Kragh et al., 2018).

Animals. All mice were housed in an AAALAC accredited, virus/specific antigen-free facility with a 12 h light-dark cycle; food (Teklad 2918, Envigo, Huntingdon, UK) and water provided *ad libitum.* Outbred CD-1 IGS mice were purchased from Charles River (Strain code 022, Charles River Laboratories, Wilmington, MA, USA), and inbred B6.129P-*Cx3cr1*^{tm1Litt}/J mice (Stock no. 005582) were purchased from Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME, USA). Experimental CD-1 mice were administered a single oral dose of *L. murinus* HU-1 (10^9 CFU) and then maintained on an antibiotic cocktail of 0.4 mg/ml kanamycin, 850 U/ml colistin, 0.215 mg/ml metronidazole (Bio-World, Dublin, OH, USA), 0.035 mg/ml gentamicin (Vet One, Boise, ID, USA), and 0.045 mg/ml vancomycin (Hospira Inc., Lake Forest, 372 IL, USA) (ABX^{HU-1}) or the above antibiotic cocktail with an addition of 0.5 mg/ml amoxicillin/clavulanic acid (Zoetis, Parsippany, NJ, USA) ($ABX^{HU-1+AC}$). Antibiotics were administered via drinking water. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and conducted under the

approval of the Virginia Tech Institutional Animal Care and Use Committee (IACUC; #17-043).

Murine fecal bacteria identification and antibiotic susceptibility. Fresh fecal pellets from mice were collected into sterile 1.5 mL microcentrifuge tubes and submitted to Virginia-Maryland College of Veterinary Medicine's Animal Laboratory Services for identification of culturable bacteria and to undergo antibiotics susceptibility testing. In brief, murine fecal pellets were immediately cultured on MacConkey and chocolate agar overnight. Colony formations were scored and identified using Bruker Microflex Biotyper 3.1 MALDI-TOF (Bruker Daltonics, Billerica, MA, USA). Additional colonies were collected from pure cultures of identified bacteria and subjected to antibiotics susceptibility testing using Sensititre^{TM} Complete Automated AST System (Thermo Fisher Scientific Solutions LLC, Waltham, MA, USA) according to manufacturer's instructions.

DNA isolation and whole genome sequencing. Genomic DNA was extracted and purified from *L. murinus* HU-1 isolates via kit (SKU D6010, Zymo Research, Irvine, CA, USA) and submitted to Beijing Genomics Institute (Shenzhen, China) for whole genome re-sequencing. In brief, the genome was sequenced using an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA). Genomic DNA was sheared randomly to construct three read libraries with lengths of 300 bp by a Bioruptor ultrasonicator (Diagenode, Denville, NJ, USA) and physiochemical methods. The paired-end fragment libraries were

sequenced according to manufacturer's protocol. Raw reads of low quality from paired-end sequencing were discarded.

Genome assembly, annotation, and genomic features. Bioinformatic analyses on *L. murinus* HU-1 were performed using Pathosystems Resource Integration Center (PATRIC) Comprehensive Genome Analysis service (Wattam et al., 2017). In brief, raw sequenced reads were assembled using SPAdes. Assembled genome was then annotated using RAST tool kit (RASTtk). Specialty genes were determined by homology to those identified as drug targets in the DrugBank database (Law et al., 2014), transporters in the Transporters Classification Database (TCDB) (Saier et al., 2016), and virulence factors in the Virulence Factor Database (VFDB) (Chen et al., 2016). Antibiotic resistance genes, their functional annotation, mechanism of antibiotic resistance, and drug class were identified using the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013) and a curated database of representative antibiotic resistance gene sequence variants available on PATRIC (Wattam et al., 2017). Subsystems analysis depicting biological processes or structural complexes of specific genes was based on SEED subsystems annotations (Overbeek et al., 2005). A comprehensive genome analysis was similarly performed for the representative strain, *L. murinus* ASF361 (SRR769344), to provide a basis for comparison.

Phylogenetic tree of *L. murinus* strains. Phylogenetic tree of *L. murinus* HU-1 and 10 publicly available *L. murinus* whole genome sequences (strains: ASF361 [representative strain], 510-9, CR141, CR147, DSM 20452 = NBRC 14221, EF-1, KM-1, UBA3408,

UBA3411, UBA7190) was constructed using PATRIC codon tree method utilizing PATRIC PGFams as homology groups and analyzing aligned proteins and coding DNA from single-copy genes using the program RAxML version 8.2.11 and fast bootstrapping to provide support values in the tree (Davis et al., 2016).

Proteomic analysis. Assessment of protein-coding genes in *L. murinus* HU-1 was constructed using the Protein Family Sorter Service (PATtyFams) tool in PATRIC. In brief, protein families were generated based on k-mer functional assignments using RAST and Markov Cluster algorithm (MCL) (Davis et al., 2016). PATRIC genus-specific families (PLfams) option was used to provide comparative assessment of protein families between *L. murinus* HU-1 and relevant strains due to the stringent criteria used (MCL inflation = 3.0), which allow for greater specificity when comparing genomes within the same species.

Data Availability Statement. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession VMDX00000000. The version described in this paper is version VMDX01000000.

4.4 Results

Genomic features of *L. murinus* HU-1

To ascertain whether *Lactobacillus murinus* HU-1 was a novel strain, we conducted whole genome sequencing and performed comprehensive genome analysis

using PATRIC (Wattam et al., 2017). Genome assembly analysis estimated genome length to be 2,408,429 bp, average GC content of 39.84%, and 232 contigs. Taxonomy was confirmed as *L. murinus*. Annotated genome analysis revealed 2,597 protein coding sequences (CDS), 55 transfer RNA (tRNA) genes, and 4 ribosomal RNA (rRNA) genes. Of these, 875 represented hypothetical proteins and 1,722 proteins with functional assignments, including 535 with Enzyme Commission (EC) numbers, 441 with Gene Ontology (GO) assignments, and 355 mapped to KEGG pathways. Investigation of specialty genes resulted in 2 potential drug targets (Law et al., 2014), 2 transporter genes (Saier et al., 2016), 23 potential antibiotic resistance genes (McArthur et al., 2013; Wattam et al., 2017), and no known virulence factors (Chen et al., 2016). These genomic features are visualized in a circular graphic in Fig. 1A.

Comparison against representative strain, *L. murinus* ASF361

Next, we conducted comparative genome analysis against the representative genome, *L. murinus* ASF361 (SRR769346) ("representative strain"). We determined *L. murinus* ASF361 to be the representative strain based on its inclusion as one of the eight microbes making up the Altered Schaedler Flora, a defined collection of gut bacteria deemed to be necessary for maintaining murine health, and thereby its endemic nature in most laboratory mice (Wymore Brand et al., 2015). Investigation of specialty genes in the representative strain revealed 1 potential drug target, 1 transporter gene, 22 potential antibiotic resistance genes, and no known virulence factors. The representative strain genomic features are visualized in Fig. 1B.

The specialty genes expressed in the representative genome were also shared by *L. murinus* HU-1. Specifically, these included *ptsH*, which encodes a potential drug target, phosphocarrier protein, Hpr (Jia et al., 1993), and a copper transporter, *tcrB* (Hasman, 2005). Potential antibiotic resistance genes were broadly determined by PATRIC as any sequence variant whose presence/absence/mutation were related to antibiotic resistance and categorized according to the following mechanisms: antibiotic target in susceptible species (*alr*, *ddl*, *EF-G*, *EF-Tu*, *folA*, *dfr*, *gyrA*, *gyrB*, *inhA*, *fabI*, *iso-tRNA*, *kasA*, *murA*, *rho*, *rpoB*, *rpoC*, *s10p*, *s12p*); antibiotic target modifying enzyme (*rlmA[II]*); gene conferring resistance via absence (*gidB*); and protein altering cell wall charge conferring antibiotic resistance (*mprF*, *pgsA*) (Wattam et al., 2017). Notably, assessment of antibiotic resistance genes according to Comprehensive Antibiotic Resistance Database (CARD) identified only *EF-Tu* as a potential antibiotic resistance gene (McArthur et al., 2013). In addition to the specialty genes identified in the representative genome, *L. murinus* HU-1 differentially possessed a multiple sugar ABC transporter gene, *msmG* (Webb et al., 2008), a potential drug target related to galactose metabolism, *lacG* (Wiesmann et al., 1997), and an extra copy of the potential antibiotic resistance gene, *inhA*/*Fabl* (Lu and Tonge, 2008).

Comparative characterization of subsystems categories

Subsystems analysis of *L. murinus* HU-1 and the representative genome showed similar categorization of biological processes and pathways, including the majority of gene functions allocated to metabolism and protein processing (Figs. 1C-D). *L. murinus* HU-1 genes included additional energy-related genes specific to dihydroxyacetone kinase

(DhaK) with purported functional involvement in central metabolism (Erni et al., 2006), as well as a cell wall-related gene specific to dTDP-rhamnose synthesis (van der Beek et al., 2019). In contrast, the representative genome differentially included additional metabolism-related genes specific to biotin synthesis and utilization (Satiaputra et al., 2016), NAD and NADP cofactor biosynthesis (Gazzaniga et al., 2009), and thiamin transport (Rodionov et al., 2002).

Proteomic assessment of *L. murinus* HU-1

To elucidate potential functional differences found in *L. murinus* HU-1 in comparison to the representative strain, we conducted comparative examination of the distribution of protein families in the two respective genomes via PATRIC genus-specific families (PLfams) (Davis et al., 2016). We observed the presence of 378 protein families in *L. murinus* HU-1 that were not identified in the representative genome; 259 of these were for hypothetical proteins. Of the attributed protein families only, approximately 51.5% were functionally related to phage-specific activities and the remaining protein families were distributed across mobile element protein, integrase, alcohol dehydrogenase, and beta-galactosidase activity (Fig. 2A). The latter is a critical enzyme produced by infant gut bacteria and is a common feature of probiotic *Bifidobacteria* (Milani et al., 2017). Prophage proteins identified in this genome (Lp2 protein 4, Lp4 protein 7, ps1 protein 14, and ps3 protein 13) were previously found in other probiotic strains, *L. reuteri*, *L. plantarum*, and *Lactococcus lactis* (UniProt, 2019). Lp2 and Lp4 were considered non-inducible prophages, whereas ps1 and ps3 were predicted to be related to DNA packaging (Bolotin et al., 2001; Ventura et al., 2003). In contrast, we

observed 163 protein families in the representative genome that were not identified in *L. murinus* HU-1, including 125 hypothetical proteins. Of the attributed protein families found in the representative strain only, approximately 30% were functionally related to gram positive anchor domain and the rest distributed across assorted activities (Fig. 2B).

Comparison against other sequenced *L. murinus* strains

Previously, we and others described *L. murinus* as phylogenetically closest to other probiotic strains, *L. animalis* and *L. salivarius* (Pan et al., 2018; Lebovitz et al., 2019). To contextualize *L. murinus* HU-1 within its subspecies, we constructed a phylogenetic tree using whole genomes of 10 publicly available *L. murinus* strains, including the representative strain (Fig. 2C). The tree formed three main branches with *L. murinus* HU-1 clustering with the representative strain, albeit several nodes apart (Fig. 2A). Protein families analysis comparing *L. murinus* HU-1 to these other 10 genomes revealed that *L. murinus* HU-1 possessed 55 protein families not found in the other strains. The majority of these genes belonged to unattributed hypothetical proteins, although the second largest proportion of genes encoded for proteins relevant to phages (approximately 46%) while the rest belonged to ABC transporters, beta-lactamase binding protein, choline binding protein, and methyltransferases (Fig. 2D). In contrast, *L. murinus* HU-1 was shown to be just missing 7 hypothetical protein families otherwise found in the 10 other strains. These were mostly of unattributed or unknown function, but several were purported to be related to a conserved domain protein or regulatory competence proteins (Fig. 2E).

Assessment of antibiotic susceptibility of *L. murinus* HU-1

In support of the potential antibiotic resistance genes identified in the above genomic analysis, we isolated *L. murinus* from antibiotics-treated experimental and conventionally-raised CD-1 mice feces for antibiotic sensitivity testing via disk diffusion method. Only *L. murinus* could be isolated from experimental mice harboring *L. murinus* HU-1 ($ABX^{HU\text{-}1}$) and these isolates exhibited antibiotic resistance to amikacin and gentamicin. Introducing amoxicillin/clavulanic acid to the antibiotic cocktail for two weeks ($ABX^{HU\text{-}1+AC}$) still resulted in *L. murinus* growth, however, it no longer exhibited antibiotic resistance according to the disk diffusion assay (Supplemental Table 1). Meanwhile, native *L. murinus* isolates from conventionally-raised control mice did not exhibit antibiotic resistance. Interestingly, native *L. murinus* isolates from conventionally-raised B6.Cx3cr1 mice, which were a different strain and purchased from a different vendor than the CD-1 mice, exhibited widespread resistance to amikacin, cefazolin, chloramphenicol, clindamycin, erythromycin, gentamicin, and imipenem. Furthermore, cross-rearing conventional B6.Cx3cr1 offspring with conventional CD-1 mice resulted in *L. murinus* isolates that no longer maintained antibiotic resistance traits (Supplemental Table 1). Thus, *L. murinus* HU-1 isolated from experimental mouse feces exhibited antibiotic resistance as predicted in the genomic analysis, but this trait was malleable under additional antibiotic therapy and was not unique as native *L. murinus* found in conventionally-housed mice also exhibited antibiotic resistance from the outset.

4.5 Conclusion

Lactobacillus murinus represents a promising probiotic candidate with a wide range of potential health applications. Here, we sequenced and analyzed the whole genome of a novel strain, *L. murinus* HU-1, previously reported to confer neurodevelopmental benefits in a murine model of maternal microbiome dysbiosis. Notably, *L. murinus* HU-1 expressed genes specific to beta-galactosidase production, which may counteract the microglial accumulation of this enzyme typically found in neurological disease models of premature cellular senescence. Beta-galactosidase production is also a common trait of commensal bacteria found in the healthy infant gut, as it is a key enzyme for proper digestion of mammary milk. Compared to other publicly available *L. murinus* strains, *L. murinus* HU-1 shared important traits of probiotics, such as expression of genes related to bacteriocin activity and resistance to a variety of environmental stresses. However, *L. murinus* HU-1 uniquely expressed genes specific to prophage activity, potential antibiotic resistance, and select biological processes. The impact of phages in probiotic genomes remain a nascent area of study; some have been credited with enhanced fitness to the gastrointestinal niche while others are considered problematic for the fermentative dairy industry due to potential phage predation. As the phages identified in *L. murinus* HU-1 were not associated with virulence, it is possible that their presence may contribute to host health through yet unknown adaptive advantages. Additional study into *L. murinus* HU-1 interactions with the host, as well as detailed conditions for its growth and scalability, will be needed to demonstrate probiotic efficacy and safety in the future.

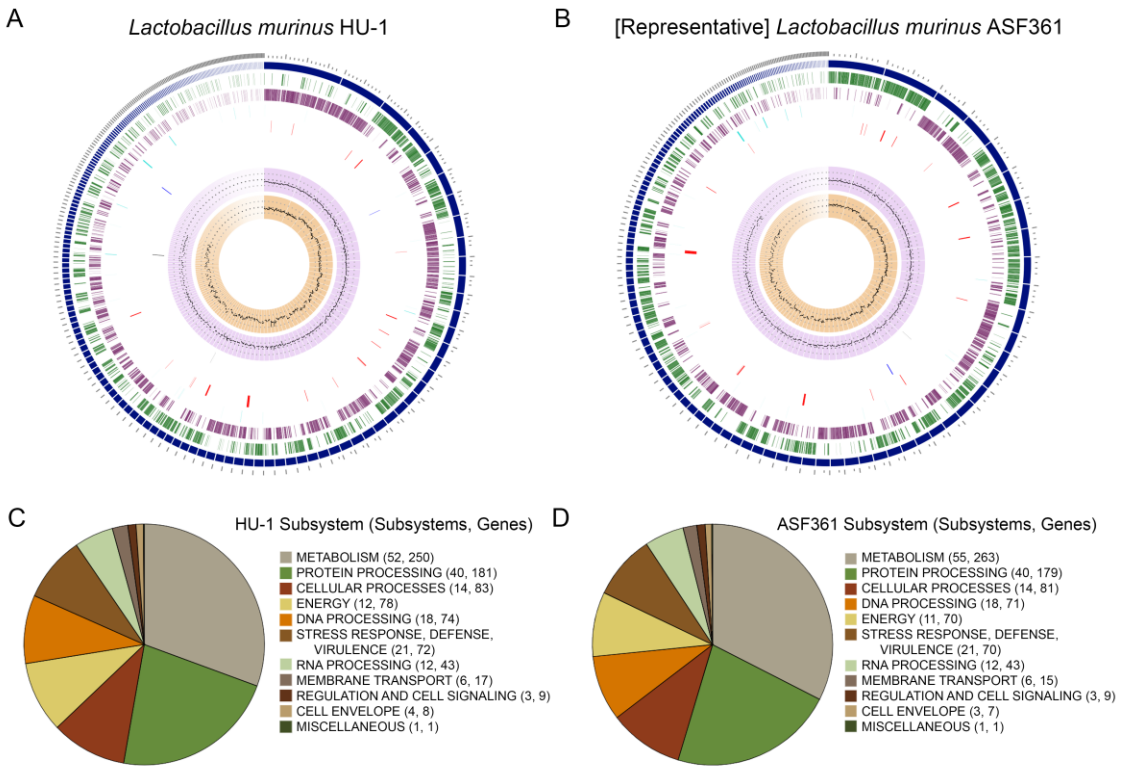

Figure 1. Comparative genomic characterization of novel strain, *Lactobacillus murinus* HU-1. (A-B) Circular graphic of novel strain, *L. murinus* HU-1, (A) and representative strain, *L. murinus* ASF361 (B). From outer to inner rings are: contigs, coding sequence (CDS) on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known transporters, CDS with homology to known drug targets, GC content, and GC skew. (C-D) Pie charts indicating major subsystems and number of genes in each category for *L. murinus* HU-1 (C) and *L. murinus* ASF361 (D). *L. murinus* HU-1 contained additional energy- and cell envelope-related genes specific to central metabolism and cell wall synthesis, while the representative strain contained additional metabolism-related genes specific to biotin synthesis, NAD and NADP cofactor biosynthesis, and thiamin transport.

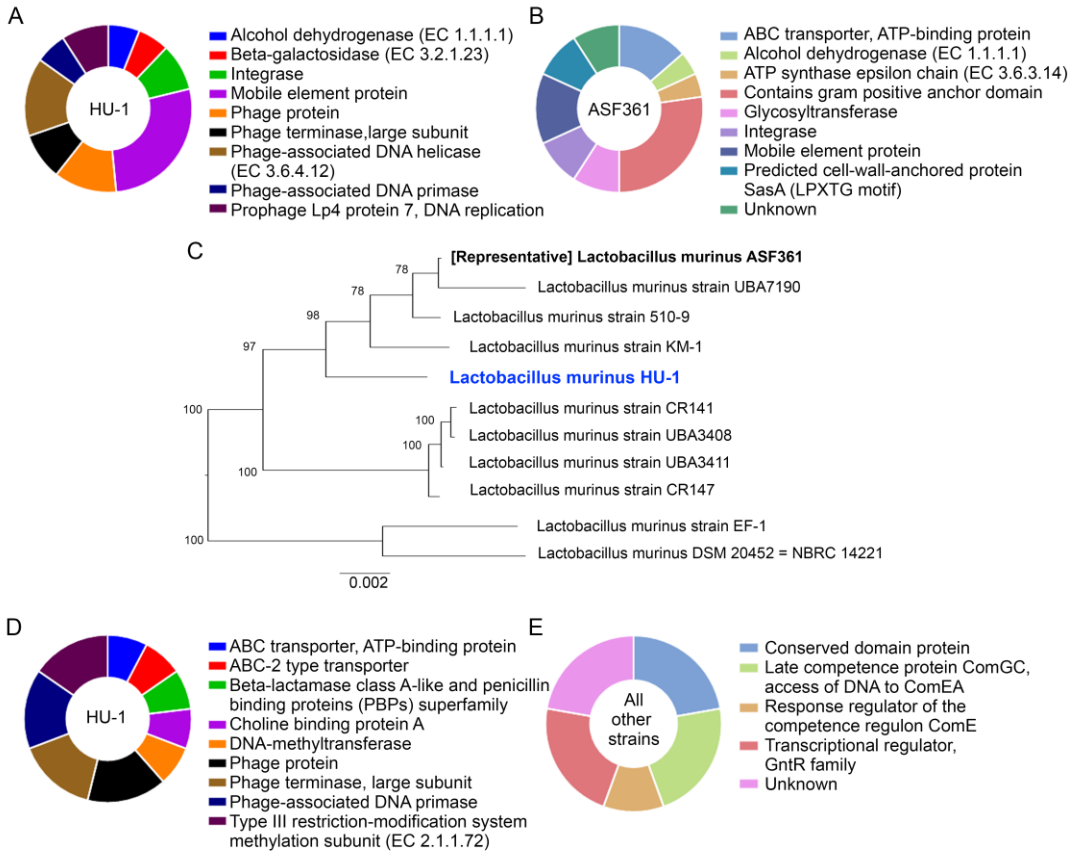

Figure 2. *L. murinus* HU-1 maintains features distinct from other strains of *L. murinus*. (A) Attributed protein families identified in *L. murinus* HU-1 only that were not in the representative strain, *L. murinus* ASF361. Pie chart excludes hypothetical proteins, which were in the majority. (B) Attributed protein families identified in the representative strain only that were not in *L. murinus* HU-1, excluding hypothetical proteins. (C) Phylogenetic tree of *L. murinus* HU-1 compared to 10 other publicly available *L. murinus* strains on NCBI database. (D) Attributed protein families identified in *L. murinus* HU-1 that were not in the 10 other strains, excluding hypothetical proteins. (E) Only hypothetical proteins were found to be missing from *L. murinus* HU-1 compared to 10 other strains, but some of these proteins possessed hypothetical functional attributions.

Antibiotic	ABX^{HU-1} CD-1		ABX$^{HU-1+AC}$ CD-1		CONV CD-1		CONV B6.Cx3cr1		CONV B6.Cx3cr1 offspring reared by CONV CD-1	
1. Amikacin	>32.00	R	<=4.00	S	<=4.00	S	>32.00	R	<=4.00	S
2. Amoxicillin/ Clavulanic Acid	0.25	S	<=0.12	S	1	S	>1.00	NI	<=0.12	S
3. Ampicillin	<=0.12	S	<=0.12	S	N/A	N/A	N/A	N/A	1	S
4. Cefazolin	<=1.00	S	<=1.00	S	4	I	8	R	<=1.00	S
5. Cefovecin	2	NI	<=0.25	NI	>4.00	NI	>4.00	NI	<=0.25	NI
6. Cefoxitin	8	NI	<=2.00	NI	16	NI	>16.00	NI	<=2.00	NI
7. Cefpodoxime	<=2.00	NI	<=2.00	NI	>16.00	NI	>16.00	NI	<=2.00	NI
8. Ceftiofur	>4.00	NI	<=0.25	NI	<=0.25	NI	>4.00	NI	<=0.25	NI
9. Cephalothin	<=2.00	S	<=2.00	S	4	S	8	S	<=2.00	S
10. Chloramphenicol	<=4.00	S	<=4.00	S	16	I	>16.00	R	<=4.00	S
11. Clindamycin	<=0.50	S	<=0.50	S	<=0.50	S	>4.00	R	<=0.50	S
12. Doxycycline	4	NI	<=2.00	NI	8	NI	8	NI	<=2.00	NI
13. Enrofloxacin	1	NI	<=0.25	NI	1	NI	>2.00	NI	<=0.25	NI
14. Erythromycin	<=0.50	S	<=0.50	S	<=0.50	S	>4.00	R	<=0.50	S
15. Gentamicin	>8.00	R	<=1.00	S	<=1.00	S	>8.00	R	<=1.00	S
16. Imipenem	<=1.00	S	<=1.00	S	2	I	8	R	<=1.00	S
17. Marbofloxacin	2	NI	<=0.25	NI	2	NI	>2.00	NI	<=0.25	NI
18. Oxacillin + 2% NaCl	1	NI	<=0.25	NI	>4.00	NI	>4.00	NI	<=0.25	NI
19. Penicillin	0.5	S	<=0.06	S	>8.00	NI	8	S	<=0.06	S
20. Rifampin	<=1.00	NI	<=1.00	NI	2	NI	>2.00	NI	<=1.00	NI
21. Ticarcillin	<=8.00	NI	<=8.00	NI	<=8.00	NI	>64.00	NI	<=8.00	NI
22. Ticarcillin/ Clavulanic Acid	<=8.00	NI	<=8.00	NI	<=8.00	NI	>64.00	NI	<=8.00	NI
23. Trimethoprim/ Sulfamethoxazol	<=0.50	NI	<=0.50	NI	<=0.50	NI	>2.00	NI	<=0.50	NI

Supplemental Table 1. Comparative antibiotic susceptibility of *L. murinus* HU-1 versus native *L. murinus* isolated from mice feces. Disk diffusion antibiotic susceptibility assay results performed on *L. murinus* cultures isolated from feces of experimental mice (ABX^{HU-1}, ABX$^{HU-1+AC}$) and conventionally-raised controls (CONV). *L. murinus* isolated from ABX^{HU-1} mice showed antibiotic resistance to amikacin and gentamicin. Supplementing ABX^{HU-1} mice with amoxicillin/clavulanic acid (ABX$^{HU-1+AC}$) for two weeks resulted in loss of antibiotic resistance in *L. murinus* isolates. Isolates collected from CONV mice showed variable antibiotic resistance based on mouse strain and vendor origin. Assay results are interpreted accordingly: S=Sensitive; I=Intermediate; R=Resistant; NI=Not Interpreted; N/A=Not available.

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Chapter 5: Summary and Future Directions

Despite increasing reports of significant correlations between maternal microbiota and neurodevelopmental outcomes, studies to reveal causal mechanisms between maternal gut and fetal brain remain challenging in part due to the complexity and heterogeneity presented in both the gut and brain. Here, we contribute to a small but growing number of reports identifying maternal microbiota as a powerful modulator of behavioral and microglial development. First, we describe the impact of antibiotics-based maternal microbiome dysbiosis (MMD) on the onset of autism-like endophenotypes in murine offspring, specifically with regard to developmental delays in neonatal gross motor and reflex abilities followed by impaired social behavior at weaning. We attribute these detrimental effects to atrophied microglia in the prefrontal and parietal cortex with a transcriptomic signature resembling a premature senescence-like phenotype. We observe increased postsynaptic protein, PSD-95, in the cortex of MMD offspring, which have been previously attributed to impaired synaptic pruning by microglia (Diaz Heijtz et al., 2011; Paolicelli et al., 2011; Zhan et al., 2014). Second, we demonstrate the presence of *Lactobacillus murinus* HU-1, a lab-generated commensal gut bacterium that is able to persist in murine gut despite antibiotic regiment, in pregnant MMD dams enables rescue of behavioral and microglial dysfunction in offspring. Third, we uncover temporal susceptibility during the earliest neonatal period wherein *L. murinus* HU-1 administration, but not general recolonization with conventional gut microbiota, can partially rescue behavioral deficits in MMD offspring. Fourth, we utilize transgenic mice lacking functional Cx3cr1, a chemokine receptor necessary for microglia-neuron

crosstalk, and demonstrate rescue of behavioral and microglial dysfunction in MMD offspring. We further propose Cx3cr1 receptor pathway as a mechanism of maternal-gut-to-fetal-brain communication due to its immunogenic role in both microglia and peripheral macrophages (Verheijden et al., 2015). Lastly, we conduct bioinformatic analysis of *L. murinus* HU-1 genome to reveal prophage, central metabolism, and antibiotic resistance features unique to this particular strain that likely account for its survivability in the MMD murine gut.

The scope of the research presented in this dissertation is nevertheless restricted to an experimental model of MMD and a neurodevelopmental trajectory specific to mice. Additional limitations of this study are related to longitudinal assessments, which were confined to the period between parturition and weaning only. Microglia analyses occurred at embryonic day 17 and postnatal day 21 only, whereas behavior tests were conducted at postnatal days 1, 11, and 21. While this experimental design was so that the number of animals used were kept at minimum, future studies would benefit from thorough investigation of microglia at these additional time points to accompany the developmental changes in behavior. Likewise, given the spontaneous recovery of gross motor deficits observed in this study and the transient nature of neonatal microglial dysfunctions noted elsewhere (Paolicelli et al., 2011), the addition of adult-aged assessments would be useful in further validating this model and confirming results from similar studies conducted in adult animals only (Buffington et al., 2016; Leclercq et al., 2017).

Perhaps the most exciting discovery from this study is the identification of a premature senescence-associated genes in MMD microglia. This is in parallel with

emerging evidence of impaired microglial metabolism in autism spectrum disorders (Kim et al., 2017), as well as studies of microglial senescence in neurodegenerative diseases (Rawji et al., 2016; Koellhoffer et al., 2017). Interestingly, Cx3cr1 signaling is heavily implicated in the senescence-associated microglial dysfunction in Alzheimer disease (Liu et al., 2010; Mecca et al., 2018; Gabande-Rodriguez et al., 2019), which poses an intriguing counter example for our model of disordered neurodevelopment. As lysosomal accumulation of beta-galactosidase is a common marker for cellular senescence (Debacq-Chainiaux et al., 2009), the unique expression of beta-galactosidase-specific genes in our novel *L. murinus* HU-1 strain also raise questions regarding possible contributions of gut microbiota on brain function. Future studies should pursue validation of premature senescence phenotype in MMD microglia (e.g., intracellular and extracellular levels of beta-galactosidase), as well as consider additional experiments to characterize the involvement of Cx3cr1's sole ligand, Cx3cl1, in the neurodevelopment of MMD and *L. murinus* HU-1 supplemented offspring.

The contribution of secondary by-products of gut microbial metabolism to brain function is a popular hypothesis behind the gut-brain axis. Although we observed no differences in gut bacteria-associated fatty acids between MMD mice and those supplemented with *L. murinus* HU-1, there are many other microbial metabolites that serve as potent neuroactive molecules, such as indole and tryptophan, which were not examined in this study (Yirmiya et al., 2015; Wlodarska et al., 2017). Notably, the eponymous metabolite of *Lactobacillus* species, lactate, is the preferred energy substrate for neurons with demonstrable uptake across the blood-brain barrier (Proia et al., 2016). While L-lactate is the most abundant isoform of lactate found in the brain, previous

studies using radiolabeled D-lactate—the isoform associated with bacteria-derived lactic acid—lend evidence for its ability to diffuse across the brain as well (Ball et al., 2010). Accordingly, the possibility of *L. murinus* HU-1-derived lactate influencing neurobehavioral outcomes in our model of MMD cannot be discounted and warrant further investigation.

Also not addressed in this study are vagus nerve and oxytocin signaling pathways for gut-brain axis communication (Bravo et al., 2011; Buffington et al., 2016; Sgritta et al., 2019). These pathways have been well-characterized in past studies utilizing other *Lactobacillus* species, which have been reported to elicit neurobehavioral changes in both clinical and animal studies (Bravo et al., 2011; Wang et al., 2015; Marotta et al., 2019). As such, future studies should endeavor to include additional probiotic controls, a wider array of microbe-related metabolites, and address the more immediate modes of gut-brain signaling through the use of vagotomy and/or oxytocin-specific transgenic animal models.

Conclusion

Given the common medical need for antibiotic use during pregnancy, the goal of this dissertation was to identify protective factor(s) that reduce the severity of neurodevelopmental consequences and to explore preventive measures for maintaining a healthy maternal gut-fetal brain axis during pregnancy. The scientific novelty herein lies in our multipronged approach to delineate causal pathways and rescue mechanisms between maternal gut microbiota and atypical behavioral and microglial development in

the offspring. The unique focus on a relatively understudied bacterial species, *L. murinus*, as a prophylaxis to broad-spectrum antibiotic administration presents opportunities for developing new standard of care protocols for human and/or animal use. Results from this study can then form the foundation for future hypotheses on maintenance of healthy maternal gut-fetal brain axis during pregnancy—akin to existing public health strategies, like folic acid and choline supplementation. The inferences made from this study will improve our understanding of microbial impact on maternal and child health with special attention to specific microbial agents that contribute to long-term neurological and behavioral disorders, as well as dietary supplements that mitigate these symptoms. If such beneficial products are identified and proven through this study, then we have the potential to provide powerful, non-invasive supportive therapies—potentially as simple as an oral supplement—for ensuring healthy environments in early neurodevelopment.

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Appendix A: Academic and Professional Honors

2018	Fellowship, Dannon Yogurt, Probiotics and the Gut Microbiome Grant
2018	James C. Bradford Memorial Award for Best Predoctoral Poster Presentation, Teratology Society
2018	Poster Selected for Judge Panel on Maternal Health, Central Virginia Chapter of Society for Neuroscience
2018	Travel Award, Teratology Society
2017	Student Fellow of the Year, Virginia Tech Center for Autism Research
2017	Research Grant, Edward Via College of Osteopathic Medicine and Virginia-Maryland College of Veterinary Medicine Center for One Health Research
2017	Travel Award, American Society for Neural Therapy and Repair
2017	Poster Presentation 2nd Place, Central Virginia Chapter of Society for Neuroscience
2017	Graduate Research Development Program Award, Virginia Tech Graduate Student Assembly
2017	Travel Award, Virginia Tech Graduate Student Assembly
2016-2017	Selected PhD Student Blogger, National Institutes of Health Broadening Experiences in Scientific Training (NIH-BEST)
2016	Student Research Award, Virginia Tech Center for Autism Research
2015	Travel Award, Virginia Tech Broadening Experiences in Scientific Training (VT-BEST) Program

Appendix B: Publications and Presentations of Dissertation

Publications

Lebovitz, Y., Kowalski, E. A., Wang, X., Kelly, C., Lee, M., McDonald, V., Ward, R., Creasey, M., Mills, W., Gudenschwager-Basso, E. K., Hazy, A., Hrubec, T., and Theus, M. H. (2019). Lactobacillus rescues postnatal neurobehavioral and microglial dysfunction in a model of maternal microbiome dysbiosis. *Brain Behav Immun,* doi:10.1016/j.bbi.2019.07.025

Lebovitz, Y., and Theus, M.H. (2019). Molecular phenotyping and genomic characterization of a novel neuroactive bacterium strain, *Lactobacillus murinus* HU-1. *Front Pharmacol*, Under review.

Lebovitz, Y., Ringel-Scaia, V. M., Allen, I. C., and Theus, M. H. (2018). Emerging Developments in Microbiome and Microglia Research: Implications for Neurodevelopmental Disorders. *Front Immunol*, 9, 1993. doi:10.3389/fimmu.2018.01993

Okyere, B., Creasey, M., Lebovitz, Y., and Theus, M. H. (2018). Temporal remodeling of pial collaterals and functional deficits in a murine model of ischemic stroke. J *Neurosci Methods*, 293, 86-96. doi:10.1016/j.jneumeth.2017.09.010

Presentations

Lebovitz, Y. (2018, November). *Maternal gut-fetal brain axis: Potential pathways for microbial influence on neurodevelopment.* VMCVM Biomedical and Veterinary Sciences – Seminar Series. Blacksburg, VA. Oral presentation.

Lebovitz, Y., Creasey, M., Lee, M., Kelly, C., Ward, R., and Theus, M.H. (2018, March). *Developmental influence of maternal microbiome dysbiosis on neurobehavioral outcomes.* Central Virginia Chapter of Society for Neuroscience. Richmond, VA. Poster presentation.

Lebovitz, Y. and Theus, M.H. (2018, March). *A role of maternal gut microbiome influence on neuroimmune development.* 4th Annual Graduate Student Assembly Research Symposium. Blacksburg, VA. Poster presentation.

Lebovitz, Y. (2018, March). *The gut-brain interface: New roles in neurodevelopmental disorders and therapeutic potential.* Regenerative Medicine Forum Series. Blacksburg, VA. Oral presentation.

Fisher, C., Lebovitz, Y., McDonald, V., Hrubec, T., and Theus, M.H. (2018, February). *Impact of antibiotic use in pregnancy and subsequent gastrointestinal flora disruption on behaviors in mice offspring.* Edward Via College of Osteopathic Medicine Research Recognition Day. Poster presentation.

Lebovitz, Y. (2017, November). *Influence of antibiotics during pregnancy on maternal gut microbiome and fetal brain development.* Advancing the Human Condition Symposium: One Health Panel. Blacksburg, VA. Oral presentation.

Lebovitz, Y., Brabender, J., Creasey, M., and Theus, M.H. (2017, October). *The role of maternal gut microbiome in perinatal neurodevelopment: Implications for*

neurodevelopmental disorders. Virginia Tech Translational Biology, Medicine, and Health Open House and Research Symposium. Blacksburg, VA. Poster presentation.

Lebovitz, Y., McDonald, V., Hrubec, T., and Theus, M.H. (2018, June). *Balancing the maternal microbiota-perinatal neuroimmune axis: Implications for neurodevelopmental disorders.* The Teratology Society. Clearwater, FL. Poster presentation.

Lebovitz, Y. (2017, September). *The role of the maternal gut microbiome in neuroimmune development: Implications for autism spectrum disorders*. Northern Virginia Research Symposium. Falls Church, VA. Invited talk.

Lebovitz, Y., Brabender, J., Creasey, M. and Theus, M.H. (2017, April) *Role of maternal gut microbiome on neuroimmune development: Implications for neurodevelopmental disorders.* Poster presentation for American Society for Neural Therapy and Repair, Clearwater, FL. Poster presentation.

Lebovitz, Y., Brabender, J., Creasey, M. and Theus, M.H. (2017, April) *Role of maternal gut microbiome on neurodevelopment: Implications for neurodevelopmental disorders.* Poster presentation for Central Virginia Chapter of Society for Neuroscience, Roanoke, VA. Poster presentation.

Lebovitz, Y. (2016, August). *Unexpected role of interferon-y in regulating neuronal connectivity and social behavior.* Oral presentation for Glial Biology in Health, Disease, and Cancer Center Journal Club, Roanoke, VA.

Lebovitz, Y. (2016, May). *Gut microbiome research for regenerative medicine*. Seminar presentation for Regenerative Medicine Interdisciplinary Graduate Education Program Data Blitz, Blacksburg, VA.

Patent Related Activity

Lebovitz, Y. and Theus, M.H. Intellectual Property Disclosure, "Novel probiotic *Lactobacillus* strain and methods of use." October 2018.